**EFFORTS TO IDENTIFY LARGE OFFSPRING SYNDROME DURING
PREGNANCY THROUGH ULTRASONOGRAPHY AND MATERNAL BLOOD
TRANSCRIPTOME ANALYSES**

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By
Bhaumik Narendrabhai Patel
Dr. Rocío Melissa Rivera, Thesis Supervisor
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Presented by Bhaumik Narendrabhai Patel

A candidate for the degree of Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Rocío Melissa Rivera

Dr. Martha Sofía Ortega

Dr. Christine Spinka

Dr. Darren Hagen

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ABSTRACT

Large offspring syndrome (LOS) is a fetal overgrowth condition observed in some bovine offspring produced *in vitro*. The main phenotypes of LOS is large body size due to overgrowth during development which can result in difficult birth, can lead to death of the dam and/or calf and can lead to financial losses to producers. Currently, no method exists that can be used to identify LOS during pregnancy. The goal of this study is to investigate if ultrasonographic measurements at day 55 of gestation and maternal blood biomarkers at day 55 and day 105 can be helpful to detect LOS carrying pregnancies.

Chapter 1, the literature review, will introduce LOS its phenotypes and molecular associations as well as the conditions that can potentiate LOS. In addition, two examples of spontaneous LOS and their associated financial losses are discussed. Chapter 1 will also introduce the usefulness of ultrasonographic fetometry and maternal blood biomarkers in bovine. Chapter 2, the research chapter, was set to determine if LOS in cattle is identifiable in the maternal blood during pregnancy and/or if fetal ultrasonographies of day 55 gestation may be used for the same purpose. Data included are: characterization of phenotypes associated with LOS, description of maternal white blood cell transcriptomes and information pertaining fetal size as determined by ultrasonographies and after collection of day 56 and 105 fetuses. This chapter has been submitted as a manuscript and it is currently under review.

NOMENCLATURE

100PE	100bp paired end
AD	Abdominal diameter
AH	Abdominal height
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
ANRIL	Antisense non-coding RNA in the INK4 locus
AOS	Abnormal offspring syndrome
AI	Artificial insemination
ART	Assisted reproductive technologies
BWS	Beckwith-Wiedemann syndrome
BGI	Beijing Genomic Institute
BPD	Biparietal diameter
BSA	Bovine serum albumin
CA	Carbohydrate antigens
CV	Coefficient of variance
CIDR	Controlled internal drug release
CPM	Counts per million
CAU	Cross-section of the abdomen at the insertion of the umbilical-cord
CRL	Crown rump length
DE	Differentially expressed
DEG	Differentially expressed genes
DMR	Differentially methylated regions
DPBS	Dulbecco phosphate buffered saline
ET	Embryo transfer
eGFP	Enhanced green fluorescent protein

FDR	False discovery rate
FL	Forelimb length
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
HC	Head circumference
HL	Head length
HF	Heart failure
HG	Heart girth
IVP	In vitro production
ISG-15	Interferon stimulated gene-15
IETS	International embryo technology society
LIPCAR	Long non-coding cardiac associated RNA
LOS	Large offspring syndrome
LV	Left ventricle
MOET	Multiple ovulation embryo transfer
MI	Myocardial infarction
MU	University of Missouri
PBMCs	Peripheral blood monocytes
PVA	Polyvinyl alcohol
PAGs	Pregnancy associated glycoproteins
PG	Prostaglandin
PGF2 α	Prostaglandin F2 alpha
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
TD	Thoracic diameter
TH	Thoracic height
U	Ultrasound
UF	University of Florida

WBC

White blood cell

Chapter 1 - Literature Review

1.1 Large offspring syndrome (LOS)

Technologies like somatic cell nuclear transfer (SCNT) and assisted reproductive technologies (ART) are useful to improve the genetic potential of the herd by selective breeding. ART or *in vitro production* (IVP) of embryos involves a variety of laboratory techniques to fertilize the oocyte and sperm to obtain large number of offspring from genetically superior animals or to obtain offspring from infertile animals. Collection of oocytes can be done either from the ovaries of live animals through transvaginal ovum retrieval method by piercing ovarian follicles through the vagina guided by ultrasonography, or from slaughterhouse ovaries. Collected oocytes are first matured in maturation medium *in vitro* and then fertilized *in vitro* with capacitated sperm and then zygotes are cultured to the desired stage of development up to the blastocyst stage. If desired, embryo transfer (ET) is done to synchronized animals or embryos are stored after freezing for later use.

If superovulation has been used to get multiple oocytes from live animals, it is generally followed by artificial insemination (AI) and then embryos are collected by flushing the uterus. Embryos then may be frozen or immediately transferred to recipient animals. This procedure is called multiple ovulation embryo transfer (MOET).

In SCNT, however, embryos are produced using the DNA of somatic cells which is injected into enucleated oocytes instead of by *in vitro* fertilization. Compared to SCNT, IVP is a less invasive method which more closely mimics natural fertilization and embryo development.

Since the inception of ART, various reports of offspring with various abnormalities have been noted. The first such case of a “large calf” was reported in cattle in an SCNT study by Willadsen *et al.* in 1991 (1), while Behboodi and coworkers reported the first large calf produced by ART in 1995 (2). In subsequent years, more cases of large calves were reported (3–5) and the phenomenon was described as “large offspring phenomenon” by Walker and coworkers (6) and then coined as “large offspring syndrome” (LOS) in a review by Young (7). Besides the overgrowth phenotype, other abnormalities such as large organs and placental abnormalities were also observed in ART/SCNT-produced offspring of ruminants (cow and sheep; (1–3, 5, 8–13) and the syndrome was then also classified as abnormal offspring syndrome (AOS; (14).

1.2 Characteristics of large offspring syndrome

1.2.1 Phenotypes

As the name implies, the main characteristic of LOS is fetal or calf overgrowth. In some instances, this fetal overgrowth leads to difficulty in birth

and in some cases, caesarean section or assisted delivery is required (15). Another feature of LOS in ruminants is visceromegaly (large internal organs) which was observed in sheep produced *in vitro* (11). In that study, the liver, heart, kidneys and plantaris and tibialis anterior muscle masses of day 61 fetuses and the liver and heart of fetuses at day 125 of gestation produced *in vitro* were larger than the control fetuses (11). The control fetuses for that study were produced *in vivo* after embryo transfer of day 6 blastocysts harvested from superovulated donor ewes which suggests that the embryo transfer technique does not affect fetal development, although it is difficult to determine this fact in the absence of controls produced from artificial insemination. In some instances, LOS calves or fetuses also show ear malformation (16), musculoskeletal deformities, abnormal spinal cords and limbs (8) and paresis of hind limbs or ataxia (5, 15).

Notable placental abnormalities such as hydro-allantois (1), polyhydramnios, umbilical hernias, allantois development defects and abnormal placental vasculature (3, 5, 8) had been also observed in LOS fetuses. In addition, SCNT LOS fetuses have also been shown to have placental overgrowth (17) with the ratio of fetal to total placentome weight being lower in the SCNT group than in the control group at 220 days of gestation. Furthermore, in that study, stereological analysis of the placenta showed deregulation of the growth of cotyledons which led to the thinning of the maternal epithelium within the placentomes and increased trophoblastic surface in the placenta (17). They suggested that abdominal ascites, the larger heart and umbilical cord observed in

those SCNT fetuses may have been the consequences of these placental abnormalities and named LOS as “large placenta syndrome” (17). This suggests that the level of perturbation in placental development can define the outcome of the fetuses. However, the rudimentary or marginal chorioallantois development during day 35 to day 60 of gestation of cloned fetuses can lead to higher embryonic mortality when compared to *in vivo* or *in vitro* derived fetuses (18) which may partially explain those findings.

1.2.2 Molecular Associations

Besides, phenotypic abnormalities, LOS also has epigenetic irregularities such as loss-of-imprinting (16). Imprinted genes are genes which show monoallelic parental-specific expression in a developmental stage-specific, cell-specific or species-specific manner. Imprinting disorder are disorders which occurs due to misregulation of imprinted gene expression resulting from perturbations in epigenetic mechanisms. In Chen *et al* (2013) they observed that seven out of 27 fetuses produced by ART along with serum supplementation were overgrown (>97 percentile of the average weight of AI [control] fetuses) at day ~105 of gestation and two out of these seven overgrown ART fetuses had shown biallelic expression of the imprinted gene *KCNQ1OT1* in various organs (16). Furthermore, the loss of monoallelic expression of *KCNQ1OT1* was shown to be associated with loss of methylation on the maternal allele at the KvDMR1 locus, an imprinting control region (16). Furthermore, other imprinted genes

were also misregulated in LOS fetuses and this loss-of-imprinting was positively associated with fetal overgrowth (19). In addition, when transcriptome analyses of muscle, liver, kidney and brain were compared between LOS and AI fetuses, multiple differentially expressed genes (DEG) in LOS fetuses were associated with distinct gene pathways, including pathways associated with myoblast proliferation and fusion into myotubes in skeletal muscle (20). Furthermore, DNA methylome analysis of skeletal muscle of LOS fetuses found multiple differentially methylated regions (DMR) but most of them were not associated with identified DEGs (20). However, misregulation of *IGF2R* in LOS fetuses was associated with altered DNA methylation at DMR1 and DMR2 of the *IGF2R* locus (20) which is similar to the loss-of-imprinting at *IGF2R* observed in sheep LOS fetuses (21). This suggests that LOS in sheep and cattle share misregulation of the *IGF2R* locus.

1.3 Beckwith Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS), a congenital epigenetic overgrowth disorder in humans, has several phenotypic and epigenetic similarities with LOS (16). In 2013, there was an epidemiological prevalence study of BWS which suggested that in northwest Italy the prevalence of BWS is 1 in 10,340 live births (22). Use of ART has been associated with an increased incidence of BWS. When populations of naturally occurring BWS and ART-induced BWS in Piemonte Italy were compared, the relative risk of BWS in the ART group was 10.7 (23). Major cardinal phenotypes of BWS are macrosomia, exomphalos, lateralized overgrowth and multifocal and/or bilateral Wilms tumor

or nephroblastomatosis (24). Exomphalos is the condition when the herniation of abdominal organs occurs through a central abdominal wall defect.

Furthermore, secondary phenotypes of BWS are ear creases, nephromegaly, transient hypoglycaemia, facial nevus flammeus, and umbilical hernia (24). The main molecular defects which lead to BWS in humans, is on chromosome 11p15 which includes defects in DNA methylation, incorrect expression of imprinted genes, changes of chromosomal contents and gene mutations (25). Loss-of-imprinting at KvDMR1, leading to downregulation of the maternally-expressed gene *CDKN1C*, has been shown to occur in BWS (26), which is similar to what has been shown for LOS (16), making LOS an appropriate animal model to study BWS.

1.4 Conditions that can potentiate LOS

It is not yet clear how ART induces LOS nor the mechanisms which can lead to LOS. However, possible factors which can lead to this syndrome are serum and/or co-culture of embryos with somatic cells during in vitro embryo culture, asynchronous uterine environment, and high urea diet during the pregnancy.

1.4.1 Serum supplementation and/or co-culture with somatic cells

1.4.1.1 Preimplantation stage embryos

ART procedures can cause cellular differences in growing embryos. When preimplantation stage embryos cultured in serum-free and serum-

supplemented media along with bovine cumulus/granulosa cells were analyzed using electron microscopy, the ultrastructural features of morula stage and blastocyst were different (27). For example, serum-supplemented cultured embryos had large lipid droplets and fewer lysosome-like vesicles at the morula stage and in the cytoplasm of trophoblast and inner cell mass cells at the blastocyst stage compared to serum-free cultured embryos (27). Further, culturing embryos in the presence of high concentrations of serum or bovine serum albumin (BSA) show an increased number of cells on day seven and they are larger on day 12 when compared to their *in vivo* counterparts (28).

Supplementation of serum during culture has been also shown to alter mRNA expression in preimplantation stage embryos. For example, the relative abundance of transcripts such as *Hsp* and *Dc III* were higher in morula stage *in vitro* produced embryos compare to *in vivo* controls (29). In addition, developmentally important genes such as *Hsp70.1*, *Cu/Zn-SOD*, *Glut-3*, *Glut-4*, *bFGF*, and *IGFI-R* were also been reported as misregulated in day 12 *in vitro* produced blastocysts compare to *in vivo* produced embryos (28). From the aforementioned studies, it may be concluded that the differences in mRNA expression might be due to the serum, however, one cannot eliminate the possibility that the effects observed may be the result of culture itself, and not serum, since all the comparisons were made with *in vivo* produced embryos. To this effect, comparison of mRNA abundance of morula, blastocyst and hatched blastocyst cultured in two different culture media (i.e. serum or polyvinyl alcohol (PVA)), showed differences in specific mRNA transcripts between those cultured

systems (30). For example, connexin43 mRNA expression virtually disappeared from the morula stage onward in serum-supplemented group, but then reappeared in hatched blastocyst, whereas it was present in all three stages in PVA medium grown group (30). Thus, one can hypothesize that these perturbations in gene expression during preimplantation development, may lead to deviations in embryo and fetal development and may lead to an increased incidence of oversized fetuses or calves. However, to date no data exists that can make this correlation since no studies have been done in which experimental embryos are followed to later stages of pregnancy or birth, to arrive at those conclusions.

1.4.1.2 Fetal and placental growth

During the 1990's, various IVF and ET studies produced fetuses with serum supplementation and/or coculture embryos with somatic cells derived from buffalo rat liver cells or bovine granulosa or oviductal epithelial cells and they observed increase incidences of higher fetal or calf weight, dystocia and calf mortalities (2, 3, 31). For example, fetal weight of day 125 sheep fetuses derived from co-cultured embryos were 34% heavier than controls (11). Similarly, in cattle, there was a report of birth of larger calves derived from co-cultured embryos compare to calves derived from AI (2). At first, LOS was thought to be due to extended gestation, but when comparison of gestation length between large calves derived from ART ((271 ± 2.4) days) and normal weighing AI calves ((277 ± 1.2) days) was done, it showed that there was no association between the birth of large calves and increased gestation length (2).

However, not all fetuses produced by ART lead to an oversized fetus. For example, when using a granulosa cell coculture *in vitro* system in sheep, only four out of 20 day 61 and three out of 13 day 125 were oversized (greater than 3 SD of mean of control fetal weight) when compared to their control counterparts (11). In addition, the variations observed in LOS phenotypes varies between laboratories and may depend on the type of ART procedure used to produce the embryos (7). For example, when comparing fetuses produced from embryos grown with 5% vs. 10% serum supplementation and compared to AI fetuses at day 80 of gestation, fetuses from the 10% serum-supplemented group were heavier and their internal organs such as liver and heart were larger when compared to AI fetuses, whereas fetuses from 5% serum-supplemented group didn't show any overgrowth phenotype (32).

ART has also been shown to modify the fetal musculature. For example, in sheep, when *in vivo* produced zygotes were cocultured with bovine granulosa cells for five days and transferred to synchronous ewes, an increase in day 125 plantaris muscle weights were seen when compared to control fetuses (33). Furthermore, examination of myogenesis in plantaris muscle revealed that primary and secondary muscle fiber areas of day 61 and day 125 collected fetuses from cocultured group were higher when compared to those observed in control fetuses (33). Thus, it is suggested that coculturing embryos with bovine granulosa cells alters the myogenic programming and can lead to abnormally large muscles in oversized fetuses (33). Here, control fetuses were produced from *in vivo* embryo collected at day 6 of development and transferred to

synchronous ewes which eliminates potential problems with *in vitro* oocyte maturation and *in vitro* fertilization, which supports the notion that embryo culture can affect subsequent fetal development.

Besides overgrowth, fetuses derived from embryos, cocultured with bovine oviductal epithelial cells show various placental variations when compared to their *in-vivo* counterparts (34). Placentomes of day 37 *in vitro* produced singleton or twin fetuses were smaller and thinner compared to controls, whereas placentomes of *in-vitro* derived singleton fetuses collected at day 72 and day 93 of pregnancy, were longer and thinner. However, at term, larger cotyledons were found in the *in vitro* group which were also shown to be associated with a larger cotyledonary surface area found in the fetal horn compare to the *in vivo* group (34).

As mentioned earlier, ART can alter imprinted and no-imprinted gene expression in various tissues of developing fetuses and also had been shown to be associated with fetal overgrowth (19). When comparison of placental gene expression at day 50 of pregnancy between *in-vitro* produced embryos and *in-vivo* embryos was done, several genes associated with immune system, organ and blood vessel development and extracellular matrix organization were found to be misregulated in the *in vitro* group (35). That suggests that this aberrant gene expression may lead to placental abnormalities and can have adverse impact on development and health of the offspring (35).

