ABSTRACT

FARMER, WILLIAM TAYLOR QUINTON. Expression and Analysis of the Imprinted Genes, IGF2R and AIRN, During Development of In Vivo and In Vitro Produced Bovine Pregnancies. (Under the direction of Charlotte Farin and John Gadsby).

The objectives of this study were to examine the expression of IGF2R and AIRN in cattle; and determine the effects of in vitro embryo production on expression of these imprinted genes during bovine fetal and placental development. Study 1 was conducted to determine if AIRN was expressed in bovine fetal tissues and whether or not bovine AIRN exhibited time-specific expression during development, indicative of its role in regulating imprinted expression of IGF2R. The existence of bovine AIRN was validated using RNA extracted from gestational Day 70 bovine fetal liver. The presence of AIRN in early gestation bovine fetal tissues was examined. No AIRN was detected in any blastocyst pools (n = 11). The number of conceptuses expressing AIRN increased from 1 of 9 at Day 15 of gestation to 8 of 10 at Day 18 of gestation. AIRN was expressed in all fetal livers recovered at Day 35 to 55 (n = 5) and Day 70 (n = 7). In contrast, IGF2R was expressed in all blastocyst pools, Day 18 conceptuses, Day 35 to 55 fetal livers, and Day 70 fetal livers. Only 1 conceptus out of 9 at Day 15 gestation did not express IGF2R. The relative level of AIRN expression was decreased (P < 0.05) in in vitro produced fetal livers at Day 70 of gestation compared to in vivo produced controls. Our results indicate that, in cattle, expression of AIRN is not expressed in blastocyst-stage embryos, is expressed in an increasing proportion of embryos around the time of maternal recognition of pregnancy, and is expressed following implantation. Additionally, the relative expression level of AIRN in bovine fetal liver can be altered by the method of embryo production. The objective of study 2 was to characterize the novel bovine
AIRN non-coding RNA. Total RNA was extracted from gestational day 150 bovine fetal liver and DNase-treated prior to cDNA synthesis and PCR amplification. PCR primer sets (n=16) were designed based on genomic sequences to “walk” down the predicted AIRN ncRNA sequence. All PCR amplicons were sequence verified. A putative AIRN promoter was located 441 base-pairs upstream of differentially methylated region 2 (DMR2) within intron 2 of IGF2R. A strong polyadenylation signal was found 117 kb downstream of the promoter. Primer sets designed upstream of the promoter and downstream of the polyadenylation signal yielded no PCR amplicons. In conclusion, bovine AIRN is a long non-coding RNA approximately 117kb in length with transcriptional overlap of IGF2R. These results provide further evidence that AIRN most likely plays a role in regulating imprinted expression of IGF2R. In the third and final study, the objectives were to examine the expression of IGF2R mRNA and AIRN non-coding RNA in in vivo (IVO) and in vitro produced (IVP) bovine conceptuses, and in fetal and placental tissues at early and late gestation; and to analyze the level of DNA methylation of cytosine-guanine (CpGs) repeats within differentially methylated region 2 (DMR2) of IGF2R in these tissues. Expression of IGF2R mRNA did not vary between treatments regardless of the method used for embryo production, tissue, or stage of development. The level of expression for AIRN non-coding RNA was decreased in Day 70 IVP fetal cotyledons and Day 222 IVP-AOS fetal livers, and increased in Day 222 IVP-AOS fetal cotyledons compared to IVO controls. DNA hypomethylation was observed in Day 15 IVP conceptuses, Day 70 IVP and Day 222 IVP-AOS fetal liver and cotyledons. Therefore, a mechanism proposed to regulate and maintain imprinted expression of IGF2R, expression of AIRN, was affected by the methods used for in
vitro embryo production. However, a direct correlation between the level of AIRN expression and the level of imprinted expression of IGF2R was not identified. To summarize, AIRN in cattle is a 117kb long non-coding RNA whose expression was correlated with the onset of imprinted expression of IGF2R in bovine conceptuses. Furthermore, expression of AIRN varies in a time and tissue-specific manner. DNA methylation at DMR2 of IGF2R also appears to be involved in maintaining imprinted expression of AIRN and IGF2R; and expression of these imprinted genes can be affected by in vitro embryo production.
Expression and Analysis of the Imprinted Genes, *IGF2R* and *AIRN*, During Development of In Vivo and In Vitro Produced Bovine Pregnancies

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

To my family, mentors, and friends who believed in me and helped me to endure.
BIOGRAPHY

Will Farmer completed his B.S. degree in Animal Science in 2003 before deciding to move to Texas in the pursuit of changing career interests. In 2004, between working for a TV commercial add agency and an Americana Foods in quality control, Will decided that he would once change directions, move home, and continue his pursuit of working with animals. In 2005, he enrolled in graduate level course work and earned a probationary admission to graduate school at NCSU. Dr. Charlotte Farin mentored Will in his pursuit of a 2008 M.S. degree in Animal Science and Physiology investigating the existence of a non-coding RNA that plays a role in regulating the imprinted expression of a gene important to bovine fetal growth and development. Dr. Farin encouraged him to continue his work into a Ph.D., however, Will had started a young family and decided to work as a research assistant. In 2009, after a year of working with Ohio State University to collect swine fecal samples in eastern N.C. Will decided that Dr. Farin was right and came back to her lab as a Ph.D. student to continue his investigation of developmentally important imprinted genes. During his graduate school career Will took a special interest in teaching. He taught 10 semesters as a laboratory instructor for the Biology and Animals Science Departments. In 2013, Will won the graduate student teaching award from the North American Colleges and Teachers of Agriculture (NACTA) organization. Will enjoys teaching Anatomy and Physiology this fall for Wake Technical Community College and Central Carolina Community College. Currently, he is applying to postdoctoral positions at the EPA where he hopes to study the effects of endocrine disruptors on reproductive physiology.
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INTRODUCTION

In the 1970s and 1980s non-surgical embryo transfer techniques were developed and established for cattle (Bowen et al., 1978; Elsden et al., 1976; Seidel, 1981). Valuable female donor cows could be subjected to a superovulation protocol involving multiple injections of FSH, followed by artificial insemination, and their embryos recovered 7 days later by uterine lavage. The embryos could then be transferred into less valuable recipients that had been properly subjected to an estrous synchronization protocol to match the timing between the embryo and the recipient’s intrauterine environment. Application and commercialization of this technology took off in the 1990s and by the late 1990s had reached nearly 400,000 transfers of in vivo produced embryos per year (Thibier, 2002). The number of transfers continued to rise steadily through the 2000’s, however, in recent years, the worldwide number of in vivo produced bovine embryos transferred has plateaued at about 550,000 per year (Stroud, 2012).

Commercial availability of non-surgical embryo transfer in cattle gave rise to other assisted reproductive technologies such as in vitro maturation of oocytes, in vitro fertilization, and in vitro culture of embryos. From 2001 to 2011 the number of in vitro produced (IVP) bovine embryos transferred increased ten-fold from 30,260 to 373,869 (Stroud, 2012; Thibier, 2002). In vitro production and transfer of bovine embryos offers several advantages. Embryos can be produced from highly valuable females that have damaged oviducts. Additionally, oocytes can be collected from a single female and fertilized with sperm from different sires. Pregnancy rates have also been improved in heat stressed
dairy cows that received an IVP embryo compared to artificial insemination (Block et al., 2003; Hansen, 2006; Hansen and Block, 2004). Transfer of IVP embryos in cattle offer the potential to overcome infertility due to ovulation failure, oocyte defects, fertilization failure, spermatozoa defects, and failure of the developing embryo to transit the oviduct. Furthermore, transfer of IVP embryos in dairy and beef cattle have been used to maximize genetic improvement, optimize breeding schemes, and increase fertility (Block et al., 2011; Hansen, 2006; Hansen and Block, 2004).

In vitro production of embryos is a key technology that supports other more advanced biotechnologies such as somatic cell nuclear transfer (SCNT) (Farin et al., 2010b). Cattle have been cloned for a variety of reasons. For example, some cloned cattle have been developed as bioreactors that produce pharmaceuticals or human blood components (Keefer, 2004; van Berkel et al., 2002; Wall et al., 1997; Wang et al., 2008). Cloning technology may also be used to perpetuate the genetics of superior animals (Hoshino et al., 2009; Yonai et al., 2005), create transgenic cattle for disease research (Edwards et al., 2003; Narbonne et al., 2012), or as a potential method to rescue endangered species (Gomez et al., 2009).

Once transfer of IVP embryos became a common occurrence, reports of abnormalities began to emerge. In one initial report, ovine zygotes were produced in vivo, cultured for 5 days, and then transferred (Walker et al., 1992). The resulting pregnancies exhibited extended gestation lengths, fetal overgrowth, and increased mortality (Walker et al., 1992). Early reports in cattle that investigated the effects of in vitro production on offspring documented similar abnormalities including oversized fetuses and increased perinatal mortality (Behboodi et al., 1995; Farin and Farin, 1995; Sinclair, 1995). However, it should
be noted that, in each case, in vitro maturation of oocytes prior to in vitro fertilization (IVF) and in vitro culture (IVC) of embryos was used and therefore not possible to discern which in vitro technology or combination thereof, was the dominating factor contributing to the abnormalities. Interestingly, these abnormalities were similar to those observed following the transfer of either blastomere (NT) clones or somatic cell nuclear transfer clones (Chavatte-Palmer et al., 2004; Edwards et al., 2003; Keefer et al., 1994; Panarace et al., 2007; Willadsen et al., 1991; Wilson et al., 1995).

Cumulatively, initial reports indicated that the transfer of IVP or IVM embryos resulted in aberrant development of embryos, fetuses, placentas, and offspring. The most obvious phenotype associated with the transfer of IVP or IVM embryos was fetal overgrowth, however a host of other abnormalities were observed (Behboodi et al., 1995; Farin and Farin, 1995; Keefer et al., 1994; Miles et al., 2004; Walker et al., 1996; Willadsen et al., 1991; Wilson et al., 1995). These abnormalities included increased perinatal death, extended gestation lengths, congenital deformities, and abnormalities of the placental vasculature. The occurrence of these phenotypes became known as “Large Offspring Syndrome” or “large calf syndrome” (LOS) (Farin et al., 2006). The LOS syndrome occurs more frequently in pregnancies following the transfer of SCNT embryos (Keefer et al., 1994; Willadsen et al., 1991; Wilson et al., 1995) compared to that of IVP (Behboodi et al., 1995; Farin and Farin, 1995; Sinclair, 1995). However, since the recognition that IVP and manipulation of bovine embryos can affect development, many studies have been completed that document the occurrence of more abnormalities in conjunction with or in the absence of fetal overgrowth.
ABNORMAL OFFSPRING SYNDROME (AOS)

The term, Abnormal Offspring Syndrome (AOS), encompasses all of the phenotypes observed in fetuses, placentas, and offspring following the transfer of SCNT, and to a lesser extent, IVP embryos (Behboodi et al., 1995; Farin and Farin, 1995; Farin et al., 2006; Keefer et al., 1994; Miles et al., 2004, 2005). This term was suggested as a way to encapsulate all of the phenotypes observed without placing too much emphasis on one phenotype (Farin et al., 2006). More broadly, AOS could be used as a descriptor for any aberrant phenotype in a mammalian species that results from the transfer of an IVP or SCNT embryo. Abnormalities have been observed in pigs, goats, cows, sheep, mice, and humans. Currently, these abnormal phenotypes associated with AOS are thought to arise from aberrant epigenetic modifications that regulate expression of imprinted and non-imprinted genes. Because known differences in gene regulation between species exist, it is likely that the resulting abnormal phenotypes from transfer of IVP or SCNT embryos will vary between species. For example, fetal and placental overgrowth has been observed in cattle and sheep (Behboodi et al., 1995; Bertolini et al., 2002a; Farin and Farin, 1995; Young et al., 2001); however, it has not been observed in pigs (Archer et al., 2003; Park et al., 2001). Cloned pigs exhibit other abnormalities (Archer et al., 2003; Park et al., 2001) that could be characterized as AOS and may be specific to swine. Interestingly, there is a greater risk of intra-uterine growth restriction (IUGR) in human fetuses following the transfer of IVP embryos generated from in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Zollner and Dietl, 2013). These observations lend further support to the idea that the underlying mechanisms that
generate abnormalities post-transfer of IVP or SCNT embryos may be similar between species. However, variation in manifestation of the abnormalities produced in fetuses, placentas, and offspring may indicate the subtle differences in how specific genes are expressed during development between species.

**Classification System of AOS**

The abnormalities associated with AOS have been categorized into 4 separate subtypes (Farin et al., 2006). These categories arose from the observation that there are several potential outcomes following the transfer of an IVP or SCNT embryo. Type I AOS describes embryonic and conceptus losses during the pre- and peri-attachment due to abnormal development that results in death prior to completion of organogenesis at Day 42 of gestation (Farin et al., 2006). In cattle, Type II AOS pertains to abnormalities of the fetus and placenta that result in fetal death and abortion between Day 42 and Day 280 of gestation (Farin et al., 2006). When a pregnancy results in Type III AOS, there is no evidence of a compensatory mechanism and the fetus and placenta exhibit moderate to severe developmental abnormalities (Farin et al., 2006). Parturition may be normal or difficult. Severely altered clinical, hematological, and biochemical parameters contribute to death of the fetus or calf near term or shortly thereafter during the neonatal period (Farin et al., 2006). In contrast, in Type IV AOS compensation of the feto-placental unit occurs and allows for development of a full term fetus and or placenta with only moderate abnormalities (Farin et al., 2006). Calves with Type IV AOS may have altered clinical, hematological, or biochemical parameters. Additionally, they may be abnormally sized for their breed (Farin et al., 2006).
However, despite anatomical, physiological, or metabolic abnormalities, the pregnancy is successful to term and the calf survives (Farin et al., 2006).

Study of AOS has proven to be difficult given the amount of heterogeneity of abnormal phenotypes. Specific phenotypes have not successfully been tied to any of the known variables that influence development following transfer of IVP or SCNT embryos. These variables include culture conditions (Rooke et al., 2007; Sinclair et al., 1999; Wells et al., 2003), extent of embryo manipulation (Edwards et al., 2003; Lawrence et al., 2005), maternal nutrition (Powell et al., 2006), and species. The abnormalities may vary from little or no apparent phenotypic abnormality (Piedrahita et al., 2002) to severe phenotypes that lead to abortion and peri-natal death (Hill et al., 2000a; Wells et al., 1999). However, observations of the various abnormalities can be documented at different developmental time points.

**Pre-Attachment Development**

In cattle, embryos are typically transferred on Day 7 of development as blastocysts to a synchronized recipient. In normal development following the transfer of IVO embryos or, with artificial insemination (AI), the greatest losses occur during the embryonic period of pregnancy prior to the end of organogenesis at Day 42 (Hubbert, 1974). Similarly, pregnancies resulting from the transfer of IVP embryos exhibit a high rate of embryonic loss during early gestation; however, the losses are greater than those observed after the transfer of IVO produced embryos or AI (Hasler, 2000; Hasler et al., 1995; Kruip and Den Daas, 1997). Categorically, these losses would fall under type I AOS (Farin et al., 2006).
Pregnancy rates following the transfer of IVO or IVP embryos can be influenced by numerous factors beyond how the embryos were generated. For example, pregnancy rates following the transfer of IVO and IVP embryos were similar if the embryos were grade 1; however, if they were grade 2 then IVP pregnancy rates were significantly lower (Farin and Farin, 1995). In general, pregnancy rates following the transfer of IVP embryos have been observed to vary within and between laboratories, can be lower than IVO embryos, can differ by embryo evaluator, and differ by the recipient’s characteristics (Farin et al., 1995; Farin et al., 1999; Hasler, 2000; Peterson and Lee, 2003; Pontes et al., 2009). Additional factors that influence if a pregnancy will be established and maintained following the transfer of IVP embryos include the embryo culture system, number of embryos transferred per recipient, degree of synchrony between the embryo and the recipient, whether the embryos were fresh or frozen-thawed, and the lactation status of the recipient (Farin et al., 2001; Hansen and Block, 2004; Peterson and Lee, 2003). Pregnancy rates following the transfer of IVP embryos have reached 45% or higher in large field studies (Hasler, 2000; Lane et al., 2003; van Wagtendonk-de Leeuw et al., 2000).

IVP embryos differ from IVO embryos morphologically and physiologically depending on the environmental conditions in which they were grown. If the culture medium used to grow the embryos contained serum, then they will appear dark, have a less organized ICM, and exhibit less compaction at the morula stage (Van Soom et al., 1996; Van Soom et al., 1997). IVP embryos exposed to culture conditions with and without serum exhibited increased numbers of lipid droplets and immature mitochondria (Abe et al., 1999; Crosier et al., 2001, 2000; Farin et al., 2001; Plante and King, 1994). Additionally, IVP embryos failed
to show an increase in mitochondrial volume density between the morula and blastocyst stages of development as was observed in their IVO counterparts (Crosier et al., 2001). Other differences between IVP and IVO blastocysts include fewer cells per blastocyst and decreased number of cells in the inner cell mass (Iwasaki et al., 1990; Van Soom et al., 2001). IVP embryos also exhibited a higher rate of abnormal ploidy compared to IVO embryos (Hyttel et al., 2000; Iwasaki et al., 1992; Viuff et al., 1999). Blastocysts with mixoploidy can establish a pregnancy, however, blastocysts with severe mixoploidy do not survive (Hyttel et al., 2000; Viuff et al., 2000). IVP embryos have altered ultrastructure, metabolic features, physiological features, altered gene expression, and reduced cryotolerance (Block et al., 2011; Crosier et al., 2001; Enright et al., 2000; Farin et al., 2004a; Khurana and Niemann, 2000; Lazzari et al., 2002b; Lonergan et al., 2006; Rizos et al., 2003). All of these differences contribute to lower pregnancy rates following transfer and are associated with increased birth weight of offspring (Block et al., 2011; Lazzari et al., 2002b).

In cattle, pregnancy losses after transfer of embryos occurs during the first 21 days of pregnancy and are greatest between days 8 and 16 (Farin and Farin, 1995; Sreenan and Diskin, 1983). These early gestation losses are considered to be of the Type I AOS variety (Farin et al., 2006) Conceptuses generated from the transfer of IVP embryos have been observed to be twice the length of IVO conceptuses at Days 12 and 17 of gestation (Farin et al., 2001; Lazzari et al., 2002b). However, IVP conceptuses have also been documented as being shorter than IVO produced conceptuses at Day 16 (Bertolini et al., 2002a). The difference in length of IVP conceptuses at Days 16 and 17 may indicate that the later
gestation conceptuses at Day 17 were those that had successfully navigated the maternal recognition process. Of the IVP conceptuses recovered at Day 17, 19% were degenerate suggesting that the group of shorter conceptuses at Day 16 in the Bertolini study may have had a greater proportion of conceptuses that were degenerate or were not likely to survive the process of maternal recognition of pregnancy. Apart from conceptus length, the developmental capacity of a conceptus may be correlated to its ability to create an embryonic disc at an appropriate time relative to maternal recognition of pregnancy. A high rate of early pregnancy loss in IVP and SCNT pregnancies has been associated with the absence of an embryonic disc near the time of maternal recognition (Alexopoulos et al., 2008; Fischer-Brown et al., 2004).

**Post-Attachment Development**

Pregnancies generated from the transfer of IVP embryos are associated with high losses during the late embryonic period at Day 30 through the early fetal period at Day 90 (Farin et al., 2006). These losses can be described as either Type I or Type II AOS and therefore attributed to different mechanisms. IVO pregnancy losses occur at a rate of less than 10% and are evenly distributed IVP between Day 30 and 90 (Bertolini and Anderson, 2002). In contrast, IVP pregnancies are lost at a higher rate between day 30 and 65 with the majority of them occurring between Days 30 and 44 (Bertolini and Anderson, 2002). Fetal losses of IVP pregnancies occurred at a rate of about 13% after Day 40 of gestation and may differ slightly depending on the culture system used to generate the embryos (Agea et al., 1998). Fetal abortion rate in dairy cows after transfer of IVP embryos was 24% from Day 53
to calving with the majority of losses between Day 50 and 80 (Block et al., 2003). Abortion rates are significantly higher in IVP and SCNT pregnancies compared to AI or IVO embryo transfer produced controls (Agea et al., 1998; Block et al., 2003; Edwards et al., 2003; Hasler, 2000; Hasler et al., 1995; Hill et al., 2000b). For SCNT pregnancies, fetal losses are substantially higher between Days 30 to 60 and occur at a rate of 50 to 100% (Edwards et al., 2003; Hill et al., 2000a; Hill et al., 2000b; Wells et al., 1998). In contrast to IVP pregnancies, fetal loss in SCNT pregnancies continues to be high during the second trimester and third trimesters (Edwards et al., 2003; Heyman et al., 2002; Hill et al., 2000a).

Early gestational losses due to placental defects after transfer of IVP or SCNT embryos can be categorized as Type II AOS (Farin et al., 2006). Developmental failure during the first trimester (Day 30 to 90) may occur due to placental membrane abnormalities and reduction in placental blood vessel development (Farin et al., 2006). Interestingly, the leading cause of embryo mortality at Day 30 in pregnancies from SCNT embryo transfers is improper allantoic development (Edwards et al., 2003). Additionally, SCNT placentas lack proper vascularization which may contribute to abnormalities such as hypoplasia and reduced cotyledonary development (Chavatte-Palmer et al., 2012; Constant et al., 2006; Hill et al., 2000a; Hoffert et al., 2005). Hydrallantois is a common feature of IVP pregnancies (1 in 200) compared to IVO pregnancies (1 in 7500) (Farin et al., 2006; Hasler et al., 1995), however, it is greater still in SCNT pregnancies (Edwards et al., 2003; Hill et al., 2000a). Additionally, placentas from IVP pregnancies at Day 70 of gestation exhibited placentomegaly, decreased placentome number, decreased placental efficiency, decreased placental fluid, and decreased blood vessel density (Farin et al., 2006; Miles et al., 2005).
Altered placentome weight and distribution have also been reported at Day 90 and 180 of gestation in IVP pregnancies (Bertolini and Anderson, 2002; Bertolini et al., 2004).

**Peri-Natal Development**

In late gestation, fetal abnormalities occur more frequently in IVP and SCNT derived pregnancies compared to those from AI or IVO/ET. For example, congenital malformations such as hyrdallantois and abnormal limb formation occur more frequently in IVP fetuses (3.2%) compared to IVO fetuses (0.7%) (van Wagterendonk-de Leeuw et al., 1998). Fetal overgrowth has also been observed in IVP fetuses at Days 90, 180, and 222 of pregnancy (Bertolini et al., 2002b; Bertolini et al., 2004; Farin and Farin, 1995). Similarly, fetal overgrowth was observed in late gestation fetuses and postnatal calves derived from SCNT embryos (Chavatte-Palmer et al., 2002; Hill et al., 1999; Wells et al., 1999). High rates of pregnancy loss that occur at term may result from dystocia as a result of large fetus size (Behboodi et al., 1995; Farin et al., 2006).

Additional abnormalities that follow after the transfer of SCNT embryos observed in late term or postnatal period include respiratory problems, prolonged recumbency, and contracted flexor tendons (Panarace et al., 2007). Survival of SCNT calves after birth is compromised by various abnormal metabolic states including hypothermia, hypoglycemia, severe metabolic acidosis, and hypoxia (Panarace et al., 2007). Respiratory distress syndrome is also a common feature of AOS observed in cloned calves after birth (Hill et al., 1999).
Peri-natal mortality rates in AI and IVO/ET pregnancies range from 6.2 to 9% (King et al., 1985; van Wagendonk-de Leeuw et al., 1998). By comparison, post-natal death rates of IVP calves after parturition are greater and range from 8.6% to 17.9% (Block et al., 2003; Hasler et al., 1995; van Wagendonk-de Leeuw et al., 1998). Mortality rates are even higher in late gestation fetuses and postnatal SCNT calves. In 2012, the Japanese Ministry performed a nationwide survey spanning 10 years and found that out of 594 clones 14.8% were stillborn and 95% died within 24 hours of birth (Akagi et al., 2013). Similarly, a 5 year study performed in the U.S., Brazil, and Argentina found that out of 3374 SCNT embryos transferred resulted in 388 calves (9%) out of which 42% died within 150 days of birth (Panarace et al., 2007).

In late gestation, placental abnormalities are common following the transfer of IVP and SCNT embryos (Farin et al., 2010b; Farin et al., 2006). Placentas from IVP and SCNT pregnancies differ from AI or IVO/ET pregnancies morphologically, and exhibit differences in gene expression, hormone, and protein expression (Chavatte-Palmer et al., 2002; Edwards et al., 2003; Everts et al., 2008; Farin et al., 2006; Hill et al., 2000a; Miles et al., 2005). These differences in placental development may contribute significantly to losses of IVP and SCNT pregnancies. Placental defects are most notable in SCNT pregnancies (Hill et al., 2000a; Hill et al., 1999). Interestingly, the leading cause of pregnancy failure in IVO pregnancies is fetal abnormalities which is in contrast to SCNT pregnancy failure where the main cause is placental defects (Hasler et al., 1995; Hill et al., 2000a; Hill et al., 1999).

