UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

PROMOTER-SPECIFIC EXPRESSION OF THE IMPRINTED *IGF2* GENE IN BOVINE OOCYTES AND PREIMPLANTATION EMBRYOS

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Dissertation presented as partial requirement to obtain a Master's Degree in Veterinary Sciences in the area of Animal Reproductive Technologies and Physiopathology of Reproduction

Advisor: Marcelo Bertolini

PORTO ALEGRE

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RESUMO

O *splicing* de precursores de RNA mensageiro para mRNA maduro é um componente crítico da regulação gênica. O processo pode codificar proteínas distintas ou afetar a estabilidade, localização e tradução de mRNAs. Já foram descritas diversas correlações entre uma maior mortalidade embrionária precoce, principalmente em embriões de produzidos *in vitro* (PIV), que apresentavam retardo de crescimento do concepto e expressões reduzidas de IGF2 com anormalidades placentárias e fetais durante a fase fetal. O *locus IGF2* é uma região genômica complexa que produz múltiplos transcritos com *splicing* alternativos, a partir de vários exons distintos controlados por quatro promotores diferentes. O presente estudo explorou a expressão dos diferentes promotores do *IGF2* em oócitos bovinos e embriões bovinos em estádios de pré-implantação, no intuito de esclarecer alguns aspectos da fisiologia do desenvolvimento de embriões bovinos produzidos *in vitro* (PIV). Para descrever o comportamento do gene *IGF2*, estruturas de PIV em vários estádios de desenvolvimento, desde oócitos imaturos até blastocistos expandidos, foram produzidas e coletadas em três rotinas, em *pools* de cinco estruturas por estádio. O RNA total foi extraído dos *pools* e submetido à transcrição reversa para a obtenção de cDNA, o qual foi utilizado para estimar a abundância das quatro isoformas de mRNA para o *IGF2* por PCR quantitativo em tempo real (qPCR), utilizando inicializadores específicos para cada promotor do gene *IGF2*. Os produtos de amplificação foram submetidas ao sequenciamento de DNA para confirmação molecular. Os dados de expressão quantitativa das isoformas do *IGF2* foram transformados em log para a normalização, foram analisados pelo *Mixed Procedure* do SAS, com comparações pareadas por LSM, para $P<0,05$. A expressão dirigida pelos promotores P2 e P4 seguiram o padrão observado no mRNA ativo do gene *IGF2*. Um pico inicial pôde ser visto em estádios precoces, entre oócitos maturados e o estádio embrionário de 2-células, principalmente causada por uma expressão materna e acumulação de transcritos antes da fecundação, seguido por uma diminuição da quantidade de transcritos até a ativação do genoma embrionário, no estádio embrionário de 8-células. Então, um novo aumento dos transcritos pôde ser detectado na compactação e cavitação embrionária. A expressão dirigida pelo promotor P1 do *IGF2* mostrou-se menos representativo nas fases iniciais, havendo aumento durante a compactação, após a ativação genômica, prévia à cavitação. Diferentemente, a atividade do promotor P3 não foi detectada em embriões, estando possivelmente presente apenas em estádios mais avançados de desenvolvimento. Futuros estudos genéticos focados no desenvolvimento embrionário inicial devem prestar especial atenção ao papel da expressão do promotor P4 do gene *IGF2*, com os promotores P1 e P2 tendo um aparente papel secundário no desenvolvimento inicial, enquanto o promotor P3, se geneticamente manipulado, poderia trazer mudanças fisiológicas somente posteriormente no desenvolvimento. Os achados deste estudo fornecem uma compreensão adicional da biologia do desenvolvimento de embriões derivados de PIV, bem como novas possibilidades para o uso da expressão dos diferentes promotores do gene *IGF2* para a manipulação genética durante estádios pre-implantacionais de embriões bovinos, o que pode ser um caminho para estudos que visam o aumento da viabilidade e a redução da mortalidade embrionária no início da gestação pela modulação da taxa de crescimento após a produção embrionária *in vitro*.

Palavras-chave: IGF2; mortalidade embrionária; *imprinting* genômico; bovinos.

ABSTRACT

The splicing of messenger RNA precursors to mature mRNA is a critical component of the gene regulation that can encode distinct protein or affect mRNA stability, localization, and translation. Correlations between the higher early embryonic mortality in in vitro-produced (IVP) embryos with the growth retardation of the conceptus and reduced expressions of IGF2 with placental and fetal abnormalities in the fetal phase in development have already been established. The IGF2 locus is a complex genomic region that produces multiple alternative splicing transcripts of several leader exons controlled by four distinct promoters. The present study evaluated the pattern of the promoter-specific IGF2 expression in bovine oocytes and in preimplantation embryos, as a means of unraveling some aspects of the developmental physiology of bovine embryos produced in vitro. To describe the behavior of the IGF2 gene, IVP-derived structures from distinct stages of development, from immature oocytes to expanded blastocyst, were collected in three IVP procedures, in pools of five structures per stage. Total RNA was extracted from the pools and reverse transcribed in cDNA, which were evaluated to estimate the abundance of the four different IGF2 mRNA isoforms by real time quantitative PCR system (qPCR), using IGF2 promoter-specific primers. Amplifications were DNA sequenced for confirmation. Data comprising quantitative IGF2 isoform expression were log transformed for normality and analyzed by the Mixed Procedure of SAS, with pairwise comparisons by LSM, for $P<0.05$. Promoter P2 and P4 IGF2 expression followed the pattern seen in active mRNA of IGF2 gene. An initial peak could be seen in early development, between matured oocytes and 2-cells stage embryos, mostly from accumulation prior to fertilization, followed by a decrease in abundance until embryo genome activation, at the 8-cells stage embryo. Then, a new surge in splicing variants could be detected at compaction and cavitation. Promoter P1 IGF2 expression showed to be less representative at the initial stages, having a notorious increase during compaction, after genomic activation, prior to cavitation. Differently, Promoter P3 activity was not detected at all stages, possibly being more relevant at more advanced stages in development. Future genetic studies focused on early embryonic development should pay especial attention to the role of the P4 promoter for IGF2 expression, with the P1 and P2 promoters having an apparent secondary role during early development, whereas the P3 promoter, if genetically manipulated, could possibly bring physiological changes only latter in development. The findings of this study provide some further understanding of aspects in developmental biology for IVP-derived embryos, also offering novel possibilities for the use of promoter-specific IGF2 expression for genetic manipulation during preimplantation embryo stages in cattle, which may be a path for studies seeking an increase in embryo viability and reduction of embryo mortality in early pregnancy by the modulation of growth rate after in vitro embryo production.

Keywords: *IGF2; embryo mortality; genomic imprinting; cattle.*

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND ACRONYMS

ART	Assisted reproductive technologies
AS	Antisense
DMR	Differentially methylated regions
DNMT	DNA methyltransferase
dpc	Day post-coitum
EGA	Embryonic genome activation
EGF	Epidermal growth factor
ES	Stem-like cell
GH	Growth hormone
ICR	Imprinting control region
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBP	IGF-binding proteins
IR	Insulin receptor
IVF	*In vitro* fertilization
IVP	*In vitro* production
LOS	Large offspring syndrome
MBD	Methyl-CpG binding domain protein
mRNP	Non-translatable
MSA	Multiplication-stimulating activity
NSILA	Non-suppressible insulin-like activity
NT	Nucleus transfer
P	Promoter
PcG	Polycomb group-protein
qPCR	PCR quantitativo em tempo real
PGC	Primordial germ cell
PGRC7	Primordial germ cell protein 7
SM	Somatomedin
SNP	Single nucleotide polymorphism
TET3	Translocation protein 10-11 3

SUMMARY

1. INTRODUCTION

Processes for the control of reproduction in animals have been developed and perfected by humankind since animal domestication, thousands of years ago, to facilitate animal reproduction and to increase production. Humanity started with the simplest procedures, such as animals feeding and sheltering; in the most recent centuries, once basic husbandry practices were mastered, more complex approaches were conceived, including careful animal breeding to enhance specific genetic traits. In this view, the use of assisted reproductive technologies (ART) has been of great importance in livestock production, setting off the development of four generations of ART, including: 1) artificial insemination (AI) and gamete and embryo freezing, 2) multiple ovulation and embryo transfer 3) *in vitro* fertilization (IVF) procedures and 4) cloning by nuclear transfer using either embryonic or somatic cells; genetic engineering and transgenesis, and stem cell biology (Bertolini & Bertolini, 2009). It has been more than three decades since the birth of the first bovine calf (Virgil) from *in vitro* fertilization (IVF) procedures (Brackett *et al.*, 1982), with the development of complete *in vitro* production (IVP) systems (Lu *et al.*, 1988), which not only facilitated the process but also paved the way into studies that resulted in tremendous technological advances, commercial application and novel knowledge in many related areas. With time, a dramatic enhancement in embryo production has been achieved, but unexpected problems also emerged as a consequence. Embryonic mortality, early or late, is still one of the most important causes of reproductive losses after the transfer of *in vitro*-produced (IVP) embryos, significantly more than natural breeding, artificial insemination (AI), or even for *in vivo*-derived embryos (IVD), which causes significant economic impact in the livestock industry. Therefore, a gap in knowledge regarding cause-and-effect mechanisms is yet to be filled in this regard, with the application of

procedures to promote embryo development at pre- and postimplantation stages aiming to favor the establishment and maintenance of pregnancy under physiological conditions.

Embryonic mortality is a multifactorial problem, occurring mainly in the first 16 days post-conception (early mortality) in cattle (Diskin *et al.*, 2015). Such a problem seems to be intensified after the transfer of IVP bovine embryos, which usually have lower pregnancy rates and higher gestational losses during the embryonic phase than for IVD embryos or even after AI. In cloned animals, such effects are even more pronounced, encouraging many studies in the field (Hasler *et al.*, 1995; Bertolini *et al.*, 2002ab). Pregnancy rates on Day 30 for IVF embryos are usually lower than for those obtained with IVD embryos, but still well comparable (30 to 50%). Gestational losses between Days 30 and 60, however, are significantly higher for IVP embryos than for IVD or AI, ranging from 15 to 60% (Wells *et al.*, 1999; Hill *et al.*, 2000; Bertolini & Anderson, 2002; Heyman *et al.*, 2002). Since discrepancies between losses are more drastically perceived after and during the period of initial development and placentation (from Day 30 to 60; Hill *et al.*, 2000, 2001, DeSouza *et al.*, 2001; Bertolini & Anderson, 2002), such period deserves particular attention. Pieces of evidence suggest that the high mortality of IVP-derived embryos is a consequence of an irregularity in maternal-embryonic-fetal connection and exchange, leading to embryo-fetal malnutrition, initial growth retardation and increased mortality during the first trimester of pregnancy (Bertolini *et al.*, 2002ab, Chavatte-Palmer *et al.*, 2006). Under physiological conditions, establishment of gestation and placentation must follow a strict schedule in a precise temporal development during the onset of gestation, when the maternal-embryonic connection is initiated and established. This process depends on several embryo changes, such as the elongation of the trophoblast and the formation of

the extra-embryonic membranes (yolk sac, allantoic and amniotic sac) to allow the necessary support for placentation (Bertolini *et al.*, 2007). In fact, some studies with embryonic IVP pregnancies have demonstrated developmental defects of the extra-embryonic membranes, which translate into failures in maternal recognition of gestation (early) or failures in the formation of extra-embryonic membranes and placentation (late; Bertolini *et al.*, 2007a). Other findings include the occurrence of trophoblast underdevelopment in cattle (Bertolini *et al.*, 2002a), developmental disorders of the yolk and allantoic sacs (Peterson & McMillan, 1998ab), and hypoplasia and/or underdevelopment of the placentomes (Hill *et al.*, 2000, 2001) in cattle. Therefore, the summation of altered early intrinsic and extrinsic processes resulting from the IVP of embryos may be the cause of embryonic developmental delay leading to lower pregnancy rates, higher embryonic losses and lower *ex utero* survival (Bertolini *et al.*, 2012). Interestingly, the occurrence of a biphasic conceptus growth pattern in bovine IVF-derived pregnancies has been described in detail (Bertolini *et al.*, 2002ab). Such an initial period of growth restriction observed during the embryonic phase and early fetal phase was characterized by lower pregnancy rates and a high rate of late embryonic losses attributed to failures in placental formation, possibly negatively affecting maternal-fetal nutrient and bioproduct exchange.

The progress of the fourth generation of ART in animals entails embryo cloning, genetic engineering and transgenesis, and stem cell biology. It brought forward the potential of farm animals to improve human health and, when associated with molecular tools, may further advance progress in animal breeding and genetics (Bertolini *et al.*, 2009). Hereof, the use of molecular markers will enhance the ability to manipulate animals genetically, either through breeding or by means of genetic engineering. As mankind acquired more knowledge in genomics and in gene functions, and as

procedures to manipulate gametes, cells and embryos tend to evolve to more closely mimic nature into more natural physiologic processes, all fields in biology and medicine will advance towards the resolution of current problems associated with embryo development after certain embryo manipulations. Several methods for introducing exogenous genetic material into cells are available, by means of mechanic (microinjection), electric (electroporation) and physicochemical (calcium phosphate, lipofection by cationic lipids, proteins; Lou & Saltzman, 1999) procedures. Furthermore, gene-editing techniques have recently emerged and expanded tremendously in efficiency with the discovery of new tools, such as specific endonucleases. However, living genetically engineered animals are still not widely used in commercial livestock. The difficulty in obtaining a genetically manipulated offspring is a reflection of the lack of knowledge and adequate mastery of the various stages of the process *per se*, such as the proper engineering of gene constructs and specific promoters, control of transgene expression, as well as the basic aspects of genetics on genetic interaction, and even embryo production itself that is reflected in survival rates lower than ideal, usually inferior to 50%. Therefore, studies that focus in early embryo development, or the understanding of particular gene functions, mechanisms and behavior are essential to improve genomic manipulation efficiency.

As discussed above, the high rate of embryonic mortality for IVP embryos has been related to an initial growth delay, and that, to changes in the modulation of expression of the IGF system, such as the IGF2 ligand and its receptor (IGF2R; DeChiara *et al.*, 1991; Baker *et al.*, 1993; Lui *et al.*, 1993; Bertolini *et al.*, 2002b). Such genes have a particular expression pattern called genomic imprinting, where only the specific parental chromosome allele will have active expression, while the other will be silenced by typical epigenetic processes (DeChiara *et al.*, 1991; Constância *et al.*, 2000).

The IGF2 ligand, for example, has a direct positive pleiotropic effect on development, being expressed in the paternal allele; on the other hand, the IGF2R has indirect negative pleiotropic effect, blocking the effect of IGF2, being of maternal origin. Both components of the IGF system are clearly related to the control of the regulation of embryonic, fetal and mammalian growth and placentation (DeChiara *et al.*, 1991; Lau *et al.*, 1994). According to the "parental conflict theory", genomic imprinting is a consequence of viviparity as a result of the evolution of placentation. In this theory, a biological "tug-of-war" is established between paternal and maternal genes with respect to greater or lesser allocation of maternal nutrients to the concept (Moore & Haing 1991). The IGF2 and IGF2R components are typical examples of such parental conflict (DeChiara *et al.*, 1991; Baker *et al.*, 1993; Liu *et al.*, 1993). Interestingly, previous studies have observed a close correlation between the higher early embryonic mortality in IVF embryos with the growth retardation of the conceptus and reduced expression of the *IGF2* gene, as well as higher *IGF2R* gene expression in the late embryonic phase, and with placental and fetal abnormalities in the fetal phase (Bertolini *et al.*, 2002ab). Thus, the loss of the imprinting pattern of the *IGF2* and/or the *IGF2R* genes has been postulated to be associated with major changes in IVP-derived concepti (Lau *et al.*, 1994; Young *et al.*, 2001; Suteevun-Phermthai *et al.*, 2009).