1.4.2 Asynchronous uterine environment

Asynchronous uterine environment during pregnancy can also induce fetal growth. For example, when day three ovine embryos were transferred to ewes which were three days more advanced than their chronological age, fetal growth was accelerated when compared to the synchronous controls (Wilmot and Sales, 1981). However, mortality rate of those asynchronous embryos was also higher (36). In another study, when progesterone was administered during early pregnancy (one to three days of gestation) in sheep, to induce an asynchronized uterine environment, fetuses were heavier and had a larger heart, increased skeletal muscle weight, and brain weight at day 74 of pregnancy (38). The reason behind this phenomenon may be that embryos in an advanced uterine environment might have received one or more secretory signals earlier than expected time, which led to earlier growth of those embryos. Similar to ART, transferring day three embryos into a day six uterine environment led to an increase in the total number of muscle fibers in plantaris muscles of day 110 and day 125 fetuses which suggested a prolonged hyperplasia (39). Furthermore, they also observed alterations in the ratio of secondary-to-primary fibers in plantaris muscle from the asynchronous group (39). However, they did not see a difference in fetal weight at day 110 or 125 of gestation (39).

1.4.3 High urea diet during pregnancy

High urea diet has been shown to be associated with an increase in fetal growth in sheep. For example, ewes fed excess amounts of urea from 21 days

before mating to day 63 of gestation, showed increased embryo mortality and fetal growth in the surviving embryos, as well as a higher birth weight (40). Furthermore, they observed increased urea and ammonia in the plasma and uterus of ewes which might have caused up regulation in metabolism in some embryos which could have affected either the oocyte or embryo (or both) leading to enhanced fetal growth (40).

1.5 LOS-associated financial burden to the industry

Since LOS calves can have higher bodyweight and greater size than control calves, it can lead to dystocia and can require surgical fetal extraction (15). Furthermore, LOS calves have been associated with lower neonatal viability (2). However, there is no published data on the economic loss experienced by the industry.

As oversized calves can lead to dystocia, one can relate the consequences of dystocia to LOS. Often cesarean section or assisted delivery is required to deal with dystocia, and this could have adverse impact on cows and newborn calves. A study by *Linden et.* showed that higher calf birthweight and calf size can increase the chances of lameness in cows after delivery (41). It has been also shown that calf mortality rate (12.4%) after caesarean section is higher than calves delivered from normal delivery (5.2%) (42). In addition, severe dystocia increases the chances of stillbirth by approximately 20 times (43) and also increase the respiratory and digestive disorders in calves born from these cows (43). In addition, dystocia can also have an adverse impact on milk production and increase the calving to conception interval. For example, dystocia

leads to an average loss of 4.9 kg of milk per day in high yielding cows (44). Furthermore, It can also increase 2 to 3 days from calving to next service in cows (45) and decrease the conception rate at first service after it which together increase calving to conception interval 6 to 12 days (45). In short, economic losses also depend on consequences after birth of LOS calves.

Next are two examples of estimated economic losses as a result of LOS:

1.5.1 Example 1 (Oklahoma, 2019)

Six continental large calves were produced through embryo transfer by MOET and they weighed 51 to 65 kg and needed induced delivery or cesarean section. Furthermore, four out of six recipient cows also died after delivery. Below is the figure summarizing the estimated economic losses incurred by this embryo transfer technician. The estimated total loss in terms of dollars was \$28,832.37 (Figure 1.1A).

1.5.2 Example 2 (Missouri, 2019)

Another case of LOS is from a local farm in Missouri. A dead Charolais calf weighing 78 kg was delivered at the University of Missouri Veterinary hospital through cesarean section. Figure 1.1 B shows the estimated economic loss (\$29,200) suffered by this local producer.

Figure 1.1

Figure 1.1 Estimated economic loss. (A) Case of Oklahoma LOS. Cow related loss = \$5000 (4 x \$1250 to replace with open 6 y/o cows). Calf related loss = \$7200 (4 x \$1800 for embryo calves). Medical related loss = \$1782.37 (Veterinary service etc.). Future loss = \$14,850 (4 cows x 4 more calves per cow x 450lbs x \$1.65). (B) Case of Missouri LOS. Cow related loss (current + predicted) = \$14,500 (dam valued at \$2600, sold for salvage \$500 + value of next four calves as yearlings were expected -\$12400). Calf related loss (77 kg) = \$2600 (average selling price of their yearling bulls). Sire related loss = \$11,000 (sire cost – had to sell to market after SLOS son). Medical related loss = \$1,100 (Veterinary services).

1.6 Ultrasonographic morphometry as a non-invasive method to identify LOS

1.6.1 Use of transrectal ultrasonography to identify fetal abnormalities

Since 1953, it has been clear that ultrasonography can be useful for clinical purposes such as the identification of tumors (46) although this technology can be used for purposes beyond the identification of diseased tissue. Ultrasonography is the most convenient and economic non-invasive method to identify fetal developmental abnormalities during pregnancy. In humans, ultrasonography can detect various fetal abnormalities such as lethal muscle and skeletal disorders, spina-bifida, hydrocephalus, Down's syndrome, complex cardiac malformations, non-lethal dwarfing syndromes, anterior abdominal wall defects, diaphragmatic hernia, congenital myotonic dystrophy, haemoglobinopathy and many others (47). Although the overall accuracy of those detections was 55%, it helped to decrease overall prevalence of those abnormalities up to 20% by terminating some of those pregnancies (47). Current advancements in ultrasonographic techniques have led to increased accuracy of detection of fetal developmental defects. For example, when various body malformations including macrocrania, cerebellar hypoplasia, enlarged kidneys and others were accessed through ultrasonography in three different trimesters, the overall accuracy of those structural anomalies were 93.73%, 94.43% and 93.95% for first, second and third trimester of pregnancy, respectively (48). In addition, when comparison of various fetal fat body mass measurements between mothers with gestational diabetes and normal pregnant women, all

ultrasonographic measurements such as anterior abdominal wall thickness, subscapular thickness, mid-upper arm and mid-thigh subcutaneous areas fat mass, were higher in fetuses of mothers with gestational diabetes (49). These data suggest that ultrasonography can be useful to identify the disproportionate development of human fetuses (49).

1.6.2 Relationship between fetal development and fetal morphometry

As in humans, ultrasonography is extensively used in cattle. Fetal age can be estimated in cattle by measuring fetal crown-rump length (50, 51) and fetal crown-nose length during the first trimester of pregnancy (51) with crown-rump length being most precise for age estimation on gestation day 30 (50). However, other measurements such as uterine diameter, head, trunk and nose diameter can also be useful to estimate fetal age in cattle (50). Furthermore, transrectal ultrasonography of various organs and body parts of developing bovine fetuses during the first seven months of pregnancy showed that head, thorax, abdomen and pelvis regions can be readily accessible during the first four months of development, whereas, during five to seven months of gestation, thoracic, abdominal and pelvic regions is not easily accessible but fetal head can be accessible for ultrasonography in 80% of developing fetuses (52).

Furthermore, ultrasonography has also been used to better characterize variations of conceptus development in *in vivo* and *in vitro* derived bovine pregnancies based on fetal measurements (34). For instance, ultrasonographic

examinations from gestation day 30 to term found that *in vitro* produced fetuses were smaller than *in vivo* ones during early pregnancy from day 37 to day 58 (34).

Since the main phenotype of LOS is gigantism, there is a possibility that ultrasonography can help identify this fetal overgrowth in LOS cases during early pregnancy. In past, ultrasonography has been used to measure variations in growth in the developing fetuses. For example, weekly comparisons of fetal development from day 35 to day 108 days of gestation using fetal ultrasound dimensions such as biparietal diameter of the cranium (BPD) and cross-section of the abdomen at the insertion of the umbilical cord (CAU) between three different methods of embryo production (i.e. MOET, IVP co-culture or IVP SOF medium) have been done. Investigators found no differences between groups, thus, it was suggested that there is no difference in fetal growth in fetuses produced from the aforementioned techniques during early pregnancy (53). However, in that study, fetal growth was compared among three different procedures in which embryos are manipulated, not to an AI group, so it is not clear that ART produced fetuses follow the same fetal growth patterns as AI fetuses would or not. That question was answered by Bertolini *et al* (2002) who compared the fetal morphometry between IVP fetuses and *in vivo* produced fetuses from day 30 of gestation to term and found that fetal crown-rump length of *in vivo* produced fetuses at day 37 to day 58 of gestation was smaller than *in vivo* controls (34). Furthermore, *in vitro* produced calves were higher in their birth-weight compare to *in vivo* produced calves and researchers suggested that

it may be due to the placenta trying to erase the effect of early growth retardation which led to aberrant placental development and compensatory fetal growth leading to LOS (34). Those authors suggested that IVP calves show biphasic growth, a period of growth retardation during early gestation followed by a placenta-driven growth overcompensation later in development (34). However, a SCNT study suggests that placental deficiencies in cloned fetuses during early pregnancy can lead to developmental retardation and increase in embryo mortality (54).

1.7 Fetal-maternal communication and their influence on pregnancy associated markers

1.7.1 Fetal-maternal exchange

The placenta transfers nutrients and oxygen to the developing fetus and removes the waste products from the fetus. There are four types of placenta in eutherian mammals which are defined on the basis of invasiveness of the fetal chorioallantois membranes to the maternal uterine tissue (55). Humans have hemochorial placenta which allow fetal chorioallantois membranes to be in direct contact with the maternal blood circulation (56). This type of placenta allow fetal DNA, RNA or other fetal products to easily pass to the maternal blood (57). The presence of fetal cell and other components in maternal blood is referred to as microchimerism (58).

Cattle have very distinct type of placenta, namely syndesmochorial, in which the trophoblast cells do not invade the maternal uterine tissue as deeply as

occurs in hemochorial type placentas (59). In syndesmochorial placentas, degradation of the epithelium of the uterine caruncles occurs through several hormones and proteases secreted by the trophoblast cells at the preimplantation period, (60, 61) and subsequently, the trophectoderm adheres to caruncles and form a cotyledonary placenta. After successful implantation, uterine remodeling helps to restore the uterine epithelium. During this stage of pregnancy and even during later stage of pregnancies, trophoblast giant cells or binucleate cells, fuse with maternal endothelial cells and form the syn-endotheliochorial cells (62), which allows better transportation of nutrients to the fetus. These placental regions are called as syncytial regions and this placentation is called as “syn” epitheliochorial placenta.

Early on it was thought that syndesmochorial placentas do not allow the passage of any fetal contents into the maternal circulation. However, several studies have shown the presence of fetal DNA and other fetal factors in the maternal blood (63–65). Furthermore, when maternal blood of enhanced green fluorescent protein (eGFP) expressing transgenic bovine embryos was analyzed at day 60 of gestation, eGFP was detected in all pregnancies and testis-specific Y-encoded protein was also detected in male-specific pregnancies (66). Furthermore, eGFP protein and mRNA were also observed in the intercaruncular chorion and intercaruncular endometrium on day 60 of pregnancy (66). Thus, authors concluded that fetal DNA and protein can transfer to the maternal circulation. The exact mechanism for this microchimerism in cattle is still not clear but it was suggested that transfer of fetus derived molecules into maternal

circulation may be possible through nonclassical protein secretion pathways, through transtrophoblastic-like channels and/or by placenta apoptotic processes (66).

1.7.2 Blood Biomarkers

Biomarkers are defined as “*cellular, biochemical or molecular alterations that are measurable in biological media such as tissues, cells, or fluids*” (67). Biomarkers can be used to diagnose diseases and can also be used to predict the cause and progression of disease as well as regression or outcome of treatment. In the past few decades, technological advancements in biomedical technologies have led to a better understanding of the pathogenic mechanism of multiple diseases that have allowed to access the risk associated with them through various biomarkers especially blood biomarkers. In support of that, earlier publications brought the concept on how these transcriptomic bio-signatures of blood cells can be useful indicators of prognosis in the oncology field (68, 69). For example, detection of three carbohydrate antigens (CA) CA50, CA242 and CA199 in serum helped to increase accuracy and predictability of early stage pancreatic ductal adenocarcinoma in human (70). In addition, when gene expression analysis of approximately 13,000 unique genes of acute myeloid leukemia (AML) patients peripheral blood or bone marrow was done, unsupervised hierarchical clustering revealed 16 different clusters that helped to better classify AML (69). In the aforementioned study, gene expression was measured using GeneChips which was costly and time

consuming. However, recent advancements in RNA biology associated technologies such as mRNA sequencing, make it easier and more economical to perform whole transcriptome analysis that identify transcript beyond known protein coding genes. For example, when whole transcriptome of the plasma RNA of patients with or without left ventricular (LV) remodeling after myocardial infarction (MI) was done, the long non-coding RNA (lncRNA) named Long non-coding cardiac associated RNA (LIPCAR) was found to be downregulated in the patients with LV remodeling one year after MI, whereas this gene became upregulated during the development of heart failure (HF). In another study, the novel circular lncRNA, ANRIL (antisense non-coding RNA in the INK4 locus), which regulates gene expression of the *INK4/ARF* gene, was shown to be a useful biomarker to assess the risk of atherosclerotic vascular disease (71).

Throughout gestation, dynamic inflammatory changes occur in animals either by the animal's own hormones and/or under the influence of the fetus and some of them can be detected in blood. For example, in ruminants, interferon-tau secreted by the trophoblast cells of the developing conceptus (72), has been shown to induce various interferon associated genes such as interferon stimulated gene -15 (ISG-15) in maternal peripheral blood monocytes (PBMCs) at day 19 of gestation (73–76). Thus, leukocyte molecules can also be used as biomarkers for early diagnosis of pregnancy in cows (73–76). In addition, during fetal development, the placenta secretes pregnancy associated proteins (PAGs) into the maternal blood. Radioimmunoassay has been developed to identify PAGs in serum gestation day 28 (77, 78). This biotechnology is also useful for

complicated pregnancies, for example, expression of *ADAMTS2* in leucocytes is increase in women during spontaneous preterm labor when compared to same gestational aged non labor women which suggest that *ADAMTS2* can be used as a marker of premature labor in human (79). Furthermore, in preeclamptic pregnancies, various genes such as *COL1A1*, *IKBKB*, *RBI* are misregulated when compared to normal pregnant patients (80).

Since various blood biomarkers has been shown to be useful to identify different states of health in both human and cattle, it can be hypothesized that blood biomarkers can also be useful to identify the LOS pregnancies in cattle.

Rationale for Thesis

LOS is a syndrome in ruminants characterized by fetal overgrowth, visceromegaly, and various placental abnormalities. Since some LOS calves can be oversized and this can lead to dystocia, sometimes cesarean section or assisted delivery is required. Dystocia can increase the chances of death of newborn calves and dams and increase the chances of having subsequent mastitis and lameness in dams. Furthermore, dystocia can decrease milk production and increase the calving to conception interval. These consequences of dystocia can have major adverse economic impact to producers. Thus, identification of LOS early during pregnancy would help producers minimize some of these losses by terminating the pregnancy or by preparing for a difficult parturition for LOS carrying dams.

Ultrasonography is a non-invasive method which is widely used in farm animals to detect pregnancy and fetal sex. Furthermore, ultrasonography has also been shown to be useful in the identification of various developmental abnormalities during pregnancy in humans, thus, one can hypothesize that ultrasonography can be useful to identify LOS fetuses during early pregnancy in cattle.

Pregnancy leads to dynamic changes in the body of animals and those changes can be detected in the blood as exemplified by the use of serum PAGs to identify the status of pregnancy in cattle. Furthermore, interferon stimulated genes such as *ISG15* can also be detected in maternal blood leucocytes. In addition, complicated pregnancies such as preeclampsia in humans can also

alter several gene expressions such as *COL1A1*, *IKBKB* and *RBI* in maternal blood leucocytes which can be used to identify preeclamptic pregnancies in pregnant women. Although it is possible that LOS can also alter gene expression of maternal blood leucocytes during pregnancy, no evidence is available to support this hypothesis.

The main goal of the current study is to determine the usefulness of fetal morphometry through ultrasonography and maternal blood to identify LOS prior to day 105 of gestation (pregnancy in cattle = ~280 days). For this, I hypothesize that ultrasound measurements of day 55 fetuses and maternal blood biomarkers on day 55 and day 105 of gestation will be useful to detect LOS.