In Type III AOS, compensation of the placenta and fetus are insufficient and the calf is born with severe developmental abnormalities or dies near birth (Farin et al., 2006). Some
IVP placentas appear to exhibit compensation to decreased fetal villi and decreased binucleate cell volume density within the placentomes as indicated by increased blood vessel volume in the maternal caruncles and increased blood vessel volume density to placentome surface area (Miles et al., 2004).

**Potential Mechanisms Underlying of AOS**

The abnormalities observed in bovine embryos, fetuses, placentas, and offspring following the transfer of IVP or SCNT embryos were initially hypothesized to be the result of epigenetic dysregulation and subsequent aberrant mRNA expression of developmentally important imprinted genes (Farin et al., 2004b; Lazzari et al., 2002a; Reik et al., 2001; Wrenzycki et al., 2004). Imprinted genes are expressed monoallelically, in a parent-of-origin specific manner, as a result of epigenetic modifications (Prickett and Oakey, 2012). Epigenetic modifications are chemical alterations to the DNA or to histone proteins that alter chromatin structure without altering the nucleotide sequence (Barlow, 2011). Embryos derived from IVP and SCNT are exposed to the in vitro culture (IVC) environment which is thought to disrupt the acquisition or maintenance of epigenetic patterns regulating imprinted gene expression (Farin et al., 2010a; Farin et al., 2010b; Smith et al., 2012). In addition to insults from the IVC environment, SCNT embryos must also contend with rupture of the zona, loss of maternal cytoplasm and cytoskeleton, electrical or chemical fusion and activation of the enucleated oocyte with the donor cell, and epigenetic reprogramming of the donor nucleus to a totipotent state (Niemann and Lucas-Hahn, 2012). Another source of epigenetic dysregulation contributing to abnormal phenotypes associated with AOS may
arise from the aspiration and in vitro maturation (IVM) of bovine oocytes prior to IVP or SCNT manipulations. The acquisition of maternal epigenetic modifications that regulate imprinted gene expression occurs during the latter stages of follicular development, a time at which cumulus oocyte complexes (COC) are aspirated and may also become altered during IVM (O'Doherty et al., 2012). Interestingly, many imprinted genes regulate the expression of non-imprinted genes. Therefore, abnormal phenotypes may arise from aberrant expression of non-imprinted genes as a downstream effect of epigenetic dysregulation on imprinted genes (Farin et al., 2006). Alternatively, in vitro culture environments and SCNT protocols may have adverse effects on the DNA modifications regulating the expression of non-imprinted genes independent of genomic imprinting (Farin et al., 2006). Adding to the complexity of the potential mechanisms underlying AOS comes from the observation that IVC and SCNT protocols also negatively impact the expression of genes that function to regulate the acquisition and maintenance of epigenetic modifications necessary for the appropriate expression of imprinted genes.

IMPRINTED GENES

Parental genomes were once thought to be equivalent in their contribution to the development of a new individual. However, examination of parthenogenetic and gynogenetic embryos revealed that mammalian embryos derived from maternal genomes did not result in a viable embryo (Graham, 1974; Surani and Barton, 1983). Further investigation of androgenetic embryos demonstrated that the same was true for embryos
generated from only paternal genomes (Mann and Stewart, 1991). Furthermore, a series of nuclear transplantation experiments in mice showed that both parental genomes were necessary for complete fetal and placental development (McGrath and Solter, 1984; Surani et al., 1984). The parental genomes are, therefore, not equivalent in their gene expression and function in regulating growth and development (Surani et al., 1984). The process by which marks are established in the genome during gametogenesis that allow specific genes to be expressed monoallelically in a sex-specific manner from only one of the parental genomes is called genomic imprinting (Li, 2013). These imprinted genes are therefore expressed from either the maternal or paternal allele. Interestingly, it has been found that in some cases monoallelic expression of the imprinted gene is not absolute and basal levels of expression of one allele are overshadowed by dominant expression from the other allele (Latos et al., 2009). Expression of most imprinted genes is regulated in a developmental and some in a tissue-specific manner. For example, some genes only exhibit imprinted expression in the placenta such as Ascl2, Plda2, Slc22a2, and Slc22a3 (Abramowitz and Bartolomei, 2012).

In the cow, there are 34 known imprinted genes, however, in the human and mouse the number of imprinted genes has risen to more than 150 (Imprinted Gene Catalogue www.otago.ac.nz/IGC). More imprinted genes in cattle are likely to be found as limitations in technology for discovery are overcome; however, the total number of imprinted genes in mammalian species has been suggested to be limited to a few hundred (Kelsey and Bartolomei, 2012).

Imprinted genes are primarily found in co-regulated clusters (Edwards and Ferguson-Smith, 2007). The number of genes within each cluster may range from 2 to several dozen
There are 16 known clusters of imprinted genes that are controlled by an imprinting control region (ICR) which may exert its effects on imprinted genes. The distance of an ICR to the genes it may affect can vary from only a few kilo-bases up to several mega-bases (Li, 2013). ICRs are found within differentially methylated regions (DMR) that exhibit different patterns of DNA methylation on one of the parental alleles compared to the other (Reik, 2007). Another common feature of ICRs is that exhibit covalent modifications to histone tails (Trasler, 2006). Depending on the type of modification to the histone tails they may repress or promote transcription of the imprinted genes. Lastly, many imprinted gene clusters contain a non-coding RNA that is involved in regulating the parent-specific expression of the other genes within the cluster.

**DNA Methylation**

DNA methylation of cytosine and guanine dinucleotides (CpGs) within DMRs of imprint control regions are part of the regulatory control that induces allelic silencing and allowing for monoallelic expression from imprinted genes (Edwards and Ferguson-Smith, 2007). CpGs are found within distinct regions of the genome called CpG islands, which are often associated with the promoter of protein coding genes (Hackett and Surani, 2013). DNA methylation of CpGs within a DMR represses transcription by blocking access of transcription factors and other enzymes to the DNA (Scarano et al., 2005). Gametic DNA methylation imprints are established during gametogenesis by enzymes called de novo DNA methyltransferases (DNMTs). After fertilization, the parental genomes undergo erasure of
DNA methylation; however, the gametic DNA methylation imprints are maintained by maintenance DNMTs. During embryonic development, somatic DNA methylation imprints are established secondarily to the gametic imprint and may pose as a second line of epigenetic information that reinforces monoallelic expression. As cellular differentiation continues, primordial germ cells arise and migrate from the yolk sac to the genital ridge where they will become precursor cells for future gametes. During migration they undergo complete DNA demethylation and new sex-specific imprints are established during gametogenesis and are completed as the gametes mature (Holmes and Soloway, 2006).

DNA methylation is established de novo by DNMTs that transfer a methyl group from S-adenosyl methionine to the 5’ position of a cytosine creating 5-methylcytosine (5mC) (Chen and Riggs, 2011). The regions of DNA that undergo methylation are cytosine and guanine rich and are referred to as CpG islands. Interestingly, the majority of CpG islands in the genome remain unmethylated during development. In contrast, CpGs located outside of the majority of CpG islands are methylated indicating that DNA methylation is the default state and unmethylated CpG islands associated with imprinted genes are the exception (Suzuki and Bird, 2008). DNMT3A and DNMT3B are de novo methyltransferases that methylate unmethylated cytosines (Okano et al., 1999). DNMT3L does not have an active methyltransferase site, however, it does increase the activity of DNMT3A and 3B (Li, 2013). After gametic imprints are established, they are maintained by other methyltransferases such as DNMT1 and DNMT1o. DNMT1 maintains methylation of hemi-methylated DNA and faithfully copies the existing methylation pattern to newly synthesized DNA after replication (Yoder et al., 1997). Dnmt1o is an oocyte specific DNMT that is expressed only in oocytes
and preimplantation embryos (Howell et al., 2001). Deletion of the Dnmt1o promoter resulted in methylation at imprinted loci was not faithfully maintained. However, establishment of imprints remained unimpeded. These observations indicate that DNTM1o was important for methylation maintenance but not necessarily imprint establishment during early embryonic development (Dean and Ferguson-Smith, 2001; Howell et al., 2001).

**Histone Modifications**

In addition to DNA methylation, histone modifications are heritable and serve as epigenetic marks that can close or open chromatin for transcription at the gametic DMRs (gDMR) (Li, 2002). Histones make up the core unit of the nucleosome that is wrapped with DNA (Margueron et al., 2005). Each histone has an N-terminal tail that can undergo post-translational modifications such as methylation and acetylation (Peters and Schubeler, 2005). Acetylation of lysine residues on histones H3 and H4 are associated with an open chromatin state called, euchromatin. In contrast, methylation of lysine residues on H3 and H4 are associated with a closed chromatin state, called heterochromatin (Nafee et al., 2008). For example, the repressive histone modifications of histone 3 lysine 9 di/tri methylation (H3K9me2/3) recruits PGC7 which protects gDMRs from the active demethylation events that occur following fertilization (Nakamura et al., 2012). Other histone modifications associated with all of the known imprinted gDMRs in murine embryonic stem cells include H4K20me3 at methylated gDMRs and H3K4me3 at unmethylated gDMRs (Macdonald, 2012; McEwen and Ferguson-Smith, 2010; Mikkelsen et al., 2007). Interestingly, preimplantation embryos that were subjected to immunofluorescence staining for H3K9me3,
H3K4me3, and H4K20me3 revealed that only H3K4me3 and H3K9me3 were present (van der Heijden et al., 2005; Wongtawan et al., 2011). H4K20me3 was only detectable in postimplantation stage embryos. Therefore, histone modifications may serve multiple functions in resulting allele specific expression from imprinted genes. How these modifications are acquired and their exact roles in regulating imprinted expression remains to be elucidated.

**Long Non-Coding RNAs**

Recently, it has been predicted that less than 2% of the mammalian genome codes for proteins and amazingly that most of the genome gives rise to non-protein coding RNAs (Djebali et al., 2012). Long ncRNAs make up the largest group of ncRNAs. In humans, there have been over 10,000 long ncRNAs annotated. LncRNAs are >200 bases in length, transcribed by RNA polymerase II and lack an open reading frame. The function of most lncRNAs is undetermined, however, there is growing recognition that lncRNAs contribute to regulation of gene expression in growth, differentiation, and disease (Guttman et al., 2011; Kornienko et al., 2013; Pauli et al., 2011; Yap et al., 2010). LncRNAs participate in transcriptional regulation of coding genes in a tissue and developmental stage specific manner. They can negatively or positively influence transcription of protein-coding genes on the same chromosome in cis or on other chromosomes in trans (Kornienko et al., 2013). An example of a trans acting lncRNA is 7SK, a 331bp RNA that represses transcription elongation of protein coding genes through inhibiting transcription factors from phosphorylating the RNAPII carboxy-terminal domain (Peterlin et al., 2012). Another
example of a lnc RNA operating in a locus-specific manner in trans is hox antisense intergenic RNA (HOTAIR). HOTAIR lncRNA is 2.2kb spliced transcript expressed from the HOXC cluster and represses transcription in trans from the HOXD cluster (Rinn et al., 2007). Currently, there are more examples of transcription regulation in cis by lncRNAs than trans. LncRNAs can regulate transcription in cis through a variety of mechanisms that can either interfere with transcription or promote it. Transcriptional interference by lncRNAs in cis may involve promoter nucleosome repositioning, influencing promoter histone modifications and DNA methylation, or inhibiting RNAPII activity (Tufarelli et al., 2003). LncRNAs can also operate in a manner that creates an open chromatin state and encourages transcription.

**Cycle of Genomic Imprinting**

After fertilization, the maternal and paternal genomes in their respective pronuclei must undergo extensive remodeling in order for the developing zygote to reach a totipotent state (Clift and Schuh, 2013). Global DNA demethylation of the paternal genome is an active process (Kelsey and Feil, 2013). At the time of fertilization, sperm DNA is tightly packaged on protamines and highly methylated relative to the oocyte. Loss of DNA methylation from the paternal genome prior to the first DNA replication originally was interpreted to indicate that demethylation was an active process (Oswald et al., 2000). However, currently there is more evidence to suggest that the process is a passive one whereby 5mC is converted to 5hmC by DNA hydrolase ten-eleven translocation 3 (TET3) enzyme. Converted 5hmCs in the paternal genome are not remethylated during replication and therefore the DNA demethylation event is termed passive replication-coupled dilution.
However, the mechanisms behind this reaction are not yet understood. Additionally, several proteins involved in DNA repair are also involved in the demethylation process, including GADD45, MBD4, and TDG (Gehring et al., 2009; Guo et al., 2011; He et al., 2011; Wu and Zhang, 2010).

In contrast to the paternal genome, the maternal genome is protected from initial rapid demethylation. Primordial germ cell protein 7 (PGRC7) or STELLA, binds to histone H3 methylated at Lysine 9 (H3K9me2) on the maternal chromatin and inhibits demethylation. Following the initial rapid demethylation, both parental genomes undergo gradual passive demethylation leading up to the blastocyst stage (Reik, 2007). Maintenance DNMT1 and PGRC7, both play a critical role in maintaining the sex specific imprints present on the maternal and paternal genomes during this demethylation event (Hirasawa et al., 2008). Global DNA methylation erasure, in conjunction with maintenance of methylation at sex specific imprints, culminates at the blastocyst stage with establishment of Inner Cell Mass (ICM) cells that are pluripotent. De novo methylation begins to occur coinciding with the first differentiation event when cell lineages within the blastocyst give rise to the ICM and the trophectoderm (Armstrong et al., 2006). The rest of the embryonic genome is progressively methylated corresponding to the species-specific transition to transcription from the embryonic genome (Niemann et al., 2008; Reik and Walter, 2001).

In contrast to the erasure that occurs during zygotic and embryonic development, global erasure that occurs in primordial germ cells (PGCs) includes DNA demethylation of sex specific imprints (Li, 2013). PGCs are the progenitors of the future gametes that arise from the extra-embryonic mesoderm, migrate to the genital ridge, where they populate the
area that will develop into the gonads (Naee et al., 2008). Acquisition of new sex-specific methylation imprints does not occur until after sex determination has been initiated and divergence of the male and female germ lines begins (Sasaki and Matsui, 2008). If the developing fetus is male, then the PGCs will undergo DNA demethylation and acquire new paternal-specific imprints during spermatogenesis. However, if the fetus is female, the PGCs will acquire maternal-specific imprints after erasure during oogenesis. Interestingly, male specific imprints are established in utero and are nearly complete at the time of parturition (Li, 2013). In contrast, establishment of maternal specific imprints occurs post-natally in primary oocytes that are selected for growth and are not fully established until the oocyte has matured and is ready for ovulation (O'Doherty et al., 2012).

**INSULIN-LIKE GROWTH FACTOR FAMILY**

The insulin-like growth factor (IGF) family is essential for fetal and placental growth and development (Forbes and Westwood, 2008). Insulin-like growth factor-1 and -2 (Igf1 and 2) are both powerful mitogens that function as regulators of cell survival, proliferation and differentiation (Pavelic et al., 2007). The functions of the growth factors are mediated through binding to the type-1 receptor (Igf1r) (Forbes and Westwood, 2008). In contrast, the type 2 receptor (Igf2r) primarily binds Igf2 and targets it for lysosomal degradation (Forbes and Westwood, 2008). Igf1 and Igf2 are very similar to each other and to a lesser extent, proinsulin (Pavelic et al., 2007). Similarly, the type 1 receptor shares close structural homology to the insulin receptor. Igf1r binds Igf1 with a high affinity and with less affinity
to Igf2 and insulin. The IGFs are unlike insulin in that they are not restricted to production and secretion by specific cell types (Dupont and Holzenberger, 2003). Instead, almost all cell types within the body can produce IGFs (Dupont and Holzenberger, 2003). Biological activity of IGFs is modulated by six insulin-like growth factor binding proteins (IGFbp1 - 6) (Samani et al., 2007).

**Ligands**

Igf1 is a trophic factor that circulates in the bloodstream at high levels postnatally (Pavelic et al., 2007), and at low levels during embryonic development (Pollak et al., 2004). Therefore, it is thought that Igf1 is more essential for postnatal development (Pavelic et al., 2007). However, Igf1 has been shown to be necessary for organogenesis (Baker et al., 1993). The main source of Igf1 in circulation is the liver (Dupont and Holzenberger, 2003). In mice, targeted disruption of the Igf1 gene in the liver resulted in a 75% reduction in circulating Igf1 (Sjogren et al., 1999; Yakar et al., 1999). Interestingly, loss of hepatic Igf1 did not dramatically decrease postnatal growth illustrating that local production of Igf1 plays a major role in tissue growth (Dupont and Holzenberger, 2003). Serum Igf1 levels are primarily regulated by growth hormone (GH) produced from the anterior pituitary (Dupont and Holzenberger, 2003). A negative feedback loop forms as serum Igf1 levels rise and exert a negative response on the hypothalamus and pituitary gland to inhibit secretion of GH from the anterior pituitary (Dupont and Holzenberger, 2003).

Igf2 is a mitogen that is highly expressed during fetal development (Pavelic et al., 2007). Synthesis of Igf2 is mostly independent of GH regulation (Pavelic et al., 2007). The
Igf2 gene is imprinted and expression only occurs from the paternal allele while the maternal allele is silenced (DeChiara et al., 1991). During early embryonic development Igf2 is essential at the fetomaternal interface for development and function of the trophoblast (Dupont and Holzenberger, 2003). Loss of Igf2 expression results in placental insufficiency and low fetal weight (Constancia et al., 2002). In addition, Igf2 null mice are 60% smaller than the wild type (Baker et al., 1993; DeChiara et al., 1991). Interestingly, these mice grow normally after birth indicating that Igf2 regulates intrauterine growth (Baker et al., 1993; DeChiara et al., 1991).

Receptors

The IGF ligands (Igf1 and Igf2) exert their cellular effects on growth by binding to the type-1 receptor (Forbes and Westwood, 2008). Igf1r is a member of the tyrosine kinase receptor family and exists at the cell surface as a heterotetrameric glycoprotein (Pavelic et al., 2007). The receptor consists of two extracellular α-subunits and two transmembrane β-subunits that are joined together by disulfide bonds (Hawkes and Kar, 2004). When one of the ligands binds to the receptor a conformational change occurs resulting in autophosphorylation of the receptor and activation of tyrosine kinase activity (Hawkes and Kar, 2004). Subsequent tyrosine phosphorylation of specific substrates including, insulin receptor substrate proteins (IRS) 1 – 4 and the Src-homology collagen protein (Shc) stimulates several intracellular signaling cascades (Samani et al., 2007). Activation of the phosphoinositide 3’-kinase (PI-3K) pathway leads to activation of several downstream substrates that regulate anti-apoptotic effects (Dupont and Holzenberger, 2003). In addition,
phosphorylation of IRS-1 or Shc leads to activation of the mitogen activated protein kinase (MAPK) system as well as nuclear factors that stimulate cellular proliferation (Grey et al., 2003; Hermanto et al., 2000). The IGF type-2 / mannose 6-phosphate receptor (Igf2r/M6PR) is different from Igf1r in structure and function (Hawkes and Kar, 2004). Igf2r/M6PR consists of 15 homologous extracytoplasmic domains, a single transmembrane region and a carboxy-terminal cytoplasmic tail (Byrd et al., 2000). The receptor has two binding sites for M6P bearing ligands, like lysosomal enzymes, and one binding site for non-M6P bearing ligands such as Igf2. Igf2r binds Igf2 with an affinity about 100 times greater than that for Igf1 and does not bind insulin at all (Hawkes and Kar, 2004). Igf2r/M6PR binds and targets Igf2 for lysosomal degradation and also functions in lysosomal enzyme trafficking, regulation of apoptosis and growth, and tumor suppression (Kornfeld, 1992; Lau et al., 1994; Ludwig et al., 1996; Motyka et al., 2000; Wang et al., 1994). Furthermore, Igf2r plays an essential role in regulating fetal growth as shown by loss of Igf2r expression in fetal mice resulting in overgrowth and neonatal death (Wylie et al., 2003). Similarly, fetal overgrowth phenotypes in sheep are associated with reduced Igf2r expression (Young et al., 2001).

**Binding Proteins**

In the circulation and other biological fluids, IGFs are found bound to one of six high affinity insulin-like growth factor binding proteins (Igfbp1 – 6) (Dupont and Holzenberger, 2003). The binding proteins have a higher affinity for the IGFs than the receptors (Jones and Clemmons, 1995). Therefore, Igfbps can modulate the biological accessibility and activity of
the IGFs (Forbes and Westwood, 2008). The binding proteins accomplish this by
transporting the IGFs from circulation to the peripheral tissues, sequestering a surplus of
IGFs in circulation, and by inhibiting or contributing to the activity of the IGFs (Jones and
Clemmons, 1995).

_IGF2R/AIRN IMPRINTING CLUSTER_

The insulin-like growth factor type 2 receptor (Igf2r) is also known as the cation-
independent mannose 6-phosphate receptor (M6PR) and functions as a fetal and placental
growth suppressor (Byrd and MacDonald, 2000). Igf2r accomplishes this task primarily by
binding Igf2 at the cell surface and targeting it for lysosomal degradation (Oka et al., 1985).
In mice, Igf2r is an imprinted gene that is maternally expressed and paternally imprinted
(Barlow et al., 1991). The Igf2r gene is part of a 400 kb cluster that contains two other
maternally expressed imprinted genes, solute carrier family 22a (Slc22a2 and Slc22a3) , and
one paternally expressed antisense non-coding RNA, Air (Lyle et al., 2000; Zwart et al.,
2001). The promoters for Slc22a2 and Slc22a3 are 190 kb and 260 kb downstream of the
Igf2r promoter (Schweifer et al., 1997). Neither Slc22a2 or Slc22a3 are expressed in the
embryo, however, both exhibit imprinted expression in the placenta and biallelic expression
in adult tissues (Regha et al., 2006; Zwart et al., 2001). In contrast, Igf2r is biallelically
expressed in the preimplantation embryo (Szabo and Mann, 1995) and maternally expressed
in all postimplantation tissues (Lerchner and Barlow, 1997), except in the brain where
biallelic expression is exhibited (Yamasaki et al., 2005). The only paternally expressed gene
in the cluster, \textit{Air}, is transcribed antisense to \textit{Igf2r} and shares 30 kb of sequence with \textit{Igf2r} (Regha et al., 2006). In addition, the \textit{Igfr2/Air} cluster contains two differentially methylated regions (DMRs) (Regha et al., 2006). DMR1 contains the promoter for \textit{Igf2r} and is methylated on the paternal allele, but not on the maternal allele (Regha et al., 2006). DMR2 is located within intron2 of \textit{Igf2r} and contains the promoter for \textit{Air} (Regha et al., 2006). On the maternal allele DMR2 is methylated and on the paternal allele it is not (Regha et al., 2006).

\textbf{Evolution of IGF2R Imprinting}

Parent-of-origin specific expression of the insulin-like growth factor type 2 receptor (\textit{Igf2r}) is thought to have first appeared in the mammalian lineage between 180 and 210 million years ago (MYA) (Hore et al., 2007). The appearance of imprinted \textit{Igf2r} expression is coincident with the divergence of the monotremes from the therian lineage 210 MYA and the departure of the marsupials from eutherians around 180 MYA (Hore et al., 2007; Woodburne et al., 2003). Imprinted expression of \textit{Igf2r} has been demonstrated in eutherian mammals including mice (Barlow et al., 1991), rats (Mills et al., 1998) sheep (Young et al., 2001), cows (Long and Cai, 2007), pigs (Vu et al., 2006), and dogs (O'Sullivan et al., 2007), as well as in the marsupial, opossum (Weidman et al., 2006). Animals that are more ancestral to marsupials and eutherians exhibit biallelic expression of \textit{Igf2r} including, chickens (Nolan et al., 2001) and the monotremes, platypus and echidna (Killian et al., 2000). Interestingly, all mammals in the Euarchonta clade also exhibit biallelic expression of \textit{Igf2r} including the tree shrew, flying lemur, ringtail lemur, and humans (Killian et al., 2001b).
Genomic imprinting of *Igf2r* and *Igf2* appear to have evolved in mammals in accordance with different reproductive strategies (Hore et al., 2007). *Igf2r* and *Igf2* are not imprinted in monotremes such as the platypus and echidna (Killian et al., 2000; Killian et al., 2001a), which lay eggs and secrete milk from their abdomens (Hore et al., 2007). Concurrent with the divergence of marsupials and eutherians from monotremes is the appearance of viviparity and imprinted *Igf2r* and *Igf2* (Hore et al., 2007). Both *Igf2r* and *Igf2* are imprinted in marsupials (Killian et al., 2000; O'Neill et al., 2000; Suzuki et al., 2005), which have a non-invasive choriovitelline placenta (Krause and Cutts, 1985), short gestation period, and give birth to altricial young (Killian et al., 2000). Similarly, imprinting of *Igf2r* and *Igf2* is exhibited by eutherians (Weidman et al., 2004; Young et al., 2001), which are true placental mammals. However, loss of imprinted *IGF2R* expression occurred in primates around 75 MYA (Vu et al., 2006), while imprinted expression of *Igf2* is maintained (Killian et al., 2001a). Interestingly, *IGF2R* imprinting in humans appears to be polymorphic in a small subset of the population (Oudejans et al., 2001; Xu et al., 1993). *IGF2R* serves as a tumor suppressor gene and studies on loss of heterozygosity or mutations of IGF2R are frequently found in early stage tumors indicating that monoallelic expression of *IGF2R* may be an early mechanism for initiating cancer growth (De Souza et al., 1995; Jamieson et al., 2003; Kong et al., 2000; Vu et al., 2006). The occurrence of polymorphic *IGF2R* imprinting in humans may be the result of ancestral imprinted alleles still in the population or the re-emergence of IGF2R imprinting (Killian et al., 2001b).