The application of procedures to stimulate embryo development at pre- and postimplantation embryo stages may favor the establishment and maintenance of pregnancy under physiological conditions. The IGF2 ligand promotes cell proliferation and differentiation, and growth of the conceptus, enhancing the transport of substrates to the conceptus (Constância *et al.*, 2005), and as discussed, being already related to conceptus development (Lau *et al.*, 1994; Young *et al.*, 2001; Suteevun-Phermthai *et al.*, 2009). The better understanding of the control for growth and gene expression

patterns at early embryo stages of development become essential to allow better protocols for the IVP of embryos and for the application of genetic engineering with higher efficiency and safety. The genetic vulnerability of the early embryo turns less precise genetic modifications a liability to the offspring, as significant changes in embryonic gene expression may have unpredictable impacts in further development. The present study aimed to investigate the promoter-specific pattern of expression of the imprinted *IGF2* gene in bovine oocytes and in preimplantation embryos, as a means to unravel safer paths to genetic engineer embryos at such early stages that may lead to a reduction in embryo mortality in early pregnancy. To describe accurately the behavior of such key imprinted gene in oocytes and in preimplantantion embryos, structures from several stages of development, from immature oocytes to expanded blastocysts (including: immature oocytes (IO), matured oocytes (MO), and 1-cell (1), 2-cells (2), 4-cells (4), 8-cells, 16-cells, morula (MO), compact morula (CM), blastocyst (BL) and expanded blastocyst (EB) stage embryos), were collected in pools of five structures each. Three procedures were performed, each of them contributing to samples from all tested stage groups. The sample pools were evaluated to estimate the abundance of the four different IGF2 mRNA isoforms by real time quantitative PCR (qPCR), defining the pattern of expression *per se* based on the promoters known to drive *IGF2* expression in cattle. The widespread qPCR analyses of all stages allowed an optimal perspective to investigate the progression of the IGF2 transcription pattern and transcript storage throughout time. Oocytes were included, as such gametes represent an interesting point for genetic alterations, especially by microinjection, and stand as a period in which transcripts are widely stored within the ooplasm, as part of the maternal inheritance to early development. The last embryonic stages included expanded blastocysts, as such embryos represent the most common stage used for the assessment of embryo

production systems, micromanipulations, freezing, and transfer to female recipients. The structures from fertilization to cavitation were elected to allow an overview of the changes over time in the pattern of IGF2 expression, also providing ampler possibilities for future micromanipulation of embryos at distinct developmental stages during *in vitro* embryo production.

2. LITERATURE REVIEW

2.1 Imprinting genes

Different species have distinct numbers of chromosomes. Yet, mammals are known to be mostly diploid, meaning that every cell has one identical pair of each chromosome, called homologous copies, that contains allele genes. One of the duo are inherited from the mother, while the other, from the father. Parental genomes were once thought to be equivalent in their contribution to the development of a new individual. In such case, both chromosomes would be expected to express most genes, the phenotype would depend basically on gene hierarchy quoted in many genetic interactions, such as the Mendelian laws (Aigner *et al.*, 1999). However, parthenogenetic and gynogenetic studies reveled the incapacity of growth with only one type of chromosome, leading to the characterization of a group of genes that do not follow somatic expression, which are called imprinted genes, or genomic imprinting. These genes are characterized by a specific expression according strictly with the parental origin of the chromosome, either maternal or paternal. There are more than 200 imprinted (Imprinted Genes Catalogue www.otago.ac.nz/IGC) genes identified, all of those belonging to the eutherians (true placentated mammals). It is considered maternal imprinting when the maternal allele is active alone, and paternal imprinting when only the paternal genome is expressed. The importance of such specific set of genes is evidenced by developmental failure of embryos with the two copies of chromosomes from the same parent, named gynogenotes (maternal) or androgenotes (paternal; Surani & Barton 1983; Barton *et al.*, 1984; Surani *et al.*, 1984). This viability reduction is caused, not by incapacity of imprinting expression, but by an unbalance in imprinted controlled genes (Surani *et al.*, 1984). Thus, androgenotes will have twice the amount of paternally imprinted genes and none of the maternal imprinted ones. The *IGF2* gene is one of the first imprinted

genes documented, and is a good representative of such situation, as this gene is typically paternally expressed. Embryos made only with oocyte (maternal) nuclear material (gynogenotes or parthenogenotes) render embryos incapable of expressing this specific IGF ligand, hence, leading to reduced viability (DeChiara *et al.*, 1990; Surani *et al.*, 1990; Rapolee *et al.*, 1992).

2.1.1 The tug-of-war theory

Diploidy is an important defense and default mechanism against deleterious effects due to mutations. Thus, the evolution from biallelic expression to genomic imprinting represents an evaluative paradox. Various theories arose to explain such phenomenon (Hurst *et al.*, 1997), with most agreeing that placental gestation with multiple paternity favored the evolution of paternal imprinting. Imprinting, most probably, developed in mammals due to the embryo direct uptake from the maternal system. The amount of nutrients switched from mother to fetuses via placenta must be regulated during development, and failure in this regulation has a catastrophic effect to the embryo, to the mother and to the siblings. At this point, a conflict of interests exists between mother and father in the extent of nutrients mobilized to the offspring. Through time, progeny of sires that were capable of demanding a bigger supply of nutrients during gestation had an advantage in perpetuating their genetic material, since they were born heavier than their siblings. On the other hand, dams capable of slowing the fetus drawing of nutrients can preserve their own reproductive health and metabolic homeostasis, favoring subsequent gestations, thus, increasing the number of decedents. Such opposite goals caused an evolutionary tug-of-war between progenitors (Moore & Haig, 1991). Therefore, mammalian selection favored a set of genes with distinct allele expression biased on the parental interest and based on the origin of chromosome: genomic imprinting. This hypothesis is reinforced by the amount of correlation

observed among imprinted genes and their growth regulation function in mice, as is the case of *Igf2*, *Igf2r*, *H19*, *Ins-2*, among others (DeChiara *et al.*, 1990; Barlow *et al.*, 1991; Leighton *et al.*, 1995a; Louvi *et al.*, 1997). Furthermore, an association within the regulation function and allelic activity can be made: a clear promotion of growth can be attributed to a paternal expression, while the maternal expression leads to delayed development. Such tendency is even more notorious in androgenic quimeras, whose muscles are hypertrophic, whereas gynogenic quimeras have an atrophic muscular development (Surani *et al.*, 1990).

2.1.2 Notions of epigenetics

The knowledge of genomic imprinting brings us to the question: In what ways are the maternal and paternal chromosomes different that allow distinct expression patterns throughout development? The genetic sequences are virtually the same throughout the cells into an organism, in the concept of genomic equivalence, except for SNP (single nucleotide polymorphisms) and V(D)J recombinations and deletions in lymphocytes. Hence, epigenetic modifications are believed to be responsible for differences in gene expression, especially DNA methylation. Biochemically, DNA methylation is a process that involves the addition of a methyl group at position 5' of cytosine residues (5mC) in the DNA. This is an important regulator of gene transcription, being a mechanism often used to silence and regulate genes without changing the original DNA sequence. It typically occurs in regions rich in dinucleotide guanine-cytosine, called CpG islands. These islands are usually present in 5' regulatory regions of various genes, which are also associated with differentially methylated regions (DMR), directly related to genomic imprinting (Monk *et al.*, 1987; Reik *et al.*, 2001).

Epigenetics is based on the assumption that the genome can be strongly affected

by external or environmental influence. This field of study focus on how genes are regulated, what factors can influence them, how genes control and are controlled in the course of development and under physiological and pathological conditions. To allow responses according to habitat status, genomic imprinting is required to be reversible, so it can respond to changes, as well as inheritable, so in mitosis the daughter cell can have the mother's identity and each division do not require expense adaptation to exterior stimulus. DNA methylation and histone modifications are the most representative examples of epigenetic alterations.

DNA methylation is the best-studied epigenetic trade. The addition of a methyl group at position 5' of cytosine is an important marker, capable of regulating gene transcription on a more permanent basis. DNA methylation has basically two ways of controlling a gene: by physically blocking the interaction of transcription factors (essential to gene expression) to gene promoters, and through a binding protein called MBD (methyl-CpG binding domain protein), which is able to recruit other proteins such as histone deacetylases and chromatin-remodeling complexes, that compact the chromatin, redeeming it inactive through fiscal inaccessibility. Throughout these characteristics, DNA methylation can suppress endogenous retroviral expression and others deleterious sequences that can incorporate themselves into the host genome trough time, as well as to protect DNA from enzymatic cleavage, since enzymes cannot bind to methyl groups. Such methyl marker also enables the same DNA sequence to be read distinctly among different tissues, as well as in gametes, which can be inherited by daughter cells after mitoses, maintaining the identity of the mother's tissue and of the offspring after fertilization (Monk *et al.*, 1987; Reik *et al.*, 2007). There are at least two different periods through development that this heritability is shunned in favor of a genome wide reprogramming of methylation patterns: in germ cells and in

preimplantation embryos, which allows a broad developmental potential (Reik *et al.*, 2001). In embryos, for example, even if feminine and masculine gametes have different methylation patters on the same genes, some of those epigenetic marks have to be maintained after fertilization (such as imprinted genes, as a mean to impose gene expression according to parental interest), while others have to be lost to ensure nuclear totipotency (so a single cell, the zygote, can express all necessary genes to form every tissue of an individual). The loss of most methyl groups in early embryo development is followed by a *de novo* methylation mechanism, that will be distinct according to tissue differentiation (Monk *et al.*, 1987; Chaillet *et al.*, 1991).

There are some areas in the genome called differentially methylated regions (DMR) involved in transcription regulation. DMRs can be used as markers of the methylation state, being currently in use to identify aberrant epigenetic patterns in cancer patients. Imprinting control regions (ICR) are also strongly related to genomic imprinting, responsible for regulating the unique imprinting expression pattern. An example of this kind of control can be seen in the *Igf2/H19 locus*, with the first gene transcribing to IGF2, while the other forming a non-coding microRNA with tumor suppression effects. Under normal circumstances the DMR located between genes is unmethylated in the maternal allele and methylated in the paternal counterpart, with both being under the effect of an enhancer downstream. The specific paternal methylation prevents the insulator CTCF protein to bind to the DMR, blocking the H19 transcription, allowing the activation of the *IGF2* promoter by the enhancer. Conversely, the unmethylated maternal DMR allows its interaction with the CTCF protein, leading to *H19* transcription, avoiding the activation of the *IGF2* promoter. Therefore, the correct methylation of the DMR sequence ensures *IGF2* transcription only by the paternal allele, thus controlling imprinting expression (Bartolomei, 1993;

Leignhton, 1995; Reik & Murrel, 2000; Ludwig, 2002).

DNA methyltransferases (DNMTs) are a family of enzymes responsible for the transfer of methyl groups to DNA, with two classes existing within this family: the maintenance DNMTs (DMNT1) and the *de novo* DNMTs (DNMT3a, DNMT3b, and DNMT3L). The first class is in charge of maintaining the methylation pattern at the CpG islands through cellular division; the second class is responsible for addition of new methyl groups to unmethylated CpG islands. The DNMT1 is the most abundant methyltransferase in mammalian cells; in mice, such class of enzymes has distinct isoforms, with the somatic form, the DMNT1b, common to all cells, and the oocyte form, DNMT1o, specific to the oocyte, synthetized and stocked in the ooplasm (Reik *et al.*, 2001). The DMNT3 class has three members: DMNT3a, DMNT3 and DMNT3L. The first two are able to establish methylation patterns in early embryogenesis, therefore, promoting differentiation. The last isoform is similar to the other DNMTs, however it does not have a catalytic domain; it interacts with DNMT3a and DNMT3b aiding to DNA binding to DNA, enhancing their activity (Reik *et al.*, 2001; Salle *et al.,* 2004; Edward & Ferguson-Smith, 2007; Reik *et al.*, 2007).

Genes that are required during development and differentiation are repressed in pluripotent and undifferentiated cells, such as embryonic stem-like cells (ES). Several genes that are required for early development or for germ-cell development only (for example, those that encode pluripotency-sustaining transcription factors, such as OCT4 and NANOG) are known to be expressed by ES cells but silenced during differentiation, by acquiring repressive histone modifications and DNA methylation. Histone modifications have a more transient profile than DNA mehtylation. In the first case, the Polycomb group-protein repressive system (PcG) marks histones associated with specific genes by methylating the lysine tales of the histone 3 (H3K27), thus depressing

genomic code prone to spontaneous differentiation. However, as it happens in DNA methylation, this mark can be either an inactivation mark (H3K27) or an activation one (H3K4), depending on the place. Interestingly, in cancer cells, genes target by PcG often become DNA methylated, which results in permanent expression of characteristics specific to pluripotency (Reik, 2007).

2.1.3 Genome wide reprograming of methyl patterns and maintenance of imprinting

2.1.3.1 Germ lines

A small cell population is selected in early development to become primordial germ cells (PGCs). Such cells arise from the extra-embryonic mesoderm, and then migrate to the genital ridge, where they populate the area that will develop into the gonads. These will have a completely different relation with imprinted genes than somatic cells. Methylation of imprinted genes can only be totally erased in this specific set of cells, which ultimately give rise to the germ line. In fact, as contra productive as it seems, PGCs acquire a genome-wide *de novo* methylation during initial development, like any other cell type in the early embryo. However, soon after the migration to the genital ridge, all methylation patterns (imprinting or not) are completely removed, thus reaching an equivalent arrangement between maternal and paternal alleles. Methylation patterns are, then, reestablished in a sex-specific manner, matching the fetal gender (Monk *et al.*, 1987; Chaillet *et al.*, 1991). In mice, the monoallelic expression regulation is remained intact during migration. The erasure of methyl marks, from both DNA and histones, takes place in a defined period (from 10.5 to 12.5 in days post-coitum, dpc) in all imprinted genes, both in the male and the female germ lines, and it probably occurs by active DNA demethylation due to its rapid loss (Hajkova *et al.*, 2002). Interestingly, establishment of maternal specific imprints occurs postnatally (in primary oocytes that are selected for development) and are not fully established until the oocyte has matured

and is ready for ovulation. In contrast, male specific imprints are established *in utero* and are nearly complete at the time of parturition (Kafri *et al.*, 1992; Obata & Kono, 2002; Lucifero *et al.,* 2004; Salle *et al.*, 2004; O'Doherty *et al.*, 2012).

The reestablishment of imprinting methylation in germ cells in both parents is gradual (Salle *et al.*, 2004; O'Doherty *et al.*, 2012). Obata & Kono (2002) showed that the female germ cell methylation pattern extends through all oocyte development in mice, from primordial germ cells to the antral phase, during which, each gene has its own specific period of time to receive the primary imprinting marks. Possibly, this phenomenon is due to distinct chromosomal positions and dependent of the chromatin status. The methylation establishment occurs in a follicular size dependent manner, in which each size marks a different group of genes, without any link with the animal's age. The *Igf2r*, *Kcnq1ot1* and *Plagl1* genes in mice, for example, are 85% hypermethylated in oocytes with diameters between 55 to 60 μm; whereas impact, *Snrpn*, *Mest* and *Grb10* genes are 85% methylated when oocytes reach 60 to 65 μm in diameter. Moreover, each allele is affected in its own time, as seen for the *Snrpn* gene, where the maternal copy acquires methylation at the preantral follicle stage, whereas the paternal allele is marked in more mature stages, as antral follicles. The oocyte completes the imprinting pattern and acquires competence to support embryo development just before reaching its full size (Lucifero *et al.,* 2004; Hiura *et al.,* 2006).

Once it has been proven that the oocyte diameter has a task in germ cell methylation, it was possible to imply the importance of the storage of some enzymes or regulating molecules as being limiting factors to the establishment of DNA methylation. Hence, the follicular dimension and the imprinting competence are linked to DNMT3a, DNMT3b and DNMT3L cytoplasmic transcript storage (Lucifero *et al.*, 2004; Salle *et al.,* 2004; O'Doherty *et al.*, 2012). Knockout mice studies were made to describe each of

these enzymes function in germ cell methylation. Kaneda *et al.* (2010) demonstrated that *DNMT3a* disruption in growing oocytes leads to hypomethylation of maternal DMRs. Embryos derived from such mutation expressed aberrant maternal imprinted genes, with mortality around the 9th dpc in mice. These consequences highlight the importance of *de novo* methylation pattern established by DNMT3a and its relevance to maternal imprinting. The DMNT3b, on the other side, seems to be unessential to imprinting oogenesis, since its absence does not affect imprinting or the methylation of repetitive elements. This same experiment proved the requirement of DNMT3L to the methylation of correct repetitive DNA elements, consistent with the high expression levels of this protein found in postnatal oocytes. The DMNT1, conversely, is situated in the nucleus of PGCs of both sexes during mitotic divisions, on 11.5 dpc in mice. As previously mentioned, the imprinting methylation erasure occurs on 10.5 to 12.5 dpc, with the highest concentration of DNMT1 at this time suggesting an activity in the maintenance methylation of repetitive DNA elements. Germ cells from both sexes move gradually into a non-proliferative state if *DNMT1* is silenced (Salle *et al.*, 2004).