Chapter 2 – Efforts to identify the large offspring syndrome during pregnancy through ultrasonography and maternal blood transcriptome analyses

Note: this Chapter has been submitted to the Federation of American Societies for Experimental Biology for consideration of publication and will include the following people as authors:

Bhaumik Narendrabhai Patel¹, Zhoulin Wu^{1,2}, Darren Erich Hagen³, Edgar Joel Soto-Moreno¹, Yahan Li¹, Chris Kim¹, Cliff Miller⁴, Anna Katherine Goldkamp³, Fred Williams III⁵, Elizabeth Jannaman⁶, Yao Xiao⁶, Paula Tribulo^{6,7}, Eliab Estrada-Cortes^{6,8}, Peter James Hansen⁶, Christine Marie Spinka⁹, Neal Martin¹⁰, Christine G. Elsik^{1,9}, Astrid Roshealy Brau-Rodriguez¹, Rocío Melissa Rivera^{1*}

¹Division of Animal Sciences, University of Missouri, Columbia, MO, ³Department of Animal and Food Sciences, Oklahoma State University, ⁴Green Hills Veterinary Clinic, MO, ⁵Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, ⁶Department of Animal Sciences, University of Florida, Gainesville, FL, ⁹Division of Plant Sciences, University of Missouri, Columbia, MO, ¹⁰Martin Veterinary Services, Centralia, MO.

Present Address: ²Key Lab of Meat Processing of Sichuan Province, Chengdu University, Chengdu, China, ⁷Instituto de Reproducción Animal, Córdoba, and CONICET, Córdoba, ARGENTINA X5000, ⁸Centro Altos de Jalisco INIFAP México,

2.1 Abstract:

In vitro production of bovine embryos can result in large/abnormal offspring syndrome (LOS), a condition characterized by macrosomia, macroglossia, exomphalos and placental abnormalities. LOS can lead to the death of dam and calf. Currently, no test exists to identify LOS pregnancies. We hypothesized that ultrasonography and/or maternal blood markers are useful to identify LOS. Bovine fetuses were generated by artificial insemination (control) or produced *in vitro*. Fetal ultrasound measurements were taken on gestation day 55 (d55) and surgical fetal collections performed on d56 or d105. Maternal blood was collected on gestation d55 and d105. On d56 and d105, fetuses produced *in vitro* weighing ≥ 97 percentiles of the AI weight were considered as LOS. D55 ultrasonographies were not useful to identify LOS on d56 fetuses, however for d105 fetuses, abdominal height and thoracic height were larger in LOS when compared to controls, and thoracic diameter and thoracic height were only larger in male fetuses. To determine whether maternal blood may be used to identify LOS on d55 and/or d105 of gestation, leukocyte mRNA from 23 pregnant females was isolated and sequenced using a short-read platform. Unsupervised clustering grouped 20 samples by animal (n=10 heifers) rather than by stage of pregnancy. Leukocyte gene expression analyses revealed >3,500 differentially expressed genes in females carrying the two largest LOS fetuses. In summary, our study suggests that abdominal height and thoracic height from d55 fetal morphometry and transcript mount of *ATF7IP* from d105 maternal leukocytes may be useful to identify extreme cases of LOS.

Keywords: Large offspring syndrome, abnormal offspring syndrome, assisted reproductive technologies, fetal morphometry, maternal blood biomarker.

2.2 INTRODUCTION

Large offspring syndrome (LOS) is an overgrowth condition observed in ruminant fetuses and neonates (6, 16). LOS was first reported in 1991 in a cloned calf produced via nuclear transfer (1). Later, in 1995, overgrowth was reported as a result of non-invasive assisted reproductive technology (ART) procedures (2). At that time, the overgrown animals were called “large calves” and the syndrome was called LOS (7). As the use of ART in ruminants increased during the next decade, so did the number of LOS report (7, 14, 32, 34, 81–83). The main characteristic of LOS is overgrowth, which in some instances can result in calves weighing twice the average birthweight of their breed (2). However, LOS in ruminants is a complex disorder with other phenotypes observed including visceromegaly, large tongue, increased incidence of hydro-allantois, abnormal limbs and spinal cord, ear malformation, hypoglycemia and umbilical hernia (14, 16, 32, 34, 63, 81, 83, 84). Because of these varied phenotypes, this syndrome is also known as abnormal offspring syndrome (AOS) (14).

Even though it is now clear that LOS/AOS is a multi-locus loss-of-imprinting (i.e. epigenetic) condition (19) it is still not known what triggers LOS and which ART (e.g. *in vitro* maturation of oocytes, *in vitro* fertilization, *in vitro*

embryo culture and embryo transfer) is involved. Most reported cases of LOS in the literature were produced using serum supplementation during oocyte maturation and/or during embryo culture, which suggest that serum may be a factor promoting the syndrome (16, 19, 20, 30, 85–88). Serum has been experimentally determined to cause LOS in sheep (89, 90) and a large portion of bovine calves derived from embryos cultured in serum containing medium can develop LOS (16). Even though published data demonstrates that serum supplementation promotes AOS in sheep (11), to date, no controlled study has been done in cattle to specifically answer this question. Further, co-culture of embryos with somatic cells as also been shown to promote LOS (5, 11). Of note, is that all studies in bovine in which LOS was associated with co-culture also used serum in their culture medium. Besides ART, particularly in sheep, asynchronous pregnancies and high urea diet during gestation have also led to fetal overgrowth (36, 37, 40). In addition, the syndrome can also occur in fetuses and calves derived from embryos cultured without serum supplementation or co-culture (91, 92) and more recently we have documented that this syndrome occurs spontaneously in cattle (93) and Rivera et al. in press). The latter is of interest as there is a similar loss-of-imprinting overgrowth syndrome in humans, namely Beckwith-Wiedemann syndrome, which occurs naturally and its incidence is increased in children conceived by ART (23).

ART is the method of choice in cattle when trying to improve genetic merit of the offspring (94) and is also used experimentally (95–97) including for the generation of genetically manipulated embryos (98). For obvious reasons,

reports on the incidence of LOS in the industry are lacking, therefore its incidence is not known (99).

Due to its large size, LOS can cause dystocia and sometimes, cesarean section may be needed for delivery (100). Dystocia can also increase the risk of metritis after parturition (101). Even if the newborn calf survives the difficult birth, the enlarged tongue or extreme body weight make suckling difficult, thus increasing the chances of postnatal death (102). In addition, to the possible death of calves and cows, veterinary costs and the associated negative economic impact in terms of losses in milk, fat, and protein yields in the subsequent lactation are financial losses as a result of LOS (41, 103). For example, two independent LOS cases have been recently reported (Rivera et al., 2021 in press), one by an embryo transfer technician in which the embryos were produced by multiple ovulation embryo transfer and another a case of LOS following natural mating (i.e. spontaneous LOS). The total estimated losses from each of those LOS pregnancies were approximately \$30,000. These monetary losses could have been minimized, if the early identification of LOS was possible. To date, however, no test exists to predict LOS pregnancies in cattle; thus, it is of particular interest to find a biomarker to identify fetal overgrowth early during gestation to help producers prepare for a difficult birth or choose to terminate the pregnancy.

Ultrasound is a valuable non-invasive and repeatable tool that has been widely used in cattle to determine fetal growth (34, 104, 105), fetal age (50, 52, 106), fetal sex (107) and clinical pathologies such as mummified fetuses or

endometritis (108). In humans, crown rump length, biparietal diameter and/or head circumference are used to determine gestational age (109, 110) and head circumference, abdominal circumference and forelimb length have been shown to be useful to estimate the fetal growth (111). Furthermore, the combination of various ultrasonographic measurements have proven useful to estimate birth weight (112–117) and to identify small-for-gestational-age and large-for-gestational-age fetuses (118, 119).

Identification of blood biomarkers have also been successfully used as a non-invasive method to determine pregnancy status or risks associated with diseases (120). Such assays have depended on both cell-free compartments like plasma or serum and white blood cells (121, 122). For example, *ISG-15* mRNA from pregnant cattle leukocytes (123, 124) and pregnancy associated glycoproteins (PAGs) from bovine maternal blood serum are useful markers of early pregnancy (77, 78, 125, 126). Since technological developments have overcome methodological constraints inherent to assessment of global RNA abundance, it is now possible to identify both coding and non-coding RNAs in human blood (127–133). Whether maternal blood components can be used to identify LOS early in pregnancy is not known.

For our study, we hypothesized that LOS can be identified during pregnancy in cattle by use of day 55 ultrasonography and/or maternal blood leukocyte mRNA biomarkers. The approach was to generate embryos by *in vitro* procedures previously shown to generate fetuses susceptible to LOS (16) and to

perform morphometric analysis of those fetuses at day 55 of gestation and transcriptome analysis of maternal blood leukocytes of gestation days 55 and 105.

2.3 MATERIALS AND METHODS

Heifers

All animal procedures were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of the University of Missouri (Protocol #9455). All animals were kept at the University of Missouri South Farm Research Center in Columbia. All heifers were purchased from two herds in October of 2018 and ranged in age from 17-19 months of age.

Estrus synchronization and selection of animals

For these experiments, fetuses were collected on three occasions. Day 56 fetuses were collected in February (Exp IA) and May 2019 (Exp IB) whereas day 105 fetuses were collected in July 2019 (Exp II)].

Experiment I: (day 56 fetal collections)

Angus crossbred heifers of approximately 18-20 months of age were synchronized and selected for breeding using the 14-day controlled internal drug release (CIDR®, Zoetis, Kalamazoo, MI) prostaglandin (PG) & timed artificial insemination (TAI) protocol (Figure 2.1). Briefly, CIDRs were placed in the

heifers (denoted as day 0) and removed 14 days later. Sixteen days after CIDR removal, 25 mg of prostaglandin F2 alpha (PGF_{2α}, (Lutalyze); Zoetis, NJ) was administered intramuscularly (i.m.). Concurrent with the PGF_{2α} administration, a breeding indicator patch (Estroject, Genex, Shawano, WI) was applied to each animal across the backbone as per the instructions of the Estroject patches. Estrus was checked three times per day (7:00 h, 12:00 h and 16:00 h/17:00 h) for three days and only animals with a heat score above two (Exp IA) or 2.5 (Exp IB) at the time of AI (13:00 h) were selected for artificial insemination (AI) or embryo transfer (ET). The breeding indicators were scored 0-4, with a score of 0 indicating no patch activation; a score of 1 signifying <25% patch activation; a score of 2 signifying >25 to <50% patch activation; a score of 3 signifying >50 to <75% patch activation; and a score of 4 signifying >75% patch activation. Regardless of group, all animals were injected with 100 mcg of gonadotropin releasing hormone (GnRH) i.m. (Factrel, Zoetis) at the time of corresponding to AI in the AI group (Figure 2.1).

In order to collect the number of fetuses required for the experiments, two sets of estrus synchronizations were performed. The first group of 120 heifers (Exp IA) were synchronized starting November 2018 and the second group of 164 heifers (Exp IB – some of these were used in Exp II) were synchronized starting February 2019.

Experiment II: (day 105 fetal collections)

For this collection, Angus heifers (n=164; some of these were used in Exp 1B) were synchronized as described above and assigned randomly to AI or ET (Figure 2.1).

***In vitro* and *in vivo* production of embryos and embryo transfer**

Media and procedures were as previously described by us (16, 134). Briefly, *B. t. taurus* (Angus/Angus-Crossbred) ovaries were collected at an abattoir and transported to Stillwater Oklahoma (Oklahoma State University; OSU) for oocyte collection. Oocytes were placed in CO₂ equilibrated maturation medium and shipped overnight at 38.5°C to the University of Missouri (MU) or the University of Florida (UF). In addition, *B. t. taurus* oocytes were also purchased from DeSoto Biosciences (Seymour, TN, USA) and processed at MU. All media for embryo production were prepared at MU by a single technician and shipped overnight to the pertinent location prior to each procedure. Two sources of oocytes and two *in vitro* production (IVP) locations were used to ensure sufficient embryos for transfer in the case of technical difficulties. Oocytes were removed from maturation medium after ~21 h of culture and inseminated with semen from one *B. t. indicus* male (Brahman breed [JDH MR MANSO 7 960958 154BR599 11200 EBS/INC CSS 2]). Putative zygotes were stripped of cumulus cells by vigorous vortexing at approximately 18 h after insemination and cultured in KSOM supplemented with amino acids in a humidified atmosphere containing 5% O₂, 5% CO₂, and 90% N₂. On day five after insemination, the culture medium was supplemented with 10% (v/v) estrus cow serum (collected and prepared in house and previously used in (16)) and embryos returned to the incubator. Day

6 embryos produced at UF were shipped overnight at 38.5°C in culture medium to MU. On day seven after insemination, blastocyst-stage embryos were selected, washed in BioLife Holding & Transfer Medium (AgTech; Manhattan), and loaded in groups of two into 0.25 cc yellow, direct transfer and irradiated straws (AgTech) and kept in a Styrofoam box until ET. Blastocysts were transferred to 52 and 106 synchronized recipient females for Exp I and II, respectively, on day 7 after GnRH injection (Figure 2.1).

Pregnancy detection and allocation to treatment

For Exp IA, pregnancy was confirmed using a breeding indicator patch. Briefly, 16 days after AI, all animals were patched and checked for heat score once daily for 11 days and scores below 1.5 were considered pregnant (Figure 2.1). Confirmation of pregnancy was not accurate when the breeding indicator patch was used because some patches became activated due to normal animal activity. Therefore, subsequent pregnancy determinations (Exp IB and Exp II) were performed on day 36 by transrectal ultrasonography (Figure 2.1).

Day 55 and day 77 fetal ultrasound morphometry

At day 55 of pregnancy, presumed pregnant animals (Exp IA - n= 39; Exp IB - n= 23; Exp II - n= 66) were checked for presence of a fetus by transrectal ultrasonography using a SonoSite EDGE ultrasound machine equipped with a L52 10.0-5.0 MHz linear-array transducer (Figure 2.1). Images acquired by the ultrasound were analyzed using software resident on the machine. Ultrasound measurements taken were abdominal height, abdominal diameter, trunk

diameter, thoracic height, crown rump length, head length and biparietal diameter (Figure 2.2.A1-A7).

Fetal sex and number of fetuses were also determined by ultrasonography on day 77 of gestation (Exp II n=55) (Figure 2.1). Furthermore, a subset of 18 animals (6 AI and 12 ART animals) were checked for fetal morphometry.

Ultrasound measurements taken were abdominal height, abdominal diameter, trunk diameter, thoracic height, crown rump length, head length and biparietal diameter.

Surgical fetal collections of day 56 and day 105 fetuses

Heifers (n = 51 for day 56; n = 48 for day 105) were fasted at least 12 h prior to surgery. Fetuses were surgically retrieved to preserve nucleic acid integrity. All surgical procedures were performed by a licensed veterinarian. Preparation of the surgical site was done by clipping hairs from the paralumber fossa with #50 clipper blade. All other organic debris was removed by scrubbing a minimum of three times alternatively with chlorhexidine and 70% ethanol. For restless animals, ~20 mg xylazine, i.m., was administered. All caesarean sections were performed under epidural anesthesia (5 ml of 2% lidocaine) by either an inverted L block or a distal paravertebral nerve block. The incision was made vertically in the middle of the paralumbar fossa, starting approximately 10 cm ventral to the transverse processes of the lumbar vertebrae and continued ventrally, far enough to allow removal of the fetuses. Linear incision was performed into the gravid uterine horn and digital manipulation was used to

remove the conceptus. Closure of the uterus and abdominal wall was done with an absorbable suture whereas closure of skin was performed with ford interlocking non-absorbable suture.

Collection of fetal tissues and fetal measurements of day 56 and day 105 fetuses

Collected fetuses and their fetal membranes were weighed and then fetal morphometry was assessed, and abnormal phenotypes noted. Measurements included crown-rump length, heart girth, forelimb length, biparietal length, abdominal height, head length and thoracic height. Subsequently, all tissues were dissected and divided in two and immediately frozen in liquid nitrogen. For all experiments, the same person measured and weighed all fetuses and fetal membranes, and another person dissected all the tissues. The average time from excision of the fetus from the uterus to when all tissues were frozen in liquid nitrogen was approximately 18 min. All tissues were stored at -86°C until further use.

Morphometric analysis of crown rump length and umbilicus diameter in day 105 fetuses

Measurement of day 105 fetuses' crown rump length (from the top of the head to base of the tail) and size of the umbilicus (cross section at the junction between the umbilicus and the body) were measured using the ImageJ's (ImageJ, NIH) freehand line function using a lateral side picture of the day 105 fetuses. The surface where the fetuses laid was squared (each square = 2.54

cm) and was used to convert all measurements to cm, and then a ratio of umbilicus diameter to crown rump length was taken (Supplementary table S2.1).

Maternal blood collection and blood processing

Maternal blood was collected via tail venipuncture on days 13, 19, 28, 55 and 105 of pregnancy into K3 EDTA tubes (BD, Franklin Lakes) and serum blood collection tubes (BD) (Figure 2.1). Blood processing was done as described earlier in (136). Briefly, both types of blood collection tubes were centrifuged at 1200 x g for 20 min at 4°C and plasma and serum were aspirated and temporarily stored at 4°C for 6 to 18 h prior to storing them at -20°C. The buffy coat from the K3 EDTA tubes were transferred to 15 ml centrifuge tubes containing 12 ml of red blood cell lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA, pH 7.0). White blood cells (WBC) containing tubes were briefly vortexed and then incubated at room temperature for 5 min and later centrifuged at 300 x g for 10 min at 4°C. After discarding the supernatant, the WBC pellet was washed once in 5 ml red blood cell lysis buffer and then with 5 ml ice-cold 1 x Dulbecco's phosphate buffered saline (DPBS), with centrifugation at 300 x g at 4°C for 5 min, at each wash. After discarding the supernatant, the WBC pellet containing tubes were placed immediately on dry ice and then stored at -86°C.

