ABSTRACT

FARMER, WILLIAM TAYLOR QUINTON. Detection of Antisense to IGF2R (*AIR*) RNA in Cattle. (Under the direction of Charlotte Farin.)

The insulin-like growth factor type 2 receptor (Igf2r/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, *Air*. In contrast, the human *IGF2R* gene is not imprinted and *AIR* ncRNA does not exist. Because it is known that *IGF2R* is imprinted in cattle, the objectives of this study were to determine if *AIR* ncRNA exists in cattle; if so, whether bovine *AIR (*b*AIR)* expression changes at developmentally important stages of gestation, and whether method of embryo production affects air expression. For objective 1, primer sets were designed for b*AIR* based on bovine genomic sequence. The primer set, bAIR3, was used to amplify a region of b*AIR* corresponding to an antisense segment within intron 1 of *IGF2R*. Primer set bAIR4 amplified a segment of b*AIR* ncRNA corresponding to an antisense region upstream of the 5'-untranslated region of *IGF2R.* Whole cell RNA was extracted from liver samples of bovine fetuses at day 70 of gestation resulting from the transfer of either in vivo-produced (n=7, IVO) or in vitro-produced (n=6, IVP) embryos. Extracted RNA was subjected to DNase treatment, reverse transcription (RT) and PCR. Control RT reactions included RT without Superscript III (Invitrogen) and RT without Superscript III or DNase. Controls confirmed that amplification products resulted from RNA present in the sample and not from genomic DNA contamination. Amplicons were obtained for both the bAIR3 and bAIR4 primer sets and were sequence verified. These results demonstrated that b*AIR* ncRNA does exist in cattle. For objective 2, conceptuses (n=9, IVO, mean ± sem length: 2.2 ± 0.6mm) were recovered from cows at day 15 of gestation and snap-frozen for RNA

extraction. Blastocysts (n=2 pools of 20 IVO embryos and n=4 pools of 25 to 27 IVP embryos) were recovered from cows on day 7 of development and snap frozen for RNA extraction. Semi-quantitative RT-PCR assays were performed to assess levels of IGF2R mRNA, H2A mRNA and b*AIR* ncRNA. Relative RNA expression was calculated as the ratio of band intensities of the RNA of interest to that of H2A. Data on levels of expression in fetal liver between IVO and IVP treatment groups were analyzed by Student's T-test. H2A mRNA was expressed in all day 70 fetal liver samples, day 15 conceptuses, and day 7 blastocyst pools. IGF2R mRNA was expressed in all fetal liver samples, in 8 of 9 day 15 conceptuses, and in all day 7 blastocyst pools. b*AIR* ncRNA was expressed in 7 of 7 samples of day 70 fetal liver. In contrast, only 1 of 9 conceptuses expressed a b*AIR* ncRNA signal based on the bAIR3 primer set whereas 8 of 9 conceptuses expressed b*AIR* ncRNA based on the bAIR4 primer set. No b*AIR* ncRNA was expressed in any blastocyst pools based on either the bAIR3 or bAIR4 primer sets. Relative levels of b*AIR* ncRNA were greater ($P<0.05$) in fetal liver generated from the transfer of in vivo-produced embryos compared to that from in vitro-produced embryos (IVO: 0.426 ± 0.090 vs. IVP: 0.112 ± 0.098). In summary, the antisense ncRNA *AIR* exists in cattle and is expressed following implantation. Furthermore, the relative level of b*AIR* ncRNA can be altered by method of embryo production. These observations are consistent with data from the mouse and suggest that b*AIR* may be involved in regulating imprinted expression of *IGF2R* in cattle.

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Detection of Antisense to IGF2R (*AIR)* RNA in Cattle

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William Taylor Quinton Farmer

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APPROVED BY:

Dr. Peter W. Farin Co-Chair of Advisory Committee	Dr. Jorge A. Piedrahita

Dr. Charlotte E. Farin
Co-Chair of Advisory Committee

BIOGRAPHY

William Taylor Quinton Farmer

Education

MS Animal Science, 2008, North Carolina State University, Raleigh, NC

BS Animal Science, 2003, North Carolina State University, Raleigh, NC

Honors and Awards

First Place award, International Embryo Transfer Society, Student Research Competition, January, 2008

Professional Affiliations

International Embryo Transfer Society

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ABNORMAL OFFSPRING SYNDROME

The transfer of in vitro manipulated and, to a lesser extent, in vitro produced (IVP) bovine embryos results in fetuses, placentas, and offspring that exhibit abnormalities [1-3]. Abnormalities observed following the transfer of IVP or somatic cell nuclear transfer (SCNT) embryos are stochastic in occurrence and duplication of all phenotypes have not been consistently linked with any single gene or mechanism [4-6]. Initially it was observed that offspring created from IVP embryos were larger than normal and were more susceptible to dystocia at birth [7]. The common occurrence of an overgrowth phenotype along with increased perinatal death, longer gestation lengths, congenital deformities, and abnormalities of placental vasculature resulting from the transfer of IVP embryos became known as Large Offspring Syndrome (LOS) [1, 7]. These developmental abnormalities occur at a higher incidence in fetuses, placentas, and offspring following the transfer of SCNT embryos compared to embryos produced in vitro [3, 8, 9]. However not all transfers of IVP or SCNT embryos result in excessive growth [10-14]. Therefore, the term abnormal offspring syndrome (AOS), better accommodates the known abnormalities that have been documented [5].

Phenotypes of AOS

IVP- or SCNT- derived fetuses, placentas and offspring exhibit abnormalities that are highly variable and may range from apparently normal with subtle phenotypes to the most obvious [5]. Unusually large or heavy offspring have been observed following the transfer of IVP or SCNT embryos in cattle [1, 9, 13, 15-17] and sheep [7, 18-20]. Increased fetal birthweight and initiation of fetal overgrowth can vary widely as has been demonstrated

in sheep where lambs twice the normal birthweight have been observed [7]. Conceptus and fetal overgrowth has also been detected as early as days 17 and 21 of gestation, respectively [21]. The increase in birthweights is also associated with a higher rate of dystocia and delivery by cesarean section [13]. Other characteristics of this syndrome include a longer gestation period [18], increased perinatal losses in the first half of pregnancy [22], and altered energy metabolism [23]. In addition, gross abnormalities have been observed in several organs. These include increased muscle mass and altered muscle fiber composition [24, 25]. Other abnormalities include skeletal malformations [7], facial malformations [7, 19, 26] and cerebellar dysplasia [27]. In cattle and sheep, placental abnormalities such as polyhydramnios, hydrallantois, and alterations in placentome number, and placental morphology have also been reported [13, 26, 28-32]. Defects in placental growth exhibited by cloned mice and cloned ruminants have been proposed to contribute to increased perinatal mortality by failure to initiate an adequate blood supply [15, 33].

To better understand the underlying mechanisms of these abnormalities, AOS has been subdivided into four classifications. Type I AOS is considered to be the abnormal development of the embryo or early conceptus resulting in early embryonic death or abortion before organogenesis can be completed at Day 42 of gestation [5]. Type II AOS describes placental and fetal abnormalities that result in fetal death or abortion between gestational Days 42 and 280 [5]. In Type III AOS, fetuses and placentas exhibit severe developmental defects that are not associated with a compensatory response [5]. In addition, dystocia may

or may not occur, and the resulting calves have severely altered clinical, hematological, and biochemical parameters resulting in death at or near the time of parturition [5]. In contrast, in Type IV AOS full term fetuses and placentas are present and exhibit only moderate abnormalities as a result of compensatory responses from the fetus and placenta [5].

Potential mechanisms of AOS in cattle

The occurrence of AOS phenotypes appears to be associated with the environmental conditions by which embryos are created [34]. Bovine IVP embryos are created from immature oocytes that are matured in vitro, fertilized in culture, and then maintained in culture medium to the blastocyst stage [34]. Cloned bovine embryos are created by maturing an oocyte to metaphase II in vitro, extracting its genetic material and then electrically fusing a donor cell with this karyoplast [35]. The resulting zygote is cultured to the blastocyst stage and transferred into a recipient animal. Both in vitro production and cloning techniques expose the developing bovine zygote to inadequate culture conditions that result in aberrant imprinted gene expression [35]. Embryos likely exhibit a high level of plasticity and an ability to adapt to stress considering all of the negative conditions associated with culture they must over come in order to develop to the blastocyst stage [36, 37]. Some of the obstacles the early embryo must combat include exposure to culture medium, serum, and coculture with other cell types [21, 38]. In vitro embryo development is also influenced by factors in the physical environment including temperature variation, embryo density, presence of oil, degree of humidity, and variations in surface area for gas exchange [39, 40]. Other hindrances to the normal development of in vitro-produced embryos are laboratory

constraints such as the stability of the incubator environment and the ability of technicians handling the embryos [4, 40].

In vitro maturation of oocytes and generation of embryos by in vitro production (IVP) or nuclear transfer result in embryos with obvious differences compared to in vivo-produced embryos. IVP embryos show differences in gross morphology when exposed to serum [4]. These differences include incomplete compaction, a darkened appearance, and less organization of the inner cell mass [4, 41, 42]. In contrast, IVP embryos not exposed to serum exhibit more complete compaction, have a lighter appearance, and their inner cell mass is more organized [4, 42]. Blastocysts generated in the presence of serum may also have a higher lipid content and incomplete junctional complexes between the inner cell mass and the trophectoderm [43-46]. Embryo fragmentation can result from high concentrations of serum in culture [20]. In addition, IVP embryos, regardless of their exposure to serum, have a more rapid development of male embryos [47, 48], increased intracellular lipid content [49, 50], fewer surface microvilli [49, 51], and altered cytoplasmic mitochondrial density [51] compared to in vivo-produced embryos [4]. Embryos created by nuclear transfer are subjected to the physical traumas of enucleation, electrofusion and reconstruction [52] as well as factors associated with the in vitro culture environment as discussed previously.

Evidence from the study of ruminants and mice is consistent with the hypothesis that perturbed epigenetic reprogramming resulting from embryo exposure to culture conditions is responsible for both abnormal DNA methylation and abnormal imprinted gene expression in

pre-implantation embryos that results in fetal and placental abnormalities [53-55]. Gene expression varies with the type of IVP system and SCNT protocols used suggesting that imprinted gene expression in the pre-implantation embryo is sensitive to alterations in epigenetic modifications [37, 56, 57]. The pre-implantation developmental program is governed by epigenetic mechanisms that, if altered or delayed, result in improper gene expression leading to abnormalities [58]. Epigenetic reprogramming errors likely occur during critical periods of pre-implantation development as a result of exposure to the culture environment during in vitro production [59, 60]. Similarly, epigenetic reprogramming errors in nuclear transfer-derived embryos likely occur from a failure to properly reprogram the donor nucleus and exposure to in vitro culture environments [59-61]. Some of the physiological aberrations associated with AOS are, in part, a result of aberrant DNA methyltransferase-1 (*Dnmt1/DNMT1*) expression [4, 62]. This enzyme regulates maintenance of methyation patterns at imprinted loci [4]. In mice, disruption of *Dnmt1* results in reduced DNA methylation, abnormal imprinted gene expression, and embryonic death [63-65]. In addition, increased *Dnmt1* expression was associated with an increase in methylation at imprint control regions of imprinted genes, also resulting in abnormal imprinted gene expression and embryonic death [66]. Interestingly, elevated levels of DNMT1 were also observed in bovine in vitro-produced embryos compared to bovine nuclear transfer blastocysts and in vivo-produced embryos [67]. These observations are consistent with the suggestion that AOS results from dysregulation of epigenetic patterns and failure of the embryonic genome to be correctly reprogrammed during gametogenesis and pre-implantation development [4, 5]. In addition, evidence that DNA methylation is altered

and imprinted genes are aberrantly expressed in fetuses, placentas and offspring following the transfer of in vitro-manipulated embryos further supports this suggestion [53, 54, 61, 68, 69].

An alternative explanation for abnormal fetal and placental development is that exposure of an embryo to culture conditions results in aberrant causal pathways [36]. Based on the aberrant causal pathway hypothesis, critical transcriptional signaling molecules are inappropriately activated during developmentally important periods of embryonic growth, such as the pre-implantation stage [36]. Improper activation of these signaling molecules is proposed to result as a response to the environment [36]. Therefore, the environment induces activation of signaling molecules inappropriately and subsequent epigenetic modifications mediate altered imprinted and non-imprinted gene expression [36] .

Another potential mechanism contributing to AOS phenotypes involves fetal-placental interaction. Many differences have been observed between bovine fetuses and placentas of IVP or SCNT embryos compared to in vivo pregnancies including placentome morphology, poor placental vascularization, hydrallantois, placentomegaly, reduction in placentome number, and increased birth weight [5, 6, 28, 32, 70]. Recently, placental overgrowth was shown to precede fetal overgrowth in late gestation fetuses following transfer of SCNT embryos [71]. Therefore, a placental compensatory mechanism resulting from placental deficiency may be a factor contributing to AOS phenotypes [28, 71].

IMPRINTED GENES

Imprinted genes were first discovered as a result of a series of nuclear transplantation experiments in mice that demonstrated the necessity of both parental genomes for complete fetal and placental development [72, 73]. These investigators were first to illustrate that parental genomes are functionally non-equivalent and designated this phenomenon as genomic imprinting [73]. Genomic imprinting is defined as the monoallelic expression of a gene or chromosomal region that is predetermined by epigenetic marks that are applied in a parent-specific manner during gametogenesis [74]. The monoallelic expression of an individual imprinted gene after fertilization can be tissue and developmental stage specific [75]. Imprinted genes infrequently occur in isolation, but rather are typically found in clusters where they share common cis-regulatory elements that can impart control over a region that can extend greater than a megabase or more [76]. In general, imprinting clusters contain several protein-coding genes and at least one gene that encodes a non-coding RNA (ncRNA) [77]. Cis-acting elements called imprinting control regions (ICRs) regulate expression of the imprinted genes within a cluster [74]. Typically, an ICR is a differentially methylated region (DMR) that is differentially methylated in the germ line and is associated with gene silencing in the rest of the cluster [78]. The three primary mechanisms by which the epigenetic silencing of gene expression occurs in imprinted genes are DNA methylation, histone-modifications, and RNA-associated silencing [79].

DNA Methylation

Imprinting control regions (ICRs) control the imprinting of genes within in a cluster by acquiring differential DNA methylation on the parental alleles during gametogenesis [77]. The methylation imprint is a modification of the DNA involving the addition of methyl groups to cytosine residues at CpG islands [74]. The term, CpG island, refers to a series of cytosine-guanine dinucleotide repeats within a genome that are ≥ 500bp in length and have a GC content ≥ 55% [80].

DNA methylation can regulate stable transcriptional repression by preventing transcription factors from binding or by recruiting methyl-DNA binding domain proteins (MBDs), histone deacteylases (HDACs) and chromatin remodeling complexes that function to alter the accessibility of DNA [81]. DNA methylation is thought to only regulate expression in a small number of genes, but may also be involved in preventing the propagation of repetitive DNA sequences and in regulating X chromosome inactivation [74]. DNA methylation imprints are acquired at differentially methylated domains (DMDs) in a sex-specific-manner during gametogenesis and are completed prior to fertilization [82]. These new patterns of DNA methylation on both imprinted and non-imprinted genes are established by de novo DNA methyltransferases (DNMTs) that methylate cytosine residues where both DNA strands are unmethylated [82]. After fertilization, genome wide demethylation occurs but imprinted genes maintain their differentially methylated domains by the action of maintenance methyltransferases that recognize hemi-methylated DNA [82].

Dnmt1 and Dnmt1o. Maintenance of DNA methylation occurs through the action of methyltransferases such as Dnmt1 and Dnmt1o. Dnmt1 maintains methylation marks on hemi-methylated DNA and methylates newly replicated DNA strands [83]. Dnmt1 null mice exhibited perturbed imprinting and died at embryonic Day 11, demonstrating the importance of this methyltransferases activity during development [63, 84]. In addition to Dnmt1, Dnmt1o is an oocyte specific maintenance methyltransferase that is expressed only in oocytes and preimplantation embryos [65]. In the mouse, Dnmt1o has been shown to translocate from the cytoplasm to the nucleus specifically at the 8-cell stage apparently to methylate imprinted genes and parasitic sequences [85]. The movement of Dnmt1o from the cytoplasm to the nucleus indicates that maintenance of methylated imprints at this stage is important [65]. The observation that deletion of the *Dnmt1o* promoter resulted in loss of methylation at imprinted loci but had no effect on the establishment of imprints indicates the importance of methylation maintenance by Dnmt1o in early embryonic development [65, 86].