Currently, there are several theories that make predictions of what evolutionary pressure may have stimulated the creation of imprinted gene expression (Hore et al., 2007;
Vu et al., 2006; Wood and Oakey, 2006). The ovarian time bomb hypothesis proposes that an allele favoring imprinted expression would suppress malignant trophoblastic disease resulting from parthenogenesis of unfertilized oocytes (Varmuzza, 1994). Based on this hypothesis only a small subset of genes necessary for embryonic development would be imprinted and therefore fails to account for genes regulating post-natal development (Wood and Oakey, 2006). The kinship theory, also known as the conflict hypothesis, arose from the observation that in the mouse Igf2r, a growth suppressor, was maternally expressed and Igf2, a growth promoter, was paternally expressed (Haig and Graham, 1991). The theory states that when the parental investment in the offspring is unequal and the degree to which litter mates are related is variable, selective pressures would act differently on the alleles of the maternal and paternal genomes in the offspring (Moore and Haig, 1991). An intra-genomic conflict arises within the offspring between the maternal and paternal sets of alleles over potential resources supplied to the offspring by the mother (Hore et al., 2007). Based on the conflict hypothesis the marsupial and eutherian ancestor may have evolved imprinted expression of Igf2r and Igf2 as a result of conflict between the parental genomes (Hore et al., 2007). Therefore, it would be advantageous for the paternal genome to increase fetal size at the expense of the mother by favoring expression of Igf2. In contrast, would be advantageous for the maternal genome to minimize fetal growth by favoring expression of $Igf2r$. The conflict theory predicts that the degree to which offspring develop in utero, gestation length, and type of postnatal care are all selective pressures influencing imprinted gene expression (Killian et al., 2000).
Imprinting Control in the \textit{Igf2r/Airn} Cluster

Parent-specific expression of \textit{Igf2r} and neighbouring genes within the \textit{Igf2r/Air} cluster is regulated by DNA methylation, expression of the non-coding RNA, \textit{Air}, and histone modifications (Regha et al., 2006; Seidl et al., 2006; Wutz et al., 1997; Wutz et al., 2001; Zwart et al., 2001). Differentially methylated region 1 (DMR1) contains the promoter for \textit{Igf2r} and acquires a methylation imprint on the paternal allele during postimplantation development as a result of imprinting and not a cause (Stoger et al., 1993). Differentially methylated region 2 (DMR2) is located within intron 2 of \textit{Igf2r} and is thought to be the imprint control region (ICR) for the cluster (Braidotti et al., 2004). In addition, DMR2 contains the promoter for the antisense non-coding RNA, \textit{Air} (Lyle et al., 2000).

Methylation of DMR2 occurs during oogenesis on the maternal allele (Stoger et al., 1993), and is maintained during embryogenesis through genome-wide demethylation and remethylation (Brandeis et al., 1993). A 113 bp imprinting box is thought to establish the methylation imprint at DMR2 by effects of a de novo methylation signal (DNS) and a allele discriminating signal (ADS) within the imprinting box of the maternal allele (Birger et al., 1999). During preimplantation development \textit{Igf2r} is biallelically expressed and the maternal methylation imprint at DMR2 is already in place indicating that the presence of methylation on DMR2 is not enough to induce imprinted expression (Lerchner and Barlow, 1997; Szabo and Mann, 1995). Furthermore, loss of methylation at DMR1 and DMR2 results in a complete lack of \textit{Igf2r} expression, indicating that the absence of methylation on the \textit{Ig2r} promoter is not enough alone to induce \textit{Igf2r} expression (Li et al., 1993). Expression of \textit{Air} ncRNA from its promoter in the unmethylated DMR2 of the paternal allele is known to
induce silencing of Igf2r, Slc22a2 and Slc22a3 (Sleutels et al., 2002). Therefore, it could be argued that the loss methylation at DMR2 allowed for biallelic expression of Air ncRNA resulting in induced silence of Igf2r expression (Regha et al., 2006). Loss of Air expression from the paternal allele by deletion of DMR2 results in biallelic expression of Igf2r, Slc22a2 and Slc22a3 (Wutz et al., 1997; Wutz et al., 2001; Zwart et al., 2001). Interestingly, imprinting control in the cluster is also lost when Air ncRNA is truncated to 3 kb even though the methylation imprint at DMR2 is still intact (Sleutels et al., 2002). Therefore, expression of full length Air ncRNA is necessary for imprinting control of the cluster. However, the 30 kb transcriptional overlap between Igf2r and Air is not necessary to silence the other genes within the cluster (Sleutels et al., 2003).

Imprinted expression of Igf2r and Air is also regulated by histone modifications (Fournier et al., 2002; Hu et al., 2000; Yamasaki et al., 2005). In murine fibroblast cells the active promoters for Igf2r and Air exhibit histone modifications that are associated with transcriptionally active chromatin, such as, tri-methylation of lysine 4 on histone 3 (H3K4me3), H3K4me2, and acetylation of lysine 9 on histone 3 (H3K9Ac) (Regha et al., 2007). The silenced promoters of Igf2r and Air exhibit histone modifications that are associated with repressed chromatin, such as, H3K9me3 and H4K20me3 (Regha et al., 2007). Previous studies on murine liver reported allele-specific histone modifications in both DMR1 and DMR2 of Igf2r including acetylation of histones H3 and H4, as well as, H3K9me2 (Fournier et al., 2002; Yang et al., 2003). In contrast, murine neuron cells that biallelically express Igf2r and do not express Air exhibit no allelic differences in histone acetylation and di-methylation at DMR1 (Yamasaki et al., 2005). However, in murine glial
and fibroblast cells $\text{Igf2r}$ is imprinted and $\text{Air}$ is expressed, histone acetylation and $\text{H3K4me2}$ were only found on the maternal DMR1 (Yamasaki et al., 2005).

DMR2 is the primary or gametic imprint of the $\text{Igf2r}/\text{Air}$ cluster, because it is established during gametogenesis and it is maintained during preimplantation development as epigenetic reprogramming occurs (Stoger et al., 1993). Acquisition of methylation at DMR2 during oogenesis signifies the first step to imprinted expression in the $\text{Igf2r}/\text{Air}$ cluster (Stoger et al., 1993). During preimplantation development Igf2r is biallelically expressed and it is assumed that $\text{Air}$ ncRNA is not expressed (Lerchner and Barlow, 1997; Szabo and Mann, 1995). Additionally, it is assumed that $\text{Air}$ ncRNA begins to be expressed around the time implantation as the paternal Igf2r allele begins to be silenced (Lerchner and Barlow, 1997; Szabo and Mann, 1995). It is unclear when $\text{Slc22a2}$ and $\text{Slc22a3}$ begin to exhibit imprinted expression in the placenta (Regha et al., 2006). However, $\text{Slc22a2}$ displays imprinted expression in placenta up to 15.5 dpc (days post coitum) (Zwart et al., 2001). Similarly, $\text{Slc22a3}$ is observed to have imprinted expression up to 11.5 dpc (Zwart et al., 2001). Interestingly, neither $\text{Slc22a2}$ or $\text{Slc22a3}$ are imprinted in adult tissues (Regha et al., 2006). Following implantation, a somatic methylation imprint is acquired at DMR1 on the paternal allele (Stoger et al., 1993). Acquisition of DNA methylation and repressive chromatin modifications at DMR1 maintain transcriptional silence of paternal $\text{Igf2r}$ (Fournier et al., 2002; Vu et al., 2004). The somatic imprint at DMR1 is not completed until after birth. It is thought that this imprint does not directly causing paternal silencing of $\text{Igf2r}$, but rather the imprint is the result of silence induced by the expression of $\text{Air}$ ncRNA (Regha et al., 2007).
Antisense to Igf2 (Air) is an antisense non-coding (nc) RNA found in the mouse regulating imprinted expression of three protein coding genes in cis (Braidotti et al., 2004). Expression of the 108 kb Air ncRNA exerts a silencing effect across more than 300 kb on the expression of Igf2r, Slc22a2, and Slc22a3 (Sleutels et al., 2002). It remains unclear how Air ncRNA induces silence in cis on the Igf2r/Air cluster. Several models have been proposed for how Air mediates gene silencing in the Igf2r/Air cluster. The expression competition model proposes that Igf2r, Slc22a2, Slc22a3 and Air are all competing for common factors required for promoter and enhancer activation (Barlow, 1997). However, this model is not accurate because the truncated version of Air can still be expressed in cis with the other genes in the cluster (Sleutels et al., 2002). The RNAi model proposes that the transcriptional overlap between Air and Igf2r could result in silencing of the Igf2r promoter by RNAi-mediated processes (Seidl et al., 2006). The silent chromatin state induced at the Igf2r promoter could then be spread to Slc22a2 and Slc22a3 by recruitment of silencing factors (Seidl et al., 2006). In opposition to this model it has been demonstrated that the transcriptional overlap between Air and Igf2r is not necessary for imprinted expression of the Igf2r/Air cluster (Sleutels et al., 2003). Similar to x-inactivation, the RNA-directed targeting model proposes that the Air ncRNA is localized to the Igf2r/Air cluster and attracts repressive chromatin proteins the other genes in the cluster (Seidl et al., 2006). However, the ability of Air to recruit silent chromatin is not indicated by studies done on DNase I hypersensitivity sites located within the Igf2r and Air genes (Pauler et al., 2005). In addition, it has recently been shown that heterochromatin formed on the silenced promoters of Igf2r and Air are limited to regions of 2-6 kb and do not spread over the length of the silenced allele.
(Regha et al., 2007). The transcriptional interference model proposes that Air induces silence of Igf2r, Slc22a2, and Slc22a3 by transcription through a domain regulatory element. Based on this model, transcription of Air would prevent binding of RNA polymerase II (RNAPII) to the promoter or binding of a domain regulator to a cis-acting enhancer resulting in silenced expression from the paternal chromosome (Seidl et al., 2006). The instability of Air ncRNA supports the TI model, but the nuclear retention of Air argues against it (Seidl et al., 2006).

DNA methylation of the Air promoter is required to silence Air expression on the maternal allele (Seidl et al., 2006). In murine embryos, loss of DNA methylation results in greatly reduced levels of Igf2r mRNA, as well as, a doubling of Air ncRNA expression (Li et al., 1993; Seidl et al., 2006). The decrease in DNA methylation allowed biallelic expression of Air and subsequent silencing of Igf2r on the maternal and paternal alleles (Seidl et al., 2006). In contrast, aging mice exhibit de novo methylation on DMR2 of the parental alleles without any change to DMR1 (Yang et al., 2000). De novo methylation of DMR2 increased with the age of the mice and was associated with a decrease in Air expression, whereas Igf2r expression was unaffected (Yang et al., 2000). Together these studies illustrate that Igf2r and Air expression can be very sensitive to changes in methylation patterns and the level expression from each other. Similarly, Igf2r and Air expression are sensitive to changes in histone modifications (Hu et al., 2000). The expressed alleles of Igf2r and Air exhibit higher levels of histone acetylation than their silenced counterparts (Hu et al., 2000). Treatment of cells with deacetylase inhibitor, trichostatin A, resulted in increased acetylation of the histones and decreased DNA methylation demonstrating that histone acetylation and DNA methylation are interdependent (Hu et al., 2000). TSA relaxed imprinting of Igf2r, but
stimulated the relaxation of *Air* imprinting to a greater degree (Hu et al., 2000). Therefore, other factors apart from DNA methylation and histone acetylation may be involved in imprinting of Igf2r and *Air* (Hu et al., 2000).

**Species Differences in Requirements for Imprinted Expression of IGF2R**

Imprinted expression of Igf2r has been widely studied in the mouse (Lyle et al., 2000; Sleutels et al., 2003; Sleutels et al., 2002). Elements required from imprinted expression of Igf2r in the mouse include methylation of the maternal DMR2, methylation of the paternal DMR1 and expression of the antisense, nc(RNA), *Air*. In addition, an imprinting box in DMR2 was identified that contains a de novo methylation signal (DNS) and an allele discriminating signal (ADS) (Birger et al., 1999). However, an imprinting box has not yet been identified in any other species (Vu et al., 2006). In contrast to the mouse, the opossum a marsupial does not have a CpG2 island comparable to the CpG2 island in DMR2 of the mouse (Weidman et al., 2006). The opossum does have a CpG1 island orthologous to the CpG1 island in DMR1 of the mouse (Weidman et al., 2006). However, CpG1 of the opossum is not differentially methylated (Weidman et al., 2006). In addition, Air ncRNA is not detected in the opossum (Weidman et al., 2006). Therefore, none of the known requirements to imprint Igf2r in the mouse exist in the opossum (Weidman et al., 2006). Notably, Igf2r in the opossum binds Igf2 with much less affinity than observed in the mouse (Dahms et al., 1993). Consistent with the conflict hypothesis is the idea that because altricial offspring of marsupials are only exposed for a limited time to a non-invasive intrauterine environment there is less selective pressure to exploit maternal resources (Hore et al., 2007).
The Artiodactyla clade contains sheep, cows, and pigs all of which exhibit imprinted expression of Igf2r and Igf2 (Weidman et al., 2004; Young et al., 2001; Young et al., 2003). The ruminants both display differential methylation on the CpG islands of Igf2r consistent with DMR1 and DMR2 of the mouse Igf2r gene (Long and Cai, 2007; Young et al., 2001). It is not yet determined if the pig Igf2r gene exhibits CpG islands and differential methylation (Vu et al., 2006). It is also currently unknown if any of the artiodactyls express Air ncRNA (Vu et al., 2006).

Recently, it was determined that Igf2r in the dog is imprinted (O'Sullivan et al., 2007). Dogs belong to the superordinal group called Laurasiatheria, which is a sister group to the one that rodents and primates are in (O'Sullivan et al., 2007). Canine Igf2r gene appears to be similar to the murine Igf2r gene in that it does have a CpG2 island that exhibits differential methylation (O'Sullivan et al., 2007). However, in contrast to the mouse, the promoter of Igf2r on the canine paternal allele is not methylated, and maternal expression of Igf2r is not accompanied by paternal expression of Air ncRNA (O'Sullivan et al., 2007).

The human IGF2R gene has been extensively studied (Brown et al., 2007; Kalscheuer et al., 1993; Lighten et al., 1997; Oudejans et al., 2001; Yamasaki et al., 2005). IGF2R in the human exhibits similar elements to the mouse for imprinted expression of Igf2r. For example, the presence of differential methylation at the CpG2 island of DMR2 in intron 2 of IGF2R (Oudejans et al., 2001; Vu et al., 2006). The human IGF2R gene also has a CpG1 island on the promoter of IGF2R, but does not display differential methylation (Vu et al., 2006). Despite similar elements necessary for imprinted expression of Igf2r in the mouse, and in contrast to all other eutherian mammals, human IGF2R is biallelically expressed in
most of the population (Kalscheuer et al., 1993; Oudejans et al., 2001). Additionally, it has also been shown the human AIR ncRNA does not exist (Oudejans et al., 2001; Vu and Hoffman, 2000).

DNA methylation at DMR2 and DMR1 are not consistently indicative of imprinted expression at the Igf2r loci. Therefore, several studies have examined histone modifications at the DMRs in different tissues in order to more precisely predict imprinted expression of Igf2r (Fournier et al., 2002; Vu et al., 2004; Yamasaki et al., 2005; Yang et al., 2003).

Histones form an octamer core for DNA to wrap around forming the nucleosome (Vu et al., 2006). Amino-terminal tails extend from the histones and are subject to post-translational modifications such as acetylation and methylation (Vu et al., 2006). The various combinations of histone modifications are thought to represent a histone code that influences chromatin structure and protein interactions thereby influencing transcription (Vu et al., 2006). Acetylation of histones H3 and H4 are associated with an open chromatin state and active transcription (Vu et al., 2006). Methylation of lysine 4 on histone H3 (H3K4me) is also associated with open chromatin, whereas methylation of lysine 9 on histone H3 (H3K9me) is associated with closed chromatin or repressed transcription (Vu et al., 2006).

The IGF2R promoter region of both human parental alleles is marked by acetylation of lysines 9 and 14 on histone H3 (H3K9ac and H3K14ac) and acetylation of lysines on histone H4 (H4K5, 8, 12, 16ac) (Fournier et al., 2002; Vu et al., 2004; Yang et al., 2003). Enriched acetylation of histones H3 and H4 on the promoters of both parental alleles corresponds to the biallelic expression of IGF2R in humans (Vu et al., 2006). Peripheral tissues in the mouse display histone acetylation enrichment at the promoter of Igf2r on the maternal allele,
which is expressed (Fournier et al., 2002). In contrast, murine neurons do not express Air ncRNA and are found to express Igf2r biallelically (Yamasaki et al., 2005). The murine neuron cells also exhibited biallelic DNA hypomethylation and biallelic histone acetylation of H3 and H4 at DMR1 on the promoter of Igf2r. In contrast, differential methylation at DMR2 was maintained in neurons, as well as differential acetylation and methylation of histones H3 and H4 (Yamasaki et al., 2005). Interestingly, murine glial and fibroblast cells exhibit differential DNA methylation and histone modifications at DMR1 and DMR2 resulting in imprinted expression of Igf2r and Air in the brain (Yamasaki et al., 2005). In humans no differential acetylation has been observed between the parental alleles at DMR2 (Yamasaki et al., 2005). Methylation of histones on the promoters of Igf2r and Air in the mouse also correspond accurately to imprinted expression (Vu et al., 2004). H3K4me is found at the promoters of the expressed alleles and H3K9me is found at the promoters of the silenced alleles (Vu et al., 2004). In the mouse, DMR2 of Igf2r, which contains the promoter for Air, is suppressed on the maternal allele and marked specifically by tri-methylation of lysine 9 on histone H3 (H3K9me3) (Vu et al., 2004). By comparison, DMR2 on the parental alleles of human IGF2R does not display differential histone methylation (Vu et al., 2004).

Histone modifications and DNA methylation may work together in marking specific promoters for expression or silence (Vu et al., 2006). Expression may result from histone acetylation and H3K4me on a promoter in combination with the loss of H3K9me3 and DNA methylation (Vu et al., 2006). Losing H3K9me3 and DNA methylation from the promoter without addition of histone acetylation and H3K4me may only result in an unrepressed chromatin state but would not allow transcription (Vu et al., 2006). For example, human
IGF2R contains an unmethylated DMR2 on the paternal allele, which lacks expression of Air ncRNA (Vu et al., 2006). Therefore, DMR2 may only lack the necessary histone modifications for active transcription of Air ncRNA (Vu et al., 2006).

Igf2r is imprinted in marsupials and all eutherian mammals except primates (Vu et al., 2006). Imprinted expression of Igf2r and Igf2 appears to have evolved with the placenta (Wagschal and Feil, 2006; Wood and Oakey, 2006). Currently, there have not been any imprinted genes discovered in egg laying animals (Hore et al., 2007). Sex-specific expression of genes appeared with the divergence of marsupials and eutherians from the monotremes (Hore et al., 2007). Placental evolution from the non-invasive type seen in marsupials to a very invasive type seen in humans may contribute to the selective forces mediating imprinted gene expression. Therefore, species specific differences, for example imprinted expression of Igf2r, may have arisen as a result of fetal competition for maternal resources through the placental membranes.

Bovine Insulin-like Growth Factor Type 2 Receptor (IGF2R) maps to Chromosome 9q27-28 (Friedl and Rottmann, 1994). Similar to murine Igf2r, bovine IGF2R contains two differentially methylated regions one surrounding exon 1 (DMR1) and between exons 2 and 3 (DMR2) (Long and Cai, 2007). DMR2 acquires DNA methylation on the maternal allele during oocyte maturation (O'Doherty et al., 2012). Expression of IGF2R appears to increase from the maternal to zygotic transition (8 to 16 cell) to the hatched blast stage and then decreases substantially at Day 16 during maternal recognition of pregnancy and conceptus elongation (Bertolini et al., 2002a; Wrenzycki et al., 2001). IGF2R exhibits imprinted expression after gestational Day 25 in cattle (Suteevun-Phermthai et al., 2009). Imprinted
expression of IGF2R appears to result from an expression bias as maternal expression is amplified 10 fold while paternal expression remains low (Bebbere et al., 2013). Additionally, the expression bias of paternal IGF2R is different between mesodermal (heart and skeletal muscle), endodermal (liver and lung), and ectodermal (placenta) tissues (Bebbere et al., 2013). Placental tissues from gestational Day 80 fetuses particularly showed a relaxation in the expression bias with paternal IGF2R accounting for 22.9 to 34.7% of total IGF2R mRNA expression (Bebbere et al., 2013).

ABERRANT EXPRESSION OF IGF2R AND AIRN DURING BOVINE DEVELOPMENT

Wrenzycki found no difference in expression of IGF2R mRNA between IVO produced Day 7 blastocysts and IVP blastocysts cultured in either SOF with BSA or TCM199 with 10% ECS (Wrenzycki et al., 2001). In contrast, a separate study found that expression of IGF2R mRNA was lower in IVP (TCM 199) Day 7 blastocysts relative to IVO blastocysts (P<0.05) (Bertolini et al., 2002a). Another study demonstrated a significant reduction in expression of IGF2R in Day 9 hatched blastocysts derived from oocytes matured in 10% fetal bovine serum and PVP40 compared to those matured with fatty acid free Bovine serum albumin (Warzych et al., 2007). In contrast, Lim and Kang found difference in expression of IGF2R mRNA at Day 8 in hatched blastocysts cultured in TCM199 with BSA or PVA (Lim et al., 2007). However, by Day 16 of gestation no difference in expression of IGF2R was detected between IVO and IVP conceptuses (Bertolini et al., 2002a). At Day 16 of gestation IVP (TCM199) conceptuses exhibited reduced embryonic disc size compared to
IVO conceptuses (Bertolini et al., 2002a). The trophoblast width was not different, however, trophoblast length was reduced in male Day 16 IVP conceptuses (P<0.05) compared to male IVO conceptuses (Bertolini et al., 2002a).

Due to the growth regulating relationship between IGF2 and IGF2R many studies have investigated the expression of these genes together. IGF2R was found not to be different between IVO and IVP Day 7 blastocysts (Wrenzycki et al., 2001). Therefore comparisons were made between IVP and differing SCNT protocols. IGF2R did not differ between IVP and SCNT blastocysts generated from Go or G1 donor cells (Wrenzycki et al., 2001). Additionally, IGF2R did not differ between SCNT blastocysts generated by two different activation protocols and IVP blastocysts at Day 7 (Wrenzycki et al., 2001).

Expression of developmentally important genes can be affected by origin of donor cells demonstrated by an increase in IGF2R in blastocysts derived from adult ear fibroblast donor cells (Jang et al., 2005). Similarly, another study compared single IVP and SCNT Day 7 blastocysts and found no difference in expression of IGF2R, however expression of IGF2 was significantly higher in SCNT embryos (P<0.05) (Han et al., 2003). SCNT calves that died perinatally were compared to IVO produced calves. Expression of IGF2R was significantly elevated in brain and bladder, whereas, expression of IGF2 was elevated in heart, kidney, lung, and spleen (Yang et al., 2005).

Some overgrowth abnormalities observed in SCNT offspring may be attributed to abnormal expression of IGF2R. Clones generated from adult and fetal fibroblasts that died within 48 hours of birth had aberrant expression of IGF2R in heart, liver, kidney, and brain (Li et al., 2007). Imprinted expression of IGF2R is regulated by DNA methylation at the
imprint control region of differentially methylated region 2 on the maternal allele. Inefficient reprogramming of these DNA methylation patterns may contribute to abnormalities in the heart, liver, lung, and brain where DNA methylation has been demonstrated to be aberrant (Long and Cai, 2007).