Selection of samples for RNA sequencing (RNAseq)

The WBC samples of 23 day 105 pregnant cows were selected for RNAseq on the basis of their group (i.e., AI or ART-normal, ART-LOS) and fetal sex. The breakdown of the samples was: 8 AI (fetuses = 4 males and 4

females), 6 ART-normal (fetuses = 3 males and 3 females), 9 ART-LOS (fetuses = 4 males and 5 females). In addition, the day 55 WBC samples of the same 23 females were also used for transcriptome analyses.

RNA isolation

Total RNA was isolated using Trizol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and stored at -86°C until use.

Library preparation and transcriptome sequencing

Three micrograms of RNA samples were submitted to BGI Americas Corporation (San Diego, CA) for library preparation and quantitative RNAseq. In brief, RNA quality and concentration were determined by Agilent 2100 analyzer. RNA quality for each sample is summarized in Supplementary table S2.2A and S2.2B. Libraries were sequenced using the DNBSEQ-G400 platform to generate 20 million 100bp paired end (100PE) reads.

RNAseq data analysis

The quality of reads of each sample were checked by FastQC (137) and MultiQC (138). All raw reads were subjected to quality trimming using the DynamicTrim and LengthSort function of SolexaQA++ (139). For a read to be included in the analyses, the phred score of at least 60 bases needed to be >19. Lastly, read quality was confirmed by FastQC and MultiQC and finally aligned to the bovine reference genome assembly ARS-UCD1.2 (140). HISAT2 v2.1.0 (141) was used to align trimmed reads to the bovine reference genome assembly ARS-UCD1.2. To minimize mismatching, --mp 6, 6 --score of HISAT2 v2.1.0

were used and paired end reads that aligned to the genome for each sample were kept for subsequent analysis. The percentage of aligned reads for each sample is summarized in Supplementary table S2.2A and S2.2B. Total read counts for each gene was calculated by using HTseq-count's default union-counting mode (142) using NCBI (GCF_002263795.1_ARS-UCD1.2) and Ensembl gtf files (GCA_002263795.2). Bioconductor packages EdgeR (145) and DESeq2 (146) were used for differential gene expression analyses. Analysis groups were: 1) AI+ART-Normal vs. ART-LOS, and 2) all animals vs. the two females carrying the two largest LOS (i.e., 604 and 664). In addition, transcript abundance analysis was done with all pregnancies and then repeated excluding pregnancies with two fetuses. Similar analyses were done for the day 55 samples.

Unsupervised hierarchical clustering

The unsupervised hierarchical clustering analysis was done using all 46 de-identified blood RNA samples using the R language hierarchical clustering function.

cDNA synthesis and quantitative RT-PCR

Total RNA was treated with DNase (Fischer Scientific, Waltham, MA) before cDNA synthesis. DNase treated 6++++ was used as template to synthesize cDNA in a 20 µl reaction with: 10 mM DTT (Invitrogen, Carlsbad, CA), 4 x Superscript IV buffer (Invitrogen), 50 ng random primer (Promega, Madison, WI), 1 mM dNTPs (Invitrogen), then incubated at 65°C for 5 minutes before

adding 200 U SuperScript™ IV reverse transcriptase (Invitrogen) and 40 U ribonuclease inhibitor (Invitrogen). The samples were incubated at 23°C for 10 min, 52.5°C for 10 min, 80°C for 10 min and stored at -20 °C until use.

In order to ensure that transcript predictions would be useful to other herds and breeds, blood from three ~day 104/105 pregnant Holstein females were collected at Foremost Dairy Farm at the University of Missouri. WBC collection and RNA/cDNA processing were as described above.

Selection of the endogenous normalizers and test genes for qRT-PCR

Genes whose false discovery rate (FDR) from EdgeR analysis was above 0.9 and padj value from DESeq2 analysis was above 0.85, consistent across both day 55 and day 105 RNA samples, were selected (Supplementary table S2.3A). Subsequently, the coefficient of variation (CV) was calculated for each gene across all samples and three genes, *SURF4*, *NECAP1* and *SMG7* were selected to be used as endogenous normalizers for qRT-PCR analysis. Standard deviation and CV values of those genes can be found in Supplementary table S2.3B. A subset of genes was chosen to corroborate expression levels of DEGs identified by deep sequencing based on differences between treatments, CV, and availability of intron-spanning TaqMan probes (ABI, Foster City, CA). The genes chosen were *BRD4* (Bt07108257_m1), *NCOR1* (Bt04314790_m1), *ATF7IP* (Bt03277239_m1), *RSG14* (Bt04296838_m1), *GPR84* (Bt03222335_m1), and *UPP1* (Bt03262341_m1). The RNAseq CV for all transcripts analyzed by qRT-PCR was <12.

Day 19 *ISG15* gene expression

Interferon-tau induces the expression of interferon stimulated gene 15 (*ISG-15*) in maternal leukocytes at day 19 of pregnancy and has been suggested as a pregnancy marker in bovine (76). To see if pregnancies with overgrown fetuses can affect the *ISG-15* expression in maternal leukocytes, *ISG-15* (BM_ISG15NM_174366BNP1) was analyzed using IDT PrimeTime® Std qPCR assay for the 23 maternal WBC RNA samples on gestation day 19 as well as 8 WBS RNA samples of females that had lost their fetuses by day 36 gestation. We used ActinB (BM_ACTB_NM_173979BNP1) as normalizer for this qPCR experiment.

Statistical analyses

The morphometric data were analyzed by analysis of variance (ANOVA) using the general linear model procedure (SAS Institute, Cary, NC). Here, the dependent variables were all fetal measurements, and the independent variable was the group (AI, ART-Normal, ART-LOS).

Statistical analyses for the RNAseq data were implemented with R (<http://www.r-project.org/>) using DESeq2 and EdgeR. For the differential gene expression analysis, DESeq2 and EdgeR both are based on negative binomial regression model. For EdgeR differential gene expression testing was performed using quasi likelihood F test (QLF) ratio test using a generalized linear model (GLM).

The mRNA levels of the target genes for the pregnant females carrying ART-LOS and the two largest LOS fetuses (604 and 664) relative to the combined AI and ART-normal groups, and control population (i.e. Holstein pregnant cows) was calculated using the comparative cycle threshold (C_T) method. Briefly, the C_T for each sample was normalized to the geometric mean of the endogenous reference genes. The average C_T for a given group was calculated by averaging the C_T of all the independent samples. These averages were then compared with the average C_T of the control by the comparative C_T method ($\Delta\Delta C_T$). The ΔC_T values were used for statistical analysis.

Data Availability

The raw FASTQ files are publicly available at Gene Expression Omnibus (accession number pending).

2.4 RESULTS

Synchronization and pregnancy results

Estrus synchronization information can be found in Supplementary table S2.4A and S2.4B. In Exp 1A, day 55 transrectal ultrasonography identified that pregnancy rates were 52.9% and 26.9% for AI and ART, respectively. For Exp IB and Exp II pregnancy rates using transrectal ultrasonography on day 36 were 67.6% and 56.6% and on day 55 were 60.4% and 50.9% for the AI and ART groups, respectively.

Fetal collections and LOS determination

Fetal overgrowth is the defining phenotype of LOS (7, 37), and weight at surgical collection was used to define LOS using criteria described earlier by us (16). A fetus was categorized as overgrown if its weight was greater than the 97th percentile weight of the AI. For day 56 fetuses, the average weight of the AI was 9.46 ± 1.5 g for male and 8.1 ± 1.08 g for females. In total, we collected 20 ART males (weight range = 6.94 - 13.77 g) and 22 ART female fetuses (weight range = 5.55 - 10.31 g). A total of eight ART males and one ART female fetus weighed > 97th percentile weight of AI (i.e., male ≥ 10.18 g and female ≥ 10.16 g; Figure 2.3A). The range in weight of the ART-LOS fetuses was 10.23 - 13.77 g for males and the largest female weighed 10.31 g (Figure 2.3B). All day 56 ART fetuses weighing $\leq 97^{\text{th}}$ percentile weight of controls were considered as ART-normal weight (referred to as “ART-normal”). In addition, several ART fetuses also showed focal hemorrhage on the brain and one fetus had abdominal wall defects (Figure 2.3C and Table 2.1).

For day 105 fetuses, the average weight of the AI was 494.3 ± 44 g for males (n=8) and 416.5 ± 36 g for females (n=4). Thus, ART-males weighing > 548.92 g and ART-females weighing > 463.16 g on day 105 of gestation were considered LOS (Figure 2.4A). A total of 8 male fetuses with LOS (weight range = 552 - 1080 g) and 9 female fetuses with LOS (weight range = 468 - 986 g) were collected. The two largest day 105 LOS fetuses (male (664): 1080 g; female (604B): 986 g) were more than twice the weight of the average AI fetal weight (Figure 2.4B). There were 25 males (442 - 542 g) and 4 females (318 -

448 g) ART-normal fetuses. In addition to oversize, various abnormal phenotypes were observed in the ART-LOS group, including protruding tongue, large umbilicus (Figure 2.4B.3 and Table 2.1), gelatinous material in the peritoneal cavity and organs and skull asymmetry (Figure 2.4C and Table 2.1).

Day 56 Fetuses

Fetal measurements at collection

In the AI group, there were 14 singleton pregnancies. For the ART group, 19 were singletons and 12 had two conceptuses. When fetal measurements of day 56 were compared between AI, ART-normal and ART-LOS, crown rump length tended to be greater in ART-LOS when compared to ART-normal ($p = 0.051$), whereas heart girth tended to be larger in AI and ART-LOS than ART-normal ($p < 0.04$) (Figure 2.5A). However, when male specific analyses were done, biparietal diameter was higher in ART-LOS compared to AI and ART-normal ($p = 0.063$ and $p < 0.02$, respectively). In addition, forelimb length tended to be smaller in ART compared to AI ($p = 0.08$), whereas heart girth tended to be greater in ART-LOS when compared to ART-normal ($p = 0.07$) (Figure 2.5B). When female ART group were compared to female AI, heart girth was smaller in the ART group ($p = 0.053$) (Figure 2.5C). Female specific analysis was not done to compare AI and ART-normal vs ART-LOS as only one female's weight was greater than the 97% threshold used to ascribe ART-LOS.

Day 55 fetal ultrasonographies

When day 55 ultrasonographic measurements of day 56 fetuses were analyzed, thoracic height tended to be greater ($p=0.066$) in the ART-LOS group compared to the ART-normal group (Figure 2.6A). Similarly, when the male specific analysis was done, thoracic height was higher ($p < 0.02$) in ART-LOS when compared to the ART-normal group. However, when female ART and female AI were compared, none of the ultrasonographic measurements were different between them (Figure 2.6B). Female specific analysis was not done to compare AI and ART-normal vs. ART-LOS as only one female in the ART group weighed more than the 97% threshold used to ascribe ART-LOS (Figure 2.6C).

The ratio of day 55 ultrasound measurements (biparietal diameter and crown -rump length) to their reciprocal day 56 fetal measurements was ~ 0.8 (Figure 2.7A). We did not identify any differences in biparietal diameter, crown-rump length and heart-girth were compared among four groups (AI, ART-normal, ART-LOS, and the group comprised of the four largest ART-LOS fetuses) (Figure 2.7A).

Day 105 Fetuses

At day 77 of gestation, sex verification of fetuses of 55 pregnancies (single- $n=44$: twin – $n=11$) was done using ultrasonography. Out of them, 26 fetuses were female, and 37 fetuses were male, two could not be sexed due to positioning and one fetus was no longer viable. An attempt was made to

determine fetal morphometry on day 77 of pregnancy, however this was not possible or reliable for many of the samples (Supplementary table S2.5).

Fetal measurements at collection

In the AI group, there were 12 singleton pregnancies. For the ART group, 26 were singletons and 10 had two conceptuses. Abdominal height, abdominal diameter, trunk diameter, thoracic height, crown rump length and biparietal diameter were larger ($p < 0.05$) when compared to ART-normal and AI counterparts, whereas head length was shorter ($p < 0.05$) in ART-LOS when compared to ART-normal counterparts (Figure 2.4D). However, when sex specific comparisons were done, males and females followed different patterns. For males, abdominal height, abdominal diameter, thoracic diameter, thoracic height, crown rump length, heart girth and biparietal diameter were larger ($p < 0.05$), in ART-LOS when compared to ART-normal and AI counterparts (Figure 8A). In females, abdominal height, thoracic height and crown rump length were larger in ART-LOS than other two groups ($p < 0.05$) and biparietal diameter was smaller in ART-normal than ART-LOS ($p < 0.05$), but there was no statistical difference in forelimb length, heart girth and head length measurements among any of these three groups (Figure 2.8B). In gross examination during collection of LOS fetuses, two of the day 105 ART-LOS fetuses had a noticeably large umbilicus (Figure 2.4B.3 and Table 2.1). However, when the ratio of the umbilicus to the crown rump length of the 4 largest day 105 fetuses were

compared to the controls using image analysis, no statistical difference was detected (Figure 2.9).

Day 55 ultrasonographies

Abdominal height and thoracic height were significantly greater in ART-LOS than AI ($p < 0.02$ and 0.08 , respectively) and ART-normal ($p < 0.002$ and < 0.08), whereas biparietal diameter and crown rump length tended to be smaller in the ART-normal group when compared to the AI controls ($p = 0.066$ and $p = 0.08$, respectively). Furthermore, abdominal diameter ($p < 0.03$) and trunk diameter ($p = 0.05$) were greater in ART-LOS when compared to ART-normal (Figure 2.2B). However, when analyzed on a sex-specific basis, trunk diameter and thoracic height were greater in ART-LOS male fetuses when compared to AI ($p = 0.069$ and $p < 0.05$, respectively) and ART-normal ($p < 0.03$ and $p = 0.055$, respectively; Figure 2.2C), a difference not seen in females (Figure 2.2D). Furthermore, ART-LOS males tended to have higher abdominal height ($p = 0.09$) than ART-normal males (Figure 2.2C) but there was no difference in females (Figure 2.2D). On the other hand, biparietal diameter was smaller in ART-normal females when compared to AI ($p < 0.04$; Figure 2.2D), but no difference was observed in males (Figure 2.2C).

The ratio of day 55 ultrasound measurements to their reciprocal day 105 fetal measurements showed that crown rump length was greater in the AI group than the ART-LOS and the group comprised of the four largest ART-LOS fetuses

($p < 0.002$ and $p < 0.002$), whereas crown rump length of the four largest LOS fetuses was smaller than ART-normal ($p < 0.03$) and ART-LOS tended to be smaller than ART-normal ($p=0.07$; Figure 2.7B). Abdominal height of ART-LOS was smaller than AI and ART-normal (both were $p = 0.06$; Figure 2.7B). Biparietal diameter of the four largest ART-LOS was smaller than AI and ART-normal ($p < 0.01$) and biparietal diameter of ART-LOS was smaller than AI and ART-normal ($p < 0.01$; Figure 2.7B). Furthermore, heart girth of the four largest ART-LOS was smaller than AI, ART-LOS and ART-normal ($p < 0.05$), whereas heart girth of AI was higher than ART-normal and ART-LOS ($p < 0.08$; Figure 2.7B).

When both ultrasound data sets were combined (Day 56 and Day 105) we found that abdominal height and abdominal diameter as well as thoracic height were on average larger ($P < 0.04$) in ART-LOS when compared to the AI and ART-normal groups (Figure 2.10). In addition, the abdominal height and the thoracic height for the largest LOS in each day (i.e., Day 56 - 13.77 g and Day 105 - 1080 g) were larger than all other fetuses (Figure 2.10).

Maternal blood transcriptome analysis

Only reads which aligned to known genes were used in the present study.

Unsupervised hierarchical clustering of RNAseq reads

Unsupervised hierarchical clustering of the raw reads counts after alignment to the bovine genome showed that out of 46 samples, 20 samples clustered together by animal. In addition, the RNAseq results of the females

carrying the two largest ART-LOS fetuses (604 and 664) separated from the rest of the animals (Figure 2.11).