Dnmt3a, Dnmt3b, and Dnmt3L. After implantation, a second wave of genome-wide demethylation occurs in the primordial germ cells (PGCs) as they move into the genital ridge [82]. The de novo DNA-methyltransferases, Dnmt3a and 3b, remethylate imprinted genes in a sex-specific manner to reflect the specific epigenotype of the new individual [82]. The female fetus must reprogram the methylation imprints in her germ cells to reflect the maternal state [82]. Similarly, the male fetus reprograms the methylation imprints in his germ cells to reflect the paternal state [82]. Null mutations of *Dnmt3a* and *3b* have been used to demonstrate that these specific methyltransferases are essential for de novo methylation

during embryonic development [87]. The *Dnmt3a* null mutation is lethal in mice by 4 weeks after birth and the *Dnmt3b* null mutation is lethal late in gestation [87]. The Dnmt3a de novo methyltransferase appears to be particularly important for establishing methylation imprints during gametogenesis [88]. Deletion of *Dnmt3a* in female PGCs prevented the establishment of maternal methylation imprints [88]. Similarly, *Dnmt3a* deletion in male PGCs resulted in loss of paternally methylated imprints [88].

A third member of the de novo methyltransferase family is Dnmt3L which is also required for methylation of imprinted genes [89, 90]. Dnmt3L does not have the catalytic site for methyltransferase activity [89]; however, it does associate with Dnmt3a and 3b apparently to regulate their acitivity [91-93]. Homozygous disruption of *Dnmt3L* in the female germline resulted in a failure of maternal-specific imprints to be established [89]. In the mouse, Dnmt3L is epigenetically regulated by methylation at its promoter [94, 95].

Histone Modifications

In addition to DNA methylation, imprinted gene expression is regulated by histone modifications that are heritable and serve as epigenetic marks for active and inactive chromatin [96]. The basic unit of chromatin is the nucleosome and it is composed of 146 bp of DNA wrapped around a core of histones, H2A, H2B, H3, and H4 [97]. The histones have N-terminal tails that can undergo post-translational modifications such as methylation, acetylation, phosphorylation, and ubquitination [98]. Most of the modified residues have been observed in the N-terminal tails of histone H3 and H4 [98]. There are two hypotheses proposed for histone modification. Based on the Histone Code Hypothesis, transcription can

be regulated by chromatin modulating proteins that modify chromatin structure in response to specific histone modifications such as methylation or acetylation [97]. These modifications are interpreted by effector proteins that induce either a heterochromatin or a euchromatin structure [99]. Heterochromatin is DNA that is tightly packaged with nucleosomes and is transcriptionally inaccessible to transcription factors [99]. In contrast, euchromatin is DNA that is more loosely packaged and is accessible for transcription [99]. Histone modification of a specific amino acid residue, alone or in combination with other histone modifications of the core histones forms the histone code [100]. For example, acetylation of lysine residues on histone H3 and H4 are associated with euchromatin, whereas, methylation of lysine residues on H3 and H4 are associated with heterochromatin [100].

An alternative hypothesis proposed to explain the function of histone modifications in transcription is the 'Methylation/Phosphorylation Binary Switch Hypothesis [101]. Based on this hypothesis, combinations of different modifications would dynamically alter the transcriptional state [101]. For example, phosphorylation of residues adjacent to a methylated lysine residue would result in an altered recruiting state for binding proteins. According to this hypothesis, changes made to the chromatin would affect transcription through'switch' sites that are dependent on the positional relationship between the phosphorylated residue and the methylated lysine residue. This would result in transcriptional activation if the phosphorylation preceeded the methylation or in transcriptional silencing if the phosphorylation followed the methylation [101].

Histone Methylation. Imprinted genes display allele-specific histone methylation patterns at differentially methylated regions [102]. Active transcription is associated with methylation of histone H3 at lysine residue 4 (H3K4me), whereas transcriptional silencing is associated with methylation of histone H3 at lysine residue 9 (H3K9me) [103, 104]. In addition, lysine residues can be mono-, di-, or tri-methylated and arginine residues can be mono- or di-methylated [98, 105]. This adds another level of complexity to regulation of transcription by histone methylation. Histone methylation of lysine residues appears to be irreversible because histone demethylases have not been identified yet [97]. These stable methylation marks, therefore, may provide an epigenetic mechanism by which gene expression profiles can be stably transferred to progeny cells [97].

Histone Acetylation. Acetylated lysine residues on histones H3 and H4 are generally associated with active transcription resulting from a relaxed chromatin structure [100]. The acetylation of histone residues facilitates transcription by reducing DNA nucleosomal interactions [97]. Deacetylation of histone residues reverses this effect [97]. For example, the transcribed alleles of the imprinted genes *H19*, *Snrpn*, and *U2af1-rs1* all exhibit hyperacetylation of histones H3 and H4 [106, 107]. In contrast, the silent allele of these imprinted genes displays hypoacetylation of the histones.

Non-Coding RNAs

Recently, use of full length cDNA sequencing has revealed that a significant proportion of the mammalian genome is composed of non-coding RNAs (ncRNA) [108]. The function of ncRNAs is difficult to predict from a nucleotide sequence compared to that

for proteins whose amino acid sequence reliably predicts function [109]. Interestingly, for a significant number of imprinted clusters, the parental chromosome that carries the unmethylated imprint control region is also the one that expresses the ncRNA [110]. Furthermore, in all six of the known imprinted clusters, expression of the ncRNA is associated with repression *in cis* of some or all of the protein-coding genes in the cluster [111-113]. Silencing by the imprinted ncRNA is bi-directional and affects multiple genes [111]. Silencing is imposed upon genes that overlap with the antisense ncRNA and also those that do not overlap [111]. Furthermore, silencing by antisense ncRNAs is regulated in a tissue-specific and developmental manner [111]. Models that attempt to explain mechanisms by which ncRNA silencing is mediated need to accomplish several things [111]. First, the model needs to elucidate how silencing is not induced by full expression of truncated ncRNAs [111]. Second, the model needs to determine how tissue specific imprinted expression operates [111]. Third, the model needs to explain how silencing is imposed on foreign genes inserted into an imprinted cluster [111]. Finally, a proposed model needs to describe how the silence induced by an imprinted ncRNA does not impact itself [111].

Several models have been proposed to explain how ncRNAs can silence an imprinting cluster. The first set of models describe ncRNA-based silencing mechanisms in which transcriptional regulation is mediated by the antisense RNA transcript itself [111, 114]. In the RNA interference (RNAi) model, silencing is mediated by double-stranded RNA intermediates complimentary to the gene that is silenced [115]. This mechanism is

possible if the imprinted ncRNA and the silenced imprinted gene share sequence homology [111]. The double-stranded RNA intermediates may silence expression by inducing mRNA degradation or by forming heterochromatin, effectively silencing the promoter [116]. Another mechanism by which silencing may be regulated by the ncRNA transcript is the "ncRNA-direct targeting model" of *Xist* [111]. In this model spreading of *Xist, in cis,* along adjacent sequences results in recruitment of heterochromatinizing factors that bind the DNA and result in X chromosome inactivation [113].

A second group of proposed models that describe how ncRNA function to silence gene expression suggest the transcription of the ncRNA itself is the mechanism that leads to silencing of imprinted genes within a cluster [111, 114]. In this 'expression-competition' model, it is proposed that the promoters for the ncRNA and mRNA are in competition for common *cis*-regulatory elements [117]. An alternative model is the 'transcriptional interference' (TI) model in which it is proposed that transcriptional regulation is mediated by active transcription where the elongating polymerase activity of an antisense gene directly interferes with transcription of the overlapping sense gene [111, 114]. However, within an imprinting cluster that includes several protein-coding sense genes, only one of them is overlapped by an antisense ncRNA [111]. Therefore, variations of the TI model have been suggested in which transcription of the ncRNA leads to either interference or activation of a *cis*-regulatory element regulating expression of all mRNA promoters within the imprinted cluster but has no effect on the ncRNA promoter [111].

A third proposed model involves a mechanism in which silencing is achieved by the formation of higher-order chromatin. In the transcription-based looping model, expression of

multiple genes within an imprinted cluster are dependent upon a *cis*-acting activator located in the ncRNA transcription unit [111]. A chromatin loop containing the activator forms when the ncRNA is expressed, preventing the interaction of the promoters with the activator and then silences expression of imprinted genes [111].

EPIGENETIC REPROGRAMMING OF IMPRINTED GENES

Epigenetic changes are defined as chemical alterations to the DNA or to the histone proteins associated with the DNA that change chromatin structure without altering the nucleotide sequence [118]. Genomic imprinting is the monoallelic, parent-of-origin specific gene expression that results from epigenetic modifications of imprinted genes [75]. In the mammalian genome, the primary epigenetic mark resulting in imprinted expression is DNA methylation [119]. This epigenetic modification is proposed to be the imprinting mark because it is both heritable and reversible and can be stably transmitted after DNA replication [120]. However, expression of imprinted genes is also regulated by other epigenetic modifications including covalent modification of histones and RNA silencing [118]. Epigenetic reprogramming occurs during both gametogenesis and pre-implantation development, and involves the erasure, acquisition, and maintenance of DNA methylation imprints [121].

Erasure of Imprints

In the developing embryo, inherited methylation imprints on the parental DNA must be erased in the primordial germ cells (PGCs) [102]. New sex-specific imprints can then be

acquired by the germ line as gametogenesis proceeds [100]. During early differentiation, primordial germ cells (PGCs) in the inner cell mass develop from a population of pluripotent cells within the extra-embryonic mesoderm [100]. The PGCs migrate through the allantois to the developing genital ridge where they undergo gametogenesis [100]. Before PGCs migrate, at embryonic Day 7 (E7) in the mouse, PGCs and somatic cells display the parentally inherited methylation imprints [75]. Erasure of these methylation imprints occurs between E8 and E12.5 in PGCs [122]. Genome-wide demethylation occurs in murine PGCs around E11.5 coinciding with their arrival at the genital ridge [75]. The sex of the embryo does not appear to have an effect on the timing of demethylation or on the amount of methylation lost [123]. The demethylation process is completed between Days E13 and E14 [75]. It has been suggested that this rapid loss of DNA methylation is due to an active targeted process of DNA demethylation [123]. This demethylation event corresponds to the period during which gametogenesis is arrested [100]. It has been suggested that mitotic arrest in male gametes and meiotic arrest in female gametes occurs following demethylation and may provide protection against replication of unmethylated DNA that may result in mutations from movement of unrepressed retro-transposon [124].

Acquisition of Imprints

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 [125]. Methylation imprints are acquired at different developmental periods in the germ lines [79]. Paternal-specific imprints are progressively acquired throughout gestation by male

gonocytes and are completed by the pachytene phase of meiosis [126, 127]. In contrast, maternal-specific imprints are acquired during the post-natal oocyte growth phase [128, 129]. Sex-specific imprints are thought to be established by de novo methyltransferases (Dnmts) [79]. Dnmt1, Dnmt3a and Dnmt3b are the DNA methyltransferases responsible for establishing and maintaining methylation imprints at imprinted loci [130]. Dnmt3L (Dnmt3-like) is a methyltransferase related to Dnmt3a and 3b, but lacks a catalytic subunit [130]. Expression of *Dnmt3L* only occurs in germ cells and only when there is active de novo methylation [89].

In the male germ line, DNA methylation begins to increase between 15.5 and 18.5 days of gestation corresponding to the time gonocytes initially acquire methylation imprints [79]. The exact mechanism by which methylation imprints are acquired in the male germ line is unknown [120]. It is predicted that Dnmt1 is not involved in the acquisition of paternal imprints because it is not expressed in gonocytes between E15.5 and E18.5 when paternal imprints are beginning to be acquired [131]. In contrast, *Dnmt3a* and *Dnmt3L* are expressed in gonocytes and have been identified as the predominant de novo methyltransferases that initially methylate imprinted sequences in the male germ line [88, 90, 132]. Loss of Dnmt3a or Dnmt3L in prospermatogonia resulted in spermatogenic failure and infertility [90, 133]. Expression of *Dnmt3b* is known only to be necessary for imprinting at the *Rasgrf1* (RAS protein-specific guanine nucleotide-releasing factor 1) locus [88, 133]. Interestingly, in the post-natal testis *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3L* are all expressed and developmentally regulated [131, 134, 135]. The paternal imprints established in the male germ-line are then perpetuated throughout the rest of male germ-cell development [125]

In the female germ line, DNA methylation begins to increase postnatally as primordial follicles enter the growing population [100]. Maternal specific imprints are acquired in the growing oocyte [100]. Based on bisulfite analysis, methylation patterns in the oocyte are established in a gene-specific manner [128, 136]. Expression of an oocyte specific form of *Dnmt1* (*Dnmt1o*) was thought to be an integral part of imprint acquisition in oocytes [137]. However, loss of *Dnmt1o* in mice demonstrated that Dnmt1o is not required for the establishment of methylation imprints in oocytes [65]. In contrast, loss of *Dnmt3a* or *Dnmt3L* in the female germ line by gene targeting resulted in a failure to establish maternal methylation imprints [88, 89]. Therefore, Dnmt3a and Dnmt3L have essential roles in acquisition of maternal imprints [125]

Maintenance of Imprints

In mammals, a fertilized oocyte is capable of epigenetically modifying the maternal and paternal genomes [121]. Fertilization induces the oocyte to resume meiosis forming the haploid maternal pronucleus and extruding the second polar body [58]. The sperm nucleus decondenses and protamines are exchanged for nucleohistones that are derived from the oocyte's cytoplasm [138, 139]. As the embryo develops through the early cleavage stages, global DNA demethylation occurs across most of the genome with the exception of imprinted genes and some repetitive sequence elements [79]. Active and rapid demethylation of the paternal genome occurs within hours of fertilization [140, 141], while the maternal genome appears resistant to this demethylation and, instead, is passively demethylated [142]. Active demethylation of the paternal genome occurs prior to DNA

replication of the paternal genome [143]. It has been suggested that the speed at which the paternal genome is demethylated is indicative that this process is actively mediated by unidentified DNA demethylases [143]. Passive demethylation of the maternal genome is a replication-dependent process that appears to result from exclusion of Dnmt1o from the nucleus [144]. Dnmt1o is essential for maintaining methylation imprints on imprinted genes during these demethylation events [65]. Offspring generated from Dnmt1o-deficient mothers died late in gestation with abnormalities related to methylation and expression of imprinted genes [65]. However, Dnmt1o was restricted to the cytoplasm at all stages during pre-implantation development in the mouse, except for the eight-cell stage when it is transported to the nucleus for one cell cycle [79]. Therefore, it remains unclear what enzyme or mechanism maintains DNA methylation at imprinted loci prior to expression of the somatic form of *Dnmt1* on day 7 [79, 145]. De novo remethylation begins coincidentally with the first differentiation event in which cell lineages within the blastocyst give rise to the inner cell mass (ICM) and the trophectoderm [119]. Imprinted genes are thought to be exempt from this process because their methylation imprints are maintained by Dnmt1 [119]. The rest of the embryonic genome is progressively methylated in a species-specific manner coincident with the onset of the maternal-zygotic transition [146, 147]

THE INSULIN-LIKE GROWTH FACTOR FAMILY

The insulin-like growth factor (IGF) family is essential for fetal and placental growth and development [148]. Insulin-like growth factor-1 and -2 (Igf1/IGF1 and Igf2/IGF2) are both powerful mitogens that function as regulators of cell survival, proliferation and

differentiation [149]. The functions of these growth factors are mediated through binding to the type-1 receptor (Igf1r/IGF1R) [148]. In contrast, the type 2 receptor (Igf2r/IGF2R) primarily binds Igf2 and targets it for lysosomal degradation [148]. Igf1 and Igf2 are similar to each other and, to a lesser extent, proinsulin [149]. The type 1 receptor shares close structural homology to the insulin receptor. Igf1r binds Igf1 with a high affinity and Igf2 and insulin with sequentially less affinity. The IGFs are unlike insulin in that they are not restricted to production and secretion by specific cell types [150]. Instead, almost all cell types within the body can produce IGFs [150]. Biological activity of the IGFs is modulated by six insulin-like growth factor binding proteins (IGFbp1 - 6) [151].

IGF Ligands

Igf1 is a trophic factor that circulates during post-natal development in the bloodstream at high levels [149] and at low levels during embryonic development [152]. Therefore, it was thought that Igf1 was essential for postnatal development [149]. However, Igf1 has also been shown to be necessary for organogenesis [153]. The main source of Igf1 in circulation is the liver [150]. In mice, targeted disruption of the *Igf1* gene in the liver resulted in a 75% reduction in circulating Igf1 [154, 155]. Interestingly, loss of hepatic Igf1 did not dramatically decrease postnatal growth illustrating that local production of Igf1 plays a major role in tissue growth [150]. Serum Igf1 levels are primarily regulated by growth hormone (GH) produced from the anterior pituitary [150]. A negative feedback loop forms as serum Igf1 levels rise and exert negative feedback control on the hypothalamus and pituitary gland to inhibit secretion of GH from the anterior pituitary [150].

Igf2 is a mitogen that is highly expressed during fetal development [149]. Synthesis of Igf2 is mostly independent of GH regulation [149]. The *Igf2* gene is imprinted and expression only occurs from the paternal allele while the maternal allele is silenced [156]. During early embryogenesis Igf2 is essential at the fetomaternal interface for development and function of the trophoblast [150]. Loss of *Igf2* expression results in placental insufficiency and low fetal weight [157]. In addition, *Igf2*-null mice are 60% smaller than wild type [153, 156]. Interestingly, these mice grow normally after birth indicating that Igf2 regulates intrauterine growth, but plays a lesser role during postnatal growth [153, 156].