**STATEMENT OF THE PROBLEM**

Abnormalities exhibited by fetuses, placentas and offspring derived from the transfer of in vitro produced (IVP) and nuclear transfer (NT) embryos have been well documented (Behboodi et al., 1995; Bertolini and Anderson, 2002; Bertolini et al., 2002b; Chavatte-Palmer et al., 2002; Farin et al., 2006; Wells et al., 1999; Young et al., 1998). These abnormalities may include, but are not limited to excessive fetal and placental growth (Jacobsen et al., 2003; van Wagtendonk-de Leeuw et al., 2000), altered organ growth (Jacobsen et al., 2000; McEvoy et al., 1998), altered placental structures (Bertolini et al., 2002b; Farin et al., 2001; Hill et al., 1999), increased gestation length (van Wagtendonk-de Leeuw et al., 1998; Walker, 1992), and increased perinatal mortality (Panarace et al., 2007; van Wagtendonk-de Leeuw et al., 1998). The term Abornmal Offspring Syndrome (AOS) and its associated classification system has been used to categorize these abnormalities (Farin et al., 2006). AOS is a significant obstacle for bovine clone production and to a lesser extent in vitro production of calves. Thus, a large effort is being made to understand the mechanisms that produce AOS phenotypes.
Attempts to understand the underlying mechanisms involved in producing phenotypes associated with AOS have primarily focused on aberrant expression of imprinted and non-imprinted genes in IVP and NT-derived embryos (Long and Cai, 2007; Moore et al., 2007; Yaseen et al., 2001). The insulin-like growth factor type 2-receptor (Igf2r) is an imprinted gene that is essential for normal fetal and placental development (Baker et al., 1993).

Expression of the Igf2r gene is regulated by two differentially methylated regions (DMRs) and expression of an antisense non-coding RNA (ncRNA), Airn (Sleutels et al., 2002; Wutz et al., 1997). Disruption of the methylation patterns at the DMRs are associated with altered Airn ncRNA expression which, in turn, may contribute to altered Igf2r expression (Ogawa et al., 2003; Seidl et al., 2006). Therefore, the objectives of the current study were to (1) determine the existence of bovine AIRN and its potential role in regulating imprinted IGF2R expression; (2) characterize pertinent transcriptional elements of the AIRN ncRNA; and (3) examine the effect of in vitro embryo production on expression of IGF2R and its associated regulatory mechanisms through bovine fetal and placental development.

**LITERATURE CITED**


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CHAPTER 2

EXPRESSION OF ANTISENSE TO INSULIN-LIKE GROWTH FACTOR-2 RECEPTOR NON-CODING RNA (AIRN) DURING EARLY GESTATION IN CATTLE
ABSTRACT

The insulin-like growth factor type 2 receptor (IGF2R) regulates fetal growth by removing IGF2 from circulation, thus preventing overgrowth. In mice, expression of the Igf2r gene is imprinted only after implantation and is associated with expression of the antisense non-coding (nc)RNA, Airn. The objectives of this study were, first, to determine if bovine AIRN ncRNA was expressed during developmentally important stages of gestation, and second, to determine if expression of bAIRN was affected by method of embryo production. A PCR primer set designated as bAIR3 which amplified a region of bAIRN corresponding to an antisense segment spanning intron 1 and exon 2 of IGF2R, was designed based on genomic sequence. Additional primer sets were used to assess expression of mRNA for IGF2R and histone 2A (H2AZ) in embryo and fetal tissues. Whole cell RNA was extracted from bovine fetal liver, subjected to DNase treatment, reverse transcription (RT) and PCR. Control reactions confirmed that amplification products resulted from RNA present in the sample and not from genomic DNA contamination. Amplicons obtained from all primer sets were sequence verified. Liver tissue was obtained from fetuses at gestational days 35-55 (n=5) and 70 (n=7). In addition, liver tissue of fetuses resulting from blastocysts produced either in vivo (IVO, n=7) or in vitro (IVP, n=6) were obtained. Conceptuses were recovered from cows at Days 15 (n=9) and 18 (n=11) of gestation. Pools of blastocysts from IVO or IVP production systems (n=9) on Day 7 of development were also obtained. Semi-quantitative RT-PCR was used to assess levels of IGF2R mRNA, H2AZ mRNA and bAIRN ncRNA in all tissue samples and embryo pools. IGF2R mRNA was expressed in all fetal
liver samples at Days 35-55 and 70 of gestation as well as in 8 of 9 Day 15 conceptuses, 11 of 11 Day 18 conceptuses, and in all day 7 blastocyst pools. bAIRN ncRNA was expressed in all samples of fetal liver at Days 35-55 and 70 of gestation. The proportion of conceptuses expression bAIRN ncRNA increased from 1 of 9 at Day 15 of gestation to 7 of 11 at Day 18 of gestation. No bAIRN ncRNA was expressed in any blastocyst pools. The relative level of bAIRN ncRNA was greater (P<0.05) in fetal liver from the IVO compared to the IVP group.

In summary, the antisense ncRNA bAIRN was not expressed in blastocyst-stage embryos, was expressed in an increasing proportion of embryos around the time of maternal recognition of pregnancy and was fully expressed following implantation. Furthermore, the relative level of bAIRN ncRNA in bovine fetal liver can be altered by method of embryo production.

INTRODUCTION

The insulin-like growth factor type 2 receptor (IGF2R/Igf2r) is an imprinted gene that regulates fetal and placental development in cattle and other species (Baker et al., 1993; Constancia et al., 2002). The primary function of the IGF2 receptor is to bind IGF2, a powerful mitogen, and target it for lysozomal degradation (Wang et al., 1994). The IGF2R/Igf2r gene is imprinted in opossums (Weidman et al., 2006), kangaroos (Yandell et al., 1999), pigs (McElroy et al., 2007), cattle (Long and Cai, 2007), sheep (Young et al., 2001), dogs (O'Sullivan et al., 2007), rats (Vu et al., 2006), and mice (Barlow et al., 1991).
IGF2R does not exhibit imprinted expression in monotremes (Killian et al., 2001a) or in primates (Kalscheuer et al., 1993; Killian et al., 2001b). Loss of Igf2r expression in mice results in excessive fetal and placental growth, as well as cardiac abnormalities, cleft palate, and increased perinatal mortality (Baker et al., 1993; Lau et al., 1994; Melnick et al., 1998; Wang et al., 1994). Interestingly, some of the phenotypes exhibited by fetuses, placentas and offspring of pregnancies derived from the transfer of in vitro produced (IVP) or in vitro manipulated embryos, collectively referred to as Abnormal Offspring Syndrome (AOS) (Farin et al., 2006), are similar to the phenotypes exhibited by Igf2r-deficient mice. AOS phenotypes are potentially the result of aberrant expression of imprinted and non-imprinted genes caused by the failure to properly establish or maintain epigenetic patterns (Farin et al., 2006; Wrenzycki et al., 2005a). Altered methylation and aberrant expression of many imprinted and non-imprinted genes have been observed in fetuses, placentas and offspring derived from the transfer of IVP and in vitro manipulated embryos (Long and Cai, 2007; Wrenzycki et al., 2006; Wrenzycki et al., 2005b; Wrenzycki et al., 2004). Aberrant expression of IGF2R was directly correlated to AOS in sheep (Young et al., 2001). Furthermore, bovine fetuses, placentas and offspring exhibiting AOS phenotypes also exhibited aberrant IGF2R expression (Li et al., 2007; Moore et al., 2007; Suteevun-Phermthai et al., 2009). Therefore, some phenotypes associated with AOS in cattle may also be the result of aberrant expression of IGF2R.

The bovine IGF2R gene is comparable to the mouse Igf2r gene in that it exhibits similar methylation imprints at two differentially methylated regions (DMRs) (Long and Cai, 2007; Wutz et al., 1997) as well as imprinted expression in a majority of adult tissues.
(Birger et al., 1999; Killian et al., 2001a). In both mice and cattle, DMR1 encompasses the promoter for the Igf2r/IGF2R gene and is methylated on the paternally inherited allele; whereas DMR2, located within intron 2 of the Igf2r/IGF2R gene, is methylated on the maternally inherited allele corresponding to maternal Igf2r/IGF2R expression (Long and Cai, 2007; Zwart et al., 2001). In mice, DMR2 encompasses the promoter for an antisense of Igf2r non-coding (nc) RNA, originally designated as Air, that is transcribed from the unmethylated DMR2 on the paternal allele (Wutz et al., 1997). The Air ncRNA was re-designated as Airn for antisense of Igf2r RNA non-protein coding, (Bruford et al., 2008; Stricker et al., 2008). Transcription of Airn has been identified as necessary for imprinted expression of murine Igf2r (Sleutels et al., 2002). In a recent study it was suggested that Airn may regulate imprinted expression of genes within the Igf2r/Airn cluster by accumulating at promoter sites and recruiting histone modifying enzymes to epigenetically silence transcription (Nagano et al., 2008). In addition, Airn may induce imprinted Igf2r expression by creating an expression bias between the maternal and paternal alleles (Latos et al., 2009).

In the human, the IGF2R gene is predominantly non-imprinted and AIRN is typically not expressed; however, expression was identified in association with 16-40% of Wilms’ tumors (Yotova et al., 2008). Additionally, while human AIRN ncRNA is not expressed in most tissues, a competent IGF2R/AIRN imprinting cluster has been identified (Yotova et al., 2008).

In mice, loss of the maintenance DNA methylation enzyme, de novo methyltransferase 1 (Dnmt1), resulted in reduced expression of Igf2r and increased expression of Airn (Seidl et al., 2006). In cattle, altered expression of DNMT1 has been
demonstrated in bovine embryos and fetuses in association with IVP and NT (Giraldo et al., 2007; Jang et al., 2005; Wrenzycki et al., 2001). Altered expression of IGF2R has also been observed in bovine fetuses derived from IVP and NT embryos (Li et al., 2007; Sagirkaya et al., 2007; Sawai et al., 2005; Suteevun-Phermtai et al., 2009). Furthermore, altered expression of IGF2R in conjunction with hyper- and hypomethylation of DMR2 was demonstrated in bovine clones (Long and Cai, 2007). Thus, it is possible that bovine embryos derived from IVP or from other in vitro manipulations may exhibit aberrant expression of IGF2R resulting from altered methylation patterns and altered expression of bovine AIRN. Elucidation of mechanisms regulating the imprinted expression of bovine IGF2R is necessary to understand how aberrant expression of IGF2R may potentially contribute to AOS. The objectives of this study were, first, to determine if AIRN ncRNA is expressed during developmentally important stages of gestation, and second, to determine if expression of AIRN is affected by method of embryo production.

**MATERIALS AND METHODS**

**Production of Day 70 Fetuses**

Methods used to produce in vivo and in vitro embryos for generating Day 70 bovine fetuses have been reported previously (Blondin et al., 2000). Briefly, in vivo embryos were produced using Holstein cows as embryo donors. Cows received two intramuscular (i.m.) injections of 25 mg Prostaglandin F$_{2\alpha}$ (PGF; Lutalyse; Pfizer Animal Health, USA) administered 12 days apart to synchronize estrus. Between Days 10 and 13 of the estrous
cycle (Day 0 = estrus), cows received i.m. injections of 20 to 32 mg follicle stimulating hormone (FSH; FSH-P; Schering-Plough, Piscataway, NJ) in decreasing doses over a 4-day period. Estrus was induced by i.m. injection of 25 mg of PGF on the morning and evening of the third day of FSH treatment. Cows were artificially inseminated 12 to 24 hours after the first observed standing estrus with frozen thawed semen from a Holstein bull. Embryos were collected by non-surgically on Day 7 (Day 0 = first detected estrus) and evaluated for stage of development and grade (IETS, 2005).

For production of in vitro embryos, ovaries from Holstein cows were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated, matured, and fertilized. Semen from the same Holstein bull used to produce the in vivo embryos described above was used to fertilize the matured oocytes. At 18 to 20 hours post-insemination (hpi), presumptive zygotes were washed six times in modified Tyrode’s-lactate Hepes and cultured in groups of 15 to 30 zygotes in either 1 ml of TCM-199 + 10% estrous cow serum (ECS; in vitro-produced with serum, IVPS) or 1 ml of TCM-199 + 1% BSA (in vitro-produced with serum restriction, IVPSR). At 72 hpi, IVPSR embryos were transferred into fresh TCM-199 + 10% ECS, whereas IVPS embryos had fresh medium replaced. At 120 hpi, fresh TCM-199 + 10% ECS was replaced in both treatments. At 168 hpi, blastocyst-stage embryos were harvested and assigned a morphological grade (IETS, 2005) prior to transfer.

Recipient Angus heifers were given two i.m. injections of 25 mg PGF 10 to 12 days apart to synchronize estrus. On Day 7 of the estrous cycle (Day 0 = estrus), single Grade 1 in vivo- or in vitro-produced blastocysts were transferred non-surgically into the uterus of heifers. At Day 70 of gestation, heifers were slaughtered and fetuses were recovered (n=7 in
vivo, IVO; n=6 in vitro, IVP). Fetal liver samples were collected and immediately snap frozen in liquid nitrogen and stored at -80°C for extraction of whole cell RNA (wcRNA).

**Recovery of Day 35-55 Fetuses**

Fetuses (n=5) from dams of either mixed beef or Holstein breeding were recovered in utero from a local abattoir. Fetal gestational age was estimated by crown-rump measurement and ranged from 35-55 days. Liver samples were collected and immediately snap frozen in liquid nitrogen and stored at -80°C for extraction of whole cell RNA (wcRNA).

**Production of Day 15 and Day 18 Conceptuses**

Embryos used to produce bovine conceptuses were selected from frozen-thawed in vivo-produced Charolais embryos. Prior to transfer, embryos were thawed and examined for morphology. Grade 1 (Excellent to Good) or Grade 2 (Fair) blastocysts were selected for transfer (IETS, 2005). Recipient Holstein cows were given a single i.m. injection of 25 mg of PGF to synchronize estrus. Batches of 3 to 15 blastocysts were then transferred non-surgically into the uterus of recipients on Day 7 of the estrous cycle (Day 0 = estrus).

At Day 15 or Day 18 of gestation, conceptuses were recovered non-surgically by uterine lavage using a silicone two-way catheter and flush medium (Complete Flush Medium, BioLife, Agtech, Inc; Manhattan, KS). Medium was recovered from the uterus in 1 liter sterile bottles. Conceptuses were recovered, evaluated for morphology, measured, snap-frozen in liquid nitrogen and stored at -80°C.
Production of Day 7 Blastocysts

In vivo-produced blastocysts were generated using methods reported previously (Farin et al., 2009). Briefly, Holstein cows received an intravaginal progesterone-releasing device (CIDR-B, 1.38 mg progesterone; Pfizer Animal Health, USA) on Day 0, and 100ug GnRH i.m. (Cystorelin; Merial Ltd, USA) on Day 2. Cows were given a total of 400 mg FSH (Folltropin-V; Bioniche Animal Health, USA) by i.m. or subcutaneous (s.c.) injections on Days 4-7. On Day 7, CIDR-B inserts were removed and cows received two 25 mg PGF i.m. injections given 12 hours apart. Cows were artificially inseminated with semen from the same Holstein sire noted above at 12 and 24 hours after standing estrus. Embryos were collected non-surgically 7 days after first detected estrus and evaluated for stage and grade (IETS, 2005). Blastocysts of Grade 1 or 2 were recovered and snap-frozen in liquid nitrogen in pools of 5 to 10 embryos.

For comparison with in vivo-produced blastocysts, in vitro-produced blastocysts were generated using a synthetic oviductal fluid (SOF) culture system (Miles et al., 2005). Cumulus-oocyte complexes were aspirated, matured, and fertilized as previously described (Miles et al., 2005). Groups of embryos were produced using the same Holstein sire noted above as well as with the semen from the same Charolais sires used to produce the Day 15 and Day 18 conceptuses described above. Presumptive zygotes were cultured in groups of 20 to 25 in 1 ml of SOF + 10% ECS. At 72 hpi, embryos were transferred into fresh SOF + 10% ECS. At 168 hpi, Grade 1 blastocysts were recovered and snap-frozen in liquid nitrogen in pools of 5 to 10 embryos.
RNA Extraction

**Day 70 fetal liver** Whole cell (wc)RNA of IVO- and IVP-derived Day 70 bovine fetal liver was extracted as previously described (Blondin et al., 2000). Briefly, frozen tissue was removed from -80 °C storage, weighed, placed in a mortar, covered with liquid nitrogen, and subsequently crushed to a fine powder. The powder was homogenized (Brinkmann Homogenizer PT 10/35; Westbury, NY) and dissociated in RNA isolation reagent (Tri-Reagent, Molecular Research Center; Cincinnati, Ohio) using a ratio of 100mg tissue per ml TriReagent. The wcRNA was extracted with chloroform using a ratio of 0.2 ml chloroform to 1 ml Tri-Reagent, precipitated with isopropanol at a ratio of 0.5 ml isopropanol per ml Tri-Reagent and then resuspended in diethyl pyrocarbonate-treated water. Concentration of wcRNA was determined by absorbance at 260nm. The quality and integrity of the wcRNA was assessed based on the ratio of absorbances at 260nm and 280nm and by visualization of 28S and 18S rRNA bands in 1% agarose gels. The average of the A260/280 ratios ± SEM was 1.47 ± 0.04 (n = 13).

**Day 35–55 fetal liver** wcRNA was extracted from bovine fetal liver of D35-55 fetuses using the GenElute Mammalian Total RNA Mini-prep Kit (Sigma–Aldrich; St. Louis, MO). For each fetal liver, 40 mg of tissue was extracted according to manufacturer’s instructions. The wcRNA that was then aliquoted into RNase/DNase free tubes and stored at −80 °C. The quality and integrity of the wcRNA was assessed in a manner similar to that of the Day 70 fetal livers. The average of the A260/280 ratios ± SEM was 1.52 ± 0.02 (n = 5). Day 15 and Day 18 conceptuses. wcRNA was extracted from individual whole in vivo produced Day 15 and 18 conceptuses (n = 9 Day 15; n = 10 Day 18) using the GenE-lute Mammalian Total
RNA Mini-prep Kit (Sigma–Aldrich; St. Louis, MO). The wcRNA was then aliquoted into RNase/DNase-free tubes and stored at −80 °C. Due to the collection of a minimal amount of wcRNA from these con-ceptuses assessment of the quality and integrity of the RNA was not possible.

**Day 7 blastocysts** wcRNA was extracted from Day 7 in vivo and IVP blastocysts using the GenElute Mammalian Total RNA Mini-prep Kit. Blastocysts were extracted in groups of 2–11. The wcRNA obtained was aliquoted into RNase/DNase-free tubes and stored at −80 °C. Due to the minimal amount of wcRNA from these embryos available, assessment of the quality and integrity of the RNA was not possible.

**cDNA Synthesis**

**Day 35–55 and Day 70 fetal liver** Samples of Day 35–55 and Day70 fetal liver was used to create cDNA for reverse transcription polymerase chain reaction (RT-PCR). Prior to cDNA synthesis, the concentration of wcRNA for each liver sample was calculated based on the A260 nm reading from the spectrophotometer. Then 2 µg of wcRNA from each fetal liver sample was DNase treated by incubation of the wcRNA with 1.5 µl of DNase and 2 µl of DNase buffer at 37 °C for 20 min. Following the manufacturer’s instructions, 2 µg of DNase-treated wcRNA was incubated with 1 µg of random primers (Promega; Madison, WI), 1 µl of 10 mM dNTP mix (PCR Nucleotide Mix, Roche; Mannheim, Germany) and distilled water at 65°C for 5 min. After placement on ice for 1 min, samples were incubated with 4 µl of 5X First Strand Buffer, 1 µl of 0.1 M DTT and 1 µl of reverse transcriptase
(200 U/µl); (Superscript III, Invitrogen; Carlsbad, CA) at 25 °C for 5 min. This was followed by incubation at 50 °C for 60 min and inactivation by heating to 70 °C for 15 min. The synthesized cDNA was subjected to purification using the QIAquick Purification Kit (Qiagen; Qiagen Sciences, MD) according to the manufacturer’s instructions. The purified cDNA was eluted into a 100 µl volume of elution buffer, yielding a final cDNA concentration of 20 ng/µl.

**Day 15 and 18 conceptuses** Previously extracted wcRNA from individual conceptuses recovered on Day 15 or Day 18 of gestation was used to generate cDNA for RT-PCR. For each conceptus, 0.14 embryo equivalents of RNA was used to generate cDNA. All other procedures were performed as for cDNA synthesis from fetal liver.

**Day 7 blastocysts** Previously extracted wcRNA from blastocyst groups were used to generate cDNA for RT-PCR. wcRNA from these groups were combined to yield a total of 24–32 blastocyst equivalents per 50 µl pool of RNA. From each blastocyst pool, either 2 or 3 separate cDNA synthesis reactions were prepared. For each reaction 7 µl of wcRNA was DNase treated by incubation with 1.5 µl of DNase and 2 µl of DNase buffer for 20 min at 37 °C. The RNA was then incubated with 1 µg of random primers (Promega; Madison, WI), 1 µl of 10 mM dNTP mix (PCR Nucleotide Mix, Roche; Mannheim, Germany) and distilled water at 65 °C for 5 min. After placing on ice for 1 min, samples were incubated with 4 µl of 5X First Strand Buffer, 1 µl of 0.1 M DTT and 1 µl of reverse transcriptase (200 U/µl); (Superscript III, Invitrogen; Carlsbad, CA) at 25 °C for 5 min. This was followed by incubation at 50 °C for 60 min and then inactivation by heating to 70 °C for 15 min. The individual cDNA synthesis reactions for each blastocyst pool were then combined across a
QIAquick purification spin column and subjected to purification according to the manufacturer’s protocols (Qiagen; Qiagen Sciences, MD).

**Determination of Conceptus and Fetal Sex**

Samples of non-DNase-treated whole cell RNA containing contaminating genomic (g)DNA were used for determination of conceptus and fetal sex. Fetal sex was determined by PCR analysis of gDNA using Y chromosome specific primers (Jarrell et al., 1994) that amplified a 129 bp segment of the Y-chromosome [BOV97M, base-pair position 14–142 (Miller and Koopman, 1990)]. Briefly, 2 µl of extracted nucleic acids from individual conceptus or fetal liver samples were used as template for a total of 40 amplification cycles in the presence of Y-chromosome specific sequence under conditions described previously (Farin et al., 2010). Amplification products were electrophoresed in agarose gels and sex was determined based on the presence or absence of the Y-specific amplification product. As an additional control, fetal sex was visually determined for each of the Day 70 fetuses whose liver nucleic acid samples were subjected to sex determination by PCR. In all seven cases, the determination of fetal sex based on PCR amplification products and visual assessment of fetal morphology coincided.

**Bovine AIRN Primer Design**

Bovine AIRN primers were designed using the known bovine genomic DNA sequence of *IGF2R* (AC 000166.1 primary assembly UMD-3.1) found on chromosome 9q27-28 and placement was determined by referring to the known sequence of murine Airn
The bAIR3 primer set was designed using the primer design program, Vector NTI (Invitrogen) and amplified a region within intron 1 and exon 2 of *IGF2R* (Fig. 1). This amplification product was sequence verified.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

*Day 35–55 and Day 70 fetal liver*  PCR reactions consisted of a 20 µl reaction volume that contained 5 µl (100 ng) of 20 ng/µl cDNA sample, 10 mM dNTP Mix, Taq DNA polymerase (1.25 U per 20 µl reaction), sense and anti-sense primers (20 ng of each) and PCR water. PCR reactions for all genes of interest were performed in duplicate within the same assay in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). The PCR program performed for the housekeeping gene, histone 2a family member Z (H2AZ), included a hot start at 92 °C for 2 min, denaturation for 10 s at 94 °C, annealing for 10 s at 67 °C and primer extension for 10 s at 72 °C ([Table 1](#)). The PCR program performed for *IGF2R* and bAIR3 included a hot start at 92 °C for 2 min, denaturation for 15 s at 94 °C, annealing for 15 s at 65 °C and primer extension for 15 s at 72 °C ([Table 1](#)). After the last cycle of the program for each primer set, an additional 5 min period at 72 °C was included to allow for maximum primer extension.