Maternal white blood cells transcriptome analyses of gestation day 55

No genes were identified as differentially expressed (i.e. - EdgeR, FDR <0.05 and DESeq2, padj <0.05) between day 55 WBC RNA for AI, ART-normal and ART-LOS using NCBI and Ensembl *Bos taurus* gene sets. However, when analyses excluded pregnancies with two fetuses, DESeq2 identified *LOC101903301* (Ensembl ID = *ENSBTAG00000040367*) as one DEG between ART-LOS and the combination of the other two groups. When the RNA of the two largest ART-LOS carrying heifers was compared to the rest of the samples, EdgeR and DESeq2 tools with Ensembl gene sets revealed 5 DEGs (*ENSBTAG00000015149*, *ENSBTAG00000025385*, *ENSBTAG00000006383*, *ENSBTAG00000002937* and *ENSBTAG00000034185*) and 4 (*ENSBTAG00000015149*, *ENSBTAG00000025385*, *ENSBTAG00000006383* and *ENSBTAG00000002937*), respectively, whereas DESeq2 tool with NCBI gene set revealed 2 DEGs (*ADGRE3* and *ADAM11*).

Maternal white blood cells transcriptome analyses of gestation day 105

When day 105 ART-LOS WBC RNA was compared to ART-normal and AI groups together, DESeq2 revealed 9 genes and 31 genes as DEGs with the Ensembl and NCBI gene set, respectively (Supplementary table S2.6A and S2.6B)., whereas EdgeR, didn't identify any DEGs.

However, when similar analyses were done excluding pregnancies with two fetuses, DESeq2 revealed 1 (*ENSBTAG00000040367*) and 2 (*LOC101903301* and *LOC107131224*) genes as DEG for Ensembl and NCBI gene set, respectively, whereas EdgeR, did not identify any DEGs as all together data.

When the WBC RNA of the two largest ART-LOS carrying cows was compared to the rest of the samples, EdgeR and DESeq2 tools with Ensembl gene set revealed 4354 and 3845 genes (note: available upon request) as DEG, respectively (Supplementary table S2.6C and S2.6D), whereas EdgeR and DESeq2 tools with NCBI gene set revealed 4271 and 3678 genes as DEG, respectively (Supplementary table S2.6E and S2.6F).

Corroboration of RNAseq DE genes by qRT-PCR

None of the genes selected for corroboration showed differences between groups (i.e. AI, ART-normal, ART-LOS and Holstein controls) when analyzed by real time RT-PCR analysis (Figure 2.12A). Upon closer examination of the data, we noticed that there is great variation in expressions within a group. One gene, *ATF7IP*, however, was consistently downregulated (both in RNAseq differential gene expression analysis and qRT-PCR results) in WBC RNA of the female carrying the largest LOS fetus when compared to all other females (Figure 2.12B).

Day 19 *ISG-15* gene expression by qRT-PCR

When the *ISG15* gene expression were compared between the AI, ART-normal, ART-LOS and ART-dead (pregnancies lost before day 36), we found that ART-dead were downregulated when compared to ART-LOS ($p < 0.06$) and AI ($p < 0.004$) groups but not with ART-normal group (Supplementary figure S2.1).

2.5 DISCUSSION

The goal of the present study was to determine the usefulness of day 55 fetal morphometry and maternal white blood cell transcriptome at day 55 and day 105 of gestation for the identification of fetal overgrowth during pregnancy. The current study revealed that day 55 fetal ultrasound abdominal height and thoracic height measurements may be an indicator of male LOS fetuses, whereas differential gene expression analysis of maternal blood revealed that *ATF7IP* may serve as an indicator for the largest LOS fetuses ($> 2X$ the average weight of controls) at day 105 of pregnancy. In addition, we identified that LOS fetuses had a different growth pattern (in relation to fetal weight) from AI and ART-normal fetuses after day 55 of gestation and that LOS fetuses had several developmental abnormalities such as hemihyperplasia (asymmetric growth), enlarged tongues, brain hemorrhage, enlarged umbilical cord, abdominal ascites and abdominal wall defect. In total, 36.9% of the day 105 collected ART fetuses were considered LOS (> 97 percentile weight of AI on a sex specific basis).

Collection of fetuses was done on day 56 of gestation, since organogenesis has been shown to be completed before this day in cattle (147). The reason behind this decision was to answer other aspects of the project (beyond the scope of the current study) such as questions regarding organ formation and epigenetic mechanisms associated with LOS; information that will be used to identify etiologies of fetal overgrowth in cattle and human (16, 19, 20, 148–151). Furthermore, in the current study, fetuses were also collected on day 105 of gestation as we have previously shown that fetal overgrowth is evident at this stage of gestation (16). In humans, 97% percentile criteria is used to describe macrosomia in newborn babies (152). Since macrosomia is the main characteristic of LOS, we used this weight to ascribe LOS in the present study, similar to what we have done previously (16).

Larger than normal umbilicus and presence of large amounts of fluid-gelatinous material in the abdominal cavity were observed in largest day 105 ART-LOS fetuses. This is similar, to what Constant *et al* revealed in somatic cell nuclear transfer (SCNT) LOS fetuses collected at day 220 of gestation (17). In that study, the authors also suggested that a large umbilical cord and abdominal ascites were the consequences of placental dysfunction which led to placental overgrowth, rather than fetal overgrowth (17). However, several other studies had shown association of early placental defects with loss of fetuses during early pregnancy (18, 153). These investigators also suggested that the angiogenesis and allantoic placental defects in the first trimester of pregnancy might have resulted in growth retardation and eventually death of ART fetuses (18, 153).

Fetuses with severe placental defects may be lost during early pregnancy and if those fetuses having placental defects survive, it can be speculated that they could have higher placental and fetal growth at later stages of pregnancy through compensatory mechanisms. In the current study, the conceptuses were surgically collected to allow rapid collection of tissues in order to preserve nucleic acid integrity, for other aspect of the project, therefore even though we collected the placentas, we were not able to take proper measurements. However, no other obvious placental abnormalities were evident in the collected tissues.

Enlarged tongue was also observed in day 105 ART-LOS which is similar to previous findings in our laboratory (16) and comparable to what has been observed in a similar congenital overgrowth condition in humans, namely Beckwith-Wiedemann Syndrome (BWS; Choufani et al., 2010; Weksberg et al., 2010; Brioude et al., 2018; Wang et al., 2020). Large tongues can lead to difficulty in suckling and increase the chances of prenatal death (102). In addition, the largest day 105 ART-LOS fetus showed brachycephaly and asymmetrical growth of the cranium, an interesting finding given that one characteristic of BWS is hemihyperplasia (157–160). These similarities suggest that these overgrowth syndromes share similar misregulated developmental mechanisms associated with asymmetrical growth.

A greater than two-times increase in fetal weight was observed in day 105 ART-LOS fetuses in the current study, an observation also reported by others (2, 6, 100), suggesting that if those fetuses were followed up to term, dystocia would be probable and assisted delivery or caesarean section would be required (15).

Dystocia as a result of LOS can lead to neonatal death (2) and/or death of cows. Further, dystocia has been associated with stillbirth (43), is a major contributor to perinatal mortality in cattle (161) and has also been shown to increase the chances of metritis (101) and lameness (41). Furthermore, dystocia can have an adverse impact on milk production (44) and increase the calving to conception interval in dairy cows (45). Thus, overgrown fetuses can have negative economic consequences to the producers (Rivera 2021, in press) and identification of those large calves during pregnancy would help overcome these problems.

In the current study, we captured fetal ultrasonographic measurements at day 55 for fetuses that were collected on day 56 or day 105 of gestation. In the past, it has been shown that fetometric ultrasonographic measurement such as crown rump length can be useful for the determination of fetal age (51, 162) and also to understand fetal growth patterns in cattle (17, 34, 53). In addition, it has also been shown that after day 57 of gestation, all important body structures including forelimb and hindlimb are prominently visible in cattle through ultrasonography (104). Since ultrasonography has been improved in the last couple of decades, several researchers are able to have accurate fetal measurements even by day 50 of gestation (34, 163). We found that the ratio of day 55 ultrasound measurements to day 56 fetal measurements was ~0.8 which suggests that fetometric growth parameters such as crown-rump length and biparietal diameter give accurate estimation of fetal developmental status at that age. In addition, the ratio was the same between groups which indicates no

difference for LOS fetuses at this stage for those measurements. Therefore, our data suggest that the 97 percentile AI weight criteria used to ascribe a fetus as being LOS on day 56 is not useful.

As expected, the ratio was smaller (0.14 - 0.43) when these comparisons were made between day 55 ultrasonographies and day 105 fetal measurement. However, we identified that the ratio of crown rump length, biparietal diameter, and heart girth was decreased in the LOS fetuses which were >1.5X the weight of the AI, which indicates disproportionate allometric growth in these fetuses after day 56 of gestation and previously reported for sheep LOS (11). In accordance with our findings, past studies have shown that smaller *in vitro* or SCNT fetuses in the first trimester resulted in heavier fetuses at term (34, 163). Bertolini and coinvestigators also suggested that *in vitro* produced fetuses show early growth retardation and then follow acceleration in fetal growth at later stages of gestation, showing biphasic growth pattern (34). Further, the ratio of day 55 ultrasound and day 105 fetal measurements of crown rump length and biparietal diameter showed that the normal weighing ART fetuses had a different growth pattern than the ART-LOS group.

Individual measurements of abdominal height and thoracic height of the largest day 56 and day 105 ART-LOS fetuses were greater when compared to all other fetuses. To our knowledge, this has not been reported before and may be two measurements to consider when trying to identify LOS by ultrasonography, at least for the extremely heavy fetuses. Previous research reported larger biparietal diameter value at day 63 of gestation in cloned LOS fetuses (163) and

smaller crown rump length value at day 58 of pregnancy in *in vitro* produced LOS fetuses (34) were shown to be associated with LOS pregnancies. However, they were not found as an indicator for LOS in our current study. Possibilities for the discrepancies in our findings, are 1) different definition of LOS (we used >97% weight of the AI fetuses while Bertolini used fetuses that were 33% heavier than controls for comparisons), 2) improvement in ultrasonography technology allowing for more accurate measurement, and 3) number of observations (113 fetuses in our study vs. 26 fetuses in (163))

Research has shown that male and female fetuses followed different fetal growth patterns after day 100 of gestation; males tend to be heavier than female fetuses (164). We also observed this in the current study at day 105, in which AI females were ~15.3% smaller than male AI. Sex-specific analyses of ultrasound measurements show that thoracic diameter and thoracic height can be indicators for male LOS pregnancies at day 105 of gestation whereas, for female LOS, we didn't find any measurements useful to identify LOS.

When we tried to do fetometry at day 77 of gestation, we were only able to measure head length with some accuracy. All other measurements were not reliable, suggesting that day 77 ultrasonography might be too late to try to accurately identify LOS. Given the allometric growth that occurs after day 56 and the fact that day 77 is too late to predict LOS by ultrasonography, and that fetal sex is also accurately predicted starting at approximately day 70 of gestation, we suggest that day ~65 may be a good target day to measure for LOS in a sex-

specific manner as it has been shown that fetuses can be measured at day 62 of gestation (163).

In our study, we also had the objective of determining if maternal blood could be used as a biomarker to identify LOS on day 55 and/or 105 of pregnancy. For this, we analyzed the WBC transcriptome of 23 females carrying day 105 AI, ART-normal and ART-LOS fetuses. We also analyzed the WBC transcriptomes of the same females on gestation day 55. Our initial approach was to do an unsupervised hierarchical clustering of de-identified samples to determine if obvious difference existed between the females carrying the largest fetuses when compared to the other two groups. To our surprise, the transcriptome of 20/46 samples clustered together by animal regardless of pregnancy stage (i.e. day 55 and day 105). Another interesting point is that the experiment was done over three seasons (Autumn, Spring and Summer with a range in temperature of -30°C to 43°C) which was not detected in the transcriptome given the clustering by individual. Further, some of the differential gene expression captured in our study could be explained by the number of fetuses in the uterus (one vs two). Taken together, our unexpected observations suggest that leukocyte gene expression is dependent on the individual animal and number of fetuses, rather than their environment or stage of pregnancy, at least for the days studied here. We could not find published works addressing this point but propose that these data should act as a cautionary tale when transcriptome of various gestation stage are compared in cattle.

To verify that the LOS maternal blood biomarker is consistent across all breeds, WBC from ~day 105 pregnant Holstein cows were also included in qRT-PCR corroborations. On the basis of fold change difference from AI, *RSG14*, and *GPR84* appeared to be downregulated in the WBC of females carrying the two biggest LOS fetuses. However, when cycle threshold was checked for each individual sample the variation among them is large even within treatment, especially for *RSG14* and *GPR84*, indicating that these genes are not appropriate for the identification of LOS in the maternal WBC RNA. Holstein controls have similar level of *ATF7IP* expression than the average of the females carrying the two largest LOS fetuses, however, when samples were analyzed individually, the maternal WBC of the female carrying the largest LOS has reduced transcript amounts of this gene. Perhaps this gene may be useful to identify the largest LOS fetuses at day 105 of gestation, although more samples would be needed to reach this conclusion. Alternatively, the combined use of low levels of day 105 *ATF7IP* together with increased day 55 ultrasound abdominal height and thoracic height measurements could more accurately predict LOS.

Concentrations of maternal PAG genes can be higher in the serum of the pregnancies carrying cloned fetuses that become overgrown when compared to controls (combined ART and AI groups) between day 36 to day 50 of pregnancy (163). It was suggested that the higher PAG in serum could be due to fewer smaller placentomes (163). In ruminants, interferon-tau is secreted by the trophoblast cells of the developing conceptus (72) and has been shown to induce various interferon associated genes such as interferon stimulated gene -

15 (*ISG-15*) in maternal peripheral blood WBC at day 19 of gestation (73–76). Thus, *ISG-15* has been suggested as a biomarker for early diagnosis of pregnancy in cows (73–76). Since in our study, we identified fetal and placental abnormalities, we decided to determine *ISG-15* concentrations in WBC of gestational stage day 19 of the 23 females selected for RNAseq. In addition, a group of WBCs of females carrying ART fetuses which died before day 36 (ART-dead) was also included to assess possible errors in placental development of these fetuses. However, we found that there was no difference between the ART-LOS, ART-normal and AI groups, but ART-dead was down regulated compared to ART-LOS and AI but not with ART-normal. Our limited data suggest that the trophoblast cells of fetuses destined to become LOS are not affected in their interferon-tau secretion.

In summary, here we document initial efforts to identify LOS during the first trimester of pregnancy in cattle. Data suggest that abdominal height and thoracic height may be useful to identify male ART-LOS fetuses at day 55 of gestation, whereas *ATF7IP* may be indicator for extreme fetal weight at day 105 of gestation. Here, we only tested six genes by quantitative RT-PCR which were differentially expressed by transcriptome comparisons. It should be noted that we only tested genes whose TaqMan probes were readily available, which were not necessarily the top differentially expressed genes as per transcriptome analysis. Published work shows that fetal growth varies among different breeds and that size difference can be noticeable as early as 3 months of gestation (165), therefore it is possible that fetal growth patterns may vary as early as day

55 of gestation among different breeds. This could be one of the limitations of our study since we only used Angus/Angus-Crossbred heifers; however, there is lack of published data showing that on day 55 of gestation fetuses from different breeds differ in size. Future research would have to address this question. Here, we also did sex-specific analyses for all our variables, including for ultrasonographic measurements. Since in our experiment, number of male fetuses (both in AI and ART groups) was higher (~ 61% male (day 56 ~51% and day 105 ~71%) our analysis might be skewed, which could be another limitation of this study. Finally, and importantly, it should be noted that we only used the known-mostly coding group of bovine transcripts for this study and that the non-coding and novel transcript portion of the transcriptome remains unexplored. Future work will focus on these types of transcripts.

2.6 Acknowledgements

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Figure 2.1. Experimental design. Production of day 56 and day 105 fetuses (Exp IA, IB and Exp II). Estrus synchronization and selection of animals for Exp. IA - 120 animals were divided into 3 groups and the 14-day CIDR®- PG & TAI protocol was followed. Estrus synchronization and selection of animals for Exp IB and II - 164 animals were divided into 3 groups and the 14-day CIDR®- PG & TAI protocol was followed except those animals were followed up to the last day of AI. Maternal blood was collected on D13, D19, D28, D55 & D105. CIDR: controlled internal drug release, an intravaginal progesterone releasing device. PG: prostaglandin. GnRH: gonadotropin releasing hormone. AI: artificial insemination. D0: day of AI. IVF: *in vitro* fertilization. D5, D7, D13, D16, D19, D28, D36, D55, D56, D77, D105: Day 5, 7, 13, 16, 19, 28, 36, 55, 56, 77, 105 after AI. ART: assisted reproductive technologies.

Figure 2.2

Figure 2.2. Fetal morphometry of fetuses collected on day 105 gestation.

(A1) to (A7) sagittal views of the ultrasound images taken on day 55 of gestation.