IGF Receptors

The IGF ligands exert their cellular effects on growth by binding to the type-1 receptor [148]. Igf1r is a member of the tyrosine kinase receptor family and exists at the cell surface as a heterotetrameric glycoprotein [149]. The receptor consists of two extracellular α-subunits and two transmembrane β-subunits that are joined together by disulfide bonds [158]. 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 [158]. 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 [151]. Activation of the phosphoinositide 3'-kinase (PI-3K) pathway leads to activation of several downstream substrates that regulate anti-apoptotic effects [150]. 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 [159, 160].

The IGF type-2 / mannose 6-phosphate receptor (Igf2r/M6PR) is different from Igf1r in structure and function [158]. Igf2r/M6PR consists of 15 homologous extracytoplasmic domains, a single transmembrane region and a carboxy-terminal cytoplasmic tail [161]. The receptor has two binding sites for M6P bearing ligands, like lysosomal enzymes and one binding site for Igf2, a non-M6P bearing ligand. Igf2r binds Igf2 with an affinity about 100 times greater than that for Igf1 and does not bind insulin at all [158]. Igf2r/M6PR binds and targets Igf2 for lysosomal degradation and also functions in lysosomal enzyme trafficking, regulation of apoptosis and growth and tumor suppression [162-166]. Furthermore, Igf2r plays an essential role in regulating fetal growth since loss of *Igf2r* expression results in overgrowth and neonatal death in mice [167]. Similarly, fetal overgrowth phenotypes in sheep are associated with reduced *Igf2r* expression [53].

IGF Binding Proteins

In circulation and in other biological fluids, IGFs are found bound to one of six high affinity, insulin-like growth factor binding proteins (Igfbp1 – 6) [150]. The binding proteins have a higher affinity for the IGFs than do Igf1r or Igf2r [168]. Therefore, Igfbps can modulate the biological accessibility and activity of the IGFs [148]. The binding proteins accomplish this by transporting the IGFs from circulation to the peripheral tissues, sequestering a surplus of IGFs in circulation, inhibiting IGF ligand activity or contributing to the intracellular signaling activities of the IGFs [168].

IGF2R/AIR 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 [169]. Igf2r accomplishes this task primarily by binding Igf2 at the cell surface and targeting it for lysosomal degradation [170]. In mice, *Igf2r* is an imprinted gene that is maternally expressed and paternally imprinted [171]. 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* [172, 173]. The promoters for *Slc22a2* and *Slc22a3* are 190 kb and 260 kb downstream of the *Igf2r* promoter [174]. Neither *Slc22a2* or *Slc22a3* are expressed in the embryo, however, both exhibit imprinted expression in the placenta and biallelic expression in adult tissues [173, 175]. In contrast, *Igf2r* is biallelically expressed in the pre-implantation embryo [176] and maternally expressed in all post-implantation tissues [177] except the brain, where biallelic expression is exhibited [178]. The only paternally expressed gene in the cluster, *Air*, is transcribed antisense to *Igf2r* and shares 30 kb of sequence with *Igf2r* [175]. In addition, the *Igfr2*/*Air* cluster contains two differentially methylated regions (DMRs) [175]. DMR1 contains the promoter for *Igf2r* and is methylated on the paternal allele, but not on the maternal allele [175]. DMR2 is located within intron2 of *Igf2r* and contains the promoter for *Air* [175]. On the maternal allele DMR2 is methylated and on the paternal allele it is not [175].

Imprinting Control in the Igf2r/Air Cluster

Parent-specific expression of murine *Igf2r* and neighboring genes within the *Igf2r*/*Air* cluster is regulated by DNA methylation, expression of the non-coding RNA, *Air*, and histone modifications [173, 175, 179-181]. Differentially methylated region 1 (DMR1) contains the promoter for *Igf2r* and acquires a methylation imprint on the paternal allele during post-implantation development [182, 183]. This acquired methylation is apparently a result of imprinting and not a cause [182]. Differentially methylated region 2 (DMR2) is located within intron 2 of murine *Igf2r* and is thought to be the imprint control region (ICR) for the cluster [184]. In addition, DMR2 contains the promoter for the antisense non-coding RNA, *Air* [172]. Methylation of DMR2 occurs during oogenesis on the maternal allele [182] and is maintained during embryogenesis through the period of genome-wide demethylation and remethylation that occurs during cleavage development [185]. A 113 bp imprinting box is thought to establish the methylation imprint at DMR2 by a de novo methylation signal (DNS) and a allele discriminating signal (ADS) within the imprinting box of the maternal allele [186]. During pre-implantation development, murine *Igf2r* is biallelically expressed and the maternal methylation imprint at DMR2 is already in place [182, 185]. This indicates that the presence of methylation on DMR2 is not enough to induce imprinted expression [176, 177]. Furthermore, loss of methylation at both DMR1 and DMR2 results in a complete lack of *Igf2r* expression in mice, indicating that the absence of methylation on the *Ig2r* promoter is alone not enough to induce *Igf2r* expression [64]. The loss of *Igf2r* expression indicates that a hypomethylated state at the DMRs inhibits *Igf2r* expression. In the mouse,

expression of *Air* ncRNA from its promoter in the unmethylated DMR2 of the paternal allele is known to induce silencing of *Igf2r*, *Slc22a2* and *Slc22a3* [187]. 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 [175]. In the mouse loss of *Air* expression from the paternal allele by deletion of DMR2 results in biallelic expression of *Igf2r*, *Slc22a2* and *Slc22a3* [173, 179, 180]. 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 [187]. 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 [188]. Imprinted expression of *Igf2r*, *Slc22a2* and *Slc22a3* was maintained in mice with a deleted *Igf2r* promoter and lacked any transcriptional overlap with *Air* [188].

DNA methylation at DMR1 and DMR2 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* [178, 183, 189, 190]. Histones form an octamer core around which DNA wraps forming the nucleosome [191]. Amino-terminal tails extend from the histones and are subject to post-translational modifications such as acetylation and methylation [191]. The various combinations of histone modifications are thought to represent a histone code that influences chromatin structure and protein interactions, thereby, influencing transcription [191]. Acetylation of histones H3 and H4 are associated with an open chromatin state and active transcription [191]. 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 [191].

In murine fibroblasts the active promoters for *Igf2r* and *Air* exhibit histone modifications that are associated with transcriptionally active chromatin [189]. These modifications include tri-methylation of lysine 4 on histone 3 (H3K4me3), di-methylation of lysine 4 on histone 3 (H3K4me2), and acetylation of lysine 9 on histone 3 (H3K9Ac) [189]. The silenced promoters of *Igf2r* and *Air* exhibit repressive histone modifications that include tri-methylation of lysine 9 on histone 3 (H3K9me3) and tri-methylation of lysine 20 on histone 4 (H4K20me3) [189]. In murine liver, allele-specific histone modifications in both DMR1 and DMR2 of *Igf2r* included acetylation of histones H3 and H4, as well as di-methylation of lysine 9 on histone 3 (H3K9me2) [183, 190]. In contrast, in murine neurons *Igf2r* is biallelically expressed and *Air* is not [178]. Interestingly, no allelic differences in histone acetylation and di-methylation at DMR1 are exhibited in these cells [178]. However, in murine glial cells and fibroblasts, *Igf2r* is imprinted and *Air* is expressed [178]. In this case histone acetylation and di-methylation of lysine 4 on histone 3 (H3K4me2) were only found on the maternal DMR1 [178]. Thus, histone modifications reliably mark the promoters of the active and repressed alleles of Igf2r in the mouse [191].

Acquisition of Gametic and Somatic Imprints

DMR2 is the primary, or gametic, imprint of the *Igf2r*/*Air* cluster because it is established during gametogenesis and is maintained during pre-implantation development

when epigenetic reprogramming occurs [182]. Acquisition of methylation at DMR2 during oogenesis signifies the first step to imprinted expression in the *Igf2r*/*Air* cluster [182]. During pre-implantation development, *Igf2r* is biallelically expressed and it is assumed that *Air* ncRNA is not expressed [176, 177]. Additionally, it is assumed that *Air* ncRNA begins to be expressed around the time implantation as the paternal *Igf2r* allele begins to be silenced [176, 177]. It is unclear when *Slc22a2* and *Slc22a3* begin to exhibit imprinted expression in the placenta [175]. However, *Slc22a2* is imprinted in placenta between 11.5 days post coitum (dpc) to 15.5 dpc [173]. Similarly, *Slc22a3* is observed to have imprinted expression 11.5 dpc but does not at 15.5 dpc [173]. Interestingly, neither *Slc22a2* or *Slc22a3* are imprinted in the embryo or adult tissues [175]. Following implantation, a somatic methylation imprint is acquired at DMR1 on the paternal allele [182]. Acquisition of DNA methylation and repressive chromatin modifications at DMR1 maintain transcriptional silence of paternal *Igf2r* [189, 190]. The somatic imprint at DMR1 is not completed until after birth [182]. It is thought that this imprint does not directly cause paternal silencing of *Igf2r*, but rather, this imprint is the result of silencing induced by the expression of *Air* ncRNA [192].

Antisense to Igf2r (Air)

Antisense to the Igf2 receptor (*Air*) is an antisense non-coding (nc) RNA found in the mouse that regulates imprinted expression of three protein coding genes in cis [184]. Expression of the 108 kb *Air* ncRNA exerts a silencing effect across more than 300 kb affecting the expression of *Igf2r*, *Slc22a2* and *Slc22a3* [187]. It remains unclear how *Air*

ncRNA induces silence in cis on the *Igf2r*/*Air* cluster. Several models have been proposed to explain how *Air* mediates gene silencing in the *Igf2r*/*Air* cluster. In the expression competition model it is proposed that *Igf2r*, *Slc22a2*, *Slc22a3* and *Air* are all competing for common factors required for promoter and enhancer activation [117]. Transcription of one gene in the cluster may modulate expression of the other genes in the cluster by reducing their access to the necessary common factors [117]. However, this model is likely not correct because the truncated version of *Air* can still be expressed *in cis* with the other genes in the cluster [187].

In the RNAi model it is proposed that the transcriptional overlap between *Air* and *Igf2r* could result in silencing of the *Igf2r* promoter by RNAi-mediated processes [181]. The silent chromatin state induced at the *Igf2r* promoter could then be spread to *Slc22a2* and *Slc22a3* by recruitment of unknown factors that act to suppress expression [181]. 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 [188].

Similar to X-inactivation, in the RNA-directed targeting model it is proposed that the *Air* ncRNA is localized to the *Igf2r*/*Air* cluster and attracts repressive chromatin proteins to the other genes in the cluster [181]. This is similar to the model proposed for X-inactivation, however, the ability of *Air* to recruit silent chromatin has not been demonstrated based on analysis of DNase I hypersensitivity sites located within the *Igf2r* and *Air* genes [193]. In addition, it has recently been shown that heterochromatin formed on the silenced promoters of *Igf2r* and *Air* are limited to regions of 2 to 6 kb and do not spread over the length of the silenced allele [192].

In the transcriptional interference model it is proposed 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 [181]. The instability of *Air* ncRNA supports the transcriptional interference model because instability is associated with a lack of splicing [181]. Therefore, the absence of splicing may trap the Air ncRNA close to the site of transcription on the paternal allele and prevent it from acting in trans on the maternal allele [181].

DNA methylation of the *Air* promoter is required to silence *Air* expression on the maternal allele [181]. In murine embryos, loss of DNA methylation results in greatly reduced levels of Igf2r mRNA, as well as, a doubling of *Air* ncRNA expression [64, 181]. Decreased DNA methylation allowed biallelic expression of *Air* and subsequent silencing of *Igf2r* on the maternal and paternal alleles [181]. In contrast, aging mice exhibit de novo methylation on DMR2 of the parental alleles without any change to DMR1 [194]. 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 [194]. Together these studies illustrate that *Igf2r* and *Air* expression can be very sensitive to changes in methylation patterns as well as of their corresponding levels. Similarly, *Igf2r* and *Air* expression are sensitive to changes in histone modifications [195]. The expressed alleles of *Igf2r* and *Air* exhibit higher levels of histone acetylation than their silenced counterparts [195]. Treatment of cells with the deacetylase inhibitor, trichostatin A (TSA), resulted in both increased

acetylation of histones and decreased methylation of DNA demonstrating that histone acetylation and DNA methylation are interdependent [195]. TSA relaxed imprinting of *Igf2r* but stimulated the relaxation of *Air* imprinting to a greater degree [195]. Therefore, factors apart from DNA methylation and histone acetylation may be involved in imprinting of *Igf2r* and *Air* [195].

EVOLUTION OF *IGF2R* IMPRINTING

Parent-of-origin specific expression of the insulin-like growth factor type 2 receptor (*IGF2R*) is thought to have first appeared in the mammalian lineage between 180 and 210 million years ago (MYA) [196]. The appearance of imprinted *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 [196, 197]. Imprinted expression of *Igf2r*/*IGF2R* has been demonstrated in most mammals including mice [171], rats [198] sheep [53], cows [199], pigs [191], dogs [200] and opossums [201]. Animals that are more ancestral to marsupials exhibit biallelic expression of *IGF2R* and include monotremes [202] and aves [203]. Interestingly, all mammals in the Euarchonta clade also exhibit biallelic expression of *IGF2R [204]*. These include the tree shrew, flying lemur, ringtail lemur, and humans [204].

Evolutionary Pressure for Imprinted Expression of Igf2r/IGF2R

Genomic imprinting of *Igf2r/IGF2R* and *Igf2/IGF2* appear to have evolved in mammals in accordance with different reproductive strategies [196]. *IGF2R* and *IGF2* are not imprinted in monotremes such as the platypus and echidna [202, 205] that lay eggs and

secrete milk from their abdomens [196]. Concurrent with the divergence of marsupials and eutherians from monotremes is the appearance of viviparity and imprinted expression of *Igf2r/IGF2R* and *Igf2/IGF2* [196]. Both *IGF2R* and *IGF2* are imprinted in marsupials [202, 206, 207]. These species have a non-invasive choriovitelline placenta [208], short gestation period and give birth to altricial young [202]. Similarly, imprinting of *Igf2r/IGF2R* and *Igf2/IGF2* is exhibited by eutherians [53, 209] which are true placental mammals. However, loss of imprinted *IGF2R* expression occurred in primates around 75 MYA [191] while imprinted expression of *IGF2* is maintained [205]. Interestingly, *IGF2R* imprinting in humans appears to be polymorphic in a small subset of the population [210, 211]. *IGF2R* serves as a tumor suppressor gene and 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 [191, 212-214]. 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 [204].

Currently, there are several theories that have been proposed to explain the evolutionary pressure that may have stimulated creation of imprinted gene expression [76, 191, 196]. Based on the ovarian time bomb hypothesis, it is proposed that an allele favoring imprinted expression would suppress malignant trophoblastic disease resulting from parthenogenesis of unfertilized oocytes [215]. Based on this hypothesis only a small subset of genes necessary for embryonic development would be imprinted; and therefore, this

hypothesis fails to account for genes regulating post-natal development [76]. 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 [216]. The theory states that the investment made in offspring is different between males and females resulting in different selective pressures on the parental alleles [217]. 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 [196]. Based on the conflict hypothesis, a ancestor of mammals may have evolved imprinted expression of *Igf2r* and *Igf2* as a result of conflict between the parental genomes [196]. 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 [202].

Species Differences in Requirements for Imprinted Expression of Igf2r/IGF2R

Imprinted expression of *Igf2r* has been widely studied in the mouse [172, 187, 188]. 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) [186]. However, an imprinting box has not yet been identified in any other species [191]. DMRs are composed of cytosine guanine repeats (CpGs) that are differentially methylated between the parental alleles. In the mouse, CpG1 corresponds to DMR1 and CpG2 corresponds to DMR2. In contrast to the mouse, the opossum, which is a marsupial, does not have a CpG2 island comparable to the CpG2 island in DMR2 of the mouse [201]. However, although the opossum does have a CpG1 island orthologous to the CpG1 island in DMR1 of the mouse [201], it is not differentially methylated [201]. In addition, *Air* ncRNA is not detected in the opossum [201]. Therefore, none of the known requirements to imprint *Igf2r* in the mouse exist in the opossum [201]. Notably, IGF2R in the opossum binds IGF2 with far less affinity than that observed in the mouse [218]. 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 [196]. Therefore, this would potentially result in less pressure for a strong imprinting response from the maternal allele.

The Artiodactyla clade contains sheep, cows and pigs all of which exhibit imprinted expression of *IGF2R* and *IGF2* [53, 209, 219]. The ruminants both display differential methylation on the CpG islands of *IGF2R* consistent with DMR1 and DMR2 of the murine *Igf2r* gene [53, 199]. It is not yet determined if the porcine *IGF2R* gene contains CpG islands and differential methylation [191]. It is also currently unkown if any of the artiodactyls express *AIR* ncRNA [191].

Recently, it was determined that the canine *IGF2R* gene is imprinted [200]. Dogs belong to the superordinal group called Laurasiatheria, which is a sister group to the superordinal group that contains both rodents and primates [200]. The canine *IGF2R* appears to be similar to murine *Igf2r* gene in that it does have a CpG2 island that exhibits differential methylation [200]. 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 [200].