In males, the meiotic stop is associated with maturation of germ cells in pro-spermatogonia, around 14.5 dpc, followed by an increase in methylation between 15.5 and 18.5 (Kafri *et al.*, 1992). The *de novo* methylation starts before birth and is consolidated postnatally. Once established, methylation patterns must be kept during DNA replication in spermatogonia (contrary to the female, where DNA replication ends and germs cells have a definitive number at birth). Hence, a specific mechanism has developed to that end. The DNMT profiles shows peaks of DNMT3a and DNMT3L on pre-natal testicles, suggesting an interaction between the two enzymes in establishing imprinting marks. The DNMT3L is not required to somatic cell development, being, however, an important regulator for the establishment of maternal imprinting, and

essential to a normal post-natal spermatogenesis, since mutant cells from the paternal origin are azoospermic, whereas such cells from the maternal origin die halfway through gestation. The *DNMT1* and *DNMT3b* expressions, on the other hand, revealed a role in the maintenance of DNA methylation in fast proliferating spermatogonia at the beginning of the post-natal period. The DNMT1, with a transcript additional peak, is responsible, furthermore, for the maintenance of DNA methylation at repetitive DNA elements in germ cells at moments when demethylation is widespread, at the end of the migration to the genital crest. Differently, *DNMT3b* is expressed at high levels at birth, having a role in actively methylation of centromeric regions, to ensure correct pairing and recombination between homologous chromosomes (Salle *et al.*, 2004).

Thanks to the monoallelic expression and complex regulation, imprinted genes are particularly sensitive to epigenetic modifications. Obata *et al.* (2011) showed that partial imprinting methylation of immature oocytes are not passed with fidelity to the progeny. Consequently, growing oocytes alter imprinting methylation to hyper or hypomethylated states during embryogenesis. Immature histone modifications can also be associated with abnormal embryo development produced from growing oocytes. Kageyma *et al.* (2007) reported various histones modifications with a drastically increase during oocyte growth. Therefore, loss of imprinting integrity and epigenetic mosaicism occurs in embryos when incomplete imprinting is inherited from immature oocytes, resulting in high frequency of abortions, post-natal death and growth abnormalities (Obata *et al.*, 2011).

2.1.3.2 Embryo

DNA methylation is critic to embryo development. After fertilization, a chromatin remodeling is strongly related with fast demethylation of the paternal genome. In fact, the maternal and paternal genomes in their respective pronuclei must

undergo extensive remodeling in order for the developing zygote to reach a totipotent state (Monk *et al.*, 1987; Reik *et al.*, 2001). Global genomic reprogramming occurs quickly after fertilization, with a big decrease in DNA methylation to approximately 30% of the somatic cell normal level. Interesting, when demethylation was experimentally forced below those values, a negative interference on embryo development could be seen, demonstrating the need of the maintenance of a certain degree of genome methylation (Giraldo *et al.*, 2009). Following methylation reduction in the first cleavages, a *de novo* methylation pattern is promoted by DMNT3a, 3b and 3L, beginning at the 8-cell stage in cattle, or at the blastocyst stage in mice (Monk *et al.*, 1987; Chaillet *et al.*, 1991; Reik *et al.*, 2001; Golding *et al.*, 2003; Aguiar *et al.*, 2017).

Reprogramming in early embryo occurs both by active and passive mechanisms. At the time of fertilization, sperm DNA is tightly packaged by protamines (basic proteins that are associated with DNA in sperm) and highly methylated relative to the oocyte. Loss of the protamines, acquisition of histones and DNA demethylation of the paternal genome is necessary during G1 prior to the first DNA replication. Originally, it was believed that paternal demethylation was an entirely active process (replication independent). However, current studies have shown pieces of evidence suggesting that the process is a mix of active and passive processes, where 5-methyl cytosines (5mC) are converted to 5-hidroximethyl cytosines (5hmC) by the Translocation protein 10-11 3 (TET3). Converted 5hmCs are not re-methylated during replication and therefore the DNA demethylation event is carried on passively by replication-coupled dilution and DNA repair system. The demethylation of the paternal genome is followed by passive demethylation of both maternal and paternal chromosomes, presumably caused by the exclusion of DNMT1o from the nuclei of preimplantation mouse embryos. Then, even if DMNT1 maintains specific imprinted sites, genome methylation decreases until the

blastocyst stage (Kafri *et al.*, 1992; Howell *et al.*, 2001; Reik *et al.*, 2007).

Some DNA sequences are known to be resistant to global demethylation, such as DMRs of imprinted genes and transposable elements, which are protected by specific proteins. Both DNMT1 and primordial germ cell protein 7 (PGRC7) play a critical role in maintaining the sex specific imprints present on the maternal and paternal genomes during this demethylation event. *DNMT1o* was characterized as needed during the first embryo cell cycles, as its knockdown leads to 50% reduction in normal imprinting methylation, while *DMNT1* would be responsible for protecting imprinting after genome activation (Howell *et al.*, 2001). On the other hand, large amounts of PGRC7 can be seen in the ooplasm that is translocated to both pronuclei after fertilization. Those proteins bind to histone H3 methylated at the Lysine 9 residue (H3K9me2) to protect the chromatin and inhibit demethylation (Hirasawa *et al.,* 2008). *De novo* methylation begins at different stages in a species-specific manner. In mice, for example, it occurs in the inner cell mass (ICM) of expanded blastocysts, whereas in cattle it is limited to the 8- to 16-cells stage. The rest of the embryonic genome is progressively methylated corresponding to the development of each species (Reik *et al.*, 2001; Golding *et al.*, 2003).

A few studies were carried out regarding methylation transmission pattern to the progeny. A partially *in vitro* methylated gene (with labeled methyl) was inserted in embryos to verify if they were subjected to global demethylation and *de novo* methylation (Frank *et al.*, 1991). The marked methylation was lost and the normal level of methylation replaced the *in vitro* one, suggesting that transgenes artificially methylated do not escape global demethylation in early embryonic development. In the Aigner *et al.* (1999) study, a DNA microinjection leaded to gene integration on the host chromosome, which specified its transmission according to a Mendellian pattern of

inheritance. Basically, the genomic methylations of a transgene depended mostly on the place of integration.

2.2 The IGF family

It is now well established that the Insulin-like growth factor family is part of a complex neuroendocrinal system that plays an important role in growth regulation in all vertebrates. IGF are pro-insulin-like polypeptides that act at endocrine, paracrine and autocrine levels to induce mitogenic and metabolic effects. The IGFs are unlike insulin in that they are not restricted to production and secretion by specific cell types. On the contrary, almost all cell types within the body can produce IGFs at some extent (Dupont & Holzenberger, 2003). At cellular level, IGF signaling can cause a series of biological responses such as growth, proliferation, survival, nutrient uptake, migration and differentiation (DeChiara *et al.*, 1991; Baker *et al.*, 1993; Gardner *et al.*, 1999; Byrne *et al.*, 2002; Constancia *et al.*, 2002; Pavelic *et al.*, 2007). This family activity is mediated by the interaction of two ligands (IGF1 and IGF2) with three membrane receptors (IGF1R, IGF2R and insulin receptor), although most of their effects are mediated through the IGF1R. In addition, the transport, half-life and functions are modulated by the interaction with six IGF-binding proteins (IGFBPs), present in various extracellular fluids in different proportions. The specific response is dependent on the tissue, proportion between the receptors, binding proteins, specific proteases, among others (Firth & Baxter, 2002). However, the impact of the IGF family on fetal and placental growth and development is one of the most studied aspects of this particular family.

2.2.1 Ligands

In bovine, the preproprotein of IGF1 and IGF2 have 154 and 173 amino acids, respectively (NCBI Reference Sequence: NP_001071296.1; AAI16040.1). While the mature human IGF1 and IGF2 contain 70 and 67 amino acids and molecular masses of

7.5 kDa and 74 kDa, respectively, being single-chain peptides with 70% homology between them. The IGFs contain four biochemically distinct domains, named B-C-A-D. Domains A and B are similar to insulin (49% homology), the D domain distinguishes this family from proinsulin, as it is absent in the last. Both ligands are synthesized as pre-pro-hormones, and targeted for secretion by hydrophobic signaling peptides. At the Golgi apparatus, a proteolytic removal occurs of the signal peptide containing carboxyl-terminus extensions (E domains) of 25 (pro-IGF-1) and 89 (pro-IGF-2) amino acid residues, to generate the mature four-domain IGF peptides (Dupont & Holzenberg, 2003; Wood *et al.*, 2005).

Even though the Igf1 ligand was initially considered to be synthetized e secreted exclusively by the liver (under the GH regulation, coordinated by a negative feedback loop), mice studies have demonstrated that specific disruption of *Igf1* in the liver results in a 75% reduction in the circulating ligand, characterizing a more sparsely distribution, as its synthesis can be found in most, if not all, non-hepatic tissues. Interestingly, loss of hepatic *Igf1* did not dramatically decrease postnatal growth, though it greatly disturbed circulating IGFBPs and their affinity for the ligand, illustrating that either changes in IGFBP profiles were capable of stabilizing bloodstream availability, or local production of Igf1 plays a bigger role in tissue growth than previously anticipated (Sjogre *et al.*, 1999; Yakar *et al.*, 1999). Furthermore, it was established that IGF1 synthesis and secretion is GH independent during early development that may extend to specific organs during later development, while post-natal growth is GH dependent (Le Roith *et al.*, 2001). IGF2 is a mitogen that is highly expressed during fetal development. The post-natal importance, however, seems species dependent as it was proven that it had no interference in mice, but it was necessary to humans. The imprinting trade of *IGF2* is originated from mainly the paternal allele expression, and its' synthesis is mostly

independent of GH regulation (DeChiara *et al.*, 1991; Pavelic *et al.*, 2007), shown to regulate trophoblast development and function at the feto-maternal interface (Constancia *et al.*, 2002).

The IGF biological responses can be classified as metabolic and anabolic. They include stimulation of glucose uptake and oxidation, glycogen synthesis, amino acid uptake and lipogenesis, cell proliferation, endothelial cell migration, protein and nucleic acids synthesis and suppression of protein degradation, attributed mainly to IGF1R, although some biological activity can be attributed through the interaction with the insulin receptor (Owens, 1991; Gluckman *et al.*, 1992; Rapolee *et al.*, 1992; Liu *et al.,* 1994; Zumkeller *et al.,* 2000; Kim *et al.*, 2006). The cell proliferation action relies mostly on regulating the cell cycle machinery in G1, spurring the progression to the S phase. Therefore, *Igf1r* knockout human cells are arrested prior to the S phase and cannot grow normally, specially by inducing cyclin D1 (Dupont & Holzenberg, 2003). Furthermore, an indirect effect on fetal growth can be seen on the IGF capacity in affecting placenta structure and function and, thus, influencing nutrient viability (Constancia *et al.*, 2002; Fowden *et al.*, 2003). Mainly, both ligands seem to regulate growth. While fetal growth needs both ligands to have a normal function (as neither one can completely compensate for the absence of the other, indicating non-redundant functions), their high concentrations at different stages in development, as well as their disruption consequences, highlight some specificity: postnatal growth seems largely related to *Igf1*, as dwarfism persist in mice after birth when this specific gene is knocked out and normal size is achieved in adulthood in the absence of IGF2; while IGF2 dictates mostly pre-natal growth as shown by the high concentration on fetal tissues and placental insufficiency results from its loss of function (DeChiara *et al.*, 1991; Baker *et al.*, 1993; Liu *et al.*, 1993; Constancia *et al.*, 2002; Pavelic *et al.*, 2007).

2.2.2 Receptors

As noted above, there are two main promoters responsible for the majority of the IGF actions through the IGF1R and IGF2R. The IGF1R is a transmembrane tyrosine kinase receptor, being the main mediator of the IGF signaling pathway. The IGF1R is posttranslationally modified by the elimination of 30 peptides and sectioned into 2 sets of α and β subunits, linked by disulfide bonds to form a heterotetramic glycoprotein, with a 70% homology to the insulin receptor (IR). The α subunits are extracellular domains and directly bind the ligand, whereas the β are transmembrane subunits responsible for intracellular catalytic response. When the ligands binds to the IGF1R, a conformation change occurs resulting in autophosphorylation of the receptor and activation of the tyrosine kinase activity. Subsequent tyrosine phosphorylation of specific substrates stimulates several intracellular cascades, among them two important pathways: MAP kinase ERK1/2 and phosphatidylinositol-3-OH kinase/akt kinase cascade, with each pathway depending on the cell type. The pathways lead to activation of several downstream substrates that regulate anti-apoptotic effects, resistance to stress oxidation, carbohydrate metabolism, pancreatic control of glucose homeostasis and even the influence on the lifespan, as proven by longevity studies in heterozygous Igf1r mice (Dupont & Holzenberger, 2003; Holzenberger *et al.*, 2003; Pavelic *et al.*, 2007). Even with the structural similarity with the insulin receptor, it has been shown that the IGF1R exhibit 100 – 1000 fold greater affinity to its own ligand as compared to insulin.

The second acting receptor of the IGF family is IGF2R. Due to its identical features with the cation-independent mannose 6-phosphate receptor, the IGF2R can also be known by such name. The receptor also arises from an imprinted gene that is maternally expressed and paternally repressed (Barlow *et al.*, 1991). The 250-kDa receptor binds IGF2 with an affinity about 100 times greater than that for IGF1 and does

not bind insulin at all (Kornfeld *et al.*, 1992). Basically, IGF2R is a monomeric transmembrane receptor, associated with lysosomal targeting of the extracellular ligand. The absence of catalytic domains leads to believe that it does not activate conventional signaling transduction pathways. Instead, such receptor internalizes and degrades the respective ligand and also functions in lysosomal enzyme trafficking, regulation of apoptosis and growth, and tumor suppression (Kornfeld *et al.*, 1992; Lau *et al.*, 1994; Ludwig *et al.*, 1996). Furthermore, IGF2R plays an essential role in regulating fetal growth as shown by mutant mice. Loss of this specific receptor results in overgrowth and neonatal death (Lau *et al.*, 1994). Similarly, fetal overgrowth phenotypes in sheep are associated with reduced *IGF2R* expression (Young *et al.*, 2001).

The IGF2 ligand can interact at some extent to the IR, as shown in knockdown mouse studies, especially in early development, before the IGF1R is expressed (Lovi *et al.*, 1997; Pandini *et al.*, 2002). The *IR* transcription is subjected to splicing variants at exon 11. Some studies have shown that the isoform lacking the amino acids transcribed by exon 11 (IR-A) have 2-folds more affinity for insulin that the variant containing exon 11 (IR-B). There was also shown that IR-A has a high affinity for IGF2, therefore, while IGF1 acts only through the IGF1R, IGF2 can act either through the IGF1R or the IR-A. There is also the possibility of hybridization between IGF1R and IR. In this case IGF1R/IR-A can bind IGF1, IGF2 and insulin, enhancing IGF axis actions; whereas IGF1R/IR-B have a reduced affinity for IGF1, an even lower interaction with IGF2 and does not bind insulin at all, which will reduce IGF system activity. In cells expressing high IR levels, hybrids may exceed typical IR and IGF1R contents, which can shift the major binding from insulin to IGF, with possible repercussions in metabolism (Pandini *et al.*, 2002).

2.2.3 IGF Binding Proteins

The transportation and function of IGF ligands are modulated by the interaction with at least six binding proteins (IGFBP-1 through -6), which can either promote or inhibit IGF actions, depending on each of them being dialed and on their phosphorylation state (Jones *et al.*, 1991). The binding proteins have a higher affinity for IGFs ligand than their own receptors (Jones & Clemmons, 1995). Therefore, IGFBPs can modulate the biological accessibility, half-life and activity of the IGF ligands. The binding proteins accomplish this by transporting IGFs from circulation to the peripheral tissues, sequestering a surplus of IGFs in circulation, and by inhibiting or contributing to the activity of the IGFs (Jones & Clemmons, 1995). More than 99% of circulating IGFs are bound to IGFBPs and at least 75% of the bound ligands are carried in complexes involving acid-labile subunits (ALS)/IGF/IGFBP-3. The ALSs are thought to increase the half-life of IGF/IGFBP-3 complexes, as its knockdown have shown a much lower (almost 70% of normal) circulating IGF1 concentration, resulting in post-natal growth retardation (Boisclair *et al.*, 2000; Forbes & Westwood, 2008). The IGFBP-3 is the predominant IGFBP in serum and is responsible for binding most of circulating ligands. The IGFBP-1 to -4 have similar affinity for IGF1 and IGF2; by contrast, the IGFBP-5 and -6 bind IGF2 with an affinity 10 and 100 times greater, respectively, than IGF1. The IGFBP-5 also forms a ternary complex with ALS. The IGFBP profile, apparently, is tissue-specific. Interestingly, studies that disrupted the function of one or several IGFBPs have not generated major phenotype changes, differently from those that overexpressed them, that causing either under or overgrowth (Firth & Baxter, 2002; Dupont & Holzenberg, 2003).