*Day 15 and Day 18 conceptuses*  RT-PCR was performed for H2AZ, IGF2R and bAIR3 using cDNA from Day 15 bovine conceptuses. The same procedure previously described for the fetal liver was followed for the Days 15 and 18 conceptuses except that 0.014 (Day 15) and 0.03 (Day 18) conceptus equivalents of cDNA were used in each PCR reaction.
**Day 7 blastocyst pools**  RT-PCR was performed for H2AZ, IGF2R and bAIR3 using previously generated cDNA from pools of Day 7 bovine blastocysts. PCR procedures used for analysis of the Day 70 fetal liver were followed for the Day 7 blastocysts except that 0.76–1.34 embryo equivalents of cDNA were used in each PCR reaction.

**Semi-quantitative RT-PCR (determination of linear phase of amplification)**

**Day 70 fetal liver**  For determination of the linear phase of amplification for each primer pair, PCRs were performed using a pool of 1.3 µg cDNA generated from all samples tested (n = 13, 100 ng each). Reactions were con ducted over a total of 40 cycles with two reaction tubes removed every 4 cycles starting with cycle 24. PCR products were visualized on 1.5% agarose gels and the signal intensity of individual bands was determined by an Alpha-Imager (Alpha Innotech; San Leandro, CA) imaging system. A response curve was generated for each primer pair and the exponential phase of product amplification was determined.

**Statistics**

Relative RNA expression in fetal liver at Day 70 of gestation was calculated as the ratio of band intensities of the RNA of interest to that of H2AZ. Data for relative RNA expression were analyzed by Student’s t-test (SAS, 1998). Categorical data on the numbers of embryos and concep-tuses at each stage of gestation that expressed bAIR3 were analyzed using Fisher’s Exact test (SAS, 1998).
RESULTS

Detection and Validation of AIRN ncRNA Expression

The procedural control performed on Day 70 bovine fetal liver demonstrated that the primer sets for the house-keeping gene, $H2AZ$, as well as $IGF2R$ and $AIRN$ produced PCR products that resulted from RNA within the sample and not from genomic contamination (Fig. 2). The (+/+) lanes represent samples in which RNA was DNase treated and then reverse transcribed prior to PCR amplification. PCR products in these lanes demonstrate that these amplification products resulted from RNA within the sample. The (+/−) lanes represent samples in which RNA was DNase treated but was not reverse transcribed prior to PCR amplification. PCR products present in these lanes would result from failure of the DNase to remove genomic DNA contamination. None of the PCR primer sets tested produced PCR products in the (+/−) lanes. These results demonstrate that the DNase treatment was effective in removing genomic DNA contamination. The (−/−) lanes represent RNA samples that were not DNase treated and were not reverse transcribed prior to PCR amplification. PCR products in these lanes demonstrate they are amplification products of genomic DNA contamination within the sample. All PCR primer sets produced PCR products in the (−/−) lanes except for $IGF2R$. Based on primer locations on the genomic sequence of $IGF2R$, the expected amplification product would be in excess of 30 kb in length and, therefore was not expected to be observed due to limitations of the PCR program. Collectively, these DNase treatment controls confirm that amplification products resulted from RNA present in the sample and not from genomic DNA contamination.
Effect of Stage of Development on AIRN Expression

**Post-implantation stage (Days 35–55 and Day 70 bovine fetal liver)** At Days 35–55 and Day 70 of gestation, all bovine fetal liver samples derived either from the transfer of in vivo produced embryos (n = 7 Day 70) or from recovery of fetuses at Days 35–55 of gestation (n = 5) exhibited PCR amplicons for H2AZ, IGF2R and bAIR3 (Figure 3).

**Peri-implantation stage (Day 15 and Day 18 bovine conceptuses)** At Days 15 and 18 of gestation, a total of 9 conceptuses and 10 conceptuses, respectively were recovered. The mean ± SEM length for the Day 15 conceptuses was 2.2 ± 0.6 mm. The length of the Day 18 conceptuses could not be determined because the conceptuses were too fragmented. All Day 15 and 18 conceptus cDNA samples produced PCR products for the housekeeping gene H2AZ. IGF2R amplicons were observed in 8 of 9 Day 15 bovine conceptuses and in 10 of 10 Day 18 conceptuses. Only 1 of 9 (11%) Day 15 conceptuses demonstrated bAIR3 amplification products (Figure 3) whereas 8 of 10 conceptuses (80%, P < 0.05 compared to Day 15) demonstrated bAIR3 amplification products on Day 18 of gestation (Figure 3). No correlations were identified within day of gestation between conceptus length and expression of H2AZ, IGF2R or AIRN RNA.

**Pre-implantation stage (Day 7 bovine blastocysts)** At Day 7 of gestation, each in vivo-produced blastocyst pool (n = 2) and all of the in vitro-produced blastocyst pools (n = 9) resulted in the production of PCR amplicons for the house-keeping gene, H2AZ (Fig. 4). Similarly, all in vivo- and in vitro-produced blastocyst pools rendered PCR amplicons representing IGF2R (Fig. 4). None of the in vivo- or in vitro-produced blastocyst pools rendered PCR amplicons from the bAIR3 primer set (Figures 3 and 4). Thus, at Day 7 of
gestation, no embryo pool, whether from in vivo- or in vitro-produced blastocysts, expressed AIRN ncRNA.

**Effect of Method of Embryo Production on AIRN Expression**

The ratio of AIRN ncRNA expression to H2AZ mRNA expression was significantly reduced in the livers of Day 70 bovine fetuses from IVP embryos compared to that of in vivo produced embryos (P < 0.05; Figure 5). In contrast, no significant difference was detected in the relative expression of IGF2R mRNA between the Day 70 in vivo and in vitro treatment groups.

**Effect of Sex on AIRN Expression**

There was no apparent effect of sex on expression of AIRN ncRNA. At Day 18 of gestation, of the 8 conceptuses expressing AIRN ncRNA 7 were male. However, the 2 conceptuses that did not express AIRN were also both male. At Days 35–55 there was 1 female fetus and 4 male fetuses and all 5 fetuses expressed AIRN. Similarly, at Day 70 of gestation, there were 2 female and 4 male IVP fetuses and 5 female and 2 male IVO fetuses. All of the Day 70 fetuses expressed AIRN ncRNA, regardless of sex.

**DISCUSSION**

Attempts to understand the mechanisms underlying AOS have thus far focused on aberrant expression of imprinted and non-imprinted genes in IVP and SCNT-derived embryos.
*Igf2r/IGF2R* is an imprinted gene that is essential for normal fetal and placental development (Baker et al., 1993). In the mouse, expression of *Igf2r* is regulated by two differentially methylated regions (DMRs) and expression of an antisense non-coding RNA (ncRNA), *Airn* (Sleutels et al., 2002; Wutz et al., 1997). Disruption of the methylation patterns at the DMRs is associated with altered *Airn* ncRNA expression and may contribute to altered *Igf2r* expression (Ogawa et al., 2003; Seidl et al., 2006). However, little is known about the mechanisms regulating imprinted expression of bovine *IGF2R*.

In the present study, we demonstrated that *AIRN* ncRNA is present in bovine conceptuses and fetuses. PCR primers were designed based on murine *Airn* using the known genomic bovine sequence. A procedural control for DNase treatment was performed to demonstrate that the DNase treatment was effective at removing genomic DNA contamination. Therefore, the PCR amplicons obtained using the PCR primers designed for bovine *AIRN* were the result of RNA within the sample and not genomic DNA contamination.

In mice, the *Igf2r* gene exhibits imprinted expression in post-implantation tissues coinciding with expression of *Airn* ncRNA (Sleutels et al., 2002). Similarly, it has been demonstrated that bovine *IGF2R* also exhibits imprinted expression in post-implantation tissues (Long and Cai, 2007). In the present study, *AIRN* ncRNA was expressed in post-implantation fetal tissue. All bovine liver samples from fetuses at Days 35 to 55 and at Day 70 of gestation expressed *IGF2R* mRNA, *AIRN* ncRNA and mRNA for the housekeeping gene *H2AZ*. Therefore, expression of *AIRN* ncRNA in post-implantation bovine fetal liver is similar to the pattern of *Airn* expression that has been observed in murine post-implantation
tissues (Ogawa et al., 2003; Seidl et al., 2006). Although it is likely that bovine AIRN ncRNA is being expressed from the paternal allele, the true allelic origin of AIRN ncRNA remains to be determined.

In contrast to mice, a species that exhibits hemochorial placentation, epitheliochorial placentation in cattle is non-invasive and requires more time for implantation to occur (Young et al., 2003). In the present study, an increasing proportion of peri-implantation stage bovine conceptuses, produced following the transfer of in vivo-produced blastocysts, expressed AIRN ncRNA between Days 15 and 18 of gestation. The observed expression of AIRN in only 11% of bovine conceptuses at Day 15 and 63% of conceptuses at Day 18 of gestation supports the suggestion that imprinted expression of IGF2R/AIRN is being established during the peri-implantation period, around the time of maternal recognition of pregnancy in cattle. This observation is consistent with the proposed establishment of imprinted expression of IGF2R in sheep (Thurston et al., 2008).

In mice, the Igf2r gene exhibits non-imprinted expression in the pre-implantation embryo (Lerchner and Barlow, 1997; Szabo and Mann, 1995). Murine Airn ncRNA has been assumed to be non-expressed in the pre-implantation embryo because Igf2r expression at this stage is biallelic (Regha et al., 2006). In agreement with this concept, we demonstrated that AIRN was not expressed in pre-implantation bovine blastocysts. All nine blastocyst pools, regardless of breed (Holstein, Charolais) or embryo production type (in vivo or in vitro), expressed mRNA for IGF2R and the housekeeper H2AZ. However, AIRN ncRNA was not detected in any of the blastocyst pools. This is also consistent with the observations that
*Igf2r/IGF2R* is biallelically expressed in blastocysts of both mice (Szabo and Mann, 1995) and sheep (Thurston et al., 2008).

The expression level of *AIRN* ncRNA varied with method of embryo production. At Day 70 of gestation, bovine fetal livers derived from either in vivo produced or in vitro produced embryos did not differ with respect to expression of *IGF2R* mRNA. However, expression of *AIRN* ncRNA was reduced in Day 70 bovine fetal livers derived from IVP embryos. In aging mice, DMR2 acquires de novo methylation while differentially methylated region 1 (DMR1), which contains the promoter for *Igf2r*, does not (Yang et al., 2000). Increased methylation in DMR2 resulted in reduced expression of murine *Airn* with no apparent change in the expression of *Igf2r* mRNA (Yang et al., 2000). These observations are consistent with the present study and suggest that DMR2 of bovine *IGF2R* may be hypermethylated in tissue of fetuses resulting from the transfer of IVP embryos.

Although breed effects cannot be fully ruled out in this experiment, it is unlikely that differences exist due to breed. At Days 35-55 and Day 70 of gestation fetuses of both beef and dairy breeding were examined and all were found to express *AIRN* ncRNA, regardless of genetic background. Similarly, at Day 7, blastocysts did not express *AIRN* ncRNA, regardless of whether they were of Holstein or Charolais breeding. We examined only Charolais-bred conceptuses at Days 15 and 18 of gestation and found significant differences in expression of *AIRN* ncRNA based on day of gestation. Because we did not examine any Holstein-bred conceptuses, we do not know with certainty that groups of Holstein conceptuses would demonstrate similar patterns of ncRNA expression. However, because of the similarity in responses between offspring of both beef and dairy breeding during the pre-
implantation and post-implantation periods, we anticipate that there would also be a similarity in responses during the peri-implantation period as well.

In summary, expression of AIRN was found at the post-implantation stages in bovine fetal liver. AIRN ncRNA was not expressed in Day 7 bovine blastocysts; however, at Days 15 and 18 of gestation, an increasing proportion of conceptuses expressed AIRN ncRNA as the peri-implantation period advanced. Furthermore, expression of AIRN ncRNA was reduced in livers of bovine fetuses at Day 70 of gestation derived from the transfer of IVP embryos. In conclusion, the observations are consistent with the hypothesis that imprinted expression of the IGF2R/AIRN imprinting cluster is established during the peri-implantation period around the time of maternal recognition in cattle. Furthermore, the level of bovine AIRN ncRNA in fetuses during the post-implantation period can be altered by the method of pre-implantation embryo production.

LITERATURE CITED


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\(^a\) H2A forward primer (136 - 155) bp and reverse primer (237 to 257) bp of NM_174809

\(^b\) IGF2R forward primer (6787 to 6805) bp and reverse primer (6902 to 6922) bp of NM_174352

\(^c\) AIRN forward primer (97651440 to 97651459) bp and reverse primer (97651546 to 97651566) bp of AC_000166.1
Figure 1. Illustration of the proposed regulation of the maternal and paternal alleles (within the first three exons) of bovine $IGF2R$ with identification of the relative position of the bAIR3 primer set. Differentially methylated region 1 (DMR1) encompasses the promoter for $IGF2R$ and is unmethylated on the maternal allele (clear circle). DMR1 is methylated on the paternal allele (striped circle). Differentially methylated region 2 (DMR2) is located in intron 2 of $IGF2R$ and encompasses the promoter for antisense to IGF2R noncoding ($AIRN$). DMR2 is methylated on the maternal allele indicated by the striped circle. The small black box labeled bAIR3 within intron 1 and exon 2 of IGF2R on the paternal allele depicts the region amplified by the bAIR3 primer set.
Figure 2. Ethidium bromide-stained agarose gel of H2AZ, IGF2R, and bAIR3 RNA amplification products from bovine fetal liver at Day 70 of gestation. (+/+) DNase-treated RNA that was reverse transcribed prior to PCR amplification. (+/-) DNase-treated RNA that was not reverse transcribed prior to PCR amplification. (-/-) RNA samples that were neither DNase treated nor reverse transcribed prior to PCR amplification.
Figure 3. Proportions of embryos, conceptuses or fetuses expressing *IGF2* mRNA (gray bars) and *AIRN* ncRNA (black bars) during early gestation.
Figure 4. (A) RNA expression in a subset of the bovine blastocyst pools assessed at Day 7 of gestation (pre-implantation stage). Ethidium bromide-stained agarose gel of IGF2R, bAIR3, and H2AZ amplification products from 4 of the 9 pools of in vitro-produced (IVP) and 2 of 2 pools of in vivo-produced (IVO) bovine blastocysts at Day 7 of gestation that were assayed. (B) RNA expression in the remaining subset of bovine blastocyst pools assessed at Day 7 of gestation. Ethidium bromide-stained agarose gel of IGF2R, bAIR3, and H2AZ amplification products from 5 of the 9 IVP pools of bovine blastocysts at Day 7 of gestation that were assayed. W: PCR water blank. M: 100 bp ladder marker.
Figure 5. (A) Expression of *IGF2R* mRNA in bovine fetal liver at Day 70 of gestation derived from the transfer of either in vivo- (IVO) or in vitro-produced (IVP) embryos. (B) Expression of *AIRN* ncRNA in bovine fetal liver at D70 of gestation derived from the transfer of either IVO or IVP embryos.
CHAPTER 3

CHARACTERIZATION OF BOVINE ANTISENSE TO INSULIN-LIKE GROWTH FACTOR TYPE 2 RECEPTOR NON-CODING RNA (AIRN)
ABSTRACT

Bovine insulin-like growth factor type 2 receptor (IGF2R) is an imprinted gene whose aberrant expression has been implicated in development of abnormal offspring syndrome. Bovine AIRN (AIRN) is expressed in post-implantation fetal tissues coinciding with imprinted expression of IGF2R. In the mouse, the antisense to Igf2r non-coding RNA, Airn, is a 118kb polyadenylated transcript that regulates imprinted expression of Igf2r following implantation. Although AIRN expression patterns have been reported based on PCR analysis, characteristics of this transcript are unknown. Therefore, the objective of this study was to sequence characterize the AIRN ncRNA transcript. PCR primer sets (n=16) were designed based on genomic sequence to “walk” down the predicted AIRN ncRNA sequence. Total RNA extracted from gestational day 150 bovine fetal liver was used as source material for analysis. Extracted RNA was DNase-treated prior to cDNA synthesis, PCR amplified, and sequenced. A putative bAIRN promoter was located 623 base-pairs upstream of differentially methylated region 2 (DMR2) within intron 2 of IGF2R. A polyadenylation signal was found 117kb downstream of the promoter. Primer sets designed upstream of the promoter and downstream of the polyadenylation signal yielded no PCR amplicons, suggesting that the length of AIRN is 117kb. In conclusion, bovine AIRN is similar to murine Airn in the location of its imprint control region, promoter, and transcriptional overlap with IGF2R. Both of these ncRNAs are also similar in length.
INTRODUCTION

Transfer of in vitro produced (IVP) or somatic cell nuclear transfer (SCNT) manipulated bovine embryos results in a proportion of conceptuses, fetuses, and offspring that exhibit developmental abnormalities collectively referred to as Abnormal Offspring Syndrome (AOS) (Farin et al., 2006). Live offspring are generated from 45% of transferred IVP embryos with 5% to 20% of those exhibiting abnormalities depending on the culture system used (Farin et al., 2006). Some of the abnormalities are hypothesized to be derived from the inadequacy of the in vitro culture environment and epigenetic reprogramming of a somatic cell that disrupts epigenetic patterns regulating parent-specific expression of imprinted genes (Wrenzycki et al., 2004). The number of transfers using IVP embryos has risen substantially from 30,000 in 2001 to 373,000 in 2011 (Stroud, 2012). Therefore, the importance of understanding the underlying mechanisms behind the abnormalities associated with the transfer of IVP embryos has become magnified as the numbers of embryos transferred continues to increase.

The insulin-like growth factor type 2 receptor (IGF2R) is an imprinted gene whose aberrant expression has been directly related to the overgrowth phenotype following the transfer of IVP embryos in sheep and implicated in overgrowth of bovine IVP fetuses (Farin et al., 2010; Li et al., 2007; Long and Cai, 2007; Young et al., 2001). However, little is known about how imprinted expression of IGF2R is regulated in cattle. The maternal bovine IGF2R allele is preferentially expressed and the paternal IGF2R allele is repressed in post-implantation tissues (Suteevun-Phermthai et al., 2009). Silencing of paternal IGF2R
coincides with AIRN expression and acquisition of DNA methylation at the paternal IGF2R promoter. Recently, it has been demonstrated that the mechanisms governing imprinted expression of IGF2R may be tissue specific since the degree to which paternal IGF2R is repressed differs between endodermal, mesodermal, endodermal origins (Bebbere et al., 2013).

Because the sequence characteristics of bovine AIRN are not known, the objective of the present study was to characterize bovine AIRN RNA sequence. This information is needed for future examination of the regulation of imprinted expression of IGF2R.

**MATERIALS AND METHODS**

**Production of Day 150 Fetuses**

All procedures and protocols involving the use of animals in this study were approved by the Institutional Animal Care and Use Committee at North Carolina State University. In vivo embryos were produced using superovulated Holstein cows as embryo donors. Cows received two intramuscular (i.m.) injections of 25mg Prostaglandin F2α (PGF; Lutalyse; Pfizer Animal Health, USA) administered 14 days apart to synchronize estrus. Between Days 10 and 13 of the estrous cycle (Day 0 = estrus), cows received i.m. injections of 20 to 32 mg follicle stimulating hormone (FSH; FSH-P; Schering-Plough, Piscataway,NJ) in decreasing doses over a 4-day period. Estrus was induced by i.m. injection of 25 mg of PGF$_2$α on the morning and evening of the third day of FSH treatment. Cows were artificially inseminated 12 to 24 hours after the first observed standing estrus with frozen thawed semen
from a proven Holstein bull. Embryos were collected non-surgically on Day 7 (Day 0 = first detected estrus) by uterine lavage and evaluated for stage of development and grade (IETS, 2005).

Cross-bred Angus heifers were given two i.m. injections of 25mg PGF$_{2\alpha}$ 10 to 12 days apart to synchronize estrus and were used as embryo recipients. On Day 7 of the estrous cycle (Day 0= estrus), a single Grade 1 in vivo produced blastocyst was transferred non-surgically into the uterus and at Day 150 of gestation, heifers were slaughtered and fetuses was recovered. Fetal liver samples were collected and immediately snap frozen in liquid nitrogen and stored at -80°C for extraction of whole cell RNA (wcRNA).

**RNA Extraction of Day 150 Bovine Fetal Liver**

Whole cell (wcRNA) of a gestational Day 150 bovine fetal liver was extracted as previously described (Blondin et al., 2000). Briefly, frozen tissue (25 to 40mg) was removed from -80°C storage, weighed, placed in a mortar, covered with liquid nitrogen, and subsequently crushed to a fine powder. The powder was homogenized (Brinkmann Homogenizer PT 10/35; Westbury, NY) and dissociated in lysis solution with mercapto-ethanol. RNA was extracted using a total RNA extraction kit (Sigma, GenElute total Mammalian RNA Extraction Kit) following the manufacturer’s protocols. The quality and integrity of the wcRNA was assessed by nanodrop using ratio of absorbances at 260nm and 280nm.
cDNA Synthesis

Prior to cDNA synthesis, 2µg of wcRNA from a previously extracted fetal liver sample was DNase treated by incubation of the wcRNA with 1.5µl of DNase and 2µl of DNase buffer at 37°C for 20 minutes. Following the manufacturer’s instructions, 2µg of DNase-treated wcRNA was incubated with 1µg of random primers (Promega; Madison, WI), 1µl of 10mM dNTP mix (PCR Nucleotide Mix, Roche; Mannheim, Germany) and distilled water at 65°C for 5 minutes. After placement on ice for 1 minute, samples were incubated with 4µl of 5X First Strand Buffer, 1µl of 0,1 M DTT, and 1µl of reverse transcriptase (200 U/µl); (Superscript III, Invitrogen; Carlsbad, CA) at 25°C for 5 minutes. This was followed by incubation at 50°C for 60 minutes and inactivation by heating to 70 °C for 15 minutes. The synthesized cDNA was subjected to purification using the QIAquick Purification Kit (Qiagen; Qiagen Sciences, MD) according to the manufacturer's instructions.

Bovine AIRN Primers

Bovine AIRN primers were designed using the known bovine genomic DNA sequence of chromosome 9 UMD 3.1 Primary Assembly AC_000166.1 (97,540,000bp to 97,710,000bp) (Appendices, Table 1). All AIRN primer sets (Table 1) were designed using the primer design program, Primer3Plus (Untegrasser and Nijveen 2007, Wageningen University). All amplification products were sequence verified.
Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

PCR reactions consisted of a 20µl reaction volume that contained 100ng of cDNA, 10mMdNTP mix, Taq DNA polymerase (1.25U per 20µl reaction), sense and anti-sense primers (20ng of each) and PCR water. PCR reactions were performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). PCR programs varied (Table 2) and were specific for each primer set evaluated in the primer walk.

RESULTS AND DISCUSSION

In cattle, AIRN ncRNA is transcribed from the paternal allele in an antisense direction from a transcriptional start site located in exon 2 of IGF2R to intron 1 of the neighboring protein-coding gene, MAS1 (Figure 1A). Within intron 2 lies a differentially methylated region, DMR2 (Figure 1B) that is 2620bps in length and is unmethylated on the paternal allele and methylated on the maternal one (Long and Cai, 2007). Moving in an antisense direction, a putative AIRN transcriptional start site was identified 623bp upstream of the DMR. The promoter region was identified by analyzing the genomic DNA sequence (AC_000166, 97664989 to 97664875) upstream of the DMR for essential promoter elements. A TATA-box was identified 20 bp upstream of the putative transcriptional start site (Lyle et al., 2000; Suslov et al., 2010) (Figure 2). An Activator Protein-1 (AP1) binding site, also known as a 12-O-Tetradecanoylphorbol-13-acetate (TPA) response element, was located upstream of the TATA-box (Park et al., 2003). In addition, downstream of the TATA-box we found an initiation response element (INR) and a down-stream promoter element.
Interestingly, as has been observed in the murine *Airn* sequence, there are 4 initiation sites that are clustered together in a 200bp region in the bovine *AIRN* promoter region (Lyle et al., 2000). Also intriguing, is the observation that spread out over a 500 bp region are various promoter elements. Within this region are additional TATA boxes, CAAT boxes, AP1 binding sites, INR elements, and DPEs. These observations are similar to those made of the murine promoter for *Airn* (Lyle et al., 2000). However, there is a lack of sequence homology between the bovine and murine sequences and some of the core promoter elements differ. In conjunction with experiments that have revealed that eukaryotic genomes exhibit a vast range of ncRNAs, closer examination of IncRNAs demonstrates that they may not share close sequence homology between mammalian species (Fatica and Bozzoni, 2014). Indeed these differences in sequence homology may arise from the presence of transposable elements that are common to mature IncRNAs and rarely occur in transcripts from protein-coding genes (Fatica and Bozzoni, 2014; Kapusta et al., 2013; Kelley and Rinn, 2012). Therefore, it is not surprising to find that bovine *AIRN* and murine *Airn* do not share extensive sequence homology throughout their given lengths.