The human *IGF2R* gene has been extensively studied [178, 211, 220-222]. *IGF2R* in the human exhibits similar elements to the mouse for regulating imprinted expression of *Igf2r*. *IGF2R/Igf2r* in both humans and mice is differentially methylated at the CpG2 island of DMR2 in intron 2 of *IGF2R/Igf2r* [191, 211]. Additionally, *IGF2R/Igf2r* in both humans and mice have a CpG1 island that contains the promoter of *IGF2R/Igf2r* [191]. However, the CpG1 island of human IGF2R does not display differential methylation [191]. In contrast to all other eutherian mammals and despite similar elements necessary for imprinted expression of *Igf2r* in the mouse, human *IGF2R* is biallelically expressed in most of the population [211, 221]. Additionally, it has also been shown that human *AIR* ncRNA does not exist [211, 223]. Therefore, methylation at DMR2 is not sufficient to induce imprinted expression of *IGF2R*.

Histone modifications such as methylation and acetylation may more accurately predict the imprinting status of IGF2R than DNA methylation. 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) [183, 189, 190]. Enriched acetylation of histones H3 and H4 on the promoters of both parental

alleles corresponds to the biallelic expression of *IGF2R* in humans [191]. Furthermore, differential patterns of acetylation and methylation between the parental alleles do not occur at the DMR2 of human *IGF2R* [178, 189].

Histone modifications and DNA methylation may work together in marking specific promoters for expression or silencing [191]. Expression may result from histone acetylation and H3K4me on a promoter in combination with the loss of H3K9me3 and DNA methylation [191]. Losing H3K9me3 and DNA methylation from the promoter without addition of histone acetylation and H3K4me may only result in an unrepressed chromatin state but may not facilitate transcription [191]. For example, human *IGF2R* contains an unmethylated DMR2 on the paternal allele, which lacks expression of *AIR* ncRNA [191]. Therefore, the human DMR2 may only lack histone modifications necessary to promote active transcription of *AIR* ncRNA [191].

Igf2r/IGF2R is imprinted in marsupials and in all eutherian mammals except primates [191]. Imprinted expression of *Igf2r/IGF2r* and *Igf2/IGF2* appears to have evolved with the placenta [76, 224]. Currently, there have not been any imprinted genes discovered in egg laying animals [196]. Sex-specific expression of genes appeared with the divergence of marsupials and eutherians from the monotremes [196]. Placental evolution from the non-invasive form seen in marsupials to the very invasive form seen in rodents may have contributed to the selective forces mediating imprinted gene expression of *Igf2r/IGF2R [202]*. However, humans also have invasive placentation and biallelic expression of *IGF2R [211]*. The occurrence of imprinted expression of *IGF2R* in a small subset of the human population indicates either that the imprinted expression of *IGF2R* is re-emerging or that

there are imprinted alleles still circulating in the population [191, 210]. An ancestor of primates may have had a selective advantage of biallelically expressing *IGF2R* over those that were monoallelically expressing *IGF2R*. IGF2R is a regulator of fetal growth, a suppressor of cell proliferation and is involved in T-cell mediated apoptosis [204]. A primate ancestor that inherited an imprinted *IGF2R* allele would potentially be subjected to a greater risk of fetal overgrowth and carcinogenesis due to haploinsufficiency [204]. Thus, the original evolutionary pressures that created imprinted *IGF2R* expression in the ancestor of marsupials and placental mammals may have been over come by another selective force favoring biallelic expression of *Igf2r/IGF2R*.

IMPLICATIONS OF GENOMIC IMPRINTING DYSREGULATION IN AOS

Bovine fetuses generated from the transfer of embryos that have been manipulated by techniques such as nuclear transfer and, to a lesser extent, in vitro production exhibit a variety of abnormalities including fetal overgrowth, cleft palate, altered energy metabolism, increased perinatal mortality, increased gestation length, hydrallantois, and alterations in placental morphology [11, 13, 28, 31, 68, 225]. Collectively, the abnormalities observed in fetuses and offspring generated from the transfer of in vitro manipulated embryos are called the Abnormal Offspring Syndrome (AOS) [5]. These abnormalities may arise from in vitro manipulation of embryos resulting in aberrant expression of imprinted and non-imprinted genes that are developmentally important for fetal and placental growth [226]. Several studies in mice [227, 228], sheep [53, 219] and cattle [226, 229] demonstrate that nuclear transfer techniques and embryo exposure to culture result in aberrant expression of imprinted

genes. DNA methylation and histone modifications are very dynamic during pre-implantation development and function to modulate chromatin structure and regulate transcription [143]. The pre-implantation embryo is highly sensitive to the external environment and exposure to culture or in vitro manipulation alters the pattern of DNA methylation and histone modifications of imprinted alleles, resulting in aberrant mRNA expression [230].

Disruption of DNA Methylation

Expression of imprinted genes is dependent upon DNA methylation to mark the parental alleles in a sex-specific manner [230]. During pre-implantation development, methylation imprints are maintained as the parental genomes first undergo global demethylation which is then followed by remethylation [230]. Embryo culture and in vitro manipulation can affect imprinted gene expression by disrupting DNA methylation imprints [120, 219, 227, 231, 232]. Therefore, some of the phenotypes associated with AOS may be attributed to aberrant expression of imprinted genes [37, 54].

Epigenetic reprogramming errors may occur during erasure, acquisition or maintenance of DNA methylation imprints [120]. Disruption of methylation imprints may subsequently silence an imprinted gene or induce biallelic overexpression [226]. Aberrant expression of imprinted genes within the developing embryo can abnormally influence cellular differentiation and proliferation [226]. Previously, it has been reported in IVP mice [227, 232] and sheep [53] that abnormal expression of imprinted genes was associated with AOS phenotypes. Altered methylation imprints that induce abnormal expression of

imprinted genes have also been observed following SCNT [62, 228, 233, 234]. Furthermore, cloned mouse, sheep and bovine embryos and offspring exhibit imprinting-related abnormalities that are thought to be derived from incomplete epigenetic reprogramming [53, 62, 229, 235-237].

Aberrant expression of imprinted genes associated with abnormalities in the placenta have also been described following the transfer of IVP [238] or SCNT embryos [62, 226, 239-241]. Abnormal placentation has been suggested to be the major cause of failed NT pregnancies [242]. In pre-implantation SCNT-derived embryos, aberrant methylation patterns have been mostly found in the trophectoderm, indicating that dysregulation of the extraembryonic lineages may be a major contributor to the inefficiency of SCNT [243].

Perturbed Histone Modifications

Altered histone modifications caused by the effects of in vitro culture and somatic cell nuclear transfer (SCNT) may contribute to some of the phenotypes associated with abnormal offspring syndrome (AOS). Post-translational modifications of histone tails in association with DNA methylation mediate remodeling of chromatin structure and gene expression and are crucially involved in regulating epigenetic reprogramming in the gametes and embryo [96]. Expression of imprinted and non-imprinted genes in embryos has been shown to be perturbed by the effects of in vitro culture and SCNT [25, 244-247]. This indicates that mechanisms regulating gene transcription may be impaired during epigenetic reprogramming [248]. In general, methlation of lysine residue 4 on histone H3 (H3K4me) and acetylation of the N-terminal tail on nucleosome core histones is associated with

hypomethylated DNA, an open chromatin state, and increased gene transcription [96]. In contrast, methylation of lysine residue 9 on histone H3 (H3K9me) and deacetylated core histone tails are associated with hypermethylated DNA, a closed chromatin state, and repressed gene transcription [96]. Acetylation and deacetylation of histone tail residues occurs by the actions of two groups of histone modifying enzymes, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs) [248]. Methylation of histone tail residues occurs by the actions of histone methyltransferases (HMTs) [96]. It has been demonstrated that before and after embryonic genome activation, in vitro culture environments and cloning procedures aberrantly affect the patterns of mRNA expression for histone modifying enzymes resulting in altered histone modifications [248, 249]. In murine clones, the pattern and level of histone acetylation varies according to the type of donor cells used [250]. Similarly, perturbed histone acetylation has been observed in SCNT-derived bovine embryos [234]. Additionally, bovine SCNT embryos exhibited altered histone acetylation and methylation patterns in conjunction with delayed and decreased DNA methylation [251, 252]. Thus, the effects of both in vitro culture environments and SCNT procedures can aberrantly affect patterns of histone modifications and potentially contribute to altered gene expression ultimately resulting in AOS phenotypes.

Contribution of Imprinted Expression of IGF2R to AOS

Some abnormal phenotypes associated with AOS in ruminants are similar to the abnormalities observed in mice. In the mouse, abnormal phenotypes have been associated with abnormally expressed genes that are developmentally important for fetal and placental

growth, such as the insulin-like growth factor family [153, 157, 164, 253]. Transgenic mice that overexpress *Igf1* were approximately 21% larger at birth than in vivo controls and exhibit organomegaly [253]. Similarly, murine fetuses that overexpress *Igf2* exhibit organomegaly, as well as fetal and placental overgrowth [254]. In addition, murine fetuses inheriting a nonfunctioning insulin-like growth factor type 2 receptor (*Igf2r*) gene died around the time of birth and exhibited major cardiac abnormalities, elevated circulating Igf2 levels, and were 25 to 30% larger than normal siblings [164]. Patterns of mRNA expression for members of the IGF family are also altered in bovine fetuses, placentas and offspring that are derived from the transfer of IVP or SCNT embryos [10, 54, 239, 255-257]. However, only aberrant expression of *IGF2R* in sheep has been shown to be directly correlated with AOS phenotypes [53]. Altered expression of the *IGF2R* gene has been demonstrated in embryos, fetuses, placentas and offspring in cattle [199, 226, 255, 257-261] following the transfer of IVP or SCNT embryos. Thus, aberrant expression of *IGF2R* may contribute to some of the phenotypes associated with AOS; for example, fetal and placental overgrowth.

In cattle, the *IGF2R* gene is imprinted and is expressed from the maternal allele [199]. Expression from this allele is mediated by two differentially methylated regions (DMRs) [199]. Alterations to the methylation patterns of these DMRs were observed following the transfer of SCNT-derived embryos and resulted in altered *IGF2R* expression [199]. In that study, 3 of 5 cloned fetuses were reconstructed using granulosa cells and 2 of them died near term and exhibited organomegaly, fetal overgrowth, and respiratory failure [199]. In addition, they exhibited reduced expression of *IGF2R* expression and hypermethylation of DMR2 compared to controls [199]. The methylation at DMR2 varied widely from 54.5% in

the heart to 99% in the brain, indicating that methylation patterns at DMR2 were regulated in a tissue-specific manner and may be mediated by other regulatory elements as previously reported in the mouse [183, 187, 195, 199].

In the mouse, imprinted expression of *Igf2r* is regulated by DNA methylation at two DMRs, expression of the antisense non-coding RNA, *Air*, and covalent histone modiciations [178, 187, 195]. Placental overgrowth, a common characteristic of AOS, was observed in embryonic Day 9.5 (E9.5) SCNT murine clones that exhibited significantly reduced *Igf2r* expression [240]. In a separate study, murine clones were created using an ES cell line known to produce fetal and placental overgrowth [262]. The cloned fetuses exhibited a 30% increase in weight over controls at Day 17.5 of gestation [262]. This was attributed to the elevated expression of Igf2 in the cloned fetuses at Day 9.5 and Day 12.5 of gestation [262]. In contrast, *Igf2r* expression was not different between clones and controls at Day 9.5, Day 12.5 and Day 17.5 [262]. Interestingly, expression of *Air* ncRNA was significantly higher in clones at Day 17.5 [262]. Therefore, a failure to increase *Igf2r* expression in response to elevated *Igf2* expression may result from the silencing effects of *Air* on *Igf2r* and may ultimately result in fetal and placental overgrowth late in gestation. Sheep also share similar methylation imprints at DMR1 and DMR2 as those identified in mice and cattle [199]. IVP-derived ovine fetuses exhibiting AOS showed a reduction of 30-60% in IGF2R mRNA expression relative to the controls [53]. Methylation at DMR2 in 9 of the 12 AOS fetuses was completely lost [53]. Similarly, in *Dnmt1* null mice DMR2 is unmethylated and *Igf2r* expression is significantly reduced [64]. Full-term cloned bovine fetuses have been observed

to be hypomethylated at the DMR2 of *IGF2R* in liver, brain and heart, whereas DMR2 in the lung was hypermethylated [199]. If loss methylation at DMR2 is associated with a reduction of IGF2R, then organomegaly observed in AOS fetuses may result from loss of methylation at DMR2 of *IGF2R* in those organs. Interestingly, when murine fibroblasts were cultured in the presence of trichostatin A (TSA), a deacetylase inhibitor, *Air* expression was dramatically increased whereas *Igf2r* expression was only slightly increased [195]. Increased histone acetylation at DMR1 should have allowed for an increase in Igf2r expression; however, it resulted in hypomethylation of DMR2 and a dramatic increase in *Air* expression [195]. This may have inhibited the expression of *Igf2r* despite the open chromatin state [195]. Interestingly, when a bovine kidney cell line was cultured with TSA, *IGF2R* expression was decreased [199]. These observations potentially indicate that relaxation of *IGF2R* imprinting may have been inhibited by a relaxation of imprinted expression of bovine *AIR*.

The level of *IGF2R* expression has consistently been shown not to differ between in vivo, IVP and SCNT derived bovine blastocysts [67, 226, 244, 263-266]. Recently, the level of *IGF2R* expression was shown to be increased in the placentas of gestational day 25 bovine concepti derived from SCNT and IVP embryos compared to in vivo controls [226]. The increase in *IGF2R* expression observed in the placenta at Day 25 may be in response to the high expression levels of IGF2 observed in both SCNT derived placentas and fetuses [226]. Alterations in expression of *IGF2R* within the placenta may contribute to abnormal development and function of the placenta, which has been proposed as one of the major contributing factors to loss of SCNT embryos [267] and also a major phenotype of AOS [5].

Imprinted expression of *Igf2r/IGF2R* is altered in fetuses and placentas derived from the transfer of IVP and SCNT of murine [240, 262, 268], ovine [53, 219, 269] and bovine [199, 226, 257] embryos. In the mouse, manipulation of regulatory elements that control *Igf2r* expression, including DNA methylation at DMR2 and expression of *Air*, result in aberrant expression of *Igf2r* [181, 195, 262]. In sheep and cattle, it is unknown if *AIR* ncRNA is involved in the regulatory mechanism mediating imprinted expression of *IGF2R* [53, 199]. However, DMR1 and DMR2 are present and hypo- or hyper-methylation of these two sites results in aberrant expression of *IGF2R* in sheep and cattle [53, 199]. Improper expression of *Igf2r* resulting in AOS-like phenotypes is associated with mutations or deletions of *Igf2r* in the mouse [64, 153]. In addition, repressed *IGF2R* expression was demonstrated in AOS ovine fetuses [53]. Thus, some phenotypes of AOS displayed by bovine fetuses and placentas may result from aberrant expression of *IGF2R*.

STATEMENT OF THE PROBLEM

Abnormalities exhibited by fetuses, placentas and offspring derived from the transfer of in vitro produced (IVP) and somatic cell nuclear transfer (SCNT) embryos have been well documented [1, 5, 10, 15, 21, 31, 270]. In the past, the abnormalities were characterized as being part of the Large Offspring Syndrome (LOS) [21]. However, not all fetuses and offspring derived from the transfer of IVP and SCNT embryos exhibit excessive growth [11, 271]. In addition, several other abnormalities have been observed including, but not limited to altered organ growth [17, 272], altered placental morphology [12, 28, 31, 32, 225], longer gestation [18, 273], increased perinatal mortality [273, 274]. Therefore, the term Abornmal

Offspring Syndrome (AOS) better describes the currently known abnormalities associated with the transfer of IVP and SCNT embryos [5]. AOS is a significant obstacle for production of cloned animals and to a lesser extent offspring from IVP embryos. Thus, a large effort is being made to understand the mechanisms that produce AOS phenotypes.

Attempts to understand the underlying mechanisms involved in producing AOS have primarily focused on aberrant expression of imprinted and non-imprinted genes in IVP and SCNT-derived embryos [199, 226, 258]. The insulin-like growth factor type 2-receptor (*Igf2r/IGF2R*) is an imprinted gene that is essential for normal fetal and placental development [153]. In the mouse, expression of the *Igf2r* gene is regulated by two differentially methylated regions (DMRs) and expression of an antisense non-coding RNA (ncRNA), *Air* [179, 187]. Disruption of the methylation patterns at the DMRs are associated with altered *Air* ncRNA expression which, in turn, may contribute to altered *Igf2r* expression in the mouse [181, 262]. Determination of the existence of bAIR and its potential role in regulating imprinted IGF2R expression may assist in understanding mechanisms that result in abnormal phenotypes associated with AOS. Relatively little is known about the mechanisms regulating imprinted expression of bovine *IGF2R*. Recently, it was demonstrated that the bovine *IGF2R* gene exhibits two cytosine-guanine repeat regions (CpG islands) that are differentially methylated and similar in size and placement to the DMRs that regulate imprinted expression of murine *Igf2r* [199].