Another important component of the IGF regulation are IGFBP proteases that cleave binding proteins, thereby drastically altering their binding capacity and

phosphorylation status. The consequence of the interaction between IGFs and IGFBPs, as well as its intensity, depends on the molecule's phosphorylation status. Westwood *et al.* (1994) reported a single phosphorylation status at the IGFBP-1 in bovine adult serum, contrasting with the heterogeneous IGFBP-1 profile described in amniotic fluid and fetal serum, highlighting the different profiles in distinct tissues. For example, in humans, it appears that IGFBPs produced by the placenta modulate IGF-mediated actions during placental growth and development (Zumkeller *et al.*, 2000).

2.2.4 The IGF family history

The IGFs were the first proven imprinted genes, which made them a natural target to many imprinted-related studies (DeCiara *et al.*, 1990; Bartolomei *et al.*, 1993; Constancia *et al.*, 2000; Wang *et al.*, 2009). Another alluring trade of this specific family of molecules is their indispensable involvement in animal growth (Li *et al.*, 1993; Constancia *et al.*, 2000). As such, they were extensively studied throughout time, beginning in the early 1940s to nowadays. They had a particularly popularity for studies during the 1990s. The discoveries that helped to better understand the IGF throughout history will be reviewed and summarized in the following topics. Discoveries made after the 2000s will not be put under historical events, but discussed in the specific topics.

2.2.4.1 1940s to 1980s

The characterization of growth hormone in the 1940s by Li & Evans (1944) provoked the birth of a new field of study. There was, however inconsistencies between *in vivo* and *in vitro* effects of GH that prevented progresses in the area for some time. A significant breakthrough happened 13 years later, when Salmon & Daughday (1957) experimented GH in cartilage explants. While normal rat serum or hypophysectomized rats' serum supplemented with GH significantly stimulated cell growth in culture,

purified GH had no effect. By such results, it was deduced that (an) unknown substance(s) present in the serum mediated the growth-promoting actions of GH. Thorough the following decades, such unknown substances were referred by many names, firstly as "sulfation factor" in the 1960s, latter as "somatomedins" (SM) in the 1970s, and finally as "insulin-like growth factors" in the 1980s.

The efforts for the purification of such elements in 1974, carried out by Van Wyk *et al.* (1974), lead to the identification of tree variants: a neutral polypeptide (SM-A), an acidic polypeptide (SM-B), and a basic polypeptide (SM-C). Latter, the SM-B was identified as the epidermal growth factor (EGF), and was excluded from the SM family (Heldin *et al.*, 1981). Both the SM-A and SM-C, on the other hand, were characterized as having an insulin-like action. In 1978, with the purification of the activated molecules, three lines of independent research merged (all three reported insulin-like activity, not suppressed by insulin antiserum, but partially replaced by high concentrations of insulin), showing that Froesch's NSILA (non-suppressible insulin-like activity; Froesch *et al.* 1963), Temin's MSA (multiplication-stimulating activity; Pierson & Termin, 1972) and Daughaday's SM (somatomedin; Daughaday *et al.*, 1972) were the same molecule (Rinderknecht & Humbel, 1978a,b). From this work forward, the peptides were renamed for insulin-like growth factors 1 and 2, or IGF1 and IGF2.

2.2.4.2 <u>1990s</u>

The early 1990s brought forward a series of experiments dedicated to study the IGF family (DeChiara *et al.*, 1990; Baker *et al.*, 1993; Liu *et al.*, 1993; Filson *et al.*, 1993). Thanks to several gene mutations produced in mice, it was possible to understand some of the IGFs' biological effects as well as the consequences of their interaction with specific receptors in living organisms. Those studies reported dwarfism in knockdown mice with various levels of severity that can be resumed in six possible

phenotypes 100%, 90%, 75%, 60%, 45%, 30%, 140% of normal weight at birth. These size changes began to be phenotypically evident at 11 dpc. Animals reaching 140% body weights were described by Lau *et al.* (1994), relative to a disablement of the *Igf2r*. Results were related to overstimulation of *Igf1r* by circulating IGF2, due to the erradication of the turnover mechanism, the own IGF2R, thus, leading to a lack of IGF2 elimination and generating an exacerbated availability and consequent increased growth (Ludwig *et al.*, 1996). The 30% body weight animals were characterized by the absence of both IGF1 and IGF2 actions, either by mutation of both ligands or their receptors, observed in the following mutants: *Igf2/Igf1r*, *Igf1/Igf2*, *Igf1r/Igf2r/Igf2*, *Igf1r/IR* e *Igf1r/Igf2r/IR* (Baker *et al.*, 1993; Liu *et al* 1993; Ludwig *et al.*, 1996; Louvi *et al.*, 1997). Those with 45% body growth were characteristic of the IGF1R absence, responsible for all IGF1 action and part of that of IGF2; in such case, the dwarfism is not as severe as the previous mutants thanks to the interaction with the insulin receptor, responsible for part of the IGF2 actions. Among them, the *Igf1r* and *Igf1/Igf1r* mutants are of notice. Differently, the deficiency of one of the ligands, either IGF1 or IGF2, resulted in mice with 60% body weights due to an overlap between functions and partial compensation by the remaining ligand (DeChiara, *et al.*, 1991; Baker *et al.*, 1993; Liu *et al.*, 1993). The 75% of the expected development was observed in animals *Igf2/Igf2r*, in which the lack of IGF2 in *Igf2r* mutants seemed to rescue the fetus from overdevelopment. When the ligand bioavailability was avoided, being one of the major causes of overgrowth, mice resulted similar to *Igf2* mutants (Dechiara *et al.*, 1991; Filson *et al.*, 1993). At last, animals with disrupted IR had 90% of the size of the wild types, characteristic to those with partial loss of IGF2 action. However, in some tissues, such as the placenta, the growth promotion function occurred only by the IGF2-IR interaction, with such interaction blocking some of the developmental delay, even if less

pronounced by compensatory *Igf1* response (Louvi *et al.*, 1997). There was only one case in which mutant mice presented the same weight as controls, the *Igf1r/Igf2r* model. Even though the IR was less effective in inducing growth than IGF1R, the exacerbated amount of IGF2, caused by IGF2R absence, seemed to have been sufficient to compensate for the lack of efficiency.

Taking into consideration the various levels of dwarfism at birth of the mice and the first signs of modifications, the relations between ligands and receptors were proposed. Given the fact that alterations of *Igf1* mutants were seen only from 13.5 dpc, whereas the ones in *Igf2* could be detected from 11 dpc, it was deduced that an exclusive relationship between IGF2 and IGF1R takes place in the beginning of gestation. This inference was reinforced by phenotypic similarity between *Igf2*, *Igf1r* and *Igf2/Igf1r* mutants from 12.5 dpc. From that time (12.5 to 13.5 dpc) IGF1R, previously defined as exclusive receptor to IGF2, started to be considered to interact also with IGF1. In fact, IGF1 interacts only with the IGF1R, as evidenced by the equivalence between *Igf1r* and *Igf1/Igf1r* mutants. Conversely, IGF2 seems not to have just one receptor, due de different dwarfism severity between *Igf1r*, *Igf2* e *Igf2/Igf1r* deficient animals. Thus, it was characterized the presence of another receptor simultaneous to the IGF1R that interacted with IGF2 after 12.5 dpc. Another phenomenon that sustained this hypothesis is the fact that the *Igf2* mutants had a decrease in placental size, whether the IGF1R deficient animals did not have the same alteration, suggesting, in this tissue, an interaction of such ligand with an alternative receptor (Baker *et al.*, 1993, Liu *et al.*, 1993). The second receptor was described as being the insulin receptor (IR). Animal *IR* (-/-) generated fetuses slightly smaller, mostly toward the end of gestation, whereas *IR/Igf1* allowed growth of only 30% of the normal size, with modifications from 13.5 dpc, similarly to those found in the *Igf2/Igf1r*

mutants, in which ligands were inactive (Louvi *et al.*, 1997). There is a strong interaction between IGF2 and IGF2R. However, the IGF2R action was related to IGF2 kidnapping and degradation, with the receptor's absence leading to gigantism (Filso *et al.*, 1993).

Louvi *et al.* (1997) showed pieces of evidence that IGF2 promoted growth actions during embryogenesis were mediated through two different receptors, IGF1R and IR. The *Igf1r* mutant cells grew well in culture when supplied with medium containing IGF2, with an overexpression of *IR*. The use of IGF1, however, did not have the same response, having no interaction with that receptor. Thought to be involved in growth, the IR is not capable to compensate the absence of IGF1R, as seen in the reduced weight (45% of normal) and size of the *igf1r* mutant animals. With the knockout of both *Igf1r* and *Igf2r*, IGF2 ligand was present at high amounts (due to the lack of IGF2R sequestering) to overstimulate IR to the point that the compensation was complete in *Igf1r* deficient animals, leading to normal size. The *Igf1r/Igf2/IR* mutants were identical to the *Igf1/Igf2* and *Igf1r/IR* counterparts, suggesting that only those receptors were responsible for the IGF actions (Louvi *et al.*, 1997). The absence of significant fetal growth restriction in *GH* and *GH receptor* knockout mice also suggests that the growth-promoting actions of the IGFs are GH independent during fetal development (Le Roith *et al.*, 2001). Some of the different mutant animals and the interaction between ligand and receptors are depicted in Figure 1 (from Louvi *et al.,* 1997).

2.3 IGF CLUSTERS

A characteristic feature of the imprinted genes is the methylation of CpG islands in only one of the parental alleles. The allele-specific methylation is typical for differentially methylated regions and its pattern is implicated in the regulation of

imprinting expression. Genomic imprinting is primarily found in co-regulated clusters of variable size. Each cluster is under the control of a major cis-acting elements, the imprinting control regions, or ICRs, though, other elements can influence the ICR function, for instance methylation of imprinted promoter can, in some cases, act as its ICR. They are also found within differentially methylated regions that exhibit parental patterns of DNA methylation. DNA methylation allows monoallelic expression by blocking access of transcription factors and other enzymes and proteins to the DNA (Monk *et al.*, 1987; Reik & Murrel, 2000; Edwards & Ferguson- Smith, 2007; Reik, 2007). Depending on the type of modification to the histone tails, the state can repress or promote transcription of imprinted genes (Reik, 2007). Lastly, many imprinted clusters contain a non-coding RNA that is involved in regulating the parent-specific expression of other genes within the cluster (Edwards & Ferguson- Smith, 2007).

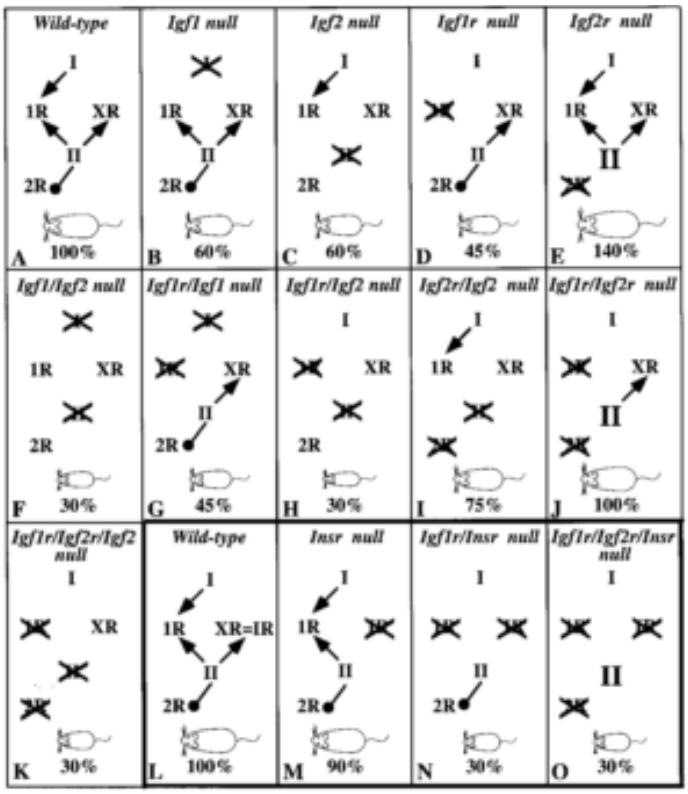

Figure 1. Summary of different phenotypes observed in mutant mice for components of the IGF axis. From: Louvi *et al.* (1996).

2.3.1 The IGF2/H19 cluster

The cluster that contains the *IGF2* gene bears also another two imprinted genes: the insulin-2 (paternally expressed in the embryo yolk sac), the *IGF2* (expressed by the paternal chromosome), and the *H19* (non-coding RNA maternally expressed) gene, positioned in this order. This cluster remains highly conserved among mammalian species, despite minor modifications: Chromosome 7 in mice (Bartolomei *et al.*, 1993), chromosome 11 in humans (Brissenden *et al.*, 1984), chromosome 2 in pigs (Nezer *et al.*, 1999), chromosome 21 in sheep (Ansari *et al.*, 1994) and chromosome 29 in cattle (Goodall *et al.*, 2003). In total, four different promoters were found to control *IGF2* expression in most of the studied species. Such variation permits a differential temporal and spatial production of unique isoforms of IGF2 mRNA, in each tissue and during distinct stages of development. Contrary to what happens to the *IGF2R/AIR* cluster, *H19* is a non-coding RNA, with a promoter that does not overlap with other genes. In addition, *H19* transcription does not seem to have a role in imprinting control, i.e., its replacement with a coding protein causes no disruption in any other genes (Regha *et al.*, 2006).

The *IGF2/H19* cluster also possesses an ICR between the *IGF2* and *H19* sequences that is activated by methylation in the paternal chromosome and silenced by CTCF boundary elements in the maternal allele, with this region being well characterized in cattle (Gebert *et al.*, 2006). The expression of both *Igf2* and *H19* in mice, the most studied model, is dependent on shared endodermal and mesodermal enhancers, located downstream the *H19* gene. The enhancers are *cis* regulatory sequences, typically between 100 and 300 bp that promotes drastic changes in transcriptional rate, depending on the methylation state of the ICR, activating or repressing a gene. Deletion of the *H19* promoter at the maternal chromosome can cause

offspring overgrowth. Based on such response, it was proposed that *H19* competed with *Igf2* for transcription factors, so its deletion would cause a loss of imprinting, allowing the enhancer to overexpress *Igf2* and *Ins-2*. In such mutants, the upstream *Igf2* promoter adopts the methylation pattern of the paternal chromosome, which seems to allow enhancer accessibility, while unmarked DNA would possess a boundary element (CTCF) that blocks interaction between *Igf2* and its enhancer. Once the endoderm-specific enhancer of *H19* was removed, it resulted in loss of expression of *H19* and *Igf2* in mice neonatal liver, straightening the enhancer competition theory and highlighting the importance of this transcriptional factor in the imprinting mechanism (Bartolomei *et al.*, 1993; Leignhton *et al.*, 1995; Reik & Murrel, 2000; Ludwig *et al.*, 2002).