U: ultrasound. AH: abdominal height. AD: abdominal diameter. BPD: biparietal diameter. CRL: crown rump length. HL: head length. TD: thoracic diameter. TH: thoracic height. **(B)** Comparison of ultrasound measurements between AI, ART-normal and ART-LOS without considering sex of fetuses. **(C) & (D)** Comparison of ultrasound measurements between AI, ART-normal and ART-LOS, males and females, respectively. **(B), (C) & (D)** AI: artificial insemination. ART: assisted reproductive technologies. * indicates $p < 0.01$. ** indicates $p < 0.05$. *** indicates $p < 0.1$. Note: sample sizes are not equal and thus there is less statistical significance for some comparisons when compared to other ones.

Figure 2.3

Figure 2.3. Day 56 fetal collections. (A) Fetal weight at day 56 of gestation.

The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they generated is shown at the bottom. The bold line represents the 97th percentile of AI day 56 fetal weight (i.e., male – 10.18 grams, female- 10.13 grams). **(B)** Overgrowth primary feature of LOS. **B1** = AI-578A (control female weighing 8.16 g which is approximate average weight of female control fetuses). **B2** = AI-615A (control male weighing 8.83 g which is the approximate average weight of male control fetuses). **B3** and **B4** show largest LOS fetuses with macrosomia (ART-LOS 678B: female weighing 10.31 g and ART-LOS 584A: male weighing 13.77 g). Each square on the background = 2.54 square cm. **(C)** Secondary phenotype observed in day 56 LOS fetus - focal hemorrhage on the brain **(C1)** and abdominal wall defects **(C2)**. LOS: large offspring syndrome. AI: artificial insemination. ART: assisted reproductive technologies.

Figure 2.4

Figure 2.4. Day 105 fetal collections. (A) Fetal weight at gestation day 105.

The X axis has no actual implication and is used to scatter the spots representing each fetus for ease of visualization. The sex of the fetuses and the way they generated is shown at the bottom. The bold line represents the 97th percentile of AI day 105 fetal weight (i.e. male – 548.92 g, female- 463.16 g). **(B)** Overgrowth primary feature of LOS. **B1** = AI-546 (control female weighing 414 g which is approximate average weight of female control fetuses) and **B2** = AI-539 (control male weighing 496 g which is the approximate average weight of male control fetuses). **B3** and **B4** show largest LOS fetuses with macrosomia (ART-LOS 604B: female weighing 986 g and ART-LOS 664: male weighing 1080 g). They also have larger umbilicus. Each square on the background = 2.54 square cm. **(C)** Secondary phenotypes observed in day 105 LOS fetuses: gelatinous material present around stomach and abdomen (**C1**), large tongue (**C2**), gelatinous material around the liver (**C3**) and asymmetrical head (**C4**). **(D)** Fetus measurements of day 105 fetus groups without considering sex of fetuses. AI= artificial insemination. ART= assisted reproductive technologies. AH=abdominal height. FL=forelimb length. BP=biparietal diameter. CR=crown rump length. HL= head length. HG= heart girth. TH=thoracic height. LOS= large offspring syndrome. AI= artificial insemination. ART=assisted reproductive technologies. * indicates $p<0.01$. ** indicates $p<0.05$

Figure 2.5

Figure 2.5. Fetal measurements of fetuses collected on day 56 of gestation.

(A) Comparison of fetal measurements between day 56 AI, ART-normal and ART-LOS fetuses without considering sex of fetuses. **(B)** Comparison of fetal measurements between day 56 AI, ART-normal and ART-LOS, males only. **(C)** Comparison of fetal measurements between two groups of day 56 female AI, ART. AI: artificial insemination. ART: assisted reproductive technologies. FL: forelimb length. BP: biparietal diameter. CR: crown rump length. HG: heart girth. ** indicates $p < 0.05$. *** indicates $p < 0.1$.

Figure 2.6

Figure 2.6. Day 55 fetal morphometry of fetuses collected on day 56 of gestation. (A) Comparison of ultrasound measurements between AI, ART-normal and ART-LOS without considering sex of fetuses. **(B)** Comparison of ultrasound measurements between male AI, ART-normal and ART-LOS. **(C)** Comparison of ultrasound measurements between female AI and ART. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome. U: ultrasound. AH: abdominal height. FL: forelimb length. BP: biparietal diameter; CR: crown rump length. HL: head length. HG: heart girth. TH: thoracic height. ** indicates $p < 0.05$.

Figure 2.7

Figure 2.7. Ultrasound/collection ratio. (A) Ratio of gestation day 55 ultrasound measurements to collection day 56 measurements. CRL, BPD among all four groups were ~ 0.8. **(B)** Ratio of gestation day 55 ultrasound measurements to collection day 105 measurements. CRL, BPD and HG of the 4 largest LOS fetuses were smaller than AI and ART-normal group whereas CRL, BPD, AH and HG of ART-LOS was smaller than AI. AI = artificial insemination. ART= assisted reproductive technologies. LOS = large offspring syndrome. AH = abdominal height. BPD = biparietal diameter. CRL= crown rump length. HL= head length. HG= heart girth. TH= thoracic height. * indicates $p<0.01$. ** indicates $p<0.05$. *** indicates $p<0.1$

Figure 2.8

Figure 2.8. Fetal measurements of fetuses collected on day 105 of gestation. Comparison of fetal measurements between day 105 AI, ART-normal and ART-LOS, males **(A)** and females **(B)** respectively. AI: artificial insemination. ART: assisted reproductive technologies. AH: abdominal height. FL: forelimb length. BP: biparietal diameter. CR: crown rump length. HL: head length. HG: heart girth. TH: thoracic height. * indicates $p < 0.01$. ** indicates $p < 0.05$.

Figure 2.9

Figure 2.9. Ratio of gestation day 105 umbilicus to CRL. Fetal pictures were used to determine the umbilicus/CRL ratio using image J. The umbilicus was measured at the base. Black circle shows AI fetus ratio, orange circle shows the ART-normal fetus's ratio, yellow circle shows ART-LOS fetus's ratio and green circle shows 4 biggest ART-LOS fetuses' ratio. CRL: crown rump length. LOS: large offspring syndrome. ART: assisted reproductive technologies.

Figure 2.10

Figure 2.10. Combined Day 55 fetal morphometry of fetuses collected on day 56 and day 105 of gestation. Comparison of ultrasound between AI, ART-normal and ART-LOS without considering sex of fetuses. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome. U: ultrasound. AH: abdominal height. FL: forelimb length. BP: biparietal diameter; CR: crown rump length. HL: head length. HG: heart girth. TH: thoracic height. ** indicates $p < 0.05$. * indicates $p < 0.1$. Green dot and blue dot represent largest day 105 and day 56 fetus's fetal measurement at day 55 of gestation, respectively.

Figure 2.11

Figure 2.11. Unsupervised hierarchical clustering of white blood cells transcriptome in day 55 and day 105 of pregnancy. Red oval denotes females (604 and 664) carrying the two largest LOS fetuses on day 105 of pregnancy. Black squares denote 10 out of 23 animals that clustered based on animal and not stage of pregnancy.

ATF7IP

Figure 2.12

Figure 2.12. Quantitative RT-PCR corroborations of genes identified as differentially expressed by RNAseq in maternal white blood cells. (A) Fold difference from AI (line at 1). **(B)** Quantitative RT-PCR corroborations of *ATF7IP* in maternal white blood cells. The figure shows the variability in transcript amount within treatment groups. The X axis denotes cycle threshold (Δ Ct). A negative Δ Ct means that the target gene amplifies sooner than the endogenous controls signifying that there is more transcript. A positive Δ Ct means that the target gene amplifies later than the endogenous controls signifying that there is less transcript. AI = Artificial insemination. ARTN = ART normal weight group. ARTLOS = ART LOS group, BigARTLOS = represent the females carrying the two largest LOS fetuses (604 and 664). HolsteinCont = Holstein females collected on day 105 of pregnancy to serve as control for the experiment.

Supplementary Figure S2.1

Supplementary Figure S2.1. Day 19 ISG15 gene expression. AI = artificial insemination. ART= assisted reproductive technologies. LOS = large offspring syndrome. ** indicates $p < 0.05$. ** indicates $p < 0.1$.

Table 2.1. LOS phenotypes observed at fetal collection.

Day 56 Fetus ID and Weight			
	584 (13.77 g)	591A (10.27 g)	607A (6.39 g)
Gross observations day 56			
Abdominal wall defects			X
Focal hemorrhage on brain	X	X	
Day 105 Fetus ID and Weight			
	664 (1080 g)	604B (986 g)	656 (704 g)
Gross observations day 105			
Macroglossia	X		
Large heart	X	X	X
Large kidney		X	
Large lung	X		X
Large pancreas		X	
Large umbilicus		X	X
Abdominal ascites	X	X	
Asymmetrical head	X		
Hemorrhage on head		X	
Hepatic cyst	X		

Supplementary Table S2.1 Ratio of day 105 umbilicus and CRL.

Group	Sex	Fetus ID	Umbilicus length (cm)	CRL (cm)	Ratio_of_umbilicus_to_CRL
AI	Female	533A	1.647409848	22.99363	0.0716
AI	Female	546A	1.469593963	22.81375	0.0644
AI	Female	609A	1.651100979	23.99733	0.0688
AI	Female	647A	1.886701166	22.41406	0.0842
AI	Male	517A	2.433474009	24.35207	0.0999
AI	Male	524A	2.064478438	22.76822	0.0907
AI	Male	527A	2.255912203	24.89788	0.0906
AI	Male	539A	1.721119514	23.90435	0.072
AI	Male	590A	2.335772769	25.02224	0.0933
AI	Male	606A	2.321687014	25.15312	0.0923
AI	Male	640A	2.01646857	23.42875	0.0861
AI	Male	648A	2.143930221	23.68326	0.0905
ART_normal	Female	519A	1.752429418	23.66431	0.0741
ART_normal	Female	522B	1.257532271	21.65827	0.0581
ART_normal	Female	688A	2.318355917	24.17182	0.0959
ART_normal	Male	522A	1.909835544	23.83783	0.0801
ART_normal	Male	530B	1.894014823	23.55227	0.0804
ART_normal	Male	532A	2.002957516	25.8395	0.0775
ART_normal	Male	540A	2.153794627	24.70058	0.0872
ART_normal	Male	542B	2.121772067	24.80472	0.0855
ART_normal	Male	547A	2.02796143	25.52157	0.0795
ART_normal	Male	547B	1.754966818	24.60583	0.0713
ART_normal	Male	557A	2.311441526	25.37462	0.0911
ART_normal	Male	559A	1.8056005	25.8542	0.0698
ART_normal	Male	560A	2.143620117	24.60551	0.0871
ART_normal	Male	576A	2.107447707	25.36457	0.0831
ART_normal	Male	580A	1.658963247	23.4185	0.0708
ART_normal	Male	585A	1.772628658	24.07664	0.0736
ART_normal	Male	585B	1.821990087	25.90078	0.0703
ART_normal	Male	605A	2.215869509	25.30408	0.0876
ART_normal	Male	611A	2.147271214	24.95087	0.0861
ART_normal	Male	624A	2.043172027	22.43629	0.0911
ART_normal	Male	628A	1.974585573	25.81613	0.0765
ART_normal	Male	628B	2.218822173	22.92348	0.0968
ART_normal	Male	631A	1.965815906	25.23815	0.0779
ART_normal	Male	633A	1.546369034	22.34727	0.0692
ART_normal	Male	652A	2.204591851	23.58467	0.0935
ART_normal	Male	666A	2.12116012	25.7349	0.0824
ART_normal	Male	688B	2.084167891	23.65453	0.0881
ART_LOS	Female	530A	1.529894939	23.21044	0.0659

ART_LOS	Female	543A	1.582656073	24.75685	0.0639
ART_LOS	Female	557B	1.656715	24.64175	0.0672
ART_LOS	Female	616A	1.883895039	26.55188	0.071
ART_LOS	Female	620A	1.661029531	25.26923	0.0657
ART_LOS	Female	663A	1.400483681	25.59089	0.0547
ART_LOS	Female	683A	1.682977702	25.22005	0.0667
ART_LOS	Male	512A	2.041310414	27.56333	0.0741
ART_LOS	Male	535A	2.140202372	25.92357	0.0826
ART_LOS	Male	542A	2.204518303	25.72483	0.0857
ART_LOS	Male	604A	2.039006599	26.25729	0.0777
ART_LOS	Male	637A	2.152399533	25.92039	0.083
ART_LOS	Male	680A	2.061112991	24.98187	0.0825
4_Biggest_ART-LOS	Female	604B	3.900653823	27.93119	0.1397
4_Biggest_ART-LOS	Male	664A	2.011625351	29.25045	0.0688
4_Biggest_ART-LOS	Female	656A	1.795230756	26.77361	0.0671
4_Biggest_ART-LOS	Male	602A	2.606172687	28.70276	0.0908

Both umbilicus and CRL were measured in centimeters from fetus pictures using ImageJ. CRL- crown-rump length.

Supplementary Table S2.2A. Gestation day 55 and day 105 maternal leucocyte RNAseq summary using NCBI Bos taurus assembly ARS-UCD 1.2.

Cow ID	Single (S) or tw in(T)	Group	Fetal weight (g)	Sex of fetus	Day of pregnancy	RIN	#reads before quality trimming	#reads after quality trimming	#total reads mapped to ARS-UCD 1.2	%total reads mapped
533	S	AI1	388	F	105	9	70494496	70486246	67374165	95.58
647	S	AI2	396	F	105	8.7	71909588	71900968	68621986	95.44
546	S	AI3	414	F	105	9.4	70369796	70361006	67410500	95.81
609	S	AI4	468	F	105	9	70317570	70308802	67279128	95.69
517	S	AI5	442	M	105	7	73996408	73987048	70654713	95.5
648	S	AI6	466	M	105	9.1	70403586	70395346	67291416	95.59
590	S	AI7	544	M	105	8.6	72009340	72000680	68727952	95.45
606	S	AI8	550	M	105	9	70077598	70068666	67018077	95.65
519	S	ART-N1	408	F	105	9.3	70125516	70118094	67332714	96.03
669	S	ART-N2	442	F	105	8.2	70173446	70165196	66962773	95.44
688	T	ART-N3	448	F	105	9.3	72435786	72427334	69233239	95.59
628	T	ART-N4	434	M	105	6.7	72180888	72172240	69107584	95.75
560	S	ART-N5	480	M	105	9	72488602	72479812	69522148	95.92
633	S	ART-N6	538	M	105	7.5	71979464	71969898	68651442	95.39
557	T	ART-LOS1	506	F	105	6.7	72106928	72097896	68845783	95.49
663	S	ART-LOS2	578	F	105	9.2	74636094	74627328	71438695	95.73
616	S	ART-LOS3	638	F	105	9.2	70201060	70192856	66924731	95.34
656	S	ART-LOS4	704	F	105	9.2	70190938	70182738	67164003	95.7
604	T	ART-LOS5	986	F	105	4.4	70307484	70299560	67518407	96.04
637	S	ART-LOS6	586	M	105	9.4	70258108	70249844	67207308	95.67
512	S	ART-LOS7	648	M	105	7.8	72169974	72161132	68719383	95.23
602	S	ART-LOS8	752	M	105	8	72386718	72377778	69236360	95.66
664	S	ART-LOS9	1080	M	105	3.6	72131696	72123546	68971848	95.63
533	S	AI1	388	F	55	8.2	72435692	72426782	69075419	95.37
647	S	AI2	396	F	55	8.5	72334552	72325372	69250131	95.75
546	S	AI3	414	F	55	8.9	72224428	72215094	69189369	95.81
609	S	AI4	468	F	55	9.4	72283066	72274632	69098021	95.6
517	S	AI5	442	M	55	8	70438806	70430230	67539053	95.89
648	S	AI6	466	M	55	8	71483452	71474136	68146852	95.34

590	S	A17	544	M	55	9.4	70376660	70368418	67541465	95.98
606	S	A18	550	M	55	7.4	74550210	74541104	71435024	95.83
519	S	ART-N1	408	F	55	9.3	72088426	72079870	69078701	95.84
669	S	ART-N2	442	F	55	9.1	70243932	70235580	67247887	95.75
688	T	ART-N3	448	F	55	9.5	72259626	72250856	69281300	95.89
628	T	ART-N4	434	M	55	6.6	74568930	74559826	71493744	95.89
560	S	ART-N5	480	M	55	9.1	72379562	72371058	69396367	95.89
633	S	ART-N6	538	M	55	9.5	72332390	72325026	69270783	95.78
557	T	ART-LOS1	506	F	55	9.5	72214470	72207666	69042385	95.62
663	S	ART-LOS2	578	F	55	8.9	70076982	70068416	67115997	95.79
616	S	ART-LOS3	638	F	55	9.3	70176532	70168294	67030106	95.53
656	S	ART-LOS4	704	F	55	9.3	72806926	72797466	69820059	95.91
604	T	ART-LOS5	986	F	55	9.6	71861400	71852960	68708083	95.62
637	S	ART-LOS6	586	M	55	9.4	70106712	70098050	67152024	95.8
512	S	ART-LOS7	648	M	55	8.7	74688290	74679108	71451930	95.68
602	S	ART-LOS8	752	M	55	7.9	70144948	70136178	67258032	95.9
664	S	ART-LOS9	1080	M	55	9.6	70174478	70165974	67298708	95.91

bp = base pairs. RIN = RNA integrity number. LOS = large offspring syndrome. AI = artificial insemination. ART = assisted reproductive technologies. F = female. M = male.