Because both bovine and murine IGF2R/*Igf2r* exhibit imprinted expression and possess similar methylation imprints, we sought to determine if bovine *AIR* (b*AIR*) exists. If

b*AIR* was found to exist, then we proposed to investigate its similarities to expression of murine *Air* and its potential role in regulating imprinted expression of *IGF2R* in cattle.

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 [153, 157]. The primary function of the Igf2 receptor is to bind Igf2, a powerful mitogen, and target it for lysozomal degradation [163]. The *Igf2r/IGF2R* gene is imprinted in opossums [201], kangaroos [275], pigs [276], cattle [199], sheep [53], dogs [200], rats [191], and mice [171]. The *IGF2R* gene does not exhibit imprinted expression in monotremes [277] and primates [204, 221].

Loss of *Igf2r* expression in mice results in excessive fetal and placental growth, as well as cardiac abnormalities, cleft palate, and increased perinatal mortality [153, 163, 164, 278]. 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), 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 [58]. 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 embyos [37, 199, 245, 279]. Aberrant expression of *IGF2R* was directly correlated to AOS in sheep [53]. Furthermore, bovine fetuses, placentas and offspring exhibiting AOS phenotypes also

exhibited aberrant *IGF2R* expression [226, 257]. Therefore, some of the 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) [179, 199] as well as imprinted expression in a majority of adult tissues [186, 277]. 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 [173, 199]. In mice, DMR2 encompasses the promoter for an antisense non-coding (nc) RNA, *Air*, that is transcribed from the unmethylated DMR2 on the paternal allele [179]. The regulatory function of *Air* is still uncertain; however, it has been identified as necessary for imprinted expression of murine *Igf2r* [187]. If regulation and maintenance of *IGF2R* expression in cattle is similar to that of the mouse, then there is potential for bovine *AIR* to be expressed in bovine fetal tissues.

In mice, loss of the maintenance DNA methylation enzyme, de novo methyltransferase 1 (Dnmt1), resulted in reduced expression of *Igf2r* and increased expression of *Air* [181]. In cattle, altered expression of DNMT1 has been demonstrated in bovine embryos and fetuses as a result of the procedures associated with IVP and NT [67, 280, 281]. Additionally, altered expression of *IGF2R* has been observed in bovine fetuses derived from IVP and NT embryos [257, 259, 265]. Furthermore, altered expression of *IGF2R* in conjunction with hyper- and hypomethylation of DMR2 was demonstrated in bovine clones [199]. Therefore, bovine embryos derived from IVP or from in vitro

manipulation may exhibit aberrant expression of *IGF2R* resulting from altered methylation patterns and altered expression of bovine *AIR*. Further elucidation of the mechanisms regulating 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 bovine *AIR* (b*AIR*) ncRNA exists; second, to determine if b*AIR* ncRNA was altered during developmentally important stages of gestation; and third, to determine if expression of b*AIR* was affected by method of embryo production.

MATERIALS AND METHODS

In Vivo Embryo Production

In vivo embryos used to produce Day 70 bovine fetuses and Day 7 bovine blastocysts were generated using Holstein cows. Cows were synchronized by intra-muscular (i.m.) injection of two doses of 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse; Upjohn Co., Kalamazoo, MI) administered 12 days apart. Between Days 10 and 13 (Day 0 = estrus), donor cows were superovulated by i.m. injection of FSH in decreasing doses over a 4-day period (20 to 32 mg FSH-P, Schering-Plough, Piscataway, NJ; or 400 mg Folltropin, Vetrapharm, Canada, London, ON). Estrus was induced by i.m. injection of 25 mg of $PGF_{2\alpha}$ on the morning and evening of the third day of FSH treatment. The donor cows were artificially inseminated 12 to 24 hours after the first observed standing estrus. Frozen thawed semen was supplied from a proven Holstein bull. Embryos were collected by nonsurgical uterine flushing on Day 7.

In Vitro Embryo Production

Methods used for generating in vitro-produced embryos and Day 70 bovine fetuses have been reported previously [54]. Briefly, ovaries from Holstein cows were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated, matured, and fertilized. Semen from a proven Holstein bull was used to fertilize the matured oocytes. At 18 to 20 hours postinsemination (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.

In vitro produced Day 7 bovine blastocysts generated for comparison with in vivo produced Day 7 blastocysts were created using oocytes from a local abattoir. Cumulus-oocyte complexes were aspirated, matured, and fertilized. Semen from a proven Holstein bull was used for fertilization. Presumptive zygotes were cultured in groups of 20 to 25 in 1 ml of synthetic oviductal fluid (SOF) + 10% ECS. At 72 hpi, the embryos were transferred into fresh SOF + 10% ECS. At 168 hpi, blastocyst stage embryos were recovered and snap-frozen in liquid nitrogen in pools of 5 to 10 embryos.

Production of Day 15 Conceptuses

In vivo embryos used to produce Day 15 bovine conceptuses were selected from frozen-thawed in vivo-produced Charolais embryos. Prior to transfer, frozen embryos were thawed in embryo transfer medium and examined for morphology. After examination blastocysts that were visibly non-viable were discarded and Grade 1 (Excellent to Good) or 2 (Fair) blastocysts were selected were selected for transfer [282].

Transfer of Embryos

Embryos Transferred to Produce Day 70 Fetuses. Embryos generated to produce Day 70 fetuses were transferred into Angus heifers that were given two i.m. injections of 25 mg of $PGF_{2\alpha}$ 10 to 12 days apart to synchronize estrus. On D7 of gestation (Day 0 = estrus) single blastocyst-stage embryos that were in vivo or in vitro produced were transferred nonsurgically into recipients. Only Grade 1 (excellent to good) blastocysts were selected for transfer.

Embryos Transferred to Produce Day 15 Conceptuses. Frozen-thawed Charolais blastocysts were transferred into Holstein cows that were given a single i.m. injection of 25 mg of $PGF_{2\alpha}$, to synchronize estrus. Batches of 10 to 15 blastocyst-stage embryos were transferred nonsurgically into recipient cows on Day 7 of the cycle (Day 0 = estrus).

Recovery of Fetuses and Conceptuses

Recovery of Day 70 Fetuses. At Day 70 of gestation recipients were slaughtered and fetuses were recovered (n=7 in vivo (IVO), n=6 in vitro produced (IVP)). 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 15 Conceptuses. At Day 15 of gestation, conceptuses were recovered by uterine lavage. A three-way catheter was used to introduce approximately 1.5 liters of medium (Complete Flush Medium, BioLife, Agtech, Inc; Manhattan, KS). Fluid recovered from the uteri of recipients was captured in 1 liter sterile bottles. Conceptuses were recovered, evaluated for morphology, measured, snap-frozen in liquid nitrogen and stored at -80°C.

RNA Extraction

Day 70 Fetal Liver. RNA of IVO- and IVP-derived Day 70 bovine fetal liver was extracted as previously described [54]. 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 a RNA isolation reagent (Tri-Reagent, Molecular Research Center; Cincinnati, Ohio) using a ratio of 100mg of tissue per 1ml of TriReagent. The wcRNA was then extracted with chloroform using a ratio of 0.2 ml of chloroform to 1 ml of Tri-Reagent. The wcRNA was precipitated with isopropanol at a ratio of 0.5 ml of isopropanol to 1 ml of Tri-Reagent and then resuspended in diethyl pyrocarbonate-treated water. Concentration of the 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 1.53 ± 0.019, n=13.

Day 15 Conceptuses. Whole cell RNA (wcRNA) was extracted from in vivo produced Day 15 conceptuses (n=9) using the GenElute Mammalian Total RNA Mini-prep Kit (Sigma-Aldrich; St. Louis, MO). Each of the conceptuses was extracted individually according to the manufacturer's specifications. wcRNA was obtained and aliquoted into RNase/DNase-free tubes and stored at -80°C.

Day 7 Blastocysts. Whole cell RNA (wcRNA) was extracted from Day 7 in vivo and IVP blastocysts using the GenElute Mammalian Total RNA Mini-prep Kit (Sigma-Aldrich; St. Louis, MO). The blastocysts were extracted in pools of 2 to 11. The wcRNA obtained was aliquoted into RNase/DNase-free tubes and stored at -80°C.

cDNA Synthesis

Day 70 Fetal Liver. Day 70 fetal liver was used to create cDNA for the purposes of reverse transcription polymerase chain reaction (RT-PCR). Following the manufacturer's instructions, 2µg of total RNA 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.

Day 15 Conceptuses. Previously extracted whole cell RNA from individual Day 15 conceptuses was used to generate cDNA for the purpose of performing RT-PCR. For each conceptus, 7µl of RNA was used to generate cDNA. All other procedures were performed the same as those for cDNA synthesis from D70 fetal liver.

Day 7 Blastocysts. Previously extracted wcRNA from blastocyst pools were used to generate cDNA for the purpose of performing RT-PCR. The blastocyst pools consisted of 2 to 11 blastocysts. For each blastocyst pool, 15µl of RNA was incubated with 2µg of random primers (Promega; Madison, WI), 2µl 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 8µl of 5X First Strand Buffer, 2µl 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 then 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). Embryos were pooled in groups of 24 to 27 for RNA extraction. A 7 µl aliquot of extracted RNA (7.6 ± 0.17 embryo equivalents) was used for cDNA synthesis. In each PCR reaction there were 0.76 ± 0.02 (mean ± SEM) embryo equivalents.

Bovine AIR Primers

Bovine AIR primers were designed using the known bovine genomic DNA sequence (NW_001495620) and placement was determined by referring to the known sequence of Igf2r (NM_010515) and Air (NR_002853) in the mouse. The bAIR primer sets were designed using the primer design program, Vector NTI (Invitrogen). The bAIR3 primer set amplified a region within intron 1 of IGF2R and upstream of the IGF2R exon 2 (Figure 1). The bAIR4 primer set amplified a region outside of the IGF2R gene approximately 1kb upstream of the IGF2R 5'UTR (Figure 1). All PCR amplicons were sequence verified.

Semi-Quantitative RT-PCR

Day 70 Fetal Liver. PCR reactions consisted of a 20µl reaction volume that contained 100ng of cDNA, 10mM dNTP Mix , Taq DNA polymerase (1.25u per 20µl reaction), sense and anti-sense primers (20 ng of each per 20µl reaction), 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, H2A, included a hot start at 92°C for 2 minutes, denaturation for 10 seconds at 94°C, annealing for 10 seconds at 67°C and primer extension for 10 seconds at 72°C (Table 1). The PCR program performed for IGF2R, bAIR3 and bAIR4 included a hot start at 92°C for 2 minutes, denaturation for 15 seconds at 94°C, annealing for 15 seconds at 65°C and primer extension for 15 seconds at 72°C (Table 1). After the last cycle of the program for each primer set an additional 5 minute period at 72°C was induced to allow for maximum primer extension.

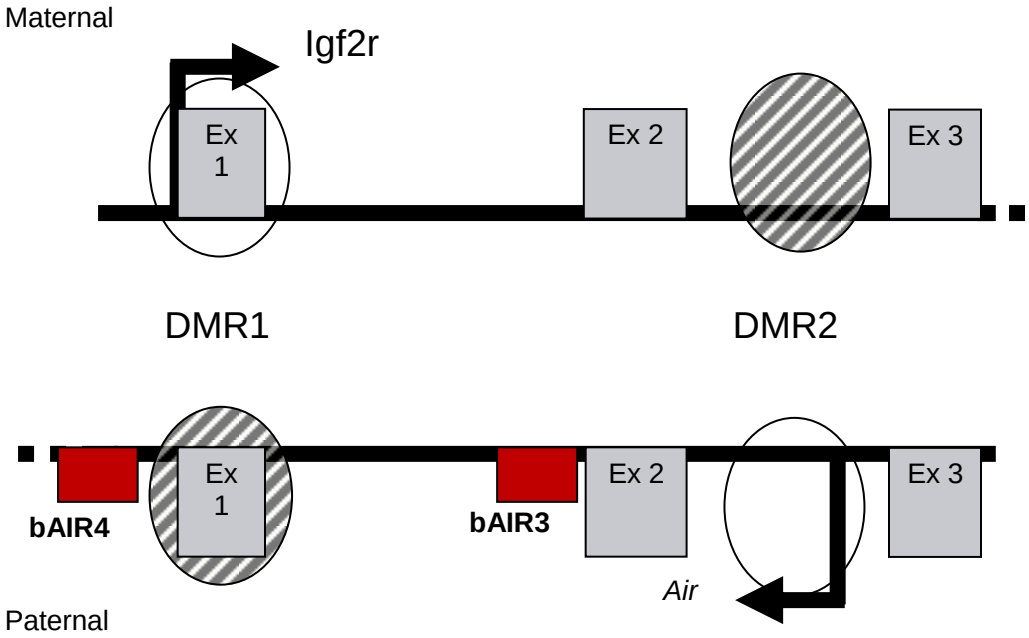

Figure 1. Regulation of the insulin-like growth factor type 2-receptor (*Igf2r*) gene in the mouse. The picture is an illustration of the maternal and paternal alleles of *Igf2r* and the first three exons of *Igf2r*. Differentially methylated region 1 (DMR1) encompasses the promoter for *Igf2r* and is unmethylated on the maternal allele as indicated by the clear circle. DMR1 is methylated on the paternal allele indicated by the striped circle. Differentially methylated region 2 (DMR2) is located in intron 2 of *Igf2r* and encompasses the promoter for antisense to *Igf2r* (*Air*). DMR2 is methylated on the maternal allele indicated by the striped circle. The small box labeled bAIR3 within intron1 of *Igf2r* on the paternal allele depicts the region amplified by the bAIR3 primer set. The small box upstream of the *Igf2r* promoter on the paternal allele indicates the region amplified by the bAIR4 primer set.

Table 1. Primers used for RT-PCR of fetal liver at Day 70 of gestation.

Genes	Primer sequences and positions	Annealing temperature (°C)	Fragment size (bp)	Cycle # for Linear Amplification		
				D7	D15	D70
Histone 2A (H2A.Z)	Forward 5' AGGACGACTAGCCATGGACGTGTG 3' [b] Reverse 5' GTTCCGATGTTAACGACCACCACC 3' [b]	67	208	40	33	30
Insulin-like growth factor 2 receptor (IGF2R)	Forward 5' GAACAGAGATGTGTCCGGGTCAGAGTC 3' [c] Reverse 5' GGGTATAAAGGTGTCTACAACT 3' [c]	65	422	40	37	38
Bovine Antisense to Igf2r – 3 (bAIR3)	Forward 5' GGTTATGGAAGTCTTAAGCTTGAAAGTGGC 3' [d] Reverse 5' TCACGACAGACATACGTCTGAACTTCTG 3' [d]	65	226	40	40	38
Bovine Antisense to Igf2r – 4 (bAIR4)	Forward 5' CCCTGGTGGTCGTGTCTAAG 3' [e] Reverse 5' ACAAACCTGTGGCAATGTGA 3' [e]	65	432	40	40	40

[a]Primer from [54].

[b]H2A forward primer (162 – 185) bp and reverse primer (347 – 370) bp of NM_174809

[c]IGF2R forward primer (768 – 794) bp and reverse primer (373 – 394) bp of NM_174352

[d]bAIR3 forward primer (809715 – 809744) bp and reverse primer (809519 – 809547) bp of NW_001495620

[e]bAIR4 forward primer (829460 – 829479) bp and reverse primer (829048 – 829067) bp of NW_001496520

Day 15 Conceptuses. RT-PCR was performed for H2A, IGF2R, bAIR3 and bAIR4 using cDNA from Day 15 bovine conceptuses. The same procedure previously described for the Day 70 fetal liver was followed for the Day 15 conceptuses except that 0.014 conceptus equivalents of cDNA were used in each PCR reaction.

Day 7 Blastocyst. RT-PCR was performed for H2A, IGF2R, bAIR3 and bAIR4 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 embryo equivalents of cDNA were used in each PCR reaction.

Determination of Linear Phase of Amplification

Day 70 Fetal Liver. For determination of the linear phase of amplification for each primer pair, PCR was performed using a pool of cDNA (100ng each) from all samples tested (n=13). Reactions were conducted for a total of 40 cycles and two reaction tubes were 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. (See Appendix A).

Day 15 Conceptuses. For determination of the linear phase of amplification for the primer pairs of H2A and IGF2R, PCR was performed using a pool of cDNA (0.028 conceptus equivalents from each conceptus, n=9). Reactions were conducted for a total of 40 cycles and two reaction tubes were removed every 5 cycles starting with cycle 25. PCR products were visualized on 1.5% agarose gels and the signal intensity of individual bands was determined using an Alpha-Imager (Alpha Innotech; San Leandro, CA) imaging system. A

response curve was generated for the 2 primer sets (H2A and IGF2R) and the exponential phase of product amplification was determined (See Appendix A). Response curves could not be generated for bAIR3 and bAIR4 because of limited availability of cDNA.

Day 7 Blastocysts. For determination of the linear phase of amplification for the H2A primer sets, PCR was performed using pooled cDNA (1.14 embryo equivalents each from each of 6 pools). Reactions were conducted for a total of 40 cycles, and two reaction tubes were 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 using an Alpha-Imager. A response curve was generated for the H2A primer pair and the exponential phase of product amplification was determined (See Appendix A). Response curves could not be generated for IGF2R, bAIR3 and bAIR4 because of limited cDNA.