Primers for *AIRN* (Sets 1 and 2) were designed to amplify regions upstream of the putative promoter and downstream of *IGF2R* exon 3 (Figure 3). These upstream primers were subjected to varying PCR conditions, including varied concentrations of cDNA template (50ng, 100ng, 150ng), varied annealing temperatures (50 to 65°C), and altered annealing and extension times (10 to 60s). All attempts to generate a PCR amplicon within this region failed. A series of primers for *AIRN* (n=16) were designed to PCR amplify regions throughout the predicted length of b*AIRN* (Figures 3, 4, and 5). The PCR amplicons
generated covered a total of 27,202 bps of the predicted \textit{AIRN} transcript. Representative gel images of amplicons produced from PCR primer sets illustrated in Figure 6.

A putative poly-A signal (AATAAA) was identified 270bps downstream of \textit{AIRN} primer set 17 (Figure 7). Two additional \textit{AIRN} primer sets (18 and 19) were designed to amplify products downstream of the poly-A signal (Figure 5). Amplicons were not obtained from PCR amplification using these primer sets. Therefore, the putative length of bovine \textit{AIRN} is 117kb. Amplification of PCR products from primers spread over a 120kb region from a putative promoter in intron 2 of \textit{IGF2R} to a putative poly-A signal in intron 1 of \textit{MAS1} suggest that AIRN is intronless. Additional poly-A signals (n=3) can be found throughout the region between the second exon of \textit{MAS1} and the first exon of \textit{IGF2R}. These additional poly-A signals may represent additional transcript termination sites.

**CONCLUSION**

Bovine \textit{AIRN} has now been characterized as a long non-coding RNA that is approximately 117kb in length. The transcriptional start site is located outside of the imprint control region located within intron 2 of \textit{IGF2R}. Transcription appears to be continuous from the putative promoter to a poly-A site within intron 1 of \textit{MAS1}. The \textit{AIRN} transcript is repeat rich and collinear with the genomic sequence. The function of \textit{AIRN} within the bovine model is still uncertain, however, it is currently assumed that \textit{AIRN} regulates imprinted expression of \textit{IGF2R}. Further studies will needed to confirm the functional role of \textit{AIRN} in regulating imprinted expression of \textit{IGF2R} or other protein-coding genes.
LITERATURE CITED


Suslov, V. V. et al. 2010. [TATA box polymorphisms in genes of commercial and laboratory animals and plants associated with selectively valuable traits]. Genetika 46: 448-457.


Figure 1. Illustration of the bovine gene region on Chromosome 9 specific to *IGF2R* and *AIRN*. A. Region illustrating the orientation of the sense genes *MAS1* and *IGF2R* to the antisense gene for the non-coding RNA, *AIRN*. Numbers indicate exons 1 and 2 or exons 1, 2, and 3 of the *MAS1* and *IGF2R* gene sequences, respectively. B. Illustration of the overlap between *IGF2R* and *AIRN*. The numbered blocks represent exons 1, 2, and 3 of the *IGF2R* gene sequence. The open rectangles indicate the location of Differentially Methylated Regions 1 and 2 (DMR1 and DMR2) with the *IGF2R* gene sequence.
Figure 2. Putative promoter for *AIRN*. Sequence showing transcription initiation site and consensus binding sites of core promoter elements. Nucleotide numbers refer to the location on chromosome 9 Primary Assembly UMD 3.1 (ACC_000166).
Figure 3. Illustration of the overlap between *IGF2R* and *AIRN* at exons 2 and 3 of *IGF2R*. Vertical Bars depict exons 2 and 3 of the *IGF2R* gene sequence connected by arrows indicating direction of transcription. Horizontal Bars 1 to 8 indicate the location of a series of PCR primer products for *AIRN* through the first 10kb of transcription. Open bars indicate that amplicons were not generated. Closed bars indicate that PCR ampiclons for *AIRN* were produced.
Figure 4. Illustration of the overlap between *IGF2R* and *AIRN* between exons 1 and 2 of *IGF2R*. Vertical bars depict Exons 1 and 2 of the *IGF2R* gene sequence connected by arrows indicating the direction of transcription. Horizontal Bars 8 to 11 indicate the location of a series of PCR primer products for *AIRN* from 10kb to 29kb of the putative *AIRN* transcript. Closed bars indicate that PCR amplicons for *AIRN* were produced.
Figure 5. Illustration of the overlap between MAS1 and AIR. A. Depiction of PCR amplicons for AIRN within the last 23kb of transcription. Vertical Bars depict the exons 1 and 2 or exon 1 of the MAS1 and IGF2R gene sequences, respectively. Horizontal Bars 12 to 19 indicate the location of a series of PCR primer products for AIRN through the first the last 23kb of AIRN transcription. Closed bars indicate that PCR amplicons for AIRN were produced. Open bars indicate that PCR amplicons were not generated. B. Poly-Adenylation signals within the last 23 kb of AIRN transcription.
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Figure 6. Representative gel images of PCR amplicons for bovine AIRN non-coding RNA. Primer sets 6, 7, and 8.
Figure 7. Putative polyadenylation (Poly-A) signal for AIRN. Nucleotide numbers refer to the location on chromosome 9 Primary Assembly UMD 3.1 (ACC_000166).
CHAPTER 4

ANALYSIS OF DNA METHYLATION AND EXPRESSION OF INSULIN-LIKE GROWTH FACTOR TYPE 2 RECEPTOR (\textit{IGF2R}) mRNA AND ANTISENSE TO \textit{IGF2R (AIRN)} NON-CODING RNA THROUGHOUT BOVINE FETAL AND PLACENTAL DEVELOPMENT
ABSTRACT

The Insulin-like Growth Factor Type 2 Receptor (IGF2R) is an imprinted gene that regulates fetal and placental growth. Expression of IGF2R is regulated by Antisense to Insulin-like Growth Factor Type 2 Receptor non-coding RNA (AIRN). Aberrant expression of IGF2R may contribute to overgrowth phenotypes observed in Abnormal Offspring Syndrome (AOS). The objectives of this study were to (1) examine the expression of IGF2R mRNA and AIRN non-coding RNA in in vivo (IVO) and in vitro produced (IVP) bovine conceptuses, and in fetal and placental tissues at early and late gestation; and, (2) compare the level of DNA methylation of cytosine-guanine (CpGs) repeats within differentially methylated region 2 (DMR2) of IGF2R in IVP and IVO produced bovine fetal and placental tissues in early and late gestation. Expression of IGF2R mRNA did not vary between treatments regardless of the method used for embryo production, tissue, or stage of development. In contrast, the level of expression for AIRN non coding RNA was decreased in Day 70 IVP fetal cotyledons and Day 222 IVP-AOS fetal livers compared to IVO controls. Expression of AIRN in Day 222 IVP-AOS fetal cotyledons was increased compared to IVO controls. DNA hypomethylation was observed in Day 15 IVP conceptuses, Day 70 IVP and Day 222 IVP-AOS fetal liver and cotyledons. In conclusion, patterns of DNA methylation which have been proposed to regulate and maintain imprinted expression of IGF2R are affected by in vitro embryo production.
INTRODUCTION

Commercial production and transfer of in vitro produced (IVP) bovine embryos is reaching a level comparable to that of in vivo (IVO) produced embryos. The number of transfers using IVP embryos has risen substantially from 30,000 in 2001 to 443,000 in 2012 (Perry, 2014). Live offspring are generated from 45% of transferred IVP embryos and 5% to 20% of those will exhibit fetal overgrowth depending on the culture system used for embryo production (Farin et al., 2006). Abnormal Offspring Syndrome (AOS) encompasses all of the abnormalities observed in fetuses, placentas, and offspring following the transfer of in vitro produced (IVP) or somatic cell nuclear transfer embryos (Farin et al., 2006). Phenotypes associated with AOS are thought to arise from improper establishment or maintenance of epigenetic patterns that regulate expression of developmentally important imprinted genes during IVP or SCNT embryo development (Farin et al., 2006; Wrenzycki et al., 2004).

The insulin-like growth factor type 2 receptor (IGF2R) is an imprinted gene whose aberrant expression has been directly related to the overgrowth phenotype following the transfer of IVP embryos in sheep and implicated in overgrowth of bovine IVP fetuses (Farin et al., 2010a; Li et al., 2007; Long and Cai, 2007; Young et al., 2001). However, little is known about how imprinted expression of IGF2R is regulated in cattle. The maternal bovine IGF2R allele is preferentially expressed and the paternal IGF2R allele is repressed in per-implantation tissues by Day 25 of gestation (Suteevun-Phermthai et al., 2009). Recently, it has been demonstrated that the mechanisms governing imprinted expression of IGF2R may
be tissue specific as indicated by tissue specific differences in the degree to which paternal
*IGF2R* is repressed (Bebbere et al., 2013).

Therefore, the first objective of this study was to compare the relative level of expression of *IGF2R* mRNA and *AIRN* non-coding RNA in bovine conceptus, fetal and placental tissues at early and late gestation from IVP and IVO production systems. The second objective of this study was to compare DNA methylation within DMR2 of *IGF2R* in these tissues to determine if DNA methylation at DMR2 is affected by in vitro production of bovine embryos.

**MATERIALS AND METHODS**

**Production of Pre-Attachment Day 15 Conceptuses**

**IVO embryo production** In vivo-produced blastocysts were generated using methods reported previously (Farin et al., 2009). Briefly, Holstein cows received an intravaginal progesterone-releasing device (CIDR-B, 1.38 mg progesterone; Pfizer Animal Health, USA) on Day 0, and 100ug GnRH i.m. (Cystorelin; Merial Ltd, USA) on Day 2. Cows were given a total of 400 mg FSH (Folltropin-V; Bioniche Animal Health, USA) in decreasing doses by i.m. or subcutaneous (s.c.) injections on Days 4-7. On Day 7, CIDR-B inserts were removed and cows received two 25 mg PGF i.m. injections given 12 hours apart. Cows were artificially inseminated with semen from a single Holstein sire at 12 and 24 hours after standing estrus. Embryos were collected non-surgically 7 days after first detected estrus and
evaluated for stage and grade (IETS, 2005). Blastocysts of Grade 1 were recovered and transferred to Holstein heifer recipients in groups of 1 to 3 embryos per transfer.

**IVP embryo production** For production of in vitro produced embryos, Holstein ovaries were recovered from a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated, matured, and fertilized in vitro as previously described (Farin and Farin, 1995; Miles et al., 2004). COCs were aspirated from follicles that were 2- to 10-mm in diameter and then washed five times in modified Tyrode medium (TL-HEPES). The COCs were grouped into pools of 20 to 30 and in vitro matured for approximately 22 h in M-199 supplemented with 10% heat-inactivated estrus cow serum (ECS), 10 mg/ml LH, 5 mg/ml FSH, 1 mg/ml estradiol, 250 mM sodium pyruvate, and 50 mg/ml gentamicin. All cultures were incubated at 5% CO2 in air with 100% humidity. After maturation, COCs were washed once and placed in fertilization medium that consisted of heparin-supplemented Tyrode albumin lactate pyruvate medium with 6 mg/ml fatty-acid-free BSA. In vitro fertilization was performed using thawed frozen semen from the same Holstein bull used for artificial insemination of donor cows. Motile spermatozoa were collected using the swim-up procedure [20] and a final concentration of 1X10⁶ spermatozoa per ml was used for fertilization in 0.75 ml of fertilization medium. Spermatozoa and COCs were coincubated for 18–20 h. Following incubation, presumptive zygotes were washed six times with TL-HEPES. The treatments were 1) IVPS (in vitro produced with serum): TCM-199 with 10% ECS and 50 mg/ml gentamicin; 2) SOF (synthetic oviductal fluid) with (0.6%) BSA and 50 mg/ml gentamicin. IVPS embryos were cultured in an incubator at 38.5°C in an atmosphere of 5%
CO₂ and 100% humidity. SOF embryos were cultured in an atmosphere of 90% N₂, 5% O₂ and 5% CO₂ while being held at the same humidity and temperature. At 72 and 120 hpi, IVPS embryos had fresh medium replaced. At 168 hpi, blastocyst-stage embryos were harvested and assigned a morphological grade (IETS, 2005) prior to transfer.

**Transfer and Recovery of Conceptuses**  Recipient Holstein heifers were given two i.m. injections of 25 mg PGF 10 days apart to synchronize estrus. On Day 7 of the estrous cycle (Day 0 = estrus), single Grade 1 in vivo- or in vitro-produced blastocysts were transferred non-surgically into the uterus of heifers. At Day 15 of gestation (8 days post transfer), conceptuses were recovered non-surgically by uterine lavage using a silicone two-way catheter and flush medium (Complete Flush Medium, BioLife, Agtech, Inc; Manhattan, KS). Medium was recovered from the uterus in 1 liter sterile bottles. Physical measurements including length, width, and presence of an embryonic disc were recorded for each recovered conceptus. A snip (1 to 3mm) was excised from each conceptus for sex determination. The conceptuses and their respective snips were then snap-frozen in liquid nitrogen and stored at -80°C.

**Production of Post-Attachment Day 70 Fetuses and Late Gestation Day 222 Fetuses**

**IVO embryo production**  Methods used to produce in vivo and in vitro embryos for generating Day 70 bovine fetuses have been reported previously (Blondin et al., 2000). Briefly, in vivo embryos were produced using Holstein cows as embryo donors. Cows received two intramuscular (i.m.) injections of 25 mg Prostaglandin F₂α (PGF; Lutalyse; Pfizer Animal Health, USA) administered 12 days apart to synchronize estrus. Between
Days 10 and 13 of the estrous cycle (Day 0 = estrus), cows received i.m. injections of 20 to 32 mg follicle stimulating hormone (FSH; FSH-P; Schering-Plough, Piscataway, NJ) in decreasing doses over a 4-day period. Estrus was induced by i.m. injection of 25 mg PGF on the morning and evening of the third day of FSH treatment. Cows were artificially inseminated 12 to 24 hours after the first observed standing estrus with frozen thawed semen from a Holstein bull. Embryos were collected by non-surgical uterine lavage on Day 7 (Day 0 = first detected estrus) and evaluated for stage of development and quality grade (IETS, 2005).

**IVP embryo production** For production of in vitro embryos, ovaries from Holstein cows were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated, matured, and fertilized as described previously (Blondin et al., 2000; Farin and Farin, 1995). Semen from the same Holstein bull used to produce in vivo embryos was used to fertilize the in vitro matured oocytes. At 18 to 20 hours post-insemination (hpi), presumptive zygotes were washed six times in modified Tyrode’s-lactate Hepes and cultured in groups of 15 to 30 zygotes in either 1 ml of TCM-199 + 10% estrous cow serum (ECS; in vitro-produced with serum, IVPS) or 1 ml of TCM-199 + 1% BSA (in vitro-produced with serum restriction, IVPSR). At 72 hpi, IVPSR embryos were transferred into fresh TCM-199 + 10% ECS, whereas IVPS embryos had fresh medium replaced. At 120 hpi, fresh TCM-199 + 10% ECS was replaced in both treatments. At 168 hpi, blastocyst-stage embryos were harvested and assigned a morphological grade (IETS, 2005) prior to transfer.

**Embryo transfer** Recipient Angus heifers were given two i.m. injections of 25 mg PGF 10 to 12 days apart to synchronize estrus. On Day 7 of the estrous cycle (Day 0 = estrus), single
Grade 1 in vivo- or in vitro-produced blastocysts were transferred non-surgically into the uterus of heifers.

**Day 70 issue collection** At Day 70 of gestation, heifers were slaughtered and fetuses were recovered (n=7 in vivo, IVO; n=6 in vitro, IVP). Samples of liver tissue were obtained, as well as, samples of cotyledonary tissues which were obtained by careful manual separation of these tissues from their associated caruncular tissues. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C for extraction of whole cell RNA (wcRNA).

**Day 222 tissue collection** At Day 222 of gestation (215 days after transfer), a total of 46 pregnant recipients (n = 14, 18, and 14 for IVO, IVPS, and IVPSR, respectively) were killed. Fetuses and their placentas were removed from the reproductive tracts and physical measurements including fetal weight, liver weight, wet placental weight, and number of placentomes were taken. Samples of liver tissue and cotyledonary tissues were obtained. Tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for whole-cell RNA (wcRNA).

**RNA Extraction of Bovine Conceptus, Fetal, and Placental Tissues**

Whole cell (wc)RNA was extracted from all conceptus, fetal, and placental tissues using the GenElute mammalian total RNA extraction kit (Sigma-Aldrich, St. Lous, MO) according to the manufacturer’s instructions. Briefly, frozen tissue (25 to 40mg) was removed from -80 °C storage, weighed, placed in a mortar, covered with liquid nitrogen, and subsequently crushed to a fine powder. The powder was homogenized (Brinkmann
Homogenizer PT 10/35; Westbury, NY) and dissociated in lysis solution with mercapto-
ethanol. The resulting lysate was filtered through a column in a centrifuge to remove cellular
debris. The filtered lysate was then added to 500µl of 70% ethanol and centrifuged through
an RNA binding column. The bound RNA was then subjected to three 500µl washes and
then eluted into 100µl of elution buffer. Concentration, quality, and integrity of the wcRNA
was assessed by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific,
Wilmington, DE) using the ratio of absorbances at 260nm and 280nm. The average of the
A260/280 ratios ± SEM for Day 15 conceptuses, Day 70 fetal liver and cotyledon, and Day
222 fetal liver and cotyledon was 2.11 ± 0.04, 2.12 ± 0.004, 2.13 ± 0.004, 2.09 ± 0.026,
and 2.14 ± 0.028, respectively.

cDNA Synthesis

Samples of Day 15 bovine conceptuses, as well as, Day 70 and Day 222 fetal liver
and cotyledons were used to create cDNA for reverse transcription polymerase chain reaction
(RT-PCR). Prior to cDNA synthesis, 2µg of wcRNA from each sample was DNase treated
by incubation of the wcRNA with 1.5µl of DNase (1.5 units of RNase-free DNAse, Promega,
Madison, WI) and 2µl of DNase buffer at 37°C for 20 minutes. Following the
manufacturer’s instructions, 2µg of DNase-treated wcRNA was incubated with 1µg of
random primers (Promega; Madison, WI), 1µl of 10mM dNTP mix (PCR Nucleotide Mix,
Roche; Mannheim, Germany) and distilled water at 65°C for 5 minutes. After placement on
ice for one minute, samples were incubated with 4µl of 5X First Strand Buffer, 1µl of 0.1 M
DTT and 1µl of reverse transcriptase (200 U/µl, Superscript III, Invitrogen; Carlsbad, CA) at
25°C for 5 minutes. This was followed by incubation at 50°C for 60 minutes and inactivation by heating to 70°C for 15 minutes. The synthesized cDNA was subjected to purification using the QIAquick Purification Kit (Qiagen; Qiagen Sciences, MD) according to the manufacturer’s instructions.

Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

PCR reactions consisted of a 25μl reaction volume that contained 100ng of cDNA, 12.5μl of iQ SYBR Green Supermix (BioRad, Hercules, CA), sense and anti-sense primers (final concentration of 200 nM) and PCR water (Table 3). PCR reactions for all genes of interest (IGF2R and AIRN) and the house keeping gene, H2AZ, were performed in duplicate within the same assay in a iQ5 thermocycler (BioRad, Hercules, CA). The PCR program performed included a hot start at 95°C for 3 minutes, denaturation for 20 seconds at 95°C, and annealing with extension for 20 seconds at 60°C. The denaturation, annealing, and extension cycle was repeated 40 times.

Extraction of Genomic DNA

Extraction of genomic DNA from all tissues was performed using the DNeasy Blood and Tissue Kit (Qiagen, Redwood City, CA) according to the manufacturer’s instructions. For Day 15 conceptuses, 1 to 4 mm from the total length of the conceptus was extracted. For Day 70 and Day 222 tissues, 20 to 25 mg of tissue was subjected to DNA extraction. Briefly, for each sample 180 μl of buffer along with 20 μl of proteinase K (40 mAU/ mg protein) were added, vortexed, and incubated at 56 °C until the sample was fully lysed. Additional
buffer (200 µl) and 200 µl of 70% Ethanol were added and then centrifuged at 8,000 g across a binding column. A series of three 500 µl washes were performed and the binding column was centrifuged at 20,000 g to dry the column. The final genomic DNA sample was eluted into 100 µl of elution buffer and analyzed for concentration and quality by the NanoDrop 1000 spectrophotometer. Snips from Day 15 conceptuses yielded an average of 100 ± 20 ng/µl (2.1 ± 0.04 A260/280) gDNA per conceptus. Day 70 fetal liver and cotyledonary tissues yielded 489.2 ± 60.5 ng/µl (2.02 ± 0.01 A260/280) and 217.7 ± 24.4 ng/µl (2.02 ± 0.01 A260/280) of gDNA, respectively. Additionally, Day 222 fetal liver and cotyledonary tissues yielded an average of 352.2 ± 64.1 ng/µl (2.04 ± 0.02 A260/280) and 186.3 ± 16.4 ng/µl (2.04 ± 0.02 A260/280) of gDNA, respectively.

**Bisulphite Conversion of gDNA**

Genomic DNA from Day 15 conceptus, Day 70 and Day 222 fetal liver and cotyledons were aliquoted into DNase/RNase free 1.5ml tubes and shipped to EpigenDx for bisulphite conversion and pyrosequencing analysis. The concentration of gDNA was substantially lower for Day 15 conceptuses, therefore, gDNA from these samples were not diluted prior to shipping. Samples of gDNA from Day 70 and 222 tissues were diluted to 50ng/µl prior to shipment. For DNA methylation analysis, 500 ng of sample DNA was bisulfite treated by EpigenDx using a proprietary bisulfite salt solution. DNA was diluted to 45 µL and 5 µL of 3N NaOH was added followed by 30 minute incubation at 42 ºC to denature the DNA. 100 µL of Bisulfite salt solution was added to the DNA and incubated for
14 hours at 50°C. Bisulfite treated DNA was purified using Zymogen DNA columns and was eluted 20 μl of T1E0.2 pH 8.0 and 1 μl of it is used for each PCR.

**Pyrosequencing Analysis**

Pyrosequencing of all tissues tested was performed by EpigenDx. For pyrosequencing, PCR primers were designed to amplify two regions within DMR2 of IGF2R (Figure 1). The primer sequences were deemed proprietary information by EpigenDx. However, PCR conditions and the specific sequences for each amplicon analyzed were made available. The first amplicon amplified a region of chromosome 9 UMD 3.1 that was 141 bp in length (97661544 to 97661685) and covered 4 CpGs. The second amplicon amplified a region that was 187 bp in length (976611563 to 97661750) and covered 6 CpGs. The PCR was performed with 0.2 μM of each primer and one of the PCR primers was biotinylated to detect purification of the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and rewash used using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen) as recommended by the manufacturer. Pyrosequencing primer (0.2 μM) was annealed to the purified single-stranded PCR product and 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Biotage AB) following the manufacturer’s instructions (Pyrosequencing, Qiagen).

For each developmental time point and tissue examined, DNA methylation analysis was performed on DMR2 of *IGF2R* (Figure 1). Two amplicon regions within DMR2 were
analyzed by pyrosequencing and quantitative DNA methylation data was generated for 10 CpGs, collectively, between the two amplicons. The percent DNA methylation at each CpG was compared between the treatments. In addition, the average percent DNA methylation for each amplicon was compared.

RESULTS

Morphometric Analysis

**Day 15 bovine conceptuses**  At Day 15 of gestation, there was no effect of IVP method on conceptus length (P = 0.58; M199, 33.1 mm ± 9.4 and SOF, 48.9 mm ± 17.2). Therefore, for purposes of comparison with IVO produced conceptuses, the two IVP groups were combined. Overall, IVP conceptuses were significantly longer than their IVO counterparts (42.9 mm ± 8.8 vs 24.3 mm ± 3.2; respectively, P < 0.05).

**Day 70 bovine fetal and placental measurements**  Physical data collected for Day 70 bovine fetuses (Table 2) demonstrated that IVP fetuses were longer than their IVO counterparts based on measurements of crown-rump length (CRL; P = 0.02). Additionally, the ratio of CRL to body weight (BW) kg was significantly (P = 0.02) greater in IVP fetuses compared to IVO. However, no differences for body weight or liver weight were detected. Morphometric analysis of the placenta at Day 70 of gestation following the transfer of either IVP or IVO embryos did not show any differences between groups for the placental measurements analyzed (Table 2).
**Day 222 bovine fetal and placental measurements**  At Day 222 of gestation, physical measurements were taken of IVP fetuses that exhibited the overgrowth phenotype associated with AOS (IVP-AOS) for comparison to those generated from IVO fetuses (Table 3). Predictably, IVP-AOS fetuses exhibited significantly increased BW (P = 0.0001) and CRL (P = 0.002) compared to IVO fetuses. The ratio of CRL to BW was significantly reduced (P = 0.002) in IVP-AOS fetuses compared to IVO produced controls. Overgrown IVP-AOS fetuses also had increased liver weight (P = 0.0001), as well as, an increase in the ratio of liver weight to BW (P = 0.03). Placental measurements were compared between pregnancies from overgrown IVP-AOS and normal-sized IVO fetuses at Day 222 of gestation (Table 3). Mean placental weights were greater in the IVP-AOS group (P = 0.002) compared to that of the IVO group. However, the placental growth was proportional to fetal growth as indicated by a lack of difference between treatments for the ratio of placenta weight to birth weight. Placentome number was also not significantly different between groups. In contrast, IVP-AOS placentas demonstrated a significant decrease in the number of placentomes per kg of fetal body weight (P = 0.0003) compared to that of IVO pregnancies at Day 222 of gestation (Table 3).