Four DMRs were documented in the *Igf2/H19* cluster: DMR0 and DMR1, between *Ins-2* and *Igf2*; DMR2, within exon 6 of the *Igf2* gene; and *H19*-DMR (Moore *et al.*, 1997; Gebert *et al.*, 2006). DMR0 is highly methylated in both parental chromosomes, transcribed in both directions and is placenta-specific in mice (Moore *et al.*, 1997). DMR1 is normally paternally methylated and has a mesodermal silencer effect on *Igf2*, with its deletion resulting in biallelic expression in mesodermal-derived tissues. DMR1 has a role in *H19*-independent expression and imprinting control over *Igf2* (Constancia *et al.*, 2000). DMR2 is demethylated prior to the 4-cells stage embryos (where 6% of this region has metylation) and partially remethylated in the paternal allele prior to the blastocyst stage (up to 10%). From these data, it was inferred that re-methylation starts right before blastocyst and may be completed prior to implantation. Female blastocysts suffer less methylation (15%) than male blastocysts (29%; Gebert *et al.*, 2009). Perhaps such differences are sufficient to induce differences in size seen in elongated bovine concepti seen by Bertolini *et al.* (2002b). Finally, the *H19*-DMR is located upstream of *H19*, and is methylated in the paternal allele, acting as the ICR for

that region. The *H19*-DMR deletion in the paternal chromosome caused up-regulation of the repressed non-coding mRNA and reduction of *Igf2* expression in the same chromosome, as the absent methylation caused by the disruption is incapable of silencing *H19* promoter, adding competitiveness for the enhancer. Alteration on the maternal allele leads to *H19* underexpression and concomitant *Igf2* activation, as there is no ligand to CTCF and, therefore, no barrier between the enhancer and the *Igf2* promoters (Thorvaldsen *et al.*, 1998).

2.3.2 The IGF2R/Air cluster

As previously reported, the IGF2R influences growth by retrieving IGF2 from circulation and targeting it for lysosomal degradation. The *Igf2r*, as tested in mice, is maternally expressed and paternally imprinted (Barlow *et al.*, 1991). The receptor shares a cluster of 40 kb with two solute carrier family *22a* (*Slc22a2* and *Slc22a3*), maternally expressed in the placenta, and one antisense non-coding RNA (*Air*), paternally expressed, as well as the biallelic expressed *Slc22a1* (Barlow *et al.*, 1991; Zwart *et al.*, 2001). The promoters for *Slc22a2* and *Slc22a3* are 190 kb and 260 kb downstream of the *Igf2r* promoter, respectively. Both of them are maternally expressed and with no expression in the embryo, but are present as monoallelic in the placenta and as biallelic in adult tissues (Zwart *et al.*, 2001). In contrast, *Igf2r* expression is biallelic in embryos and maternally-expressed in postimplantional tissues.

This cluster contains two DMRs. The DMR1 contains the *Igf2r* promoter and maintains the paternal allele silent by methylation. The DMR2 is located within intron2 of the *Igf2r*, it is the cluster imprinting control region, and contains the *Air* promoter (Wutz *et al.*, 2001; Zwart *et al.*, 2001; Regha *et al.*, 2006). The DMR2 is commonly methylated at the maternal allele from oogenesis, and it is maintained through the wide-genome demethylation in *de novo* methylation. Air is the only gene in the cluster that is

paternally expressed, sharing a 30 kb sequence homology with *Igf2r* (Regha *et al.*, 2006).

The presence of methylation at the DMR2 in different *Igf2r* allelic profiles suggest that such epigenetic mark is not enough to induce imprinting expression in the *Igf2r*, nor it is to induce expression, as its loss results in a complete absence of transcription (Liu *et al.*, 1993; Szabo & Mann, 1995; Barlow *et al.*, 1997). The expression of *Air* ncRNA induces silencing of the *Igf2r*, *Scl22a2* and *Scl22a3* genes, and loss of *Air* gene expression, by paternal allele deletions, results in biallelic expression of the other genes, as similar to what happens with truncated form of *Air* (Wutz *et al.*, 2001; Zwart *et al.*, 2001; Reagha *et al.*, 2006). Thus, it was assumed that the *Air* ncRNA is not expressed in preimplantation embryos having a biallelic *Igf2r* expression (Szabo & Mann, 1995). Although the role of *Air* in the imprinting process is clear, it is undetermined whether the Air RNA *per se* or the transcription through this region is responsible for silencing the cluster. The *Air* gene does not have a role in mouse growth, since its absence allows development of a normal offspring, as observed by Wutz *et al.* (2001). Taken together, the key features of the imprinting expression at the *Igf2r/Air* cluster are the presence and methylation of the DMR1, and the expression of a functional Air ncRNA.

2.4 Embryo development

Failures in early physiological events can lead to both early (with higher rates of return to estrus) and late (with delay in the estrus cycle) embryo mortality. It is, therefore, appropriate to briefly describe aspects of normal prenatal development, since many deviations from normality are observed after some embryonic manipulations, such as IVP of embryos. Gestation or the prenatal period, from fertilization to birth, can be divided into embryonic and fetal stages, according to the pattern of growth and

physiological development of the conceptus (Sloss & Dufty 1980; Roberts, 1986; Ménézo & Renard, 1993; Bertolini *et al.*, 2007).

The embryonic phase in cattle begins immediately after conception (1-cell stage embryo) and continues until the end of organogenesis (Day 42 of gestation), being a period of differentiation. This phase can be subdivided into two stages: (1) Preimplantation or early embryonic stage, including: (A) the initial cleavage period from conception to the activation of the embryonic genome (Days 1 to 4); (B) early embryonic differentiation, when cell differentiation, compaction, cavitation, hatching and expansion start (Days 4 to 10-12); and (C) elongation, with the stretching of the trophoblast (Dias 10-12), embryonic disc formation and morphogenesis, early formation of primitive extra-embryonic membranes, and maternal recognition of pregnancy (Dias 16-18); and (2) implantation stage, including the period of embryonic folds, placentation, and organogenesis, when the major tissues, organs, systems and extra-embryonic membranes are formed (Days 18 to 42). The implantation stage in turn is a gradual and continuous process divided into three consecutive events: pre-contact (~Day 17), apposition (Days 18 to 19), and adhesion, starting around Day 22. At 30 days of gestation, primitive cotyledons appear in juxtaposition regions with the caruncles. On Day 33, trophoblastic cones form villi that penetrate the caruncular crypts, producing lateral branches. By Day 45, crypts and villi are easily recognizable.

The fetal phase, from Day 42 until the birth, composes a period of rapid growth of the conceptus, with modest morphological changes until birth (Sloss & Dufty 1980; Roberts 1986). Embryo death at preimplantation (prior to maternal recognition of pregnancy) or implantation (between maternal recognition of pregnancy and fetal stage) is usually referred to as early or late embryonic mortality, respectively.

In vitro culture includes the initial cleavage and early embryonic differentiation, from Day 0 (fertilization) to Day 8 (hatching). As the phase that allows the most manipulation so far, several studies and parameters have been set to facilitate embryo handling. During the early years of conventional IVP, the impossibility of evaluating oocyte quality, coupled with a lack of prognostic criteria at the zygote stage, led to the introduction of embryo grading systems developed by experts in the subject and concretely related to pregnancy rate (Cummins *et al.*, 1986; Veeck *et al.*, 1990; Farin *et al.*, 1995; Shoukir *et al.*, 1997). Since then, the most widely used indicators of embryo quality are morphological criteria published by the International Embryo Transfer Society (Stringfellow & Seidel, 1998), for *in vivo*-derived (IVD) embryos. The selection of a critical time point is essential, in this case, to maximize differences between embryos in order to avoid misleading in the categorization of each development stage and timeline development. In such evaluations, a few traits are commonly used such as: number of cells, vitelline membrane (plasma membrane), perivitelline space, homogeneity of cytoplasm, polar body, fragmentation, blastocoele and zona pellucida (Ebner *et al.*, 2003). Embryos are segregated in stages of development and the morphologic quality is partially determined by the capability of achieving such stage at the appropriate timeline. The dividing tendency of the blastomeres leads to an expected development based on multiples of two, but odd numbers of cells can be found in some embryos, which may imply in some disturbances in normal development (Van Royen *et al.*, 1999). Recently, however, novel and more effective screening methods to evaluate embryo developmental capacity have been devised. A relationship between embryo metabolism and viability has been established and is now been considered along with morphokinetic data to create algorithms for embryo selection (Gardner *et al.*, 2015).

Transcripts in preimplantation embryos can be of maternal or embryonic origin.

The beginning of embryo development is controlled by maternal transcripts and proteins produced and stored during oogenesis. After fertilization, the control is switched from maternal to embryo-derived transcripts and proteins at genome activation. Such process involves the depletion of maternal transcripts by degradation and translation, and their replacement by embryonic transcripts. Therefore, transcripts that are found during early cleavage stages in cattle remain constant or, most likely, decrease during this early phase of embryo development, until the 8-cells embryo stage, increasing after the embryonic genome activation (EGA). Timing and extent of such decrease and increase varies between the different transcripts (Wrenzycki *et al.*, 1998). Major embryonic genome activation, that redeem nucleic material of the embryo apt to transcribe genes and replace the rapidly decreasing maternal transcripts, has been described to occur at the 8- to 16-cells embryo stages. However, the onset of the process has varied depending on the techniques used for detecting embryonic transcription throughout the years (Graf *et al.*, 2014a). Just recently has it been more precisely defined by an RNA-Seq study (Graf *et al.*, 2014b). Nonetheless, most studies agree that, in bovine embryo, a minor EGA occurs prior to the 4-cells embryo stage and a major one takes place between 8- and 16-cells stage (Camous *et al.*, 1986; Memili *et al.*, 1998; Graf *et al.*, 2014a,b). Figure 2, from Graaf *et al.* (2014a), exemplifies the variation in maternal end embryonic mRNA abundance from oocytes through preimplantation embryo stages in cattle.

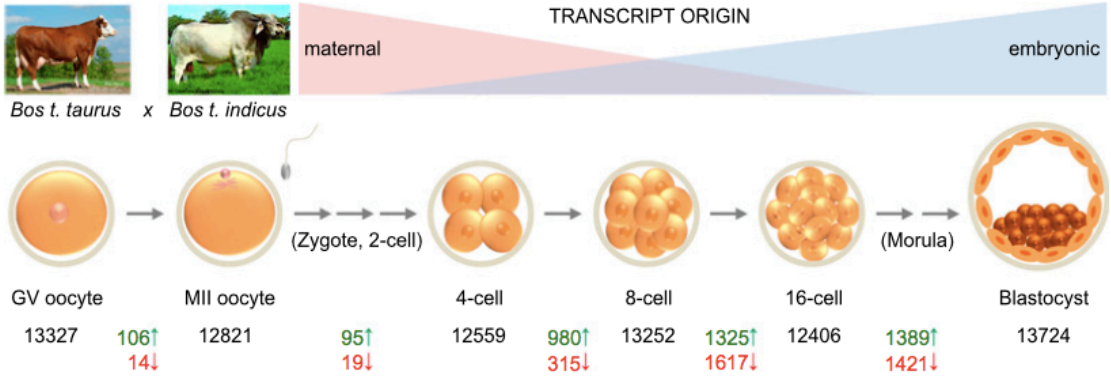

Figure 2. Amounts and origin of mRNA in oocytes and in preimplantation embryos in cattle. For each stage the total number of genes with detectable transcripts is indicated in black. The numbers of differentially abundant transcripts between two stages are shown in green (increased abundance) and red (decreased abundance in the subsequent *vs.* the previous stage). From: de Graf *et al.* (2014b).

2.4.1 The IGF system effect on embryo development

The presence of IGF1R, IGF-2 and IGF2R transcripts in all preimplantation bovine structures from the immature oocyte to the hatched blastocyst stage indicates that maternal transcripts occur in the oocyte and that persists at cleavage stages prior to major EGA. Furthermore, the mRNA concentrations seem to follow a similar pattern of most genes expressed during oocyte maturation and embryo development. The increase in transcript accumulation in oocytes may also indicate that maturation may depend on the amount of stored mRNA. After fertilization, IGF concentrations decrease gradually up to EGA. After the genomic maternal–embryonic transition, concentrations of IGF2, IGF2R and IGF1R mRNA increase up to the hatched blastocyst stage. IGF1R, more specifically, has been reported to be active in offspring genome between 16-cells and blastocyst embryo stages (Graf *et al.*, 2014a). The greatest increase in the IGF family mRNA amounts are observed as the bovine embryo reaches the hatched blastocyst stage may indicating an important role of these growth factors in elongation, when embryo becomes filamentous in shape (Yassen *et al.*, 2001). Regarding IGF1, its presence in

embryos is still under scrutiny. While a few studies detected the ligand in preimplantation embryos (Schultz 1992; Yoshida *et al.*, 1998), others could not find detectable levels of *IGF1* expression (Yaseen *et al.*, 2001; Bertolini *et al.*, 2002; Wang *et al.*, 2009; Moore *et al.*, 2007). The location of this particular family of transcripts has also been subject of studies. Prior to the 8-cells embryo stage, IGF expression is limited to the plasma membrane, while in further development it can be seen throughout the whole cell (Wang *et al.*, 2009). In mouse embryos, on the other hand, Igf2 transcripts become detectable in the nuclei during the 2-cells stage, and a transition from biallelic to monoallelic expression can be seen throughout development to the blastocyst stage, evidencing a progressive silencing of the maternal allele (Ohno *et al.*, 2001).

Concerning the IGF actions in embryo development, the ligands have roles in several metabolic responses such as growth, proliferation, survival, nutrients uptake, migration and differentiation, specially IGF2, as IGF1 presence is not still irrevocably proven (DeChiara *et al.*, 1991; Baker *et al.*, 1993; Gardner *et al.*, 1999; Byrne *et al.*, 2002; Constancia *et al.*, 2002; Pavelic *et al.*, 2007;). Studies regarding IGF2 supplementation and subtraction showed interesting responses. The enrichment with IGF2 into the maturation medium improved the number of embryos that achieved the blastocyst stage and increased the number of cells per blastocyst. Hypothetically, proper oocyte maturation would provide a good reserve of such ligand and its transcripts for embryo development (Wang *et al.*, 2009). The depletion in IGF2 by antisense oligonucleotides, on the other hand, reduced blastocyst rate and the number of cells per structure (Rappolee *et al.*, 1992). Furthermore, high concentrations of IGF2 in the medium stalls its beneficial effect and causes a decrease in blastocyst yield, similar to that observed with IGF1 in the mouse embryo, causing extensive apoptosis within the ICM (Chi *et al.*, 2000). The detrimental effect of high concentrations of IGF1 on

preimplantation embryos is associated with an increase in apoptosis and changes in cell allocation towards the ICM, not related to compensative downregulation of *IGF1R*, as once hypothesized (Velazquez *et al.*, 2011). Furthermore, not just the amount of IGFs can suffer from different culture parameters, but the imprinting expression as well. In mice, *in vitro*-cultured embryos showed a strong bias to maternal transcription of *Igf2* in the 8-cells to the morula stages (Ohon *et al.*, 2001).

The high rate of embryonic mortality has been previously related to initial growth retardation, and this, to changes in IGF system gene expression (DeChiara *et al.*, 1991; Baker *et al.*, 1993, Lui *et al.*, 1993). Growth disorders and embryonic mortality are also being associated with several others pregnancy and fetal disorder, such as the Abnormal Offspring Syndrome (AOS). Studies have been carried out to compare cloning by nuclear transfer (NT) to IVF-derived and *in vivo*-produced embryos. Regarding the IGF family, a higher abundance of IGF1 mRNA could be seen in NT and IVP concepti than *in vivo* ones, while a greater amount of IGF2 mRNA in bovine embryos and IGF2R in placentas of NT-derived concepti could be detected (Moore *et al.*, 2007; Pandey *et al.*, 2009). Young *et al.* (2001) also reposted disturbances in IGF2R as causing fetal overgrowth and possibly AOS in sheep concepti. Such studies are consistent with the hypothesis raised by several authors, that perturbations in the nuclear transfer procedure and/or the *in vitro* culture conditions alter the expression of components of the IGF family and may contribute to embryonic losses during early placental attachment. Therefore, disorders in the expression of imprinting genes, especially IGF2 and IGF2R, can lead to embryonic, fetal and placental developmental abnormalities (Lau *et al.*, 1994; Young *et al.*, 2001; Bertolini *et al.*, 2002a, 2004; Suteevun-Phermthai *et al.*, 2009).