Statistics

Relative RNA expression was calculated as the ratio of band intensities of the RNA of interest to that of H2A. Data for relative RNA expression were analyzed by student's t-test [283]. Categorical data on the numbers of embryos and conceptuses at each stage of gestation that expressed bAIR3 and bAIR4 were analyzed using Fisher's Exact test [283].

RESULTS

Detection and Validation of bAIR

The procedural control performed on Day 70 bovine fetal liver demonstrated that the primer sets for the housekeeping gene, *H2A*, as well as *IGF2R* and b*AIR* produced PCR

products that resulted from RNA within the sample and not from genomic contamination (Figure 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 30kb in length and would not be resolved on a 1.5% agarose gel. 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 b*AIR* Expression

Post-Implantation Stage (Day 70 Bovine Fetal Liver). At Day 70 of gestation, all bovine fetal liver samples derived from the transfer of in vivo produced embryos (n=7) exhibited

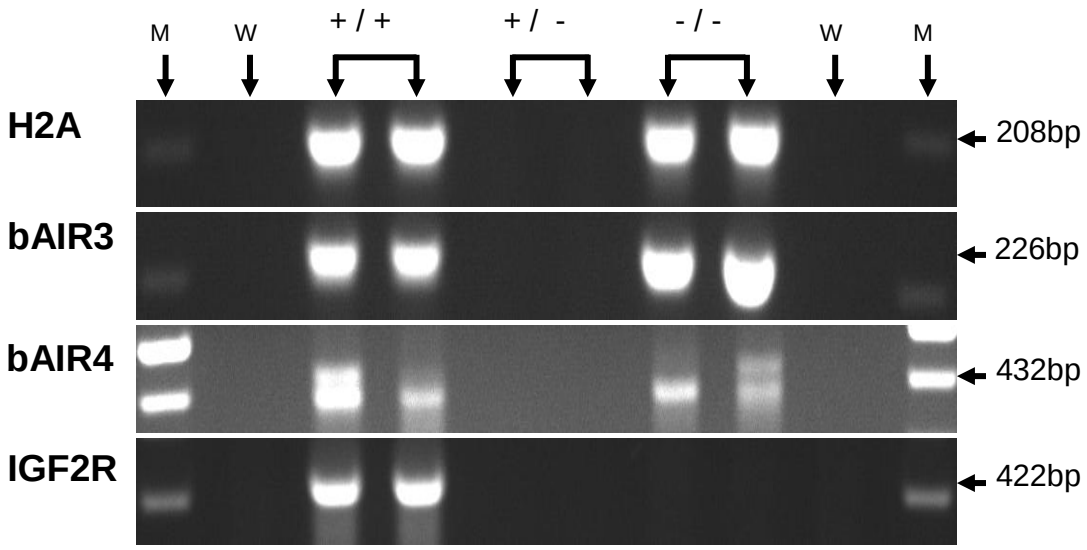

Figure 2. Ethidium bromide-stained agarose gel of H2A, IGF2R, bAIR3 and bAIR4 amplification products from bovine fetal liver at Day 70 of gestation. The (+/+) lanes depict samples of RNA that were DNase treated and were reverse transcribed prior to PCR amplification. The (+/-) lanes depict samples of RNA that were DNase treated were not reverse transcribed prior to PCR amplification. The (-/-) lanes depict samples of RNA that were not DNase treated and were not reverse transcribed prior to PCR amplification.

PCR amplicons for H2A, IGF2R, bAIR3 and bAIR4 (Figure 3). Similarly, all bovine fetal liver samples derived from the transfer of in vitro-produced embryos (n=6) exhibited PCR amplicons for H2A, IGF2R, bAIR3, and bAIR4 (See Appendix B).

Peri-Implantation Stage (Day 15 Bovine Conceptuses). At Day 15 of gestation, a total of 9 conceptuses were recovered. The mean ± SEM length for these conceptuses was 2.2 ± 0.6 mm (Figure 4). All conceptus cDNA samples produced PCR products for the housekeeping gene *H2A* (Figure 5). *IGF2R* amplicons were observed in 8 of the 9 D15 bovine conceptuses (Figure 5). However, only 1 of the 9 conceptuses demonstrated bAIR3 amplification products (Figure 5). In contrast, 8 of 9 bovine conceptuses exhibited PCR products for the bAIR4 primer set (Figure 5).

Pre-Implantation Stage (Day 7 Bovine Blastocyst Pools). At Day 7 of gestation, each in vivo produced blastocyst pool (n = 2) and all of the in vitro produced blastocyst pools (n=4) resulted in the production of PCR amplicons for the housekeeping gene, *H2A* (Figure 6). Similarly, all in vivo- and in vitro-produced blastocyst pools rendered PCR amplicons representing *IGF2R* (Figure 6). However, none of the in vivo- or in vitro-produced blastocyst pools rendered PCR amplicons from the bAIR3 and bAIR4 primer sets (Figure 6).

Effect of Method of Embryo Production on bAIR Expression

The ratio of b*AIR* ncRNA to H2A mRNA expression was significantly reduced in the livers of fetuses from IVP embryos compared to that of in vivo produced embryos ($P<0.05$; Figure 7).

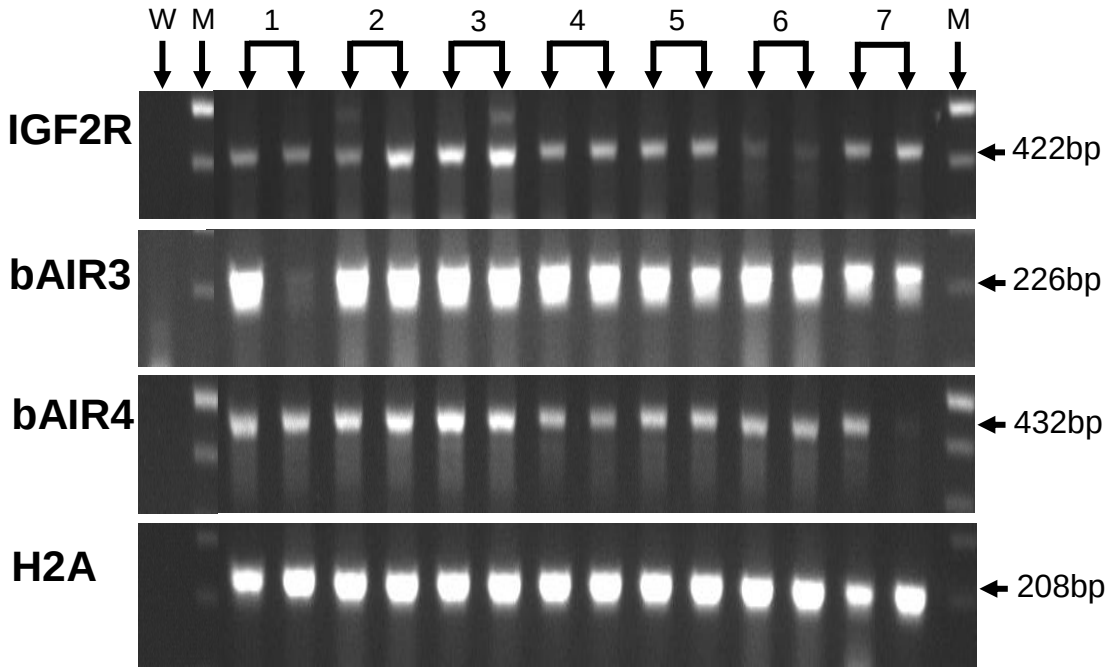

Figure 3. Post-implantation stage gene expression in Day 70 bovine fetal liver. Ethidium bromide-stained agarose gel of IGF2R, bAIR3, bAIR4 and H2A amplification products from bovine fetal liver at Day 70 of gestation derived from the transfer of in vivo (IVO) produced embryos. (W) represents the PCR water blank. (M) represents the 100bp ladder marker. IVO samples are depicted in lanes 1 to 7, and were assayed in duplicate. Each of the 7 IVO fetal liver samples depict amplication products for all RNAs of interest.

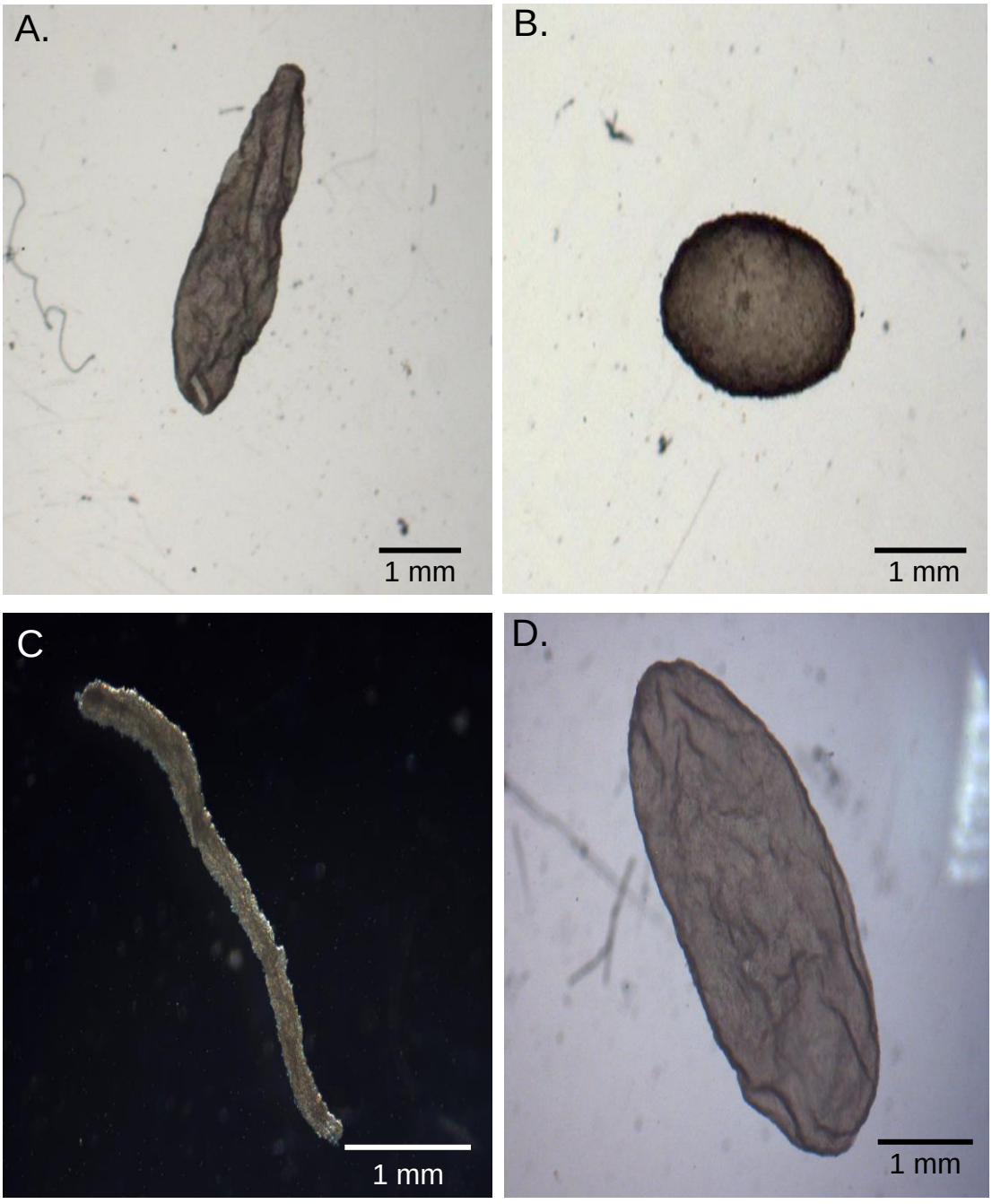

Figure 4. Bovine conceptuses at Day 15 of gestation. The mean ± SEM length of the conceptuses was 2.2 ± 0.6 mm. A) A large conceptus that measured 3.19 mm in length and corresponds to conceptus 2 in Figure 5. B) Small round conceptus measured 1.87 mm in length and correspondes to conceptus 3 in Figure 5. C) Elongated conceptus that measured 3.28 mm in length and corresponds to conceptus 4 in Figure 5. D) A large well defined conceptus that measured 3.83 mm in length and corresponds to conceptus 5 in Figure 5.

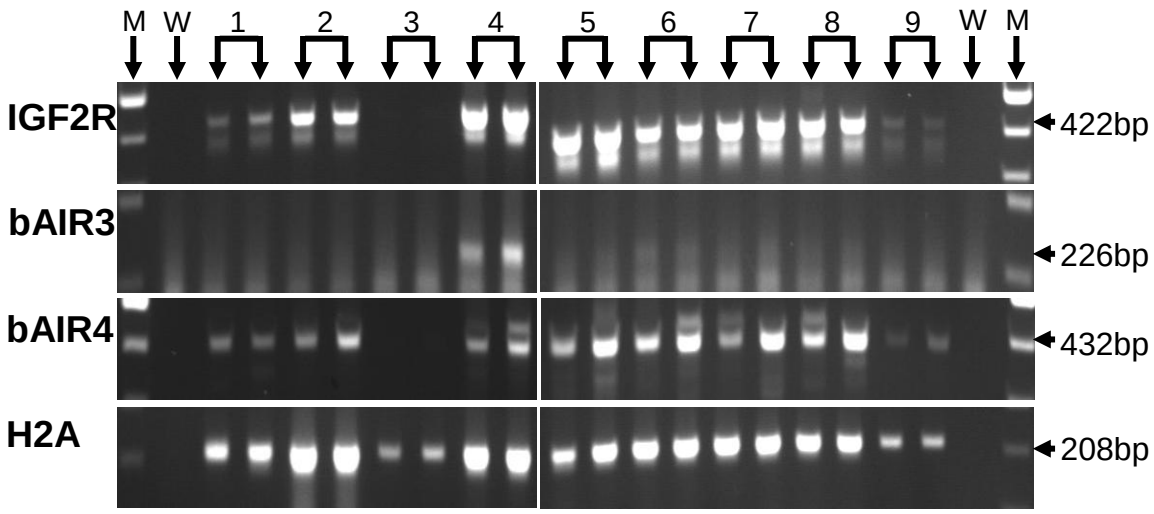

Figure 5. Peri-implantation-stage RNA expression in Day 15 bovine conceptuses. Ethidium bromide-stained agarose gel of IGF2R, bAIR3, bAIR4 and H2A amplification products from bovine conceptuses at Day 15 of gestation derived from the transfer of in vivo-produced embryos. M) 100bp ladder marker. W) PCR water blank. Duplicate PCR reactions for the IVO conceptuses are represented in lanes 1 to 9. All 9 conceptuses demonstrated amplification products for the house keeping gene, H2A. Eight of 9 conceptuses demonstrated amplification products for IGF2R. One of 9 conceptuses had amplification products for bAIR3. Eight of 9 conceptuses show amplification products for bAIR4.

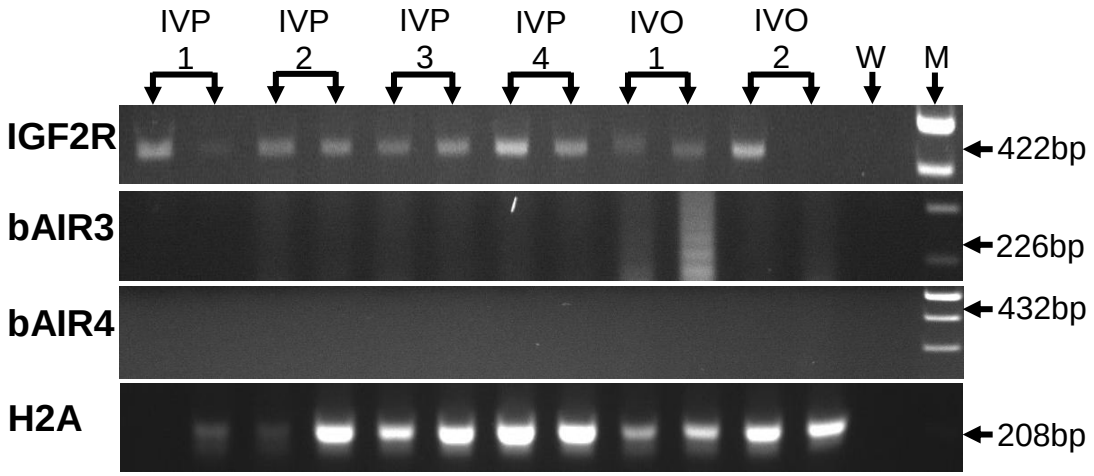

Figure 6. Pre-implantation stage bovine blastocysts at Day 7 of gestation. Ethidium bromide-stained agarose gel of IGF2R, bAIR3, bAIR4 and H2A amplification products from pools of in vitro-produced (IVP) and in vivo-produced (IVO) bovine blastocysts at Day 7 of gestation. Each of the IVP and IVO blastocyst pools examined displayed amplification products for the housekeeping gene H2A as well as IGF2R. None of the IVP or IVO blastocyst pools displayed amplification products for bAIR3 and bAIR4. W) PCR water blank. M) 100 bp ladder marker.