**Expression of IGF2R mRNA and AIRN ncRNA**

**Day 15 bovine conceptuses**  At Day 15 of gestation, IVP and IVO bovine conceptuses did not differ in the expression of either IGF2R mRNA or AIRN ncRNA (Figure 2B and C).

**Day 70 bovine fetal liver and cotyledon**  No difference in IGF2R mRNA and AIRN ncRNA was observed between fetal livers recovered from IVP and IVO fetuses at Day 70 of
gestation (Figure 3A and B). Expression of *IGF2R* mRNA was not different between IVP and IVO treatments in Day 70 bovine fetal cotyledons (Figure 3C). However, *AIRN* ncRNA was significantly lower in IVP Day 70 cotyledons compared to those that were IVO (P < 0.001; Figure 3D).

**Day 222 bovine fetal liver and cotyledon** Gestational Day 222 fetal livers from over-grown IVP-AOS fetuses were compared to that of IVOs, *AIRN* ncRNA was significantly reduced (P < 0.05; Figure 4B). However, expression of *IGF2R* mRNA in fetal liver of overgrown IVP-AOS fetuses was not different from that of livers produced from IVO fetuses (Figure 4A). Similarly, at Day 222 of gestation over-grown IVP-AOS cotyledons did not show a difference in *IGF2R* mRNA (Figure 4C). In contrast to the reduced expression of *AIRN* in IVP-AOS fetal livers, *AIRN* ncRNA in Day 222 IVP-AOS cotyledons was significantly higher than that observed in the IVO cotyledons (P < 0.05; Figure 4D).

**DNA Methylation Analysis of IGF2R DMR2**

**Day 15 bovine conceptus** At Day 15 of gestation, there were no significant differences in methylation in the first 4 CpGs comprising Amplicon Region 1. However, the percent DNA methylation was lower for IVP bovine conceptuses in Amplicon Region 2 at CpGs 6, 7, 9, and 10 compared to those CpGs in IVO conceptuses (P < 0.05; Figure 5A). The overall percent DNA methylation was not significantly different between treatments for Amplicon Region 1, but was significantly lower for Amplicon Region 2 in IVP conceptuses at Day 15 of gestation (P < 0.05; Figure 5B).
**Day 70 bovine fetal liver and cotyledon**  At Day 70 of gestation, no differences in percent DNA methylation were detected between the IVP and IVO fetal livers for any of the CpGs analyzed (Figure 6A). Furthermore, the overall percent DNA methylation for Amplicon Regions 1 and 2 were not different between groups (Figure 6B). Similarly, no differences in DNA methylation were observed between IVP and IVO produced cotyledons for individual CpGs (Figure 7A) or Amplicon Regions (Figure 7B).

**Day 222 bovine fetal liver and cotyledon**  In IVP-AOS Day 222 bovine fetal livers recovered from over-grown fetuses percent DNA methylation was significantly reduced at all 10 CpGs (Figure 8A) and overall amplicon regions (Figure 8B) observed compared to those from IVO fetuses. Interestingly, only CpG10 displayed a significant reduction in percent DNA methylation (P < 0.05; Figure 9A) in IVP-AOS Day 222 cotyledons relative to that observed in the IVO cotyledons. However, CpGs 1, 5, and 9 showed a tendency for reduced percent DNA methylation (P < 0.1; Figure 9A). There were no significant differences between IVP-AOS and IVO cotyledons for percent DNA methylation in the overall amplicon regions (Figure 9B).

**DISCUSSION**

**Day 15 Bovine Conceptus**

Physical differences between conceptuses produced from IVP and IVO embryos have been observed during the late pre-attachment period (Days 12 to Day 19). IVP bovine conceptuses at Days 12 and 17 of gestation were longer than the IVO produced controls
(Farin et al., 2001; Farin et al., 1999; Lazzari et al., 2002). However, one group did observe that at Day 16 of gestation IVP bovine conceptuses were shorter and had smaller embryonic discs relative to those produced in vivo (Bertolini et al., 2002a). It has been suggested that this difference may be due to the presence of degenerating IVP conceptuses at Day 16 which may have been healthy if they had been recovered at Day 12 or perhaps would have been fully degenerated by Day 17 of recovery (Farin et al., 2004). In the present study, we found that gestational Day 15 IVP conceptuses were longer than the IVO controls. Therefore, our data are consistent with the proposal that IVP methods appear to accelerate the rate of trophoblast expansion that occurs during conceptus elongation.

Igf2r functions as a growth suppressor through targeted removal of the mitogenic growth factor, Igf2 (Wang et al., 1994). Interestingly, despite the differences in conceptus length between IVP and IVO conceptuses at Day 15 of gestation, the expression of IGF2R mRNA and AIRN ncRNA was not different between these groups. The expression level of IGF2R mRNA has previously been shown to be negatively correlated with bovine conceptus growth (Bertolini et al., 2002a). In this study, although anecdotal, the mean expression level of IGF2R mRNA was greater in IVO conceptuses (0.46 ± 0.26) than that observed in the IVP conceptuses. However, the variances were too large and heterogeneous for the differences to become significant.

The function of AIRN ncRNA in the bovine model is not yet clear. In the mouse, Airn regulates imprinted expression of Igf2r by creating a transcriptional bias towards the maternal allele through repression of Igf2r from the paternal allele (Latos et al., 2009). At Day 15 of gestation, expression of AIRN ncRNA was not significantly different between IVP
and IVO conceptuses. However, it should be noted that expression of AIRN ncRNA in both groups was remarkably low relative to the housekeeping gene, H2AZ. It may be possible that expression of AIRN ncRNA at Day 15 in bovine conceptuses may not be sufficiently high enough to actively repress paternal expression of IGF2R mRNA. However, at day 15 of gestation the bovine conceptus is primarily trophectoderm and regulation of IGF2R may differ between fetal and placental tissues. At Day 80 of gestation, Bebbere et al. (Bebbere et al., 2013) found high paternal expression of IGF2R in placental tissues relative to fetal tissues. Furthermore, at Day 17 of gestation conceptuses generated from a Bos taurus and Bos indicus cross showed high levels of paternal expression for IGF2R mRNA indicating that imprinted expression of IGF2R had not been fully established in IVO, IVP, and SCNT conceptuses (Smith et al., 2010). Therefore, at Day 15 of gestation, it is reasonable to suggest that expression of AIRN ncRNA is detectable, but not sufficient to repress paternal expression of IGF2R.

At Day 15 of gestation DNA hypomethylation was observed at CpGs located within one of the amplicons investigated for IVP conceptuses compared to the IVO controls. The gametic DNA methylation imprint established during oocyte maturation on the maternal allele is necessary to maintain imprinted expression of Igf2r (Stoger et al., 1993; Wutz et al., 1997). In vitro maturation of bovine oocytes has been shown to alter methylation pattern of the imprint control region within DMR2 of IGF2R (O'Doherty et al., 2012). In the present study, the absence of SNPs prevents our ability to differentiate between DNA methylation on the maternal and paternal alleles. However, we were able to determine quantitative changes to DNA methylation observed at individual CpGs and across amplicons. Therefore, the
percent DNA methylation at each CpG is essentially an average of DNA methylation between the maternal and paternal alleles. IVO produced bovine fetuses exhibit an extremely hypomethylated paternal DMR2 and a very hypermethylated maternal DMR2 (Bebbere et al., 2013). Therefore, loss of methylation from CpGs within Amplicon Region 2 would likely indicate that the maternal allele in Day 15 IVP conceptuses is hypomethylated relative to the IVO controls. Because no differences in $IGF2R$ mRNA and $AIRN$ ncRNA were detected between IVO and IVP conceptuses at Day 15, it may indicate that altered DNA methylation levels at the imprint control region within DMR2 of $IGF2R$ may precede changes to imprinted gene expression.

**Day 70 Bovine Fetal Liver and Cotyledon**

Gestational Day 70 was selected as a time point of interest for analysis of expression of the imprinted genes, $IGF2R$ and $AIRN$, and DNA methylation at the imprint control region for $IGF2R$ because this stage marks the end of the first trimester and is immediately prior to a period of rapid fetal growth (Eley et al., 1978; Farin et al., 2010a). Thus, alterations to expression of genes that regulate fetal growth, such as $IGF2R$, might be expected to be apparent.

The current study demonstrates that at Day 70 of gestation bovine fetuses produced from IVP embryos exhibit increased crown-rump length (CRL) but not increased body weight or liver weight compared to IVO produced fetuses. In addition, there were no observed differences between placentas generated from IVP pregnancies relative to measurements of placenta weight, number of placentomes, or cotyledon weight compared to
IVO controls. Previous work from our lab on a larger group of gestational Day 70 fetuses produced from IVP embryos cultured with serum did not show a difference in fetal bodyweight or CRL compared to IVO produced controls (Farin et al., 2010a). Similarly, fetuses at Day 50 and 100 of gestation generated from IVP embryos were also not different in BW or CRL compared to artificial insemination controls (Lee et al., 2004). Interestingly, in another study in vitro fetuses generated from IVP embryos cultured in M199 with bovine oviduct epithelial cells were smaller than their in vivo counterparts from gestational Days 37, 44, 51, and 58 based on CRL measurements obtained by ultrasonography (Bertolini et al., 2002b). These authors found that by Day 72 the differences in growth between IVP and IVO fetuses had disappeared, and by Day 93 the IVP fetuses had a tendency to be larger than the IVO controls (Bertolini et al., 2002b). Furthermore, placentas from IVP-derived pregnancies displayed smaller placentomes at Day 37, were comparable in size on Day 44, and increased in size after Day 51 (Bertolini et al., 2002b). These observations support the suggestion that IVP-derived fetuses may exhibit compensation for growth retardation near the end of the first trimester and that compensation by the placenta may occur before that event.

At Day 70 of gestation, we did not find that expression of IGF2R mRNA was altered in IVP-derived fetal liver or cotyledon. In addition, expression of AIRN ncRNA was not different in fetal livers from IVP fetuses compared to IVO controls; however, it was significantly reduced (P = 0.0009) in IVP-derived fetal cotyledons. Previously, our lab reported that at Day 70 of gestation expression of IGF2R mRNA was decreased in fetal liver and skeletal muscle of IVP-derived fetuses compared to IVO produced controls.
(Farin et al., 2010a). The difference between what was previously reported and the current study may be attributed to the different PCR methodologies used and the number of observations. Our results did show that the expression of \textit{IGF2R} was anecdotally lower in IVP-derived fetal livers and cotyledons, however, the level of variation prevented those differences from being significant. In a different study at Day 75 of gestation, fetuses generated from the transfer of SCNT and IVO embryos were compared and found that expression of \textit{IGF2R} mRNA and \textit{AIRN} ncRNA was not different in fetal liver and cotyledon between groups (Suteevun-Phermthai et al., 2009). While the expression pattern of \textit{IGF2R} mRNA was similar to what we observed at Day 70, \textit{AIRN} expression was not. This difference may be derived from the different methods used for embryo production, the number of observations used in each study, and variation in PCR methodologies.

At Day 70 of gestation, we examined DNA methylation at DMR2 of \textit{IGF2R} by pyrosequencing of 10 CpGs. We found that in IVP-derived fetal liver methylation at these sites was unaffected, whereas, in IVP-derived fetal cotyledon tissue hypomethylation was observed at CpGs of the second amplicon compared to that of the IVO-derived controls. Our results are similar to those observed at Day 80 of gestation where DNA methylation at DMR2 of \textit{IGF2R} was unaffected in normal-sized and overgrown IVP-derived fetuses compared to AI controls (Bebbere et al., 2013). Interestingly, at Day 80 of gestation the global DNA methylation pattern observed in IVP-derived fetal liver was previously observed to be higher in one IVF group and lower in the second compared to AI controls (Hiendleder et al., 2006). These results indicate that global DNA methylation can be affected by methods.
used for in vitro embryo production while alterations to DNA methylation specific sites like imprint control regions may not agree.

Overall, at Day 70 of gestation, few of the physical parameters we measured were different between IVP and IVO–derived fetuses and placentas. Taken together with the observation that the expression of *IGF2R* mRNA was unaffected in fetal cotyledon and liver tissue by IVP embryo production supports the suggestion that compensation by the fetus and placenta may not be evident until after the first trimester. Furthermore, the regulatory mechanisms involved in establishing and maintaining imprinted expression of *IGF2R* in cattle are not abundantly clear. We did not observe a direct correlation between level of *IGF2R* mRNA and *AIRN* ncRNA expression with DNA methylation at DMR2. Imprinted expression of *IGF2R* is regulated in a time and tissue-specific manner based on differences in the level allelic expression at different gestational time points and between tissues (Bebbere et al., 2013; Suteevun-Phermtai et al., 2009). At Day 70 of gestation, IVP-derived bovine fetal liver did not exhibit a difference in expression of *IGF2R* mRNA or *AIRN* ncRNA expression, and none of the CpGs investigated exhibited hypomethylation relative to IVO produced controls. These observations, when taken into consideration with a lack of physical differences, indicates that compensation by the fetus has not yet occurred. In contrast, hypomethylation at DMR2 and decreased expression of *AIRN* was apparent in IVP-derived fetal cotyledons without a difference in *IGF2R* mRNA expression. These observations, when considered with a lack of physical differences in the placenta, indicate that the regulatory mechanisms governing imprinted expression of *IGF2R* may be subject to change prior to significantly measureable differences in expression of *IGF2R* and subsequent changes to
physical parameters. These observations are also in agreement with the suggestion that placental compensation occurs prior to fetal compensation.

**Day 222 Bovine Fetal Liver and Cotyledon**

Gestational Day 222 was selected as a point of interest because during late gestation the physical differences between IVP- and IVO-derived fetuses and placentas become more apparent (Crosier et al., 2002; Farin and Farin, 1995; Miles et al., 2004). Additionally, altered expression of IGF2R in late gestation ovine fetuses with hypomethylation of DMR2 are associated with fetal overgrowth in IVP-derived ovine fetuses compared to IVO controls (Young et al., 2001). Therefore, at Day 222 of gestation, we compared IVP-derived fetuses exhibiting the fetal overgrowth phenotype associated with AOS (IVP-AOS) to IVO-derived controls.

Fetal and placental overgrowth has been observed in perinatal calves at a rate of 5 to 20% in pregnancies generated from the transfer of IVP embryos (Bertolini et al., 2002b; Crosier et al., 2002; Farin and Farin, 1995; Farin et al., 2006; Kruip and Den Daas, 1997; Lazzari et al., 2002; Sinclair, 1995). This overgrowth phenotype of AOS has been correlated with the presence of serum in the in vitro culture medium and with co-culture (Farin et al., 2010b; Farin et al., 2006). In contrast, studies utilizing a serum free culture system have found reduced numbers of fetuses exhibiting phenotypes associated with AOS (Block et al., 2011; Constant et al., 2006; Hansen et al., 2010; Hasler, 2000). In the current study, IVP-derived fetuses were generated from embryos cultured in a defined medium with serum. Individuals exhibiting the overgrowth phenotype were selected for comparison with IVO
controls and exhibited several significant differences in physical parameters including increased body weight, CRL, and liver weight. Furthermore, the livers of IVP-AOS fetuses displayed organomegaly. These observations are consistent with earlier reports in sheep and cattle studies comparing fetuses generated from IVP and IVO-derived ovine or bovine embryos (Bertolini et al., 2004; Sinclair et al., 1997; Sinclair et al., 1998).

Placentas recovered from IVP-AOS derived pregnancies at Day 222 of gestation exhibited increased weight. However, the ratio of placental weight to body weight did not differ indicating that the placenta growth remained in proportion to the overgrowth of the fetus. These observations are consistent with previous reports of placental overgrowth in IVP-derived pregnancies (Constant et al., 2006; Miles et al., 2004). The number of placentomes was not significantly altered in IVP-AOS placentas compared to IVO controls; however, the ratio of the number of placentomes to body weight was decreased. Therefore, the IVP-AOS placentas may exhibit a decrease in feto-maternal contact and placental efficiency relative to their IVO-derived counterparts. Placental abnormalities have also been observed in pregnancies derived from cloned embryos during late gestation (Cibelli et al., 1998; Constant et al., 2006; Edwards et al., 2003; Hill et al., 2001).

At Day 222 of gestation IVP-AOS fetuses did not exhibit altered expression of IGF2R mRNA in fetal liver compared to IVO-derived controls. These results are in contrast to those observed by Young et al. 2001 in which overgrown IVP-derived lambs exhibited decreased expression of IGF2R mRNA and protein in fetal liver relative to IVO produced controls (Young et al., 2001). The differences between the studies may be attributed to species differences or that in the experiment performed by Young et al they had a greater
number of observations yielding a statistical advantage. We had expected that given the extreme physical differences between the IVP-AOS and IVO groups expression of \textit{IGF2R} would have been significantly decreased in fetal liver regardless of the number of observations. This leaves open the possibility that there are other factors involved in regulating the organ growth of the liver apart from \textit{IGF2R}.

Altered expression of \textit{IGF2R} has been observed in fetuses derived from nuclear transfer that also exhibited fetal overgrowth and organ abnormalities (Gong et al., 2012; Li et al., 2007; Long and Cai, 2007; Su et al., 2011; Yang et al., 2005). Specifically, altered expression of \textit{IGF2R} was observed in liver, heart, kidney, brain, and bladder of clones relative to IVO controls. Interestingly, one study found that in clones that died near term exhibited increased expression of \textit{IGF2R} in liver, lung and kidney compared to clones that lived (Gong et al., 2012). Increased expression of \textit{IGF2R} in abnormal organs of near term fetuses may indicate an attempt to compensate for overgrowth.

Expression of \textit{AIRN} ncRNA in fetal liver of IVP-AOS fetuses was decreased compared to IVO controls. Our observations contrast earlier work examining expression of \textit{AIRN} in late gestation SCNT-derived fetuses that did not find any differences (Suteevun-Phermthai et al., 2009). However, these differences observed in expression of \textit{AIRN} may be attributed to the different protocols used for embryo production, SCNT or IVP. The extent to which SCNT and in vitro culture of embryos influences regulation and expression of specific imprinted genes is yet to be determined. Therefore, differences in expression of specific imprinted genes like \textit{AIRN} between studies that compared SCNT or IVP embryos to those derived from IVO methods cannot be directly compared to each other. Apart from the
differences in embryo production of SCNT and IVP embryos, cloned embryos are genetically identical, whereas, IVP embryos are half-siblings resulting in a more diverse genetic background and possible differences in expression of imprinted genes. Interestingly, we did not find a correlation between the level of expression for AIRN and IGF2R in fetal liver. These findings are consistent with the study of SCNT-derived late gestation fetuses in which expression level of AIRN did not appear to be directly correlated with expression level of IGF2R (Suteevun-Phermthai et al., 2009). In the mouse, AIRN regulates imprinted expression of IGF2R by transcriptional interference whereby transcription of the long non-coding RNA product interferes with transcription of IGF2R from the paternal promoter (Santoro et al., 2013). Our observations indicate that altered expression of AIRN does not seem to have a direct impact on the level of expression for IGF2R. Therefore, suppression of IGF2R from the paternal allele may rely on continuous expression of AIRN and may not be influenced by up or down regulation in expression of AIRN.

At Day 222 of gestation, expression of IGF2R mRNA in the cotyledons of IVP-AOS-derived placentas did not differ compared to IVO controls; however, expression of AIRN ncRNA was significantly elevated. Expression of IGF2R was remarkably similar between groups as indicated by identical means and only a slight difference in variance. Given the amount of placental overgrowth observed in the IVP-AOS group, we had expected that IGF2R mRNA would be significantly reduced. In near term pregnancies, placentomes and cotyledons from IVP or SCNT-derived fetuses were shown to exhibit increased size and weight indicating compensation of the placentome unit had occurred at that stage of gestation to meet the demands of a compromised placenta with fewer placentomes available for feto-
maternal exchange (Bertolini et al., 2002b; Cibelli et al., 1998; Hill et al., 2000).

Interestingly, the lack of apparent down-regulation in expression of \( IGF2R \) may indicate that compensation within the placentome with respect to increased demands for adequate fetomaternal exchange had not occurred by Day 222 of gestation. Due to the highly tissue-specific manner in which expression of \( IGF2R \) is regulated it is not surprising that studies do not agree on up or down-regulation of \( IGF2R \) in the placenta. Expression \( IGF2R \) is known to vary considerably between tissues. Therefore, its expression may not be the same between the different tissues that make up the placenta (amnion, chorioallantois, cotyledon, caruncle, placentome).

At Day 222 of gestation, we found that overgrown IVP-AOS-derived fetal livers exhibited DNA hypomethylation at all 10 CpGs analyzed compared to IVO controls. In contrast, only 1 CpG of 10 exhibited DNA hypomethylation in IVP-AOS-derived fetal cotyledons. These differences in DNA methylation maybe attributed to differences in tissue type or may indicate how IVP methods affect tissues differently. Recently, it has been observed that bovine placental tissues exhibit a more relaxed state of imprinting for \( IGF2R \) as indicated by a higher level of paternal expression (Bebbere et al., 2013). In studies investigating DNA methylation at ICR2 of \( IGF2R \) in bovine fetal liver and placenta generated from the transfer of in vitro manipulated embryos, DNA hypomethylation was observed in both tissues (Long and Cai, 2007; Su et al., 2011). However, a direct correlation between the level of DNA methylation at the imprinting control region 2 (ICR2) within DMR2 and the expression level of \( IGF2R \) has not been made. Altered DNA methylation has been observed in other studies analyzing the level of methylation at imprint control regions
for other imprinted genes in fetal tissues derived from IVP and SCNT methods compared to IVO controls (Couldrey and Lee, 2010; Curchoe et al., 2009; Hori et al., 2010; Suzuki et al., 2009). Interestingly, in the study by Sutevuun-Phermthai et al (Suteevun-Phermthai et al., 2009), clones exhibited altered allelic expression of IGF2R ranging from dominant maternal to dominant paternal expression without loss or change in the level of expression for AIRN. However, they did not determine if AIRN had undergone allelic switching in conjunction with that observed for IGF2R or analyze DNA methylation at either of the DMRs involved in regulating imprinted IGF2R expression. Given the observation that expression of AIRN in a variety of tissues and gestational time points may be altered without affecting the level of expression for IGF2R, AIRN may play a functional role in establishing imprinted expression of IGF2R and a lesser role in maintaining it.

**SUMMARY AND CONCLUSIONS**

To summarize, we investigated the expression of IGF2R and AIRN in pre-attachment, post-attachment, and late gestation bovine fetal and placental tissues generated from the transfer of either IVO or IVP bovine embryos. In addition, we have examined the DNA methylation patterns of those tissues at the DMR that is thought to participate in regulating imprinted expression of those genes. At Day 15 of gestation, IVP conceptuses were longer, but did not exhibit a difference in expression of IGF2R or AIRN compared to the IVO produced. DNA hypomethylation was observed at amplicon region 2 in IVP compared to IVO. At Day 70 of gestation, IVP fetuses were longer but not heavier than IVO fetuses and
no differences in expression of \textit{IGF2R} and \textit{AIRN} or altered DNA methylation at DMR2 were observed in bovine fetal liver. In addition, IVP bovine placental tissues did not exhibit physical differences or altered expression of \textit{IGF2R} and \textit{AIRN} compared to those produced from IVO pregnancies; however, DNA hypomethylation at DMR2 in fetal cotyledons generated from IVP pregnancies was observed. At Day 222 of gestation, overgrown IVP-AOS fetuses and placentas exhibited several physical differences compared to their IVO counterparts. IVP-AOS fetal liver did not exhibit a difference in expression of \textit{IGF2R} compared IVO livers; however, expression of \textit{AIRN} was decreased and DNA hypomethylation was observed at DMR2. IVP-AOS fetal cotyledons did not exhibit a difference in expression of \textit{IGF2R} compared to IVO cotyledons; however, expression of \textit{AIRN} was increased and DNA hypomethylation was observed at only one of 10 CpGs within DMR2.