2.5 IGF2 isoforms

The splicing of messenger RNA precursors to mature mRNA is a flexible and dynamic process that has a high impact in cell biology. It is a critical component of the regulation of gene expression pathways. Formation of different patterns from alternative exons can expand the number of possible proteins and contributes significantly to the identity, development, and diversity of cells, tissue, and organs. Alternative pre-mRNA processing can result in mRNA isoforms that encode distinct protein products, or may differ exclusively in untranslated regions, potentially affecting mRNA stability, localization, or translation. A gene with isoforms that code for the same protein may be subject to complex regulation to maintain a certain level of output in the face of changes in expression of its transcription factors, for example (Trapnell *et al.*, 2010). Alternatively, isoforms that encode different proteins could be functionally specialized for different cell types or stages (Trapnell *et al.*, 2010). In fact, most tissues possess a conserved gene isoform expression signature that suggests a conserved expression responsible for tissue identity in mammals, as well as species-specific divergence (Merkin *et al.*, 2012). The mRNA isoforms can be achieved by different pre- and post-transcriptional mechanisms; in many cases alternative promoters are present and they work by the inclusion of alternative first exons. Such is the case with several genes, including the *IGF2* gene (Van Dijk *et al.*, 1991; Davuluri *et al.*, 2008). Somatic mutations in the genetic code encoding well-studied splicing factors are correlated with at least two types of cancer, indicating that aberrant splicing patterns are directly linked to a diseased phenotype (Taniguchi *et al.*, 1995; Davuluri *et al.*, 2008; Lee & Rio, 2015). The application of assisted reproductive technologies, such as *in vitro* fertilization and culture, is frequently associated with aberrant mRNA expression patterns in the resulting embryos, greater epigenetic disturbances and a higher risk of

aberrant phenotypes, as previously reported (Moore *et al.*, 2007; Lee & Rio, 2015). Access to genome and cDNA sequences, oligonucleotide microarrays, and especially cDNA sequencing brought forward a genome-wide assessment of alternative splicing patterns, with those highlighting such possible disruptions. In addition, preference for a particular spliced isoform was observed for several genes in degenerated embryos. Besides, the frequent occurrence of alternative splicing in IVF-derived embryos provides a repository for regulation of expression (Huang & Khatib, 2010).

In late 1990s, experiments demonstrated that the type of promoter that activated the transcription could impact the level of alternative splicing of a downstream exon (Cramer *et al.*, 1997). The insulin-like growth factor 2 is a complex genomic region that produces multiple alternative splicing transcripts of several leader exons controlled by various promoters. In total, four different promoters were found at homologous chromosomal locations in the *IGF2* (P0 – P3 in mice or P1 – P4 for other species), tissue- and developmental stage-specific (Von Horn *et al.*, 2002). The gene has ten exons in most species studied to date and is translated in a pre-pro-form peptide. The mature molecule is composed by the last three exons (8, 9, 10). The remaining exons are expressed in a tissue- and developmental stage-specific manner. Differentiation of leaders' exons, as seen for the *IGF2* gene, can contain sequences that regulate post-transcriptionally, so to, for example, keep the mRNA temporarily out of translation during certain stages of development; in such case, mRNA is present in cells as free messenger ribonucleoprotein particles (mRNP; De Moor *et al.*, 1994). Besides human studies, mostly interested in defining profiles in liver and cancer cells (Taniguchi *et al.*, 1995; Issa *et al.*, 1996; Li *et al.*, 1996; Mineo *et al.*, 2000), multiple other species had its isoform characterized, even if at less extent, including mice, pigs, sheep and cattle, with various differences in gene structure, expression and actions being reported (Van Dijk *et*

al., 1991; Boulle *et al.*, 1993; Ohlsen *et al.*, 1994; Amarger *et al.*, 2002; Courchoe *et al.,* 2005).

In terms of gene structure, bovine, ovine and swine follow basically the same profile, presenting ten exons and four promoters (P1 to P4), starting in exon 1, 4, 6 and 7, respectively (Boulle *et al.*, 1993; Ohlsen *et al.*, 1994; Amarger *et al.*, 2002). In mice, until recently, four promoters were characterized, just like most species, named P0 to P3. In this species, P1 to P3 are homologous to P2 to P4 in most animals. While there was an absence of the promoter that acted as liver-specific (P1), as seen in others species, an additional promoter was found (P0) and characterized as placenta-specific, unreported in any other animal (Constancia *et al.*, 2002). A fifth regulatory region was identified, a novel *Igf2* promoter (Pm) related to a trans-activation of *Igf2* by sense and antisense *H19* non-coding RNA (Tran *et al.*, 2012). Also, differently from other animals, only eight exons were identified in mice (U1, U2, 1, 2, 3, 4, 5, 6). However, common to others, the last 3 exons (4, 5, 6) are common in the mature mRNA. The human gene also has a similar 10-exons structure and promoters P1 to P4. A fifth promoter was recently identified, called P0, with this sequence being similar to the one found in mice, though no relation to placental function was described (Monk *et al.*, 2006).

The specific biological actions of each alternative splicing have not been extensively studied yet, with only a few exceptions. In mice, a P0 mutation leads to null production of its particular mRNA isoform composed by exon 4 to 6. It can be observed a particularly drastic reduction of placental Igf2 transcripts, even with normal function of others promoters. The consequence of this singular promoter silencing is a restrict growth in placental tissue (68% of normal weight) similar to animals used to study complete *Igf2* knockouts. The offspring are born with 70% of normal weight, with the

most restriction found in late pregnancy, due to a smaller nutritional support through the mutant placenta. A reduction in passive permeability could be seen, especially to hydrophilic solutes, accompanied by an upregulation of amino acids through an active secondary transport. However, this compensation fails to provide the fetus the nutritional needs, leading to growth restrictions after some days (Constancia *et al.*, 2002). In humans, a similar promoter to mice P0 was characterized *in vitro* by Monk *et al.* (2006), located 258 bp upstream the exon 1, in a *locus* similar to that found in mice. There are, nonetheless, dissimilarities: while in mice the P0 transcribes solely in the placenta, in humans, it is expressed at high levels in muscle and posteriorly, in lesser extent, it can be found in adult tissues and in the pre-labor placenta. The human *Igf2*-P0 transcript does not seem to have the same role as the one in mice. The P1, in humans, is related to adult liver function as well as cancer development, as it is the only promoter not hypermethylated and has a biallelic profile consistent with carcinogenous expression (Issa *et al.*, 1996). In mice, however, *Igf2* gene is no longer expressed in liver after birth and no liver-specific promoter homologous to human P1 has been detected so far.

Regarding imprinting expression, in mouse and cattle, all four promoters direct a monoallelic expression of the Igf2 gene in fetal tissues, with a paternal expression and some *leakage* of the maternal allele, except for the brain, where a biallelic expression can be seen in all species. Additionally, aging leads to a general promoter-specific loss of imprinting in both species. However, while in mice all promoters suffer this change, in cattle a loss of imprinting was detected in some organs linked to specific promoters (Constânca *et al.,* 2002; Curchoe *et al.*, 2005). Majorly, a switch was observed in P3 and P4, that were detected with strong leaky or nearly biallelic expression in all tissues after birth, including calves and adults. Unlike humans and sheep, where P1 is

biallelicaly expressed, this promoter in adult cattle loses its imprinting expression only in specific tissues, such as in the liver and sometimes in the kidneys, while retaining the monoallelic pattern in all other organs (placenta, lungs, bladder, spleen and heart; Ohlsen *et al.*, 1994; Constânca *et al.,* 2002; Curchoe *et al.*, 2005). In humans, on the other hand, the promoters P2, P3 and P4 are contained in a CpG island and are subjected to parental imprinting, such that a monoallelic paternal allele is expressed in all stages of development, while P1 is located upstream of the CpG island and, different from the formers, it escapes imprinting, having a biallelic expression in several tissues, including the adult liver (Issa *et al.*, 1996).

Relative to promoter-derived transcript abundance in key organs, P1, in humans, is active exclusively in the adult liver, where the onset and upregulation seems linked with the down-regulation of P3, with the peak of P3 usage culminating in the P1 onset, shortly after birth. The expression of P1 is gradually elevated with age until finally reaching 50 to 70% of total hepatic Igf2 transcripts (Li *et al.*, 1996). This is also the case in sheep, where P1 action is represented by transcripts from 1-3-8 or 1-8 exon arrangements. In cattle, contraire to other animals, P1-derived transcripts were found in nearly all tissues from fetuses, calves and adults, with the exception of the brain (Ohlsen *et al.*, 1994; Churchoe *et al.*, 2005).

The P2 promoter presents two distinct isoforms due to alternative splicing, one containing exon 5 and another without it. It has a low abundance in all tissues and cell lines in humans. In the human liver, for instance, there is a peak at two months of age, showing a relatively even abundance from 18 months onward. It represents only 10% of the produced transcripts, but high concentrations are seen in certain tumor cells (Ikeiriji *et al.*, 1991; Li *et al.*, 1996; Van Horn *et al.*, 2002). Nonetheless, it is not yet known its specific action in the organism, nor if the low concentration is due to low transcription,

or rapid processing. In sheep and cattle, this promoter could be detected in adult and fetal liver in small amounts (Ohlsen *et al.*, 1994). However, differently from the former, in cattle, other tissues from fetuses and calves also had some levels of this transcript, such as in the lungs and kidneys. A truncated form was confirmed in cattle, from promoters P2 to P4, where part of exon 10 was absent, disabling the E domain. This truncated from seems to be present in tissues were its complete form is missing (Curchoe *et al.*, 2005).

The P3 and P4 are the major promoters used in fetal and non-hepatic adult tissues. In sheep, transcripts were present in both fetal and adult liver (Ohlsen *et al.*, 1994). The P3 promoter, actually, is responsible for a big proportion of the Igf2 mRNA activity in fetal and placental tissues. As previously reported, this alternative splicing variant is of great importance in fetal liver. However, after finding its highest concentration at two months of age in humans, it starts to gradually decrease, while P1 starts to increase in importance, to the point of taking P3's functional place in the liver. In adult life, only P1, P2 and P4 are active in the liver; in other cells, however, P3 and P4 predominate in adult life (Li *et al.*, 1996). In cattle, on the other hand, this promoter assumes a total different pattern, as it appears to be present in almost all fetal, neonatal or adult tissues in its complete form. Only in fetal and neonatal brains, as well as in the placenta, the truncated form alone could be seen (Curchoe *et al.*, 2005). The activity of the P4 promoter is relatively high during human development. While P1 to P3 reach the highest peak in liver at two months of age, P4 shows its highest transcription at the fetal stage (Li *et al.* 1996), and in the adult liver it accounts for the production of 25–60% of total Igf2 mRNA (Von Horn *et al.*, 2002). In cattle, expression of full-length P4 transcripts are present in al tissues and appears to decrease with age, while the truncated form increases, until complete mRNA is primarily detected only in small amounts in

adult tissues (Curchoe *et al.*, 2005). In mice, the liver highest expression levels were observed for the P3 (80%) and P2 (35%) promoters, followed by the P1 (20%), with the lowest being the P0 (7%; Tran *et al.*, 2017).

Considering promoter expression regulation, the post-transcriptional differences in isoforms can occur by two meanings: influencing the availability of specific mRNA for translation, and by causing differences in translation efficiency (by secondary structure stability, blocking small ribosome subunit coupling and forming a 100S particle). Translation efficiency with P4-transcripts seems higher than P2 and P3 alternative splicing variants. This last two seem to initiate translation with similar efficiency. Temporarily non-translatable RNA was also detected among the IGF family mRNAs (De Moor *et al.*, 1994). Previous studies reported a biological effect of GH in activating P2 promoter transcription and inducing a switch in P2-derived transcripts from a non-translatable (mRNP) to a translatable protein complex, as P2 activity increases (Von Horn *et al.*, 2002). Some authors have also reported a large portion of P3-derived transcripts as being non-translatable, especially in cell lines and the fetal liver. Nielsen *et al.* (1995) proved that the rate of cell proliferation can switch P3-derived transcripts the same way as GH can with P2 transcripts, from mRNP to translatable (by S6 kinase signaling pathway), with levels 2.6 times higher in growth-arrested cells than in exponentially growing cells. Furthermore, Igf2 mRNA probably has an increased half-life in 100S particle. Thus, this phenomenon of accumulation of non-transcriptional particles possibly accounts for the great Igf2 concentration seen with such alternative splicing variant (Moor *et al.*, 1995; Nielsen *et al.* 1995).

Transcripts for the *H19* gene have also been related to Igf2 transcription for years (Bartolomei *et al.*, 1993). However, the exact action mechanism that such non-coding RNA uses to regulate IGF2 expression still remains elusive. Interestingly, in

humans, recent studies revealed an antisense H19 transcript, called 91H RNA, which opposed to H19 to favor tumor progression. Expression of 91H RNA, produced by the same endodermic enhancer that regulates the cluster in *cis*, is sufficient to trans-activate Igf2 through the Pm promoter, at the transcriptional level, despite hypomethylation of the H19 ICR. Furthermore, a large excess of H19 seems to counteract this effect. The specific characterization from this promoter transcript is yet to be made, as well as its presence and functions in other species (Taniguchi *et al.*, 1995; Tran *et al.*, 2012).

An additional form of expression was suggested as antisense (AS) transcripts were discovered in the P0 region, in mice, with potential peptides derived from this IGF2-AS that is yet to be confirmed (Moore *et al.*, 1997). Some studies suggested that these AS might interfere with IGF2 transcripts derived from P1 and P2 in pig muscle (Brauschweig *et al.*, 2004), while others could not find a direct interaction between antisense and sense transcripts (Duart-Garcia and Braunschweig, 2014). Either way, their biological functions and genetic interactions with IGF remains uncertain.

In summary, the *IGF2* gene plays a key role in embryo development. Its complex regulation includes pre- and posttranscriptional systems, such as imprinting, CTCF, IGFBPs and alternative splicing variants (DeChiara *et al.*, 1991; Firth & Baxter, 2002; Curchoe *et al.*, 2005; Tran *et al.*, 2012). As the IGF system is responsible for many cellular responses, such as growth, differentiation and migration, the better understanding of the role of the promoters in the *IGF2* expression in IVP-derived embryos could provide an interesting insight to developmental biology, assisting in creating more suitable IVP protocols to prevent or avoid biological disturbances (DeChiara *et al.*, 1991; Baker *et al.*, 1993; Constancia *et al.*, 2002; Pavelic *et al.*, 2009). In previous references, the four *IGF2* promoters reported in cattle did not appear to have an association in expression with one another, as their functions and activities

seem to behave differently between tissues and at distinct developmental stages (Curchoe *et al.*, 2005). However, a gap exists regarding their influence and activity in oocytes and in IVP-derived preimplantation embryos. Even though this specific gene could be easily associated with fetal and placental development and abnormalities (Liu *et al.*, 1993; Young *et al.*, 2001; Constancia *et al.*, 2002), it has been rare its relationship with specific tissues or biological processes. An association between the *IGF2* gene and key biological events in time and space, such as maturation, fertilization, minor or major embryonic genome activation (EGA), compaction, and cavitation, could provide important insights towards the refinement of ART in animals and even in humans. Some of the possible answers to such questions were attempted to be explored in this study.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

H_1: The *IGF2* promoters profiles and activities can be determined throughout embryo development in cattle by means of molecular tools.

H_2: The four *IGF2* promoters reported in cattle (P1, P2, P3 e P4) express distinct patterns in bovine oocytes and in preimplantation IVP-derived embryos.

H_3: A relationship can be established between the promoter-derived *IGF2* expression patterns and key events during embryo development such as maturation, fertilization, embryonic genome activation, compaction, and/or cavitation.

3.2 General Objectives

a) To assist in the better understanding the process of growth and development of *in vitro*-produced bovine preimplantation embryos to derive optimal *in vitro* embryo production systems that better mimic conditions as existent in nature for *in vivo*-derived embryos, also allowing the development of strategies for more precise genetic manipulations of *in vitro*-produced embryos.

3.3 Specific Objectives

a) To determine the temporal and spatial differential activity of the four known bovine *IGF2* promoters prior to and during the *in vitro* embryo development, through the quantification of the relative abundance of distinct *IGF2* mRNA isoforms, specific to each promoter.

b) To correlate the levels of expression of the *IGF2* isoforms with the developmental outcome after the *in vitro* production of bovine embryos.