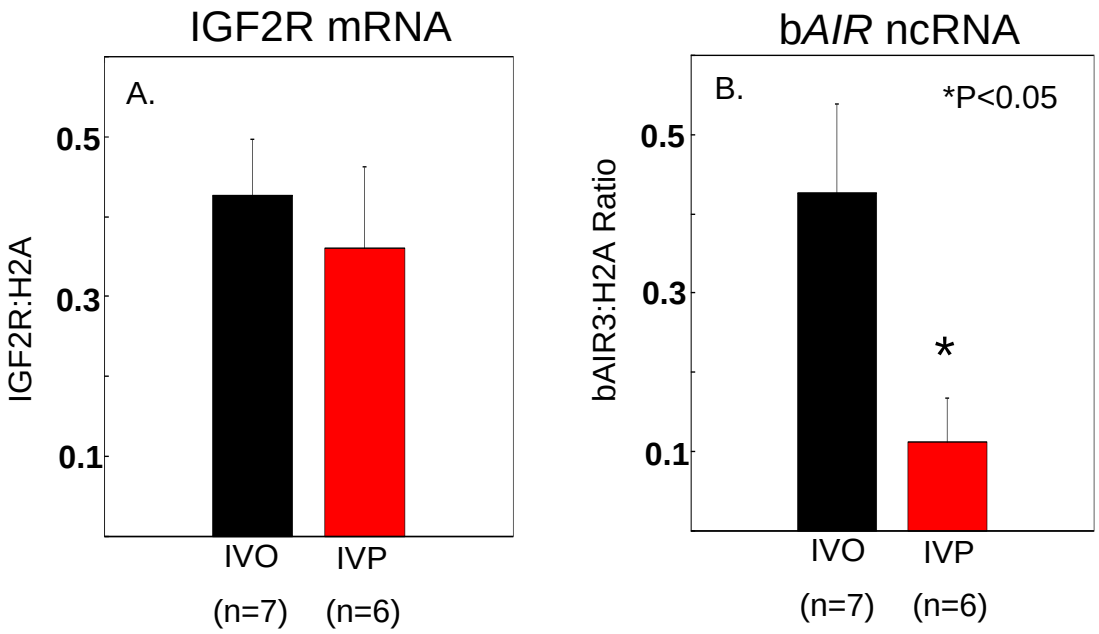

Figure 7. A) Expression of IGF2R mRNA in bovine fetal liver at Day 70 of gestation derived from the transfer of either in vivo- (IVO) or in vitro- produced (IVP) embryos. B) Expression of bAIR ncRNA in bovine fetal liver at D70 of gestation derived from the transfer of either IVO or IVP embryos. Relative RNA expression was calculated as the ratio of band intensities of the RNA of interest to that of H2A.

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 [199, 226, 258]. The insulin-like growth factor type 2-receptor (*Igf2r*) is an imprinted gene that is essential for normal fetal and placental development [153]. In the mouse, expression of *Igf2r* is regulated by two differentially methylated regions (DMRs) and expression of an antisense non-coding RNA (ncRNA), *Air* [179, 187]. Disruption of the methylation patterns at the DMRs is associated with altered *Air* ncRNA expression, which may contribute to altered *Igf2r* expression [181, 262]. However, little is known about the mechanisms regulating imprinted expression of bovine *IGF2R*.

In the present study, we have demonstrated for the first time that bovine *AIR* (b*AIR*) non-coding RNA (ncRNA) does exist. PCR primer sets were designed based on murine *Air* 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 *Air* 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 the ncRNA, *Air* [187]. Similarly, it has recently been demonstrated that the bovine *IGF2R* gene also exhibits imprinted expression in post-implantation tissues [199]. In the present study we have shown that (b*AIR*) ncRNA is expressed in post-implantation tissue at Day 70 of gestation in bovine fetal liver. All of the bovine fetal livers generated from the transfer of in vivo produced embryos expressed IGF2R

mRNA, b*AIR* ncRNA and mRNA for the housekeeping gene *H2A*. Two different PCR primer sets for b*AIR* ncRNA were used to detect b*AIR* ncRNA within these samples. The first primer set, bAIR3, amplified a region within intron 1 of IGF2R and upstream of exon 2 of *IGF2r*. The second primer set, bAIR4, amplified a region 1kb upstream of the 5'UTR of the *IGF2R* gene. All Day 70 bovine fetal livers exhibited PCR amplicons for both of the b*AIR* primer sets. Therefore, expression of b*AIR* ncRNA in post-implantation bovine fetal liver is similar to the pattern of *Air* expression that has been observed in murine post-implantation tissues [181, 262]. However, further study will be necessary to determine which of the parental alleles are producing *AIR* ncRNA in cattle.

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 [219]. In the present study, peri-implantation stage bovine conceptuses at Day 15 of gestation generated from in vivo-produced embryos partially expressed b*AIR* ncRNA. PCR amplicons were produced using the bAIR3 primer set from only one of the nine bovine conceptuses. In contrast, PCR amplicons were produced from 8 of the 9 conceptuses using the bAIR4 primer set. These primer sets amplify different regions of the b*AIR* ncRNA which may explain why the bAIR4 set detects a stronger signal for b*AIR* than the bAIR3 set. Transcription of murine *Air* is known to result in one intact ncRNA and several splice variants [181]. These splice variants are exported to the cytoplasm, whereas, the full length *Air* transcript remains localized in the nucleus [181]. The splice variants are not believed to be involved in silencing of *Igf2r* for two reasons [181]. First, each of the splice variants consists of different sequences except for an initial 53bp sequence that is conincidental with intron 2 of *Igf2r*

[181]. Furthermore, none of the variants shares sequence homology with the mature Igf2r transcript. This observation is interpreted to indicate that these variants would not be involved in post-transcriptional regulation of cytoplasmic Igf2r mRNA [181]. Second, these splice variants are exported to the cytoplasm indicating that they may not be able to target the paternal allele for silencing [181]. It is interesting to speculate that if b*AIR* splice variants exist, then they may not share sequence homology with *IGF2R*. Given our observations that bAIR4 amplicons, but not bAIR3 amplicons were produced from D15 conceptus RNA, it may be possible that bAIR4 amplifies a region that is within the splice variants located outside of the *IGF2R* sequence. In contrast, the bAIR3 primer set amplies a region that is only within the mature b*AIR* transcript (Figure 8). Expression of b*AIR* indicated by visualization of bAIR3 PCR amplicons in only 1 of 9 peri-implantation bovine conceptuses may be interpreted to mean that at this stage the mature b*AIR* transcript is not actively transcribed and that *IGF2R* is still biallelically expressed. Expression of b*AIR* ncRNA based on production of bAIR4 PCR ampicons in the majority of peri-implantation bovine conceptuses may be interpreted to mean that bAIR4 amplifies a region that is actively transcribed as part of a b*AIR* splice variant that is not directly involved in inhibiting *IGF2R* expression. To verify this hypothesis, it will be necessary to determine if b*AIR* silences paternal expression of *IGF2R* and if, indeed there are b*AIR* splice variants in cattle.

In mice, the *Igf2r* gene exhibits non-imprinted expression in the pre-implantation embryo [177]. Murine *Air* ncRNA has been assumed to be non-expressed in the pre-implantation embryo because *Igf2r* expression at this stage is biallelic [175]. In agreement

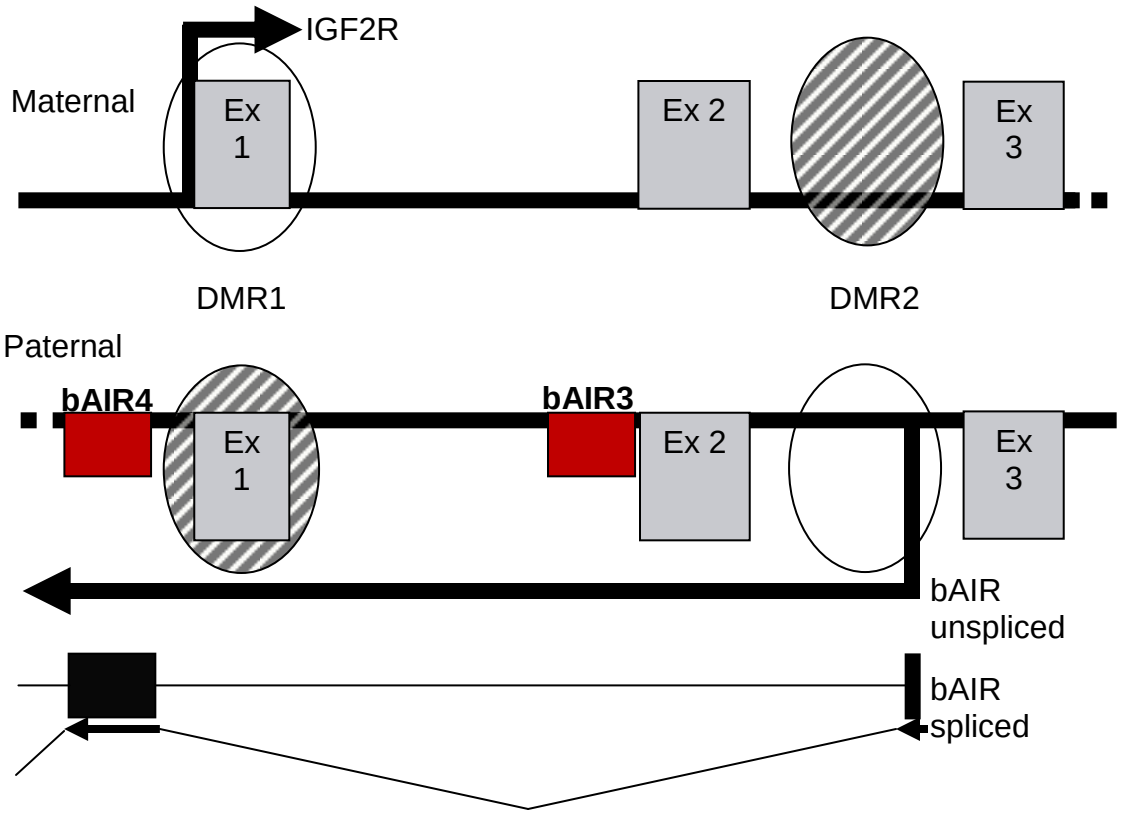

Figure 8. Potential bovine AIR splice variants. The illustration depicts the maternal and paternal alleles of IGF2R. Indicated below are the full-length unspliced AIR and a potential splice variant of bovine AIR. Black arrows: exons; thin black lines: introns: red boxes; regions amplified by bAIR primer sets.

with the concept that *Air* ncRNA is not expressed in murine pre-implantation embryos, we demonstrated that b*AIR* was not expressed in pre-implantation bovine blastocysts. All six blastocyst pools, regardless of in vivo or in vitro origin, expressed mRNA for *IGF2R* and the housekeeper *H2A.* However, b*AIR* ncRNA was not detected in any of the blastocyst pools when either the bAIR3 or bAIR4 primer sets were used. Therefore, *AIR* ncRNA is not expressed in bovine pre-implantation embryos, a stage at which *IGF2R* is thought to be biallelically expressed [191], as has been suggested in the mouse [175].

The expression level of b*AIR* 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 did not differ with respect to expression of IGF2R mRNA. However, expression of b*AIR* 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 [194]. Increased methylation in DMR2 resulted in reduced expression of murine *Air* with no apparent change in the expression Igf2r mRNA [194]. These observations are consistent with the present study and suggest that DMR2 of bovine *IGF2R* may be hypermethylated. Additional studies evaluating methylation levels of bovine DMR2 are needed to determine if this hypothesis is correct.

In summary, here we have demonstrated for the first time that bovine *AIR* does exist. Our observations are consistent with murine data and suggest that b*AIR* may be involved in regulating imprinted expression of *IGF2R* in cattle. Expression of b*AIR* was found at the post-implantation stages in bovine fetal liver. At Day 15 of gestation, b*AIR* is partially expressed in peri-implantation bovine conceptuses and b*AIR* is not expressed in Day 7

bovine blastocysts. Furthermore, we found that b*AIR* ncRNA expression is reduced in livers of bovine fetuses at Day 70 of gestation derived from the transfer of IVP embryos. These observations indicate that b*AIR* ncRNA expression is altered with the method of embryo production. Taken together, these data provide evidence that bovine *AIR* may be involved in regulating imprinted expression of *IGF2R*. Future studies will be necessary to determine if this is true; however, it is interesting to speculate that, if expression of b*AIR* is altered in bovine embryos that are in vitro produced or manipulated, then imprinted expression of *IGF2R* may become perturbed and contribute to some of the abnormal phenotypes associated with AOS.

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APPENDICES

APPENDIX A

RESPONSE CURVES FOR THE DETERMINATION OF THE LINEAR PHASE OF AMPLIFICATION

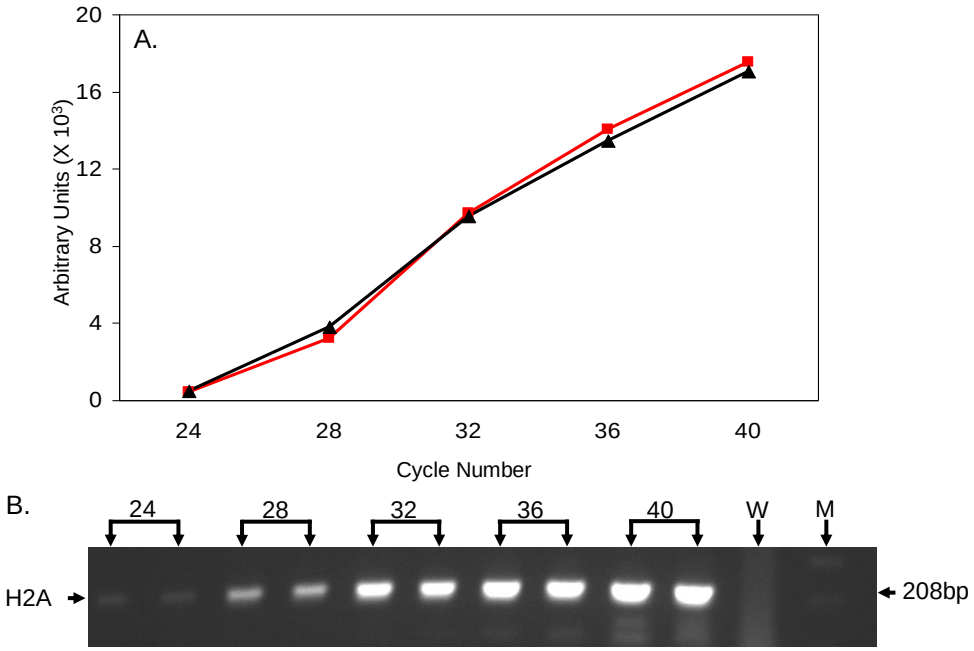

Figure A1. Response Curve for H2A in Day 70 bovine fetal liver. Figure A: Graphic representation of the response curve for H2A. Figure B: Ethidium bromide-stained agarose gel of H2A amplification products from Day 70 bovine fetal liver. W) PCR water blank. M) 100bp ladder marker.