Supplementary Table S2.2B. Gestation day 55 and day 105 maternal leucocyte RNAseq summary using Ensembl Bos taurus assembly ARS-UCD 1.2

Cow ID	Single (S) or tw in(T)	Group	Fetal weight (g)	Sex of fetus	Day of pregnancy	RIN	#reads before quality trimming	#reads after quality trimming	#total reads mapped to ARS-UCD 1.2	%total reads mapped
533	S	A11	388	F	105	9	70494496	70486246	67358493	95.56
647	S	A12	396	F	105	8.7	71909588	71900968	68606903	95.42
546	S	A13	414	F	105	9.4	70369796	70361006	67394667	95.78
609	S	A14	468	F	105	9	70317570	70308802	67264081	95.67
517	S	A15	442	M	105	7	73996408	73987048	70642124	95.48
648	S	A16	466	M	105	9.1	70403586	70395346	67274662	95.57
590	S	A17	544	M	105	8.6	72009340	72000680	68713538	95.43
606	S	A18	550	M	105	9	70077598	70068666	67003185	95.63
519	S	ART-N1	408	F	105	9.3	70125516	70118094	67317812	96.01
669	S	ART-N2	442	F	105	8.2	70173446	70165196	66947644	95.41
688	T	ART-N3	448	F	105	9.3	72435786	72427334	69219589	95.57
628	T	ART-N4	434	M	105	6.7	72180888	72172240	69094450	95.74
560	S	ART-N5	480	M	105	9	72488602	72479812	69506630	95.9
633	S	ART-N6	538	M	105	7.5	71979464	71969898	68638621	95.37
557	T	ART-LOS1	506	F	105	6.7	72106928	72097896	68832768	95.47
663	S	ART-LOS2	578	F	105	9.2	74636094	74627328	71420288	95.7
616	S	ART-LOS3	638	F	105	9.2	70201060	70192856	66907474	95.32
656	S	ART-LOS4	704	F	105	9.2	70190938	70182738	67147700	95.68
604	T	ART-LOS5	986	F	105	4.4	70307484	70299560	67505767	96.03
637	S	ART-LOS6	586	M	105	9.4	70258108	70249844	67190457	95.64
512	S	ART-LOS7	648	M	105	7.8	72169974	72161132	68703633	95.21
602	S	ART-LOS8	752	M	105	8	72386718	72377778	69219151	95.64
664	S	ART-LOS9	1080	M	105	3.6	72131696	72123546	68958743	95.61
533	S	A11	388	F	55	8.2	72435692	72426782	69060642	95.35
647	S	A12	396	F	55	8.5	72334552	72325372	69232796	95.72
546	S	A13	414	F	55	8.9	72224428	72215094	69172091	95.79
609	S	A14	468	F	55	9.4	72283066	72274632	69084757	95.59
517	S	A15	442	M	55	8	70438806	70430230	67525397	95.88
648	S	A16	466	M	55	8	71483452	71474136	68131827	95.32

590	S	AI7	544	M	55	9.4	70376660	70368418	67527200	95.96
606	S	AI8	550	M	55	7.4	74550210	74541104	71421842	95.82
519	S	ART-N1	408	F	55	9.3	72088426	72079870	69061153	95.81
669	S	ART-N2	442	F	55	9.1	70243932	70235580	67232175	95.72
688	T	ART-N3	448	F	55	9.5	72259626	72250856	69266448	95.87
628	T	ART-N4	434	M	55	6.6	74568930	74559826	71480204	95.87
560	S	ART-N5	480	M	55	9.1	72379562	72371058	69381685	95.87
633	S	ART-N6	538	M	55	9.5	72332390	72325026	69254472	95.75
557	T	ART-LOS1	506	F	55	9.5	72214470	72207666	69025723	95.59
663	S	ART-LOS2	578	F	55	8.9	70076982	70068416	67101535	95.77
616	S	ART-LOS3	638	F	55	9.3	70176532	70168294	67013207	95.5
656	S	ART-LOS4	704	F	55	9.3	72806926	72797466	69804481	95.89
604	T	ART-LOS5	986	F	55	9.6	71861400	71852960	68689763	95.6
637	S	ART-LOS6	586	M	55	9.4	70106712	70098050	67136174	95.77
512	S	ART-LOS7	648	M	55	8.7	74688290	74679108	71433702	95.65
602	S	ART-LOS8	752	M	55	7.9	70144948	70136178	67242662	95.87
664	S	ART-LOS9	1080	M	55	9.6	70174478	70165974	67281245	95.89

bp = base pairs. RIN = RNA integrity number. LOS = large offspring syndrome. AI = artificial insemination. ART = assisted reproductive technologies. F = female. M = male.

Supplementary Table S2.3A. Read counts of normalizers as per RNAseq.

Day 105																														
AI														ART-normal						ART-LOS										
Female							Male							Female						Male										
Cow ID	.	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	604	637	512	602	664						
Single (S) / Twin(T)	.	S	S	S	S	S	S	S	S	S	S	T	T	S	S	T	S	S	S	T	S	S	S	S	S					
Fetus ID	.	533	647	546	609	517	648	590	606	519	669	688A	628B	560	633	557B	663	616	656	604B	637	512	602	664						
Fetal weight (g)	.	388	396	414	468	442	466	544	550	408	442	448	434	480	538	506	578	638	704	986	586	648	752	1080						
NCBI_gene_name	padj	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.						
SURF4	1.00	48.2	49.8	51.7	41.0	41.7	39.9	44.0	42.9	38.1	46.3	47.2	36.0	42.4	43.3	41.5	48.6	47.2	43.9	32.6	43.6	38.5	44.4	32.1						
NECAP1	1.00	54.6	65.4	61.5	58.6	60.9	60.1	63.2	60.8	59.2	61.2	58.0	68.1	62.3	55.3	60.1	58.6	60.3	58.0	59.7	59.9	63.7	64.2	51.3						
SMG7	1.00	247.9	234.8	238.0	249.6	237.4	246.5	263.7	252.8	217.3	258.2	236.9	227.7	255.3	229.8	266.6	251.6	234.0	243.5	221.6	260.6	259.5	254.8	239.5						

Genes whose false discovery rate (FDR) from EdgeR analysis was above 0.9 and padj value from DESeq2 analysis was above 0.85, consistent across both day 55 and day 105 RNA samples, were selected as endogenous control. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome.

Supplementary Table S2.3B. Selection of normalizers for RT-PCR.

Day of pregnancy	Ensembl_Gene_ID	padj	FDR	Average read counts	SD	CV%
	ENSBTAG00000051352	1	1	97.97	9.13	9.31
105	ENSBTAG00000020237	1	1	61.78	5.92	9.58
	ENSBTAG00000008003	1	1	253.42	24.09	9.51
	ENSBTAG00000051352	0.94	0.89	107.16	7.86	7.34
55	ENSBTAG00000020237	0.93	0.85	64.54	3.78	5.86
	ENSBTAG00000008003	0.97	0.95	258.96	16.05	6.2
Day of pregnancy	NCBI_Gene_name	padj	FDR	Average read counts (CPM)	SD	CV%
	<i>SURF4</i>	1	0.88	42.82	4.97	11.6
105	<i>NECAP1</i>	1	0.96	60.22	3.52	5.85
	<i>SMG7</i>	1	0.91	244.25	12.81	5.24
	<i>SURF4</i>	0.96	0.87	48.39	5.09	10.51
55	<i>NECAP1</i>	0.95	0.88	61.87	4.29	6.94
	<i>SMG7</i>	0.98	0.98	244.97	12.91	5.27

False discovery rate (FDR), padj value, average count per million (cpm), standard deviation (SD) coefficient of variation (CV) was calculated for all annotated genes across all samples and analyses and three genes named *SURF4*, *NECAP1* and *SMG7* were selected to be used as endogenous normalizers for qRT-PCR analysis.

Supplementary Table S2.4A. Number of animals that expressed estrus and selection of animals for Exp IA

	D0G1		D1G1		D2G1		D3G1							
	6:00 PM	7:00 AM	12:00 PM	4:00 PM	7:00 AM	12:00 PM	4:00 PM	7:00 AM	12:00 PM					
G1 (n=40)	PG shot													
# new estrus animals		0	0	0	2	1	2	5	9					
G2 (n=40)														
# new estrus animals					0	0	0	1	1	0	9	1	7	3
G3 (n=40)														
# new estrus animals														

D0, D1, D2, D3 and D4: day 0, 1, 2, 3 and 4 respectively. G1, G2 and G3: group1, group2 and group3 respectively. n: number of animals.

Supplementary Table S2.4B. Number of animals that expressed estrus and selection of animals for Exp IB and II.

G1 (n=50)	D0G1	D1G1		D2G1		D3G1		D4G1		D5G1		D6G1					
	6:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM				
	PG shot																
# new estrus animals	0	0	0	0	3	1	15	9	7	4	2	0	0	2			
G2 (n=57)	D0G2	D1G2		D2G2		D3G2		D4G2		D5G2							
	6:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM						
	PG shot																
# new estrus animals			1	1	0	2	0	1	17	15	3	5	1	0	1		
G3 (n=57)	D0G3	D1G3		D2G3		D3G3		D4G3									
	6:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	6:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM/7:00 AM	12:00 PM	5:00 PM						
	PG shot																
# new estrus animals						0	0	1	0	1	3	25	9	2	3	2	5

D0, D1, D2, D3, D4 and D5: day 0, 1, 2, 3, 4 and 5 respectively. G1, G2 and G3: group1, group2 and group3 respectively. n: number of animals.

Supplementary Table S2.5. Day 77 ultrasonography.

Cow ID	AT/ART	Single(S) / Twin (T)	BPDU	THU	TDU	AHU	ADU	HLU
517	AI	S	1.97	2.72	2.33	3.18	2.81	4.15
537	AI	S	1.98	*	*	3.44	3.08	3.94
539	AI	S	2.25	3.44	2.06	3.17	3.08	3.86
603	AI	S	1.97	2.64	1.84	2.63	2.23	3.88
650	AI	S	2.03	2.8	1.86	3.12	3.02	3.91
677	AI	S	2.02	2.71	2.46	2.64	2.61	4.37
519	ART	S	2.16	3.02	2.36	2.62	2.84	3.88
522	ART	T	2.06	2.46	2.27	2.9	2.62	3.64
	ART		2.02	4.23	*	*	*	*
530	ART	T	*	*	*	*	*	*
542	ART	T	1.94	*	*	*	*	4.17
	ART		2.21	*	*	*	*	4.44
559	ART	S	1.99	3.56	2.52	3.26	2.77	4.39
585	ART	T	*	*	*	*	*	3.78
	ART		*	*	*	*	*	4.08
616	ART	S	*	*	*	2.91	3.17	4.21
624	ART	T	*	*	*	*	*	4.27
	ART		*	*	*	*	*	4.22
628	ART	T	*	2.96	2.75	3.15	3.29	4.2
	ART		*	2.99	2.52	3.48	2.96	3.53
669	ART	S	1.96	3.29	2.02	3.41	2.86	4.13
680	ART	S	1.98	3.09	2.39	3.17	2.73	3.93
683	ART	S	2.1	4.01	2.55	3.48	3.09	4

All ultrasound measurements are in centimeter. U=ultrasound. AI: artificial insemination. ART: assisted reproductive technologies. AH: abdominal height. FL: forelimb length. BP: biparietal diameter. CR: crown rump length. HL: head length. HG: heart girth. TH: thoracic height. * indicates measurements were not possible.

Day 105

Control AI												ART normal weight												ART LOS																									
Female						Male						Female						Male						Female						Male																			
533		647		546		609		517		648		590		606		519		669		688		628		560		633		557		663		616		656		604		637		512		602		664					
Single (S) / Twin(T)		S		S		S		S		S		S		S		S		S		S		T		T		S		S		T		S		S		S		T		S		S		S					
533		647		546		609		517		648		590		606		519		669		688A		628B		560		633		557B		663		616		656		604B		637		512		602		664					
Fetus ID		388		396		414		468		442		466		544		550		408		442		448		434		480		538		506		578		638		704		986		586		648		752		1080			
Fetal weight																																																	
Ensemble_gene_ID																																																	
padj																																																	
ENSBTAG00000008428 < 0.05		26.9		23.5		42.1		42.2		23.3		22.1		59.9		22.9		13.8		18.5		24.7		57.9		16.9		44.3		4.3		19.4		9.0		8.3		5.4		197.4		9.7		13.8		5.5			
ENSBTAG00000015592 < 0.05		9.1		3.0		6.2		13.3		3.0		7.2		11.6		7.6		2.3		5.2		5.3		23.4		3.9		5.6		1.1		3.1		1.8		2.0		1.3		43.0		1.7		2.5		1.6			
ENSBTAG00000009214 < 0.05		25.6		12.3		28.1		42.5		13.0		32.8		40.8		40.0		16.5		18.5		27.3		47.2		19.8		23.9		4.1		14.0		18.8		14.4		4.4		132.5		9.7		11.7		5.2			
ENSBTAG00000006156 < 0.05		14.5		7.4		26.6		36.3		8.5		19.5		38.3		19.3		8.7		10.1		17.5		36.5		14.0		10.9		3.9		9.9		9.8		6.3		1.7		135.7		5.7		7.9		1.5			
ENSBTAG00000013806 < 0.05		7.5		5.9		8.5		12.3		6.9		7.3		7.9		7.9		8.6		5.6		10.4		13.9		9.3		9.5		6.0		6.6		6.3		5.1		3.0		5.3		6.5		5.7		3.9			
ENSBTAG00000002580 < 0.05		70.0		32.1		149.6		129.7		23.8		75.2		183.6		76.6		29.3		33.1		60.0		143.7		56.3		112.5		3.1		47.8		39.7		30.5		7.0		759.4		18.1		18.2		3.3			
ENSBTAG00000020350 < 0.05		398.0		206.8		325.4		647.7		204.6		709.6		389.8		1045.5		187.5		367.2		520.4		1019.0		443.0		546.7		152.4		292.7		232.3		203.4		109.9		341.3		237.1		286.2		88.9			
ENSBTAG000000015918 < 0.05		11.3		6.3		13.8		18.9		5.4		13.1		12.1		10.4		5.5		9.1		12.5		9.5		9.9		7.9		6.6		7.0		8.5		6.2		2.7		33.5		4.8		4.5		2.1			
ENSBTAG000000051920 < 0.05		9.9		14.1		37.4		29.8		16.9		6.7		16.1		16.7		12.2		13.4		12.5		6.5		6.6		3.2		3.5		6.4		12.9		6.5		2.6		6.1		7.8		4.1		2.1			

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Supplementary Table S2.6B. Differentially expressed genes of day 105
AI, ART-normal and ART-LOS in maternal WBC transcriptome
Day 105

	Control AI						ART normal weight						ART LOS											
	Female			Male			Female			Male			Female			Male								
Cow ID	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	604	637	512	602	664	
Single (S) / Twin(T)	S	S	S	S	S	S	S	S	S	S	T	T	S	S	T	S	S	S	T	S	S	S	S	
Fetus ID	533	647	546	609	517	648	590	606	519	669	688A	628B	560	633	557B	663	616	656	604B	637	512	602	664	
Fetal weight	388	396	414	468	442	466	544	550	408	442	448	434	480	538	506	578	638	704	986	586	648	752	1080	
NCBI_gene_name	padj																							
GPR84	< 0.05	8.7	3.0	5.8	12.6	3.1	6.7	11.0	7.1	2.1	5.1	4.9	22.5	3.6	5.4	1.1	2.9	1.7	1.8	1.6	40.0	1.6	2.4	1.8
UPP1	< 0.05	26.0	23.2	40.9	41.2	24.2	21.1	57.1	22.5	13.5	18.2	23.5	56.1	16.1	44.0	4.4	18.8	8.8	8.1	6.7	188.0	9.7	13.7	6.5
ETS2	< 0.05	23.9	11.9	26.2	40.1	13.4	29.9	37.9	36.9	15.5	17.6	25.3	44.9	18.4	23.3	4.1	13.1	17.3	13.3	5.2	121.4	9.3	11.4	6.1
GLT1D1	< 0.05	3.6	3.2	7.3	5.1	2.0	4.0	16.4	5.4	2.7	3.0	4.0	10.1	2.4	7.9	1.3	2.3	1.9	1.3	1.7	35.7	2.0	2.5	1.1
MEGF9	< 0.05	44.4	34.7	77.7	50.9	16.9	25.2	106.0	71.2	28.3	25.1	29.5	131.7	34.2	102.4	12.2	26.4	17.4	22.6	10.0	358.5	22.0	14.4	5.9
LOC107131224	< 0.05	73.1	36.8	81.4	143.5	41.3	55.0	77.5	71.0	38.1	40.1	7.8	6.0	5.9	5.6	1.3	4.1	29.4	3.0	10.3	8.9	4.2	1.7	9.0
BST1	< 0.05	14.0	7.3	25.3	34.8	8.9	18.4	36.3	18.1	8.3	9.7	16.7	35.1	13.0	10.7	4.1	9.4	9.2	5.9	2.0	127.0	5.7	7.8	1.7
LOC101906463	< 0.05	8.6	6.6	11.7	17.6	3.7	14.9	14.7	16.2	2.7	9.1	12.8	21.0	9.9	13.6	5.8	8.0	5.4	5.0	3.2	4.4	5.8	5.3	1.8
EFHB	< 0.05	7.1	5.7	8.2	11.9	7.2	6.9	7.5	7.5	8.2	5.4	9.8	13.3	8.7	9.3	6.2	6.3	5.9	4.7	3.6	5.0	6.4	5.6	4.6
LOC107132783	< 0.05	11.9	5.5	8.9	15.0	3.7	18.2	11.3	17.7	5.6	6.3	11.1	25.2	9.6	7.6	1.1	6.5	4.9	5.8	1.1	7.6	2.7	3.8	1.1

ART-LOS's day 105 maternal blood compare to AI and ART-normal's day 105 maternal blood by DESeq2 using annotated NCBI *Bos taurus* gene model. This is just representation of some of differentially expressed genes. Red color shows upregulation of the gene and blue color shows down regulation of gene. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome.