4. ONLY CHAPTER: Promoter-specific expression of the imprinted IGF2 gene in bovine oocytes and preimplantation embryos

4.1 Introduction

It has been more than three decades since the birth of the first bovine calf (Virgil) from *in vitro* fertilization (IVF) procedures (Brackett *et al.*, 1982). The development of successful *in vitro* embryo production (IVP) systems in the late 1980s (Lu *et al.*, 1988) paved the way to studies that resulted in tremendous technological advances in the area in subsequent decades. Even with drastic improvement in embryo production over the years, preimplantation embryo development and viability, and early or late embryonic mortality following the transfer to female recipients are multifactorial problems that still are associated with reproductive losses and lower efficiency when compared with natural breeding, artificial insemination or the in vivo-production of embryos, representing a significant economic impact in the livestock industry. Early embryonic mortality occurs mainly during the first 16 days post-conception in cattle (Diskin *et al.*, 2015), and such a problem seems to be more common to bovine IVP-derived embryos, as gestational losses at the embryonic phase are usually reported to be higher than for *in vivo*-produced embryos (Hasler *et al.*, 1995; Bertolini *et al.*, 2002ab). Since discrepancies between losses are more drastically perceived after and during the period of initial development and placentation (from Day 30 to 60; Hill *et al.*, 2000, 2001; Bertolini & Anderson, 2002), pieces of evidence suggest that the high mortality of IVP-derived embryos is a consequence of an irregularity in maternal-embryonic-fetal connection and exchange, leading to embryo-fetal malnutrition, initial growth retardation and increased mortality during the first trimester of pregnancy (Bertolini Bertolini *et al.*, 2002ab, Chavatte-Palmer *et al.*, 2006). Such an initial period of growth restriction observed during the embryonic phase and fetal phase was characterized by

lower pregnancy rates and a high rate of late embryonic losses attributed to faulty placental formation, possibly negatively affecting maternal-fetal exchanges.

The high rate of embryonic mortality for IVP embryos has been related to an initial growth retardation, as mentioned above, and that, to changes in the expression pattern of component genes of the IGF system, such as the IGF2 ligand and its receptor (IGF2R; DeChiara *et al.*, 1991; Baker *et al.*, 1993; Lui *et al.*, 1993; Suteevun-Phermthai *et al.*, 2009). Both genes have genomic imprinting patterns, in which only one specific parental allele will have an active expression, while the other will be silenced by typical epigenetic processes (DeChiara *et al.*, 1991; Constância *et al.*, 2000; Reik *et al.*, 2001). Both components of the IGF system are clearly related to the control of the regulation of embryonic, fetal and placenta growth and development throughout pregnancy in mammals (DeChiara *et al.*, 1991; Lau *et al.*, 1994; Young *et al.*, 2001; Constancia *et al.*, 2002; Suteevun-Phermthai *et al.*, 2009). Interestingly, previous studies have observed a close correlation between the higher early embryonic mortality in IVF-derived embryos with the growth retardation of the conceptus and reduced expression levels for the IGF2 gene, and higher levels for the *IGF2R* gene at late embryonic phase, and with placental and fetal abnormalities at the phase fetal (Bertolini *et al.*, 2002ab). Thus, loss of the imprinting pattern for the *IGF2* or *IGF2R* genes has been related to major developmental changes in IVP-derived concepti (Lau *et al.*, 1994; Young *et al.*, 2001; Suteevun-Phermthai *et al.*, 2009).

The splicing of messenger RNA precursors to mature mRNA is a flexible and dynamic process that has a high impact in cell biology, being a critical component of the regulation of gene expression pathways. Alternative pre-mRNA processing can result in mRNA isoforms that encode distinct protein products, or may differ exclusively in untranslated regions, potentially affecting mRNA stability, localization,

or translation. Somatic mutations in the genetic code encoding well-studied splicing factors are correlated with at least two types of cancer, indicating that aberrant splicing patterns are directly linked to a potential disease phenotype (Taniguchi *et al.*, 1995; Davuluri *et al.*, 2008; Lee & Rio, 2015). Also, the application of assisted reproductive technologies, such as by *in vitro* production (IVP) of embryos by *in vitro* fertilization procedures, is frequently associated with aberrant mRNA expression patterns, greater epigenetic disturbances and higher risks of aberrant phenotypes in resulting embryos. The mRNA isoforms can be achieved by different pre- and post-transcriptional mechanisms, and in many cases, can be driven by alternative promoters that may define alternative first exons available for transcription. Such is the case for several genes, including the *IGF2* gene (Van Dijk *et al.*, 1991; Davuluri *et al.*, 2008).

The *IGF2* is a complex genomic region that produces multiple alternative splicing transcripts of several leader exons controlled by various promoters. In total, four different promoters were identified at homologous chromosomal locations in the *IGF2* sequence, named as P0, P1, P2, and P3 in mice, or P1, P2, P3, and P4 other species (Von Horn *et al.*, 2002). The IGF2 gene has ten exons for most studied species to date, being translated as a pre-pro-hormone. The mature molecule is composed by the last tree exons (8, 9, 10). The remaining exons are expressed in tissue and developmental stage specific manner. The differentiation of the leader expressed exons, as seen for the IGF2 gene, may contain sequences that regulate post-transcription events, for example, to keep the mRNA temporarily out of translation during certain stage of development, with the transcripts present in cells as free messenger ribonucleoprotein particles (mRNP; De Moor *et al.*, 1994).

The application of procedures to stimulate embryo development at pre- and postimplantation stages may favor the establishment of pregnancies under more

physiological conditions. The IGF2 ligand promotes cell proliferation and differentiation, and conceptus growth through the control of substrate transport to the conceptus (Constância *et al.*, 2005), being already related to major changes in development seen in IVP-derived concepti (Lau *et al.*, 1994; Young *et al.*, 2001; Suteevun-Phermthai *et al.*, 2009). The better understanding of the growth control expression pattern at preimplantation stages of embryo development becomes essential for the elaboration of more optimal *in vitro* production systems and also to allow for strategies for precise genetic manipulation of gametes and embryos. The genomic vulnerability of the early embryo turns less precise modifications a liability to the offspring, as major changes in gene expression can have unpredictable consequences to embryo development. The present study aimed to evaluate the spatial and temporal *IGF2* expression patterns driven by four known promoters in oocytes and preimplantation *in vitro*-produced bovine embryos, as a means for the better understanding of the role of such key gene in development in cattle.

4.2 Materials and methods

All chemicals were from Sigma-Aldrich Inc. (St. Louis, USA), unless stated otherwise.

4.2.1 In vitro production of bovine embryos

Three independent replicates for the *in vitro* production of bovine embryos were performed, according to our established protocols (Ribeiro *et al.*, 2009; Gerger *et al.*, 2017). *Cumulus*–oocyte complexes (COCs) obtained from bovine ovaries from a regional slaughterhouse collected by the aspiration of follicles between 2 to 8 mm in diameter were morphologically selected and *in vitro*-matured (IVM) for 20 to 24 h in IVM medium, composed of TCM-199 with Earle's salts, L-glutamine and HEPES, 0.2 mM sodium pyruvate, 26.1 mM sodium bicarbonate, 10% heat-inactivated fetal bovine

serum (FBS), supplemented with 5 IU/mL FSH-p (Folltropin, Bioniche, USA), 10 IU/mL hCG (Chorulon, Intervet, Inc., USA), and 1 mg/mL 17-β estradiol. A solution containing 105 IU/ml sodium penicillin, 10 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B (GIBCO-BRL1, Life Technologies, Grand Island, NY) was also added to the medium (1:100). Procedures for *in vitro* sperm capacitation and IVF were based on Gerger *et al.* (2017). Briefly, frozen–thawed bovine sperm cells were segregated by Percoll® gradient with Sperm-TALP medium. Following IVM, groups of 15 to 20 COCs were co-cultured with capacitated sperm cells, in microdroplets of IVF-TALP medium of 5 μL/oocite, under mineral oil, at an insemination dose of 5.000 viable sperm cells/COC, for 18 to 22 h, at 38.5ºC, 5% CO_2 and 95% humidity. At the end of IVF, all structures were denuded by pipetting and transferred to modified SOF culture medium, supplemented with amino acids, 1.5 mM D-glucose, and 0.4% BSA, in 100 μL microdroplets of in 10 μL/structure, for *in vitro* culture (IVC) up to Day 7 of development at 38.5ºC, 5% CO_2, 5% O_2, 90% N_2 and 95% humidity. Structures from different stages were collected, throughout the procedures, including immature COCs, mature oocytes (both stages were denuded from cumulus cells by pipetting), 1-cell, 2-cells (no corpuscles' selection was made), 4-cells, 8-cells, 16-cells, early and compact morula, blastocyst and expanded blastocyst. Selected structures were transferred to 0.2 μL PBS in pools of five and were immediately stored at a -80ºC in 0.2 mL identified microcentrifuge tubes, until RNA extraction.

4.2.2 RNA extraction and production of cDNA

Samples containing the pools of five structures were thawed in ice, and the total RNA extraction was performed using the RNeasy Micro Kit® (50; Qiagen, USA), following modified procedures from the manufacturer's recommendations. Cells were lysed with the aid of 1 mL insulin syringe and needle. The remaining steps followed as

recommended, with the exception of the elution step, which was done in 30 µL nuclease-free water, to adapt to the small concentration of nucleic material. Immediately after total RNA extraction, cDNA was generated using the SuperScript® II Reverse Transcriptase kit (Invitrogen, USA). In Mix 1, 27.5 µL mRNA samples were added to 1 µL of random primer and incubated 5 min at 70ºC, and an additional 5 min at 4ºC. Mix 2 contained 8 µL buffer (5x); 2.5 µL $MgCl_2$ (1.6 mM); 1 µL dNTP; 0.5 µL RNasin and 0.5 µL Reverse Transcriptase. Mix 2 were added to Mix 1 in the end of the incubation and let sit at RT for 5 min, followed by 60 min at 42ºC, and additional 15 min at 70ºC. The cDNAs were stored at -20ºC. Quantification was carried out using the NanoDrop 2000c UV-Vis Spectrophotometer (Thermo-Scientific, Wilmington, USA). However, the small cDNA concentrations from each sample impaired the proper quantification of all samples.

4.2.3 Primers and real time quantitative PCR (qPCR)

Specific primers for bovine *IGF2* isoforms were designed using the Primer-BLAST software package from the National Center for Biotechnology Information (EU518675.1; Table 1) according to Figure 5. Templates were confirmed by PCR in 1% agarose gel and by DNA sequencing. The quantitative PCR (qPCR) reactions consisted of 1 µL cDNA, 7.5 µL Power SYBR® Green PCR Mastermix (Promega, USA), 133 nM of each primer specific to P1, P2, P4, 533 nM P3, and 400 nM β-actin as the endogenous gene. Water was added to complete a 15 µL PCR reaction. The PCR cycles consisted of 2 min at 95°C, followed by 45 cycles of 15 s at 95°C, 60 s at 60°C. Melting curves were composed of 15 s at 95ºC, 60 s at 62ºC and elevation temperature of 0.3ºC/min until 95ºC, were it remained for 15 s. The Software LineGene9600 (Bioer Technology, USA) was used to analyze the amplification plot, the melting curve and for the quantitative PCR results from each bovine IGF2 promoter-specific. Primers with

more than one peak in melting curve were confirmed as dimers with the run in 1.5% agarose gel to confirm specific template for exclusion from de data.

Table 1. Sequence of PCR primers for the *IGF2* transcripts analyzed in bovine oocytes and preimplantation embryos, based on each specific *IGF2* promoter (P1, P2, P3, and P4)

Transcript isoforms by promoter	Primer sequences (5'-3')[a]
IGF2 P1	F - AGG GGA CGA AGA GTC AC
	R - TCA GTT CTG AGC AGG TGG
IGF2 P2	F - CTG CTA CGA AAG TAC CCG GA
	R - CCA GTC CGT TGG AAG ACC
IGF2 P3	F - GCC CGT CCT CCC TAA AGA AT
	R - CGG AAG CAC GGT CGT AGA G
IGF2 P4	F - AGC CTT CCA GAC TCC TCC T
	R - CGG AAG CAC GGT CGT AGA G
β-actin[b]	F - CGT GAG AAG ATG ACC CAG ATC A
	R - GGG ACA GCA CAG CCT GGA

[a]Primer orientations: F - forward; R – reverse
[b]Housekeeping gene

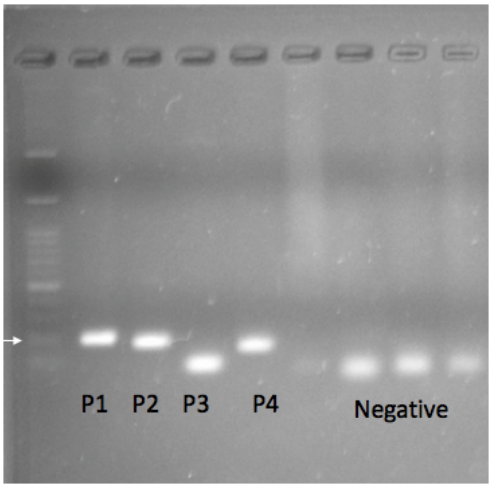

Figure 3. Representative 1% agarose gel of embryonic IGF2 isoform amplification, ran for molecular confirmation and to isolate samples for DNA sequencing. Arrow: DNA ladder, at 200 bp. Negative: negative control primers.

4.2.4 Data Analyses

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute, Cary, NC, USA), and the model included the stage of development as fixed effect. The housekeeping gene was used as a covariate in the model for gene expression data and was removed if not significant. Normality of the residuals was checked with normal probability and box plots, and data were log transformed for the analyses. LSMEANS were used to compare stages of development, for $P<0.05$.

4.3 Results

4.3.1 In vitro development of bovine embryos

A total of 301 slaughterhouse-derived bovine ovaries were used in this study, weighting a total of approximately 2,25 kg. A total of 2,257 COCs were retrieved, leading to a mean of 7.5 structures/ovary, from which, 1,340 (59.4%) were selected for IVM. As the collection followed each day, the number of structures in development was reduced at each stage. The mean cleavage rate for bovine IVP embryos on Day 2 of development was 82.8% (727/878), ranging from 78.0% to 86.0%. At 48 h post-fertilization (hpf; Day 2), 49.6% of all cleaved zygotes progressed to the 4- to 8-cells stage embryos (19.6% and 30.0% of all embryos, 173/878 and 263/878, respectively), with 17.7% (156/878) of the structures having odd numbers of blastomeres, including 12.7% (112 structures) between five and seven cells, and 5.0% (44 structures) with three cells. Then, 35.3% of all embryos reached the 16-cells stage embryo, with 10.8% reaching the 32-cells stage on Day 4 (96 hpf). The mean rate of development to early morula and compact morula stages were 33.3% (144/432) and 25.9% (112/432) at Day 5. At Day 6, 17.3% (68/394), 36.5% (144/394), 11.9% (47/394), and 5.8% (23/394) of the structures reached the early morula, compact morula, early blastocyst and blastocyst stages, respectively. Finally, at Day 7, 26.3% (72/274) and 25.5% (70/274) of structures

were at the blastocyst and expanded blastocyst stages of development. Number of structures used in the study are presented in Table 2. A control group was included to evaluate *in vitro* embryo development, with blastocyst development on Day 7 with mean of 38%.

Table 2. Total number of bovine structures used in the IVP procedures for harvesting for use in the molecular analyses

Structures	Day -1	Day 0	Day 1	Day 2	Day 4	Day 5	Day 6	Day 7
Total	2,257	1,220	1,094	878	590	432	394	274
Viable[a]	1,340	-	-	727	386	256	214	142
Harvested[b]	60	60	60	150	100	60	60	60
Stage of development[c]	OI	OM	1-cell	2-, 4-, 8-cells	16-, 32-cells	Mi, Mc	Bl	Bl, Bx

[a]Structures were considered viable when at an expected stage of development at a given chronological time in development
[b]Total number of harvested structures, which were placed in pools of five
[c]Stages of development collected at each harvesting day: bovine immature (IO) and matured (MO) oocytes, and bovine preimplantation embryos at the 1-cell (1c), 2-cells (2c), 4-cells (4c), 8-cells (8c), 16-cells (16c), morula (Mi), compact morula (CM), blastocyst (Bl) and expanded blastocyst (Bx) stages in development

4.3.2 Promoter-specific expression of the IGF2 gene isoforms in bovine oocytes and preimplantation embryos

A clear distinction in the expression of the bovine *IGF2* isoforms was observed, dependent on the specific *IGF2* promoter (Figure 4). While promoter P4 drove the *IGF2* expression in all structures analyzed, from oocytes to preimplantation embryos, promoter P2 drove the expression at all stages, except in immature oocytes, with promoter P1 driving expression at specific embryo stages that represent important landmarks in development. Interestingly, and under the conditions of this experiment, promoter P3 did not drive the expression of *IGF2* at any given stage of development. Bovine fetal liver and bovine placental tissues were used as positive control, as

previously reported in literature (Boulle *et al.*, 1993; Curchoe *et al.*, 2005). In both tissues, the alternative-splicing variant driven by P3, as for the other promoters, was found and confirmed by DNA sequencing.