In conclusion, differences in fetal and placental parameters related to growth following the transfer of IVP embryos does not directly correlate with altered expression of \textit{IGF2R} or \textit{AIRN} in bovine fetal liver or cotyledonary tissues; however, DNA hypomethylation was consistently observed across tissues and gestational time points demonstrating that methylation at DMR2 involved in maintaining imprinted expression of \textit{IGF2R} during bovine development can be influenced by in vitro production of embryos.

\textbf{LITERATURE CITED}


Lee, R. S. et al. 2004. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biology of reproduction 70: 1-11.


Santoro, F. et al. 2013. Imprinted Igf2r silencing depends on continuous Airn IncRNA expression and is not restricted to a developmental window. Development 140: 1184-1195.


<table>
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<tr>
<th>Gene Identification</th>
<th>Primer sequences and positions</th>
<th>Annealing Temp. (°C)</th>
<th>Size (bp)</th>
<th>Accession No.</th>
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| Histone 2A (H2A.Z)  | Forward: 5′ GCC GTA TTC ATC GAC ACC TG 3′<sup>a</sup>  
Revers: 5′ TCC TGC CAA TTC AAG TAC CTC 3′<sup>a</sup> | 60                  | 122        | NM_174809     |
| Insulin-like Growth Factor Type 2 Receptor (IGF2R) | Forward: 5′ TCA GTC GCA AAG TCG GAA C 3′<sup>b</sup>  
Revers: 5′ CAG TGG AAG AAG ATG GTG GAG 3′<sup>b</sup> | 60                  | 135        | NM_174352     |
| Antisense to IGF2R (AIRN) | Forward: 5′ ATC TCC AGG CAG TTG TGA TG 3′<sup>c</sup>  
Revers: 5′ GTT CCT ACC TTC CCG ATA CTG 3′<sup>c</sup> | 60                  | 127        | AC_000166     |

<sup>a</sup>H2AZ forward primer (136 - 155) bp and reverse primer (237 to 257) bp of NM_174809
<sup>b</sup>IGF2R forward primer (6787 to 6805) bp and reverse primer (6902 to 6922) bp of NM_174352
<sup>c</sup>AIRN forward primer (97651440 to 97651459) bp and reverse primer (97651546 to 97651566) bp of AC_000166.1
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<th>Physical Measurements</th>
<th>IVO</th>
<th>IVP</th>
<th>P - Value</th>
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</thead>
<tbody>
<tr>
<td>Body Wt (g)</td>
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<td>38.3 ± 1.42</td>
<td>0.21</td>
</tr>
<tr>
<td>CRL (cm)</td>
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<td>11.2 ± 0.17</td>
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<tr>
<td>CRL : BW</td>
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<td>0.02</td>
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<td>Liver WT (g)</td>
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<td>0.05 ± 0.003</td>
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<td>Placenta Wt (g)</td>
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Table 3. Physical parameters of gestational day 222 fetuses and placentas

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<th>Physical Measurements</th>
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<th>IVP - AOS</th>
<th>P - Value</th>
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<td>Liver : BWT</td>
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<td>Placentome:BW</td>
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<tr>
<td>Placenta WT (kg)</td>
<td>1.99 ± 0.12</td>
<td>3.67 ± 0.44</td>
<td>0.002</td>
</tr>
<tr>
<td>Placenta WT : BW</td>
<td>0.11 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.133</td>
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</table>
Figure 1. Location of Amplicon Regions analyzed by pyrosequencing. 

A) Depiction of $IGF2R$ and $AIRN$ promoter regions. Differentially methylated regions (DMRs) marked on the parental alleles by large rectangles. Striped rectangles indicate the region is methylated and transparent rectangles indicate the region is unmethylated. Small rectangle above DMR2 indicates location of amplicons analyzed. Arrows indicate direction of transcription for $IGF2R$ and $AIRN$ from either the maternal or paternal alleles. 

B) Pyrosequencing Amplicon Regions 1 and 2. Circles indicate location of CpGs within each amplicon. CpG ID identifies each CpG sequentially and BP identifies each CpG by its specific base pair location with the overall amplicon regions.
Figure 2. Conceptus length and real time PCR expression analysis of gestational day 15 bovine conceptuses generated from in vivo (IVO, open bars) and in vitro (IVP, closed bars) produced embryos. A) Length of whole conceptuses (IVO, n = 11; IVP, n = 9); B) IGF2R mRNA (IVO, n = 10; IVP, n = 8); C) AIRN non-coding RNA (IVO, n = 11; IVP, n = 8).
Figure 3. DNA methylation observed within DMR2 of IGF2R in gestational Day 15 bovine conceptuses generated from in vivo (IVO, open bars) and in vitro (IVP, closed bars) produced embryos. **A)** DNA methylation of individual CpGs within two amplicon regions of IGF2R DMR2; **B)** Average DNA methylation of all observed CpGs for overall Amplicon Regions 1 and 2 (IVO, n = 9; IVP n = 11).
Figure 4. Real time PCR expression analysis of \textit{IGF2R} mRNA and \textit{AIRN} ncRNA in gestational day 70 bovine fetal liver and cotyledonary tissue generated from transfer of in vivo (IVO, open bars) and in vitro produced (IVP, closed bars) embryos. \textbf{A}) Fetal Liver \textit{IGF2R} mRNA (IVO, n = 7; IVP, n = 5), \textbf{B}) Fetal Liver \textit{AIRN} non-coding RNA (IVO, n = 7; IVP, n = 4) \textbf{C}) Cotyledon \textit{IGF2R} mRNA (IVO, n = 10; IVP, n = 14), \textbf{D}) Cotyledon \textit{AIRN} non-coding RNA (IVO, n = 11; IVP, n = 14).
Figure 5. DNA methylation of observed within DMR2 of *IGF2R* in gestational Day 70 bovine fetal livers generated from in vivo (IVO, open bars) and in vitro (IVP, closed bars) produced embryos. **A.** DNA methylation of individual CpGs within two amplicon regions of *IGF2R* DMR2. **B.** Average DNA methylation of all observed CpGs for overall Amplicon Regions 1 and 2 (IVO, n = 7; IVP n = 6).
Figure 6. DNA methylation of observed within DMR2 of *IGF2R* in gestational Day 70 bovine fetal cotyledons generated from in vivo (IVO, open bars) and in vitro (IVP, closed bars) produced embryos. A.) DNA methylation of individual CpGs within two amplicon regions of *IGF2R* DMR2. B.) Average DNA methylation of all observed CpGs for overall Amplicon Regions 1 and 2 (IVO, n = 11; IVP n = 15).
Figure 7. Real time PCR expression analysis of $IGF2R$ mRNA and $AIRN$ ncRNA in gestational day 222 bovine fetal liver and cotyledonary tissue generated from the transfer of in vivo (IVO, open bars, n = 7) and in vitro produced (IVP, closed bars, n = 7) embryos.  

A) Fetal liver $IGF2R$ mRNA,  
B) Fetal liver $AIRN$ non-coding RNA,  
C) Cotyledon $IGF2R$ mRNA,  
D) Cotyledon $AIRN$ non-coding RNA.
Figure 8. DNA methylation of CpGs located within DMR2 of *IGF2R* in gestational Day 222 bovine fetal livers generated from the transfer of in vivo (IVO, open bars, n = 7) and in vitro (IVP, closed bars, n = 7) embryos. A) DNA methylation of individual CpGs within two amplicon regions of *IGF2R* DMR2. B) Average DNA methylation of all observed CpGs for overall Amplicon Regions 1 and 2.
Figure 9. DNA methylation of CpGs located within DMR2 of *IGF2R* in gestational Day 222 bovine fetal cotyledons generated from the transfer of in vivo (IVO, open bars, n = 7) and in vitro (IVP, closed bars, n = 7) embryos. A) DNA methylation of individual CpGs within two amplicon regions of *IGF2R* DMR2. B) Average DNA methylation of all observed CpGs for overall Amplicon Regions 1 and 2.
Over the last 25 years, numerous studies have documented fetal and placental abnormalities that occur after the transfer of bovine IVP embryos. Dysregulation and subsequent aberrant expression of developmentally important genes resulting in abnormal development is one of the predominant hypotheses underlying AOS. However, studies that have attempted to correlate aberrant expression of specific genes to specific abnormalities have met with limited success. The insulin-like growth factor type 2 receptor (IGF2R) is an imprinted gene whose aberrant expression has been associated with the overgrowth phenotype in late gestation IVP lamb fetuses (Young et al., 2001). Regulation and imprinted expression of Igf2r has been studied extensively in the mouse revealing that paternal expression of Igf2r is silenced by the expression of the antisense non-coding RNA, AIRN. In addition, mice with a maternal-knock out for Igf2r exhibit fetal and placental overgrowth similar to that observed in cattle following the transfer of IVP or SCNT embryos. Currently, very little is known about how imprinted expression of IGF2R in cattle is established and maintained during development in fetal and placental tissues. Furthermore, studies investigating the influence of IVP culture systems on the expression of IGF2R in cattle have not been successful at making a direct correlation between IVP of embryos and an overgrowth phenotype as has been previously demonstrated in sheep. Therefore, the first objective of these dissertation studies was to confirm the existence of bovine AIRN in cattle and demonstrate that expression of AIRN coincided with imprinted expression of IGF2R during bovine development. In addition, we sought to show that expression of AIRN during bovine development could be affected by IVP of bovine embryos. Upon verifying the existence of AIRN in cattle, our second objective was to characterize the long ncRNA bovine
AIRN transcript. Our third objective was to determine if imprinted expression of IGF2R mRNA and AIRN ncRNA was affected by IVP embryo production in early, mid, and late gestation bovine fetuses. Furthermore, we sought to determine if the extent of DNA methylation at the promoter of AIRN and the level of expression of AIRN were correlated with the level of expression of IGF2R in bovine fetal and placental tissues.

In Chapter 2 we sought to address the question “Does AIRN regulate imprinted expression of IGF2R in cattle?” After examination of bovine AIRN non-coding RNA expression during development in embryonic and fetal tissues we determined that AIRN does exhibit a temporal expression pattern indicative of a role for regulating imprinted expression of IGF2R. Bovine embryos undergo elongation between Days 14 and 21 of gestation prior to a period of placental development and attachment occurring between days 21 and 45. Currently, the timing of imprint establishment in cattle is not clear. Previous reports have demonstrated that in sheep and cattle IGF2R is imprinted and that the gene exhibits differential methylation at a cytosine-guanine island with in intron 2 of IGF2R (Long and Cai, 2007; Young et al., 2001). These observations prompted us to ask if AIRN was expressed in cattle and whether or not it was involved in regulating imprinted expression of IGF2R. After verifying the existence of bovine AIRN, we sought to examine its temporal expression pattern during early gestation. We found that day 7 blastocysts do not exhibit AIRN expression, indicating that paternal repression of IGF2R had not occurred at that time. At day 15 of gestation, we found that only a small percentage of conceptuses entering into the period of elongation exhibited AIRN expression. It is conceivable that a more advanced conceptus at day 15 could exhibit AIRN expression and imprinted expression of IGF2R. At
day 18 of gestation, we found that the majority of conceptuses (80%) exhibited AIRN expression. The small percentage of conceptuses that did not express AIRN at day 18 may represent those that are developmentally delayed in regards to establishment of imprinted expression of IGF2R. From these observations it appears as though a correlation between conceptus elongation and establishment of imprinted expression of IGF2R may exist. In a separate study examining the allele specific expression of IGF2R in bovine fetal tissues it was confirmed that imprinted expression of IGF2R is established by day 25 of gestation (Suteevun-Phermthai et al., 2009). Therefore, our findings along with the work of others indicates that imprinting of IGF2R and AIRN occurs between Day 15 and Day 25, by mechanisms that are still yet to be determined.

Expression of AIRN coincides with the imprinted expression of IGF2R, beginning of conceptus elongation, and maternal recognition of pregnancy. Interestingly, repression of the paternal IGF2R allele may contribute to conceptus elongation. IGF2R targets and removes the primary embryonic growth factor from circulation and therefore a reduction in IGF2R at this critical time point in development may influence the success rate of maternal recognition of pregnancy which is occurring during this developmentally important time period (Day 15 to 21). Maternal recognition of pregnancy in cattle is dependent upon the ability of the developing conceptus to produce and secrete interferon t (IFNT). Reduced paternal expression of IGF2R may contribute to a correlation between conceptus size and its ability to secrete necessary levels of IFNT for maternal recognition.

We also sought to determine if IVP embryo production affected expression of AIRN during development. The relative abundance of AIRN ncRNA was compared between livers
of bovine fetuses generated from the transfer of either IVO or IVP embryos at Day 70 of gestation. The reduction in AIRN ncRNA observed at Day 70 of gestation in IVP bovine fetal livers indicated that expression of AIRN can be affected by the method of embryo production. Therefore, altered expression of AIRN resulting from IVP embryo production may contribute to altered expression of IGF2R during critical points in bovine development.

In the second chapter of this dissertation we sought to characterize this long non-coding antisense RNA transcript. By performing a primer walk we determined that bovine AIRN is approximately 117kb in length. Our investigations of this transcript also revealed that the AIRN transcript is repeat rich. This characteristic suggests that the AIRN transcript is highly unstable. In our work a putative promoter was identified upstream of a differentially methylated region 2 of IGF2R, in an antisense orientation, signifying the importance of DNA methylation at this region to inhibiting maternal AIRN expression and absence of DNA methylation to allowing paternal AIRN expression. Recent studies in the mouse have demonstrated that Airn inhibits paternal expression of Igf2r by an interference mechanism at the Igf2r promoter which is dependent upon the transcriptional overlap between Igf2r and Airn. Bovine AIRN maintains transcriptional overlap with IGF2R and therefore may work to inhibit paternal expression of IGF2R through transcriptional interference.

In the third and final research chapter of this dissertation we sought to determine if IVP production of bovine embryos altered expression of IGF2R in fetal and placental tissues. Furthermore, we sought to determine if the mechanisms that regulate imprinted expression of IGF2R were affected by embryo production, AIRN expression, and DNA methylation at DMR2. We predicted that growth related parameters such as conceptus length, fetal body
weight, CRL length, placenta weight, and cotyledon size of IVP pregnancies would be greater in IVP fetuses and associated with aberrant expression of the imprinted gene, \( IGF2R \). In addition, we predicted that the mechanisms such as expression of \( AIRN \) ncRNA and DNA methylation at DMR2 involved in regulating imprinted expression of \( IGF2R \) would also be affected by IVP. We predicted that the level of expression for \( AIRN \) would be correlated to the level of expression for \( IGF2R \) and the extent of DNA methylation at DMR2 would be correlated to the expression of \( AIRN \) and possibly \( IGF2R \).

At Day 15 of gestation, we found that IVP derived conceptuses were significantly longer than IVO derived conceptuses. We expected that the longer IVP-derived conceptuses would exhibit decreased expression of \( IGF2R \) compared to that of IVO conceptuses. However, we observed no difference in expression of \( AIRN \) and \( IGF2R \) between IVP and IVO derived conceptuses. We also did not observe any correlation between the level of \( AIRN \) and \( IGF2R \) expression. Because we observed no difference in \( IGF2R \) and \( AIRN \) expression analysis in Day 15 conceptuses we also expected that methylation at DMR2 would not differ. Surprisingly, we found hypomethylation in IVP conceptuses in the second amplicon region (AR2). The observation of DNA hypomethylation at DMR2 in IVP conceptuses compared to IVOs without the presence of altered gene expression indicates that DNA methylation may not be directly tied to the level of gene expression for \( AIRN \) and \( IGF2R \). It is important to recognize that we analyzed only a 10 CpGs total out of 260 present within the entire DMR2 region. Therefore, we were limited in the range and scope of our assessment of DNA methylation within the region. The fact that we were able to demonstrate that individual CpGs within DMR2 can exhibit hypomethylation indicates that
some CpGs within region are more susceptible to environmental conditions that result in hypomethylation. Because the paternal allele is believed to be unmethylated, our results demonstrating hypomethylation at DMR2 indicate a likely loss of methylation from the maternal allele. Because expression of *IGF2R* and DNA methylation at DMR2 is known to be limited to primarily the maternal allele and expression of *AIRN* primarily from the paternal one, the technique employed provided useful information about the role of *AIRN* and DNA methylation at DMR2 in regulating imprinted expression of *IGF2R*.

We continued our investigation of the effects of IVP on *IGF2R*, *AIRN*, and DNA methylation at Day 70 of gestation which is a time point soon after completion of placental development and attachment. We selected fetal liver and cotyledon as our tissues to analyze. Fetal liver is a primary location for IGF production and cotyledons make up the fetal side of the placental attachments within placentomes. Of the physical parameters that we measured only crown rump length (CRL) and crown rump length to body weight ratio (CRL:BW) were significantly greater in IVP fetuses compared to IVOs. Liver weight and body weights were not different between treatments. Therefore, growth enhancement was not abundantly evident at Day 70 of gestation in the IVP fetuses examined. We had expected that expression of *IGF2R* mRNA would be reduced in the IVP fetuses, however, we found that neither expression of *IGF2R* or *AIRN* was different between IVP fetal livers and those that were IVO derived. Interestingly, the CRL and CRL:BW ratio parameters were significantly increased in IVP fetuses compared to IVO. Therefore, IVP fetuses exhibit increased length but not body mass at Day 70 of gestation. We found no difference in DNA methylation at any of the 10 CpGs analyzed between the two Amplicon Regions. At least in the liver, no difference in
gene expression of *IGF2R* and *AIRN* or altered DNA methylation pattern at DMR2 coincided with few recognizable differences in parameters associated with enhanced fetal growth.

At Day 70 of gestation, the placenta did not exhibit enhanced placental growth between pregnancies derived from the transfer of IVP embryos compared to those from IVO embryos. Based on the results from the physical data, we predicted that there would not be a difference in expression of the imprinted genes, *IGF2R* and *AIRN*, or altered DNA methylation patterns at DMR2. Interestingly, we found no difference in expression of *IGF2R*, but we did observe a decrease in expression of *AIRN* ncRNA between IVP fetal cotyledon tissue and that of the IVOs. In addition, IVP fetal cotyledons exhibited hypomethylation within both amplicon regions studied relative to those of IVO pregnancies. Taken together, this may indicate that the level of expression for *IGF2R* is not directly regulated by expression of *AIRN* or DNA methylation at DMR2. However, it does demonstrate that loss of methylation at DMR2 is associated with altered expression of *AIRN*. In studies by Barlow and others (Latos et al., 2012; Stricker et al., 2008), they have shown that maintenance of imprinted expression of *Igf2r* requires continuous expression of *Airn* from the paternal allele. Therefore, a reduction in expression of *AIRN* may not directly affect expression of *IGF2R* from the paternal allele. The reduction in expression of *AIRN* and hypomethylation at DMR2 in the fetal cotyledons of IVP pregnancies shows that the mechanisms involved in establishing and maintaining imprinted expression of *IGF2R* are altered at this stage of gestation in IVP pregnancies. Therefore, although we did not observe physical differences in the placentas of IVP pregnancies, we found that the mechanisms thought to regulate expression of a key gene in placental growth were altered. This opens the
possibility that \textit{IGF2R} expression could be altered later in development. Indeed, IVP fetuses and placentas did not demonstrate growth enhancement until later in gestation. Thus, subtle changes to the mechanisms regulating imprinted expression may occur prior to the physical differences that were observed later.

At Day 222 of gestation we examined the fetal livers and cotyledons of IVP-derived pregnancies that exhibited fetal overgrowth (AOS) compared to those obtained from IVO-derived pregnancies. We hypothesized that fetuses exhibiting fetal overgrowth associated with AOS would also exhibit decreased expression of IGF2R, and altered expression of \textit{AIRN} with hypomethylation at DMR2. Interestingly, we found no difference in expression of \textit{IGF2R}, decreased expression of \textit{AIRN}, and hypomethylation of both amplicon regions. The unaltered state of expression for \textit{IGF2R} between fetal livers of overgrown IVP-derived pregnancies was unexpected. Fetal growth in utero is primarily driven by the potent mitogenic growth factor \textit{IGF2}. Removal of \textit{IGF2} from circulation is performed by \textit{IGF2R}. Extreme fetal overgrowth either occurs from increased expression of \textit{IGF2} or from decreased expression of \textit{IGF2R}. By purposefully selecting individuals exhibiting the overgrowth phenotype associated with AOS we had expected to observe a decrease in expression of \textit{IGF2R}. Therefore, there is not a clear indication that altered expression of \textit{IGF2R} in cattle contributes to the overgrowth phenotype associated with AOS. Another possible explanation of these findings is that prior to this time point, reduced expression of \textit{IGF2R} contributed to enhanced fetal growth and a compensation mechanism had been activated already to transition expression of \textit{IGF2R} back to levels similar to that observed in IVO derived fetuses. Interestingly, the mechanisms known to regulate imprinted expression of \textit{IGF2R} are altered
in IVP-AOS fetal livers, including decreased expression of AIRN and hypomethylation at DMR2. However, a direct correlation between the expression level of IGF2R and expression of AIRN or DNA methylation at DMR2 could not be made. The role of these regulatory mechanisms may be necessary for establishing and maintaining imprinted expression of IGF2R, but may not be directly involved in modulating the level of expression for IGF2R in bovine fetal liver.

At Day 222 of gestation we also examined the expression of our imprinted genes of interest and DNA methylation at DMR2 in bovine cotyledonary tissue from the placentas of IVP-AOS fetuses relative to those derived from IVO. We predicted that overgrown IVP-derived fetuses would exhibit reduced expression of IGF2R in the cotyledonary tissue of their placentas relative to their IVO counterparts. However, no difference in expression of IGF2R was detected between treatments. We examined several physical parameters of the placentas and found that placentas derived from IVP-AOS pregnancies were heavier than those from IVO. However, no differences in placenta to BW ratio, placentome number, or placentome number to BW ratio were detected between treatments. Therefore, the placentas of overgrown IVP-AOS pregnancies were larger, but were proportional to the body size of the fetus. With no differences in the physical parameters measured for the placentas except placenta weight, which was proportional, perhaps it is not surprising that expression of IGF2R was not different in cotyledonary tissue derived from overgrown IVP-AOS placentas compared to that of IVOs. Interestingly, expression of AIRN was increased in IVP-AOS cotyledons compared to the IVOs. Silencing or reduced expression of IGF2R from the paternal allele is dependent upon a level of transcription driving expression of AIRN.
Therefore, an increase in expression of \textit{AIRN} may only reinforce the known mechanism inhibiting expression of \textit{IGF2R} from the paternal allele. In addition, only 1 of 10 CpGs examined exhibited DNA hypomethylation within DMR2. Therefore, the degree of DNA methylation at DMR2 was relatively unchanged between IVP-AOS cotyledons and those of IVO-derived placentas. Overall, our findings of no significant differences in expression of \textit{IGF2R} and DNA methylation at DMR2, as well as an increase in expression of \textit{AIRN} are consistent with the observation that the placentas of overgrown IVP-AOS pregnancies lacked physical differences according to the parameters we measured. These findings may indicate that compensation by the placenta had already occurred contributing to fetal survival (Type IV AOS).

Throughout the body of this dissertation we have examined the role of \textit{IGF2R} and its regulatory non-coding RNA, \textit{AIRN}, during development of IVP and IVO-derived bovine pregnancies. We have demonstrated that expression of \textit{AIRN} coincides with establishment of imprinted expression of \textit{IGF2R} during the peri-attachment period which occurs as the bovine conceptus elongates and placental attachments are established. We have characterized \textit{AIRN} and revealed that it is approximately 117kb in length and maintains a transcriptional overlap with paternal \textit{IGF2R} allele. In our investigation of the effects of \textit{IGF2R} and \textit{AIRN} expression on bovine development we were able to show that expression of \textit{IGF2R} remains unaltered between IVP and IVO derived fetal and placental tissues. Interestingly, the mechanisms thought to regulate imprinted expression of \textit{IGF2R}, expression of \textit{AIRN} and DNA methylation at DMR2, are susceptible to alteration from conditions associated with in vitro embryo production. Clearly, IVP-derived pregnancies exhibit growth related
abnormalities, however, our work was unable to provide evidence to support the idea that increased fetal or placental size is directly correlated to aberrant expression of *IGF2R*. Our overall work provides further evidence that fetuses and placentas derived from the transfer of IVP embryos do exhibit aberrant expression of imprinted genes and altered DNA methylation patterns as indicated by alterations in expression of *AIRN* and DNA methylation at DMR2.

**LITERATURE CITED**


**Figure A1.** Proportions of embryos, conceptuses (short and long), or fetuses expressing *IGF2R* mRNA (red bars) and *AIRN* ncRNA (dark red bars) during early gestation.
Figure A2. Gestational Day 15 In Vivo-produced bovine conceptuses. A. Representative short gestational Day 15 conceptus. B. Representative long gestational Day 15 conceptus.