Supplementary Table S2.6C. Differentially expressed genes of day 105 AI, ART-normal and ART-LOS in maternal WBC transcriptome.
Day 105

	Control/AI						ART normal weight						ART LOS						2 Largest ART LOS									
	Female			Male			Female			Male			Female			Male			Female	Male								
	Cow ID	Single (S) / Twin(T)	Fetus ID	Fetal weight	Ensemble_gene_ID	FDR	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	637	512	602	604
ENSBTAG000000011819	.	S	S	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	637	512	602	604	664	24.5	26.9
ENSBTAG00000002836	< 0.05	31.2	31.9	33.5	31.3	33.7	32.0	33.1	31.7	28.9	32.5	30.0	27.6	35.7	35.0	41.2	35.6	32.1	33.8	30.7	34.1	35.2	48.7	58.1	48.7	58.1	48.7	58.1
ENSBTAG000000038434	< 0.05	129.8	142.3	108.7	138.8	171.2	133.4	128.3	147.4	137.8	143.4	109.4	138.8	167.8	137.6	211.0	132.9	161.4	152.6	113.9	149.8	156.4	275.1	340.8	275.1	340.8	275.1	340.8
ENSBTAG000000008388	< 0.05	117.9	126.6	112.2	132.2	137.3	128.9	115.6	141.0	115.1	125.3	102.8	110.0	131.9	123.6	160.8	130.1	148.8	132.2	121.5	136.0	137.6	191.4	243.9	191.4	243.9	191.4	243.9
ENSBTAG000000018710	< 0.05	76.7	70.6	65.6	77.7	92.2	64.2	75.5	70.4	64.5	73.9	61.4	86.7	78.1	76.0	96.7	62.5	67.6	71.1	61.5	81.0	82.9	137.8	173.4	137.8	173.4	137.8	173.4
ENSBTAG000000015474	< 0.05	304.1	337.1	244.7	310.0	399.5	280.3	303.1	319.2	334.8	330.5	260.4	345.6	327.8	299.7	454.5	275.4	341.3	275.9	227.3	344.5	369.6	611.0	877.2	611.0	877.2	611.0	877.2
ENSBTAG000000021520	< 0.05	172.0	179.8	140.1	151.9	172.7	154.9	150.4	165.7	178.4	157.4	157.0	154.2	176.0	177.5	198.7	148.6	198.1	174.8	136.4	170.9	186.9	246.1	321.8	246.1	321.8	246.1	321.8
ENSBTAG000000002495	< 0.05	105.2	101.2	107.7	111.2	114.5	107.9	102.4	111.9	85.2	106.3	86.8	115.0	104.5	118.9	122.0	108.9	99.4	106.1	118.5	112.1	107.1	173.5	211.1	173.5	211.1	173.5	211.1
ENSBTAG000000004934	< 0.05	82.9	94.9	69.3	81.6	90.0	78.7	78.3	84.0	89.5	77.4	75.8	71.7	96.7	94.8	104.2	84.2	94.0	80.4	63.4	93.4	89.4	130.2	168.0	130.2	168.0	130.2	168.0
ENSBTAG000000017177	< 0.05	190.2	217.9	185.7	215.1	274.4	181.2	204.2	215.4	201.3	210.3	177.8	227.2	227.5	217.3	291.2	207.8	204.2	180.1	190.6	240.6	263.2	388.8	503.0	388.8	503.0	388.8	503.0

Two largest ART-LOS day 105 maternal blood compare to AI and ART-normal's day 105 maternal blood by EdgeR using annotated Ensembl *Bos taurus* gene model. This is just representation of some of differentially expressed genes. Red color shows upregulation of the gene. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome.

Supplementary Table S2.6D. Differentially expressed genes of day 105 AI, ART-normal and ART-LOS in maternal WBC transcriptome.

Cow ID	Control AI										ART normal weight										ART LOS										2 Largest ART LOS	
	Female					Male					Female					Male					Female					Male					Female	Male
	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	637	512	602	604	664	664								
Single (S) / Twin(T)	S	S	S	S	S	S	S	S	S	T	T	T	S	S	T	S	S	S	S	S	S	T	S									
Fetus ID	533	647	546	609	517	648	590	606	519	669	688A	628B	560	633	557B	663	616	656	637	512	602	604B	664									
Fetal weight	388	396	414	468	442	466	544	550	408	442	448	434	480	538	506	578	638	704	586	648	752	986	1080									
Ensemble_gene_ID	padj																															
ENSBTAG000000025385	0.00	221.2	203.7	366.7	173.4	168.0	165.7	364.6	373.9	471.5	179.1	364.1	178.3	185.7	464.6	213.9	189.8	442.3	181.2	144.0	358.1	194.6	3.7	5.2								
ENSBTAG000000011819	0.00	6.2	7.0	5.4	7.0	10.4	7.1	6.6	6.7	7.7	7.8	5.7	7.0	7.2	8.5	11.2	6.9	7.2	7.7	7.6	8.3	8.0	24.5	26.9								
ENSBTAG000000002836	0.00	31.2	31.9	33.5	31.3	33.7	32.0	33.1	31.7	28.9	32.5	30.0	27.6	35.7	35.0	41.2	35.6	32.1	33.8	30.7	34.1	35.2	48.7	58.1								
ENSBTAG0000000031940	0.00	22.2	23.2	24.2	24.6	25.1	24.5	22.9	28.4	22.1	24.6	20.7	20.8	28.8	23.7	27.1	23.9	24.6	25.2	21.4	27.5	29.0	36.7	42.5								
ENSBTAG0000000021520	0.00	172.0	179.8	140.1	151.9	172.7	154.9	150.4	165.7	178.4	157.4	157.0	154.2	176.0	177.5	198.7	148.6	198.1	174.8	136.4	170.9	186.9	246.1	321.8								
ENSBTAG0000000008388	0.00	117.9	126.6	112.2	132.2	137.3	128.9	115.6	141.0	115.1	125.3	102.8	110.0	131.9	123.6	160.8	130.1	148.8	132.2	121.5	136.0	137.6	191.4	243.9								
ENSBTAG0000000006383	0.00	4.9	7.1	226.6	4.5	7.2	4.2	278.2	6.3	6.9	4.5	4.7	7.4	5.5	7.3	6.7	4.4	5.3	3.9	175.5	5.6	6.3	5.8	317.9								
ENSBTAG0000000002495	0.00	105.2	101.2	107.7	111.2	114.5	107.9	102.4	111.9	85.2	106.3	86.8	115.0	104.5	118.9	122.0	108.9	99.4	106.1	118.5	112.1	107.1	173.5	211.1								
ENSBTAG0000000004934	0.00	82.9	94.9	69.3	81.6	90.0	78.7	78.3	84.0	89.5	77.4	75.8	71.7	96.7	94.8	104.2	84.2	94.0	80.4	63.4	93.4	89.4	130.2	168.0								
ENSBTAG0000000002972	0.00	7.4	8.1	7.1	7.3	9.8	7.3	6.4	8.3	10.0	8.0	8.1	9.1	7.6	10.4	9.3	7.4	8.7	7.5	6.3	8.7	8.1	16.3	18.2								

Two largest ART-LOS day 105 maternal blood compare to AI and ART-normal's day 105 maternal blood by DEseq2 using annotated Ensembl *Bos taurus* gene model. This is just representation of some of differentially expressed genes. Red color shows upregulation of the gene. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome.

Supplementary Table S2.6E. Differentially expressed genes of day 105 AI, ART-normal and ART-LOS in maternal WBC transcriptome.

Day 105

Control AI												ART normal weight										ART LOS								2 Largest ART LOS	
Female						Male						Female				Male				Female				Male				Female		Male	
Cow ID	533	647	546	609	517	648	590	606	519	669	688	628	560	633	557	663	616	656	637	512	602	604	664								
Single (S) / Twin(T)	.	S	S	S	S	S	S	S	S	S	S	T	S	S	T	S	S	S	S	S	S	T	S								
Fetus ID	.	533	647	546	609	517	648	590	606	519	669	688A	628B	560	633	557B	663	616	656	637	512	602	604B	664							
Fetal weight	.	388	396	414	468	442	466	544	550	408	442	448	434	480	538	506	578	638	704	586	648	752	986	1080							
NCBI_gene_name																															
FDR																															
TAF3	< 0.05	37.7	40.7	39.2	38.1	44.0	38.1	40.1	37.6	37.2	39.3	37.6	34.7	43.9	43.5	52.8	41.8	38.6	41.9	34.4	41.9	45.2	71.4	82.5							
TTF1	< 0.05	72.9	68.8	61.7	73.5	95.2	59.9	70.9	65.6	62.0	71.5	57.2	83.0	72.9	73.9	97.2	58.7	63.1	66.4	57.5	78.5	80.7	169.8	204.9							
RSBN1	< 0.05	17.9	18.6	19.3	19.3	22.9	18.6	18.4	21.3	18.0	20.1	16.2	16.8	22.9	20.0	24.5	18.2	18.5	19.0	16.2	22.7	24.3	42.1	47.2							
ATRX	< 0.05	183.4	210.8	156.3	188.2	242.9	190.7	185.6	204.7	203.4	208.2	172.0	186.0	239.9	197.2	291.3	185.9	228.6	219.2	160.3	212.9	222.4	408.1	474.8							
NEMF	< 0.05	83.4	96.6	69.8	81.5	98.1	77.4	77.8	81.6	91.8	80.4	75.2	71.6	94.8	96.8	109.0	84.2	94.1	79.5	62.5	95.7	92.9	163.2	202.6							
UPF2	< 0.05	125.8	124.6	105.7	110.6	136.4	110.5	122.3	127.0	140.7	116.0	122.8	117.0	141.8	137.0	162.7	104.2	128.5	125.3	115.2	130.8	135.5	227.4	258.0							
PHF14	< 0.05	34.5	38.2	30.2	29.8	38.1	30.3	32.3	33.4	37.2	31.5	29.6	28.1	34.5	29.8	41.1	31.3	33.9	35.4	31.0	37.2	34.4	57.3	65.7							
DDX46	< 0.05	201.4	213.5	162.7	173.1	218.0	176.8	175.6	185.1	214.7	187.4	184.1	181.8	201.9	210.8	242.1	165.7	228.8	202.9	155.2	202.4	219.8	344.1	427.6							
CHD7	< 0.05	165.8	129.9	94.6	142.1	160.9	137.0	112.4	162.6	175.8	149.7	129.6	147.0	158.2	163.3	183.1	146.8	168.9	151.9	115.0	145.8	160.8	287.7	408.7							
CHMP4A	< 0.05	46.2	47.1	37.9	39.0	58.2	43.0	43.4	40.1	52.2	44.5	37.0	55.5	48.5	68.6	52.9	40.2	45.4	44.5	30.9	47.7	51.9	114.5	103.4							

Two largest ART-LOS day 105 maternal blood compare to AI and ART-normal's day 105 maternal blood by EdgeR using annotated NCBI Bos *taurus* gene model. This is just representation of some of differentially expressed genes. Red color shows upregulation of the gene. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome.

Supplementary Table S2.6F. Differentially expressed genes of day 105 AI, ART-normal and ART-LOS in maternal WBC transcriptome.

Day 105

Cow ID	Single (S) / Twin(T)	Control AI				ART normal weight				ART LOS				2 Largest ART LOS										
		Female		Male		Female		Male		Female		Male		Female	Male									
		533	647	546	609	517	648	590	606	519	669	688	628	580	633	557	663	616	656	637	512	602	604	664
Fetus ID		S	S	S	S	S	S	S	S	S	S	T	T	S	S	T	S	S	S	S	S	S	T	S
Fetal weight		533	647	546	609	517	648	590	606	519	669	688A	628B	580	633	557B	663	616	656	637	512	602	604B	664
Fetal weight		388	396	414	468	442	466	544	550	408	442	448	434	480	538	506	578	638	704	586	648	752	986	1080
NCBI_gene_name	padj																							
TAF3	<0.05	37.7	40.7	39.2	38.1	44.0	38.1	40.1	37.6	37.2	39.3	37.6	34.7	43.9	43.5	52.8	41.8	38.6	41.9	34.4	41.9	45.2	71.4	82.5
RSBN1	<0.05	17.9	18.6	19.3	19.3	22.9	18.6	18.4	21.3	18.0	20.1	16.2	16.8	22.9	20.0	24.5	18.2	18.5	19.0	16.2	22.7	24.3	42.1	47.2
PHF14	<0.05	34.5	38.2	30.2	29.8	38.1	30.3	32.3	33.4	37.2	31.5	29.6	28.1	34.5	29.8	41.1	31.3	33.9	35.4	31.0	37.2	34.4	57.3	65.7
DDX46	<0.05	201.4	213.5	162.7	173.1	218.0	176.8	175.6	185.1	214.7	187.4	184.1	181.8	201.9	210.8	242.1	165.7	228.8	202.9	155.2	202.4	219.8	344.1	427.6
NEMF	<0.05	83.4	96.6	69.8	81.5	98.1	77.4	77.8	81.6	91.8	80.4	75.2	71.6	94.8	96.8	109.0	84.2	94.1	79.5	62.5	95.7	92.9	163.2	202.6
UPF2	<0.05	125.8	124.6	105.7	110.6	136.4	110.5	122.3	127.0	140.7	116.0	122.8	117.0	141.8	137.0	162.7	104.2	128.5	125.3	115.2	130.8	135.5	227.4	258.0
TF1	<0.05	72.9	68.8	61.7	73.5	95.2	59.9	70.9	65.6	62.0	71.5	57.2	83.0	72.9	73.9	97.2	58.7	63.1	66.4	57.5	78.5	80.7	169.8	204.9
ATRX	<0.05	183.4	210.8	156.3	188.2	242.9	190.7	185.6	204.7	203.4	208.2	172.0	186.0	239.9	197.2	291.3	185.9	228.6	219.2	160.3	212.9	222.4	408.1	474.8
THOC2	<0.05	216.3	238.4	203.5	221.3	245.7	224.8	214.0	243.6	222.6	229.4	204.3	201.1	240.1	226.0	287.0	222.6	251.8	232.0	198.4	249.3	244.8	331.3	387.1
CHD7	<0.05	165.8	129.9	94.6	142.1	160.9	137.0	112.4	162.6	175.8	149.7	129.6	147.0	158.2	163.3	183.1	146.8	168.9	151.5	115.0	145.8	160.8	287.7	408.7

Two largest ART-LOS's day 105 maternal blood was compared to AI and ART-normal's day 105 maternal blood by DEseq2 using annotated NCBI *Bos taurus* gene model. This is just representation of some of differentially expressed genes. Red color shows upregulation of the gene. All samples read counts are counted in count per million (cpm). Fetal weight is in grams. AI: artificial insemination. ART: assisted reproductive technologies. LOS: large offspring syndrome

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