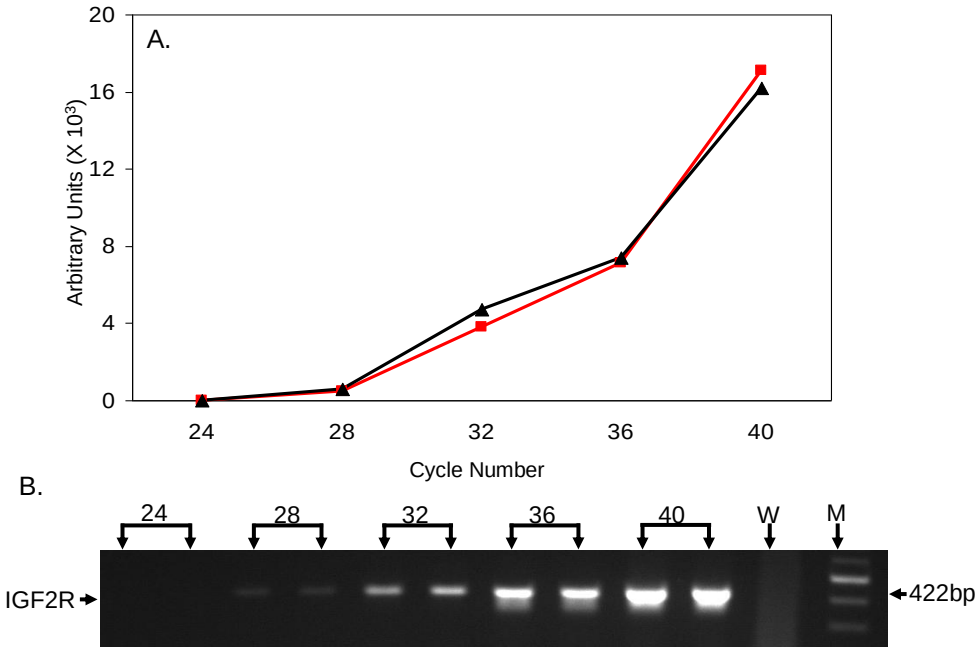

Figure A2. Response Curve for IGF2R in Day 70 bovine fetal liver. Figure A: Graphic representation of the response curve for IGF2R. Figure B: Ethidium bromide-stained agarose gel of IGF2R amplification products from Day 70 bovine fetal liver. W) PCR water blank. M) 100bp ladder marker

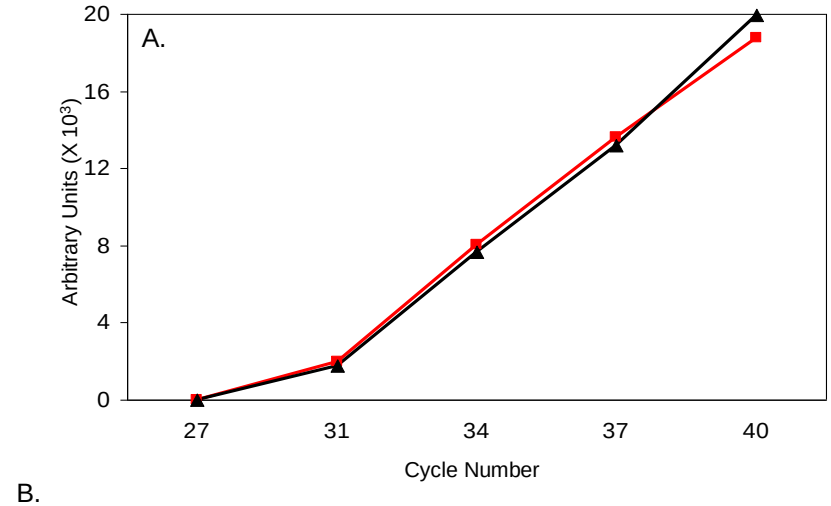

Figure A3. Response Curve for bAIR3 in Day 70 bovine fetal liver. Figure A: Graphic representation of the response curve for bAIR3. Figure B: Ethidium bromide-stained agarose gel of bAIR3 amplification products from Day 70 bovine fetal liver. W) PCR water blank. M) 100bp ladder marker

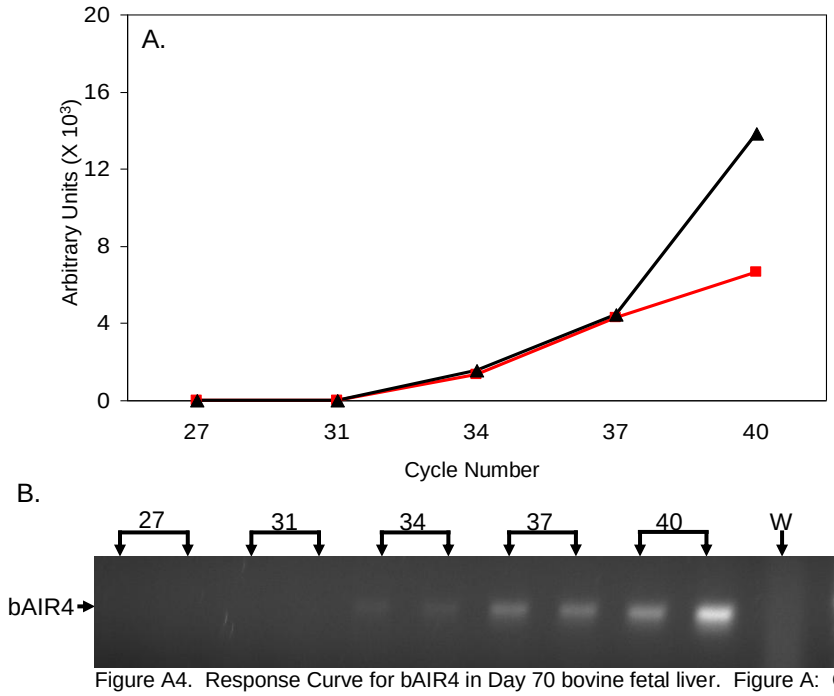

Figure A4. Response Curve for bAIR4 in Day 70 bovine fetal liver. Figure A: Graphic representation of the response curve for bAIR4. Figure B: Ethidium bromide-stained agarose gel of bAIR4 amplification products from Day 70 bovine fetal liver. W) PCR water blank. M) 100bp ladder marker

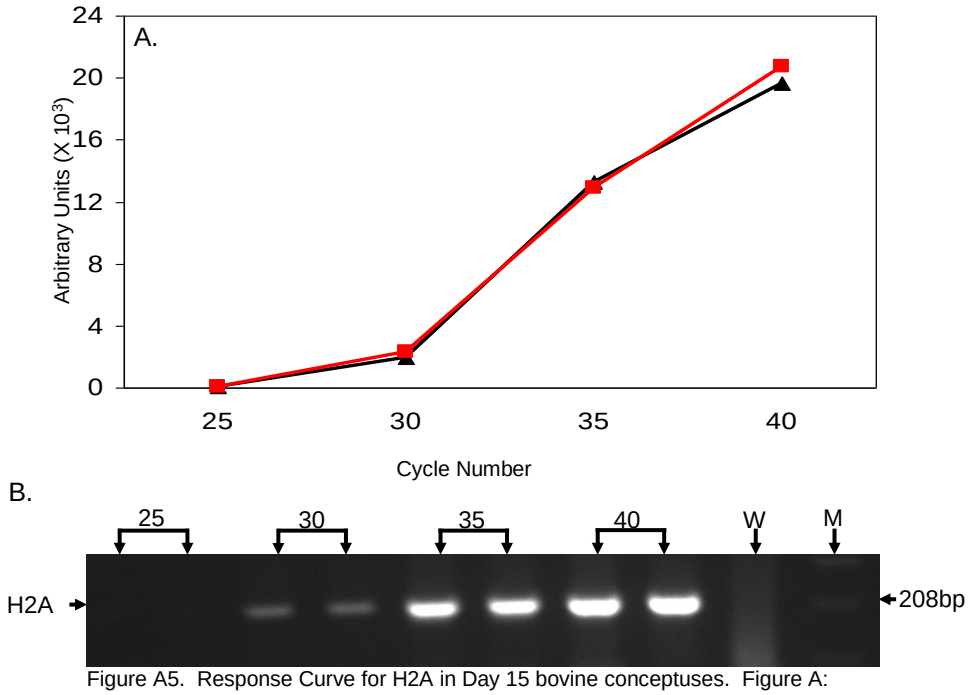

Figure A5. Response Curve for H2A in Day 15 bovine conceptuses. Figure A: Graphic representation of the response curve for H2A. Figure B: Ethidium bromide-stained agarose gel of H2A amplification products from Day 15 bovine conceptuses. W) PCR water blank. M) 100bp ladder marker

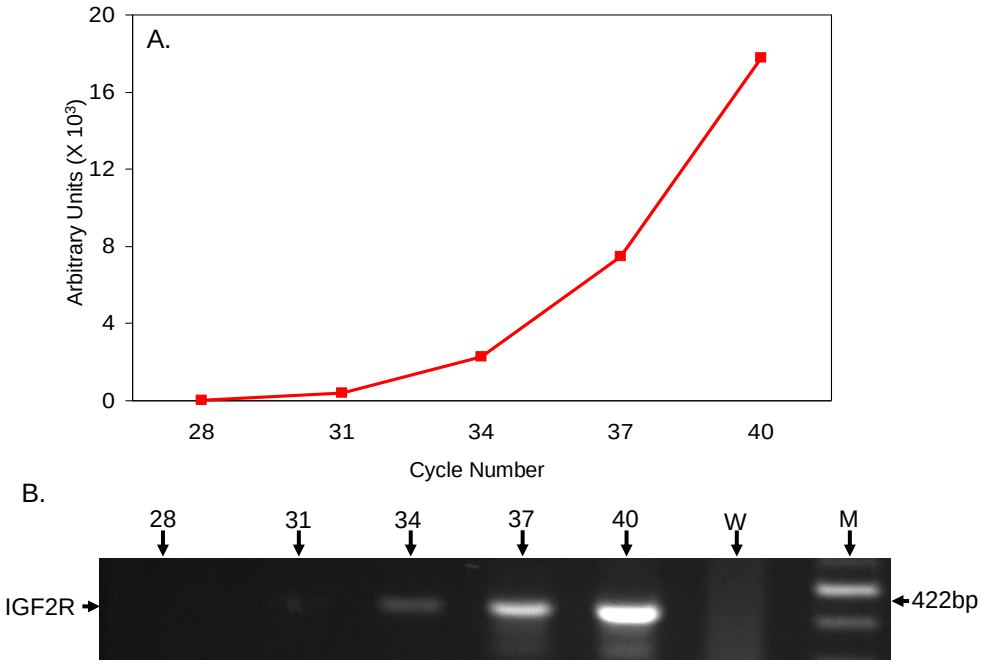

Figure A6. Response Curve for IGF2R in Day 15 bovine conceptuses. Figure A: Graphic representation of the response curve for IGF2R. Figure B: Ethidium bromide-stained agarose gel of IGF2R amplification products from Day 15 bovine conceptuses. W) PCR water blank. M) 100bp ladder marker

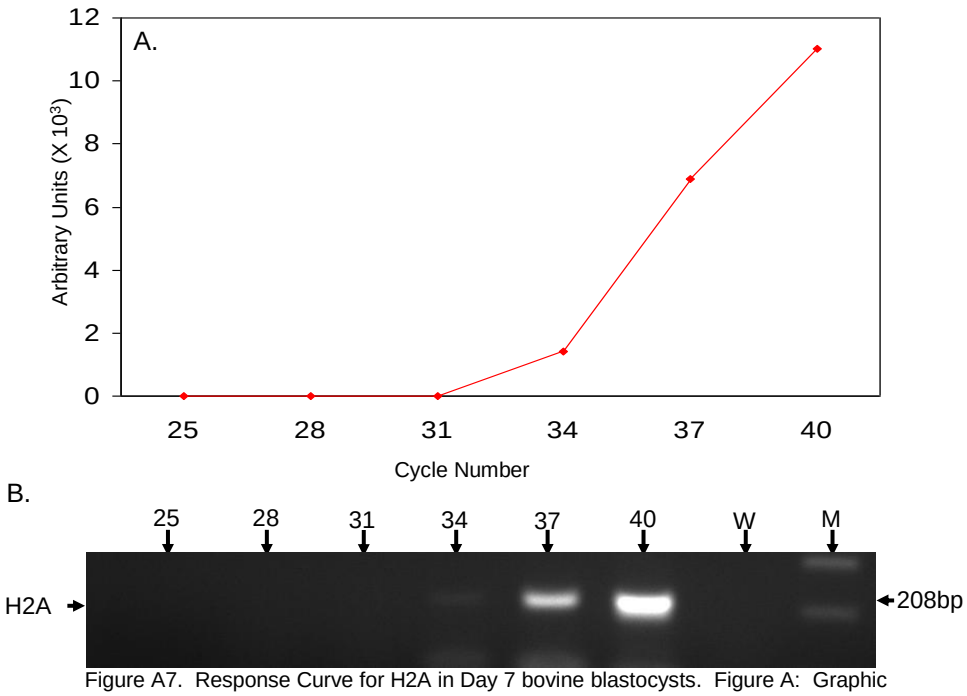

Figure A7. Response Curve for H2A in Day 7 bovine blastocysts. Figure A: Graphic representation of the response curve for H2A. Figure B: Ethidium bromide-stained agarose gel of H2A amplification products from Day 7 bovine blastocysts. W) PCR water blank. M) 100bp ladder marker

APPENDIX B

DAY 70 IVP AND IVO BOVINE FETAL LIVER AGAROSE GEL PHOTOGRAPHS OF PCR AMPLICONS FOR ALL RNAS OF INTEREST

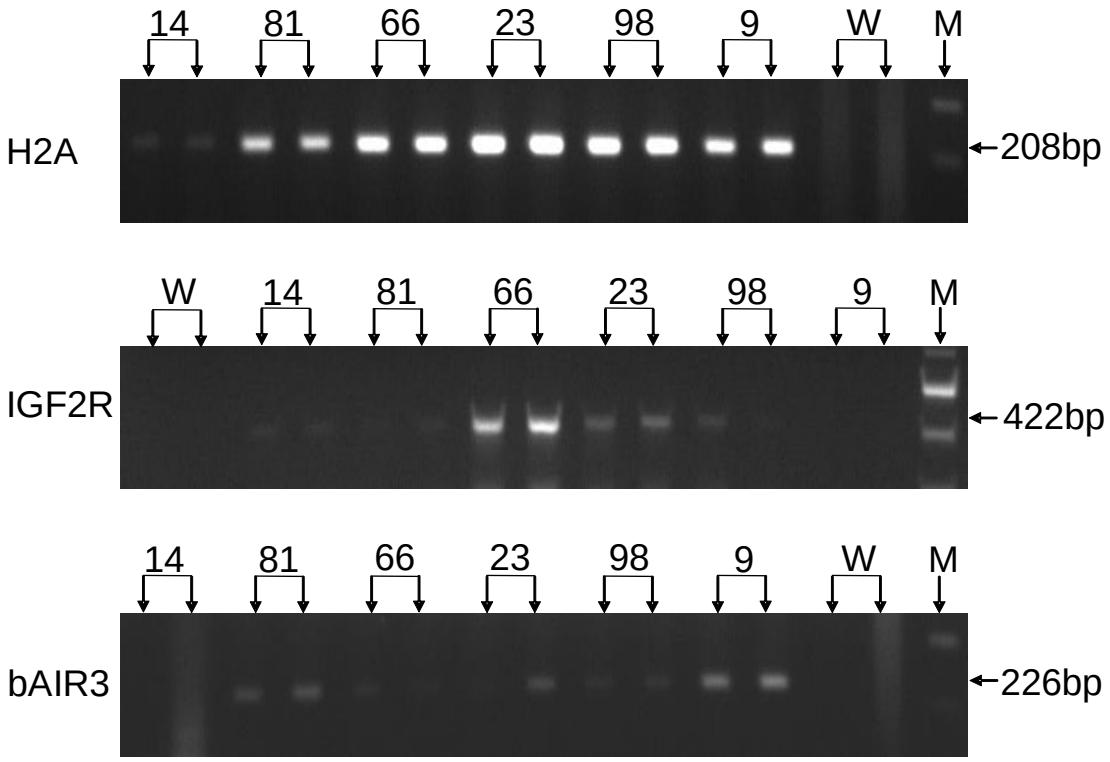

Figure B1. Post-implantation-stage RNA expression in Day 70 bovine fetal liver resulting from IVP embryos.

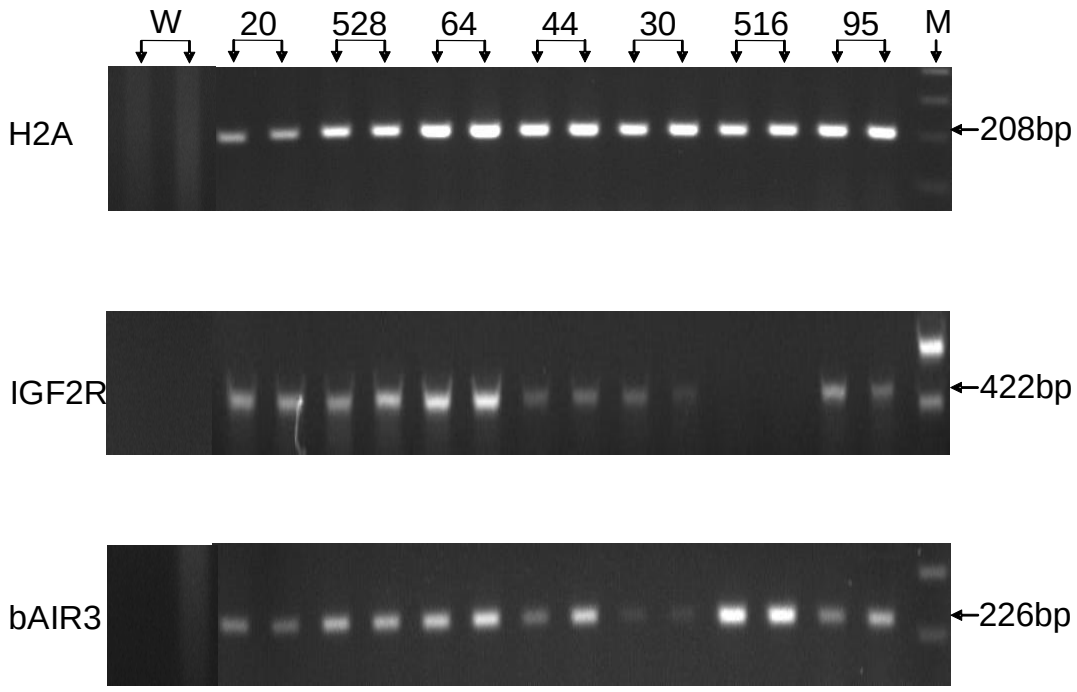

Figure B2. Post-implantation-stage RNA expression in Day 70 bovine fetal liver resulting from IVO embryos.