The IGF2 relative abundance for transcripts driven by promoter P1, was not detectable in oocytes, prior to fertilization, and after compaction or cavitation, not appearing in blastocyst stage embryos. Expression was readily detectable upon fertilization, and then became undetected up to the EGA, becoming detectable again from the 16-cell stage, reaching an apparent peak at the compact morula stage, although no differences in relative expression were observed between the stages in which expression levels were detectable (Figure 4a).

The IGF2 relative abundance for transcripts driven by promoter P2, was not detectable in immature oocytes, prior to *in vitro* maturation (IVM), becoming readily detectable at the remaining analyzed stages. Interestingly, P2-derived *IGF2* expression started after IVM, reached a peak at the 2-cell stage embryo, then falling down prior and after the EGA, to increase after compaction and cavitation, with the lowest expression pattern seen at the pre-compacting morula stage (Figure 4b).

The IGF2 relative abundance for transcripts driven by promoter P4, was very robust and was readily detectable at all analyzed stages. The P4-derived *IGF2* expression was lower in immature oocytes, reaching a peak at and after maturation, fertilization and first three cell cycles, falling at and after the EGA, reaching a peak again at compaction and during blastocyst expansion. Interestingly, the blastocyst stage presented the lowest *IGF2* mRNA relative abundance, comparable to immature oocytes, prior to IVM (Figure 4c).

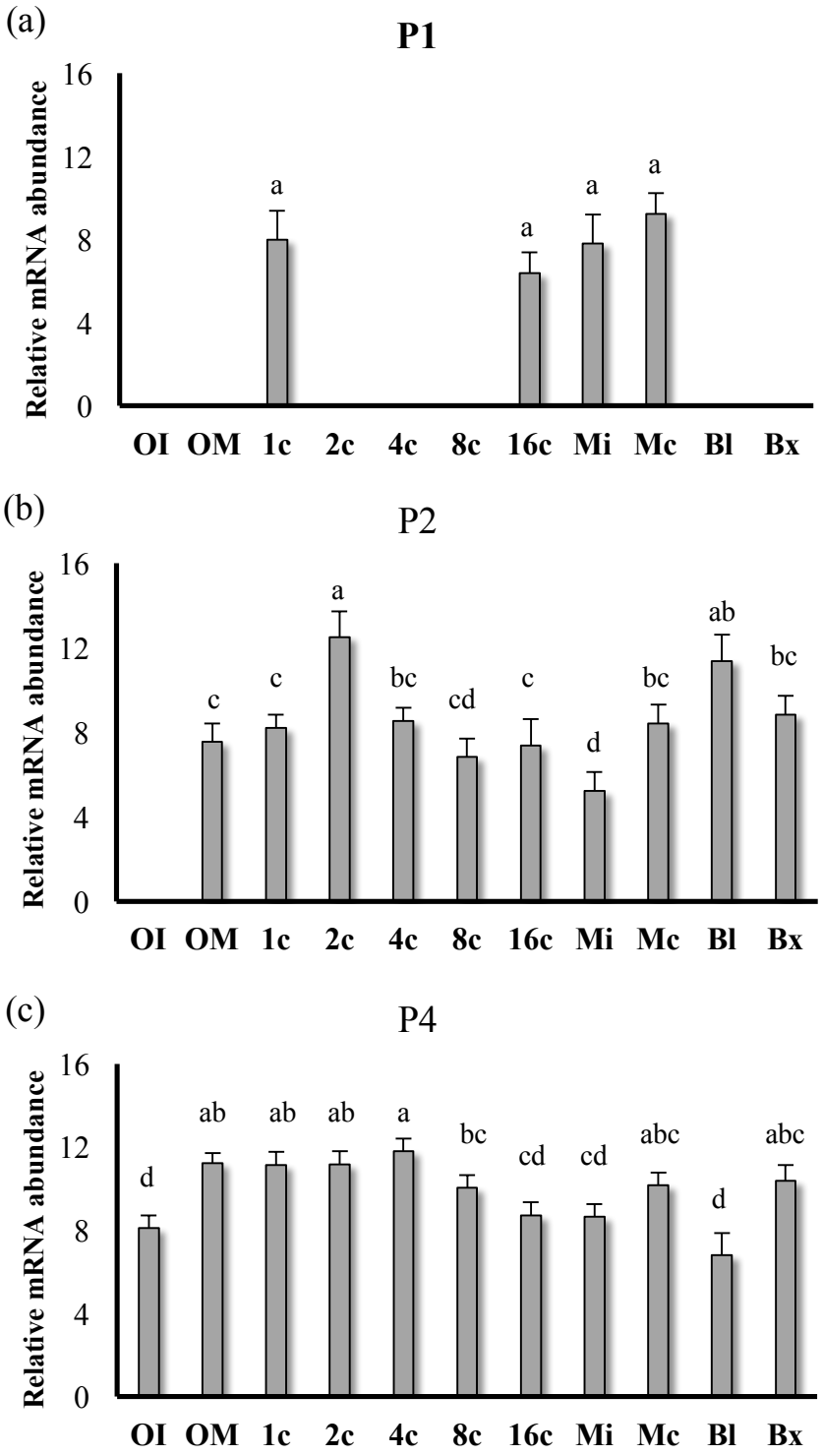

Figure 4. Relative abundance for transcripts (LSM ± SEM) for *IGF2* isoforms driven by promoters P1 (panel a), P2 (panel b), and P4 (panel c) in bovine immature (OI) and matured (OM) oocytes, and preimplantation embryos at the 1-cell (1c), 2-cells (2c), 4-cells (4c), 8-cells (8c), 16-cells (16c), morula (Mi), compact morula (Mc), blastocyst (Bl) and expanded blastocyst (Bx) stages in development. Different letters represent significate statistial difference.

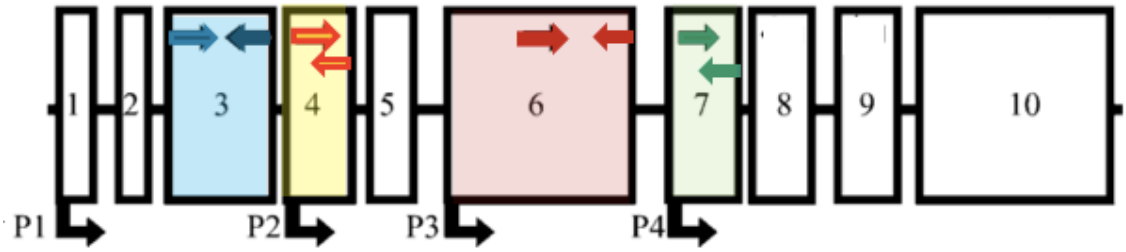

Figure 5. Gene structure and primer places. Rectangles represent exons. Lines represent introns. Broken arrows represent the beginning of mRNA expression of each promoter. Strat arrow indicate primers used.

4.4 Discussion

To accurately describe the molecular behavior of such important gene in oocytes and in preimplantation embryos, structures from several stages of development, from immature oocytes to expanded blastocysts, were collected in pools of five. Three replicates were carried out, for the evaluation of the *IGF2* expression driven by distinct promoters. The relative transcription analyses allowed an optimal perspective to investigate the progression of *IGF2* transcription throughout time. Immature and matured oocytes were included in the study as such structures represent an interesting physiological landmark for the storage of maternal transcripts at the end of folliculogenesis, prior and after maturation. Other important physiological landmarks were also evaluated, including structures subjected to fertilization, structures that develop prior to the global EGA, and therefore, that depend on maternal transcripts, and structures after the global EGA, when the embryonic genome starts driving cell differentiation and embryo growth, including phenomena such as embryo compaction, cavitation and expansion. Pools were evaluated to estimate the abundance of *IGF2* mRNA isoforms driven by four distinct known promoters by real time qPCR with the objective of drawing a molecular profile for transcription of the chosen imprinted gene.

Due to the absence of previous works that analyze the *IGF2* promoters in embryo development, studies with fetal tissues in different animals were used to enrich the paper discussion. Contraire to other already studied animals, in which P1-derived

IGF2 isoform expression seems to be specific to the liver tissue (Ohlsen *et al.*, 1994; Li *et al.*, 1996), P1-derived transcripts in cattle were shown to be widely spread over several ages, such as fetal, calf and adult animals (Curchoe *et al.*, 2005). In this study, bovine embryos seemed to accumulate such specific isoform mostly after the 16-cells stage embryo. Due to its suggestive timing, it is possible to infer that only small quantities of this specific mRNA were stored prior and after oocyte maturation, as a detectable amount could be seen at the 1-cell stage embryo, possibly to used in the early development, upon fertilization, becoming depleted at the 4- and 8-cells stage embryos. This *IGF2* splicing variant is, most likely, more representative from the 16-cells stage embryo onward, been transcribed from the embryonic genome after genome activation. The variable expression pattern during development makes P1 an unfit candidate for use in genetic engineering strategies, as its profile did not seem to be phenotypically important in early development.

Unexpectedly, transcripts driven by P2 were detected for most structures throughout development, except prior to maturation. Commonly described by the current literature as driving low levels of *IGF2* mRNA, in spite of the tissue, stage of development or species, the role of the promoter P2 in mammalian organisms have been questioned (Ohlsen *et al.*, 1994; Li *et al.*, 1996; Von Horn *et al.*, 2002; Curchoe *et al.*, 2005). However, a readily visible response in P2-driven *IGF2* expression in juvenile animals to differences in GH concentration emphasizes a link with a cause-and-effect mechanism to a known growth regulator, suggesting an actual biological and endocrinally-regulated activity (Von Horn *et al.*, 2002). The expression pattern seen in preimplantation embryos suggests an important role of this isoform in embryo development, rather than seen in other tissues. As it was not present in immature oocytes, the P2-driven *IGF2* isoform showed the commonly seen transcript abundance

in mature oocytes, as expected for imprinted genes (Obata & Kono, 2002). Yet, and surprisingly, the isoform reached its peak at the 2-cells stage embryo, which is in line with reports of minor embryo genome activation (Graf *et al.*, 2014a). The subsequent decrease in abundance preceded the global EGA, increasing again at compaction and cavitation, in stages in which the own embryonic genome starts transcribing important genes for growth and differentiation (Graf *et al.*, 2014b). As an important modifier of matured oocyte quality and competence, the *in vitro* culture conditions could possibly interfere with P2-driven *IGF2* abundance, as suggested to occur in IVP-derived embryos that fail to develop after the EGA (Bertolini *et al.*, 2002b; Moore *et al.*, 2007; Suteevun-Phermthai *et al.*, 2009; Graf *et al.*, 2014a), and consequently, alter its profile by degradation during such a phase of highly dependence on the maternal mRNA reserves. The more precise cause of such variation in abundance through early development requires further investigation, and as observed for other tissues analyzed thus far (Li *et al.*, 1996; Van Horn *et al.*, 2002; Curchoe *et al.*, 2005), the mRNA profile of such splicing variant does not encourage its use in genetic engineering in oocytes or in preimplantation bovine embryos.

The P3-driven *IGF2* relative abundance was not detected at any stage analyzed in this study. Being noted as the most abundant transcript isoform during fetal development in many species (Ohsen *et al.*, 1994), with an even bigger distribution in expression in bovine tissues, the P3 is considered the most active promoter among different tissues types, extending its activity to adulthood (Curchoe *et al.*, 2005). In embryos specifically, total *IGF2* mRNA concentrations seem to follow a similar pattern as for most genes expressed during oocyte maturation and embryo development. The greatest increase in IGF abundance was observed as the bovine embryo reached the hatched blastocyst stage (Yassen *et al.*, 2001). This surge in growth pattern could

indicate a switch in promoter profile, with P3 possibly being the one responsible for a major activity later in development. Unfortunately, this study could not confirm this hypothesis, as even the more advanced embryo stages in development did not show P3-driven *IGF2* transcripts. Then, the P3 role to actual biological actions in early development is questionable, as in other tissues; large mRNA amounts were accounted as a consequence of a untranslatable form having extended half-life (De Moor *et al.*, 1994). Therefore, efforts to use this promoter for genetic manipulations are discouraged for in early stages of developmental, being possibly relevant after the hatching stage, specially for the S6 kinase signaling pathway, as a mean to switch non-translatable forms to translatable ones (Nielsen *et al.* 1995).

The P4-driven IGF2 isoform is the major path used in fetal and non-hepatic adult tissues in most species such as mice, human and bovine (Li *et al.* 1996; Von Horn *et al.*, 2002; Curchoe *et al.*, 2005). Consistent to description of previous studies during the fetal phase, findings from our study demonstrated that P4-derived transcripts were widely spread throughout the preimplantation stages of development, including immature and matured oocytes, suggesting an early antral accumulation during folliculogenesis. This P4-*IGF2* expression coupling has already been previously related to fetal growth, while the P1-*IGF2* and P3-*IGF2* reached the highest peak in human liver at two months of age (Li *et al.* 1994). In human adult liver, the P4-*IGF2* expression coupling accounts for the production of 25–60% of total *IGF2* mRNA (Von Horn *et al.*, 2002). In mice, the P3 (correspondent to P4 in other species) was responsible for the highest expression level in the liver (80%), whereas in cattle, expression of full-length P4 transcripts are present in all tissues and appears to decrease with age, while truncated *IGF2* forms increase, until complete mRNA is primarily detected only in a small number of adult tissues (Curchoe *et al.*, 2005). In all species,

the P4-driven expression seems to be an extremely relevant pathway for the production of *IGF2*, especially during fetal development, with our study corroborating such concept. Present at all preimplantation stages in significant abundance, this splicing variant followed the pattern seen for embryo *IGF2* amounts as a whole (Yassen *et al.*, 2001), reaching a peak from oocyte maturation to the onset of global EGA, declining, and then increasing again after global EGA, in special after compaction. For its widespread and robust expression pattern seen in all structures analyzed in this study, this should be the predominant isoform in bovine embryos to be targeted in genetic engineering studies, for instance, to influence *in vitro* embryonic development.

In summary, apart from some distinct features and expression patterns, promoters P1, P2 and P4 drove the *IGF2* expression in oocytes and preimplantation bovine embryos, following a pattern already proposed (Yassen *et al.*, 2001). An initial peak can be seen in early development, between matured oocyte and 2-cells stage embryos, followed by a decrease around the time of the global embryonic genome activation (EGA), likely due its recruitment and use, degradation and/or lack or minimal transcription level. After EGA, a new surge in splicing variants can be detected through embryo compaction, cavitation and expansion. Promoter P3 was an exception, as its activity was not detected in oocytes or embryos, being possibly present only at more advanced stages of development. The P1-driven *IGF2* expression was temporally relevant after fertilization and global EGA, disappearing at cavitation, whereas P2-related *IGF2* expression was significant after oocyte maturation, early cleavages, and after compaction. The P4-driven *IGF2* expression was the most consistent and robust pathway detected at all analyzed stages, from immature oocytes through expanded blastocysts. Such results should aid in future attempts to genetically manipulate embryo

development by strategies that may use the *IGF2* gene, specifically the P4 promoter, for studies in developmental biology and prenatal physiology.

5. CONCLUSIONS

The present study proposed the unraveling of the promoter-specific activity of the *IGF2* gene, which is key for embryo and conceptus development. Based on the results observed in this study, it is possible to conclude that:

(a) It was possible to evaluate the *IGF2* promoters profiles in as few as pools of five embryos at all stages of development, including immature and matured oocytes, and 1-cell, 2-cells, 4-cells, 8-cells, 16-cells, morula, compact morula, blastocyst and expanded blastocyst stage embryos by real time qPCR.

(b) The progression of the *IGF2* transcription pattern and transcript storage throughout development followed a different fashion according to each of the four promoters, based on the *IGF2* transcript isoforms in early development in cattle, in agreement with several other tissues already evaluated, such as liver and placenta.

(c) Promoters P2 and P4 were predominant driving *IGF2* expression in oocytes and preimplantation bovine embryos, following mostly the pattern already proposed in the literature. The early development showed an initial peak, between matured oocyte and 2-cells stage embryos, followed by a decrease around the time of the global embryonic genome activation at the 8-cells stage embryo. After EGA, a new surge in splicing variants could be detected through embryo compaction, cavitation and expansion.

(d) Promoter P1 adopted similar profile as P2 and P4 in driving *IGF2* expression, however, such promoter was much less representative, having significant transcription mostly around Day 4 and 5 of development.

(e) Promoter P3 was an exception, as its activity was not detected in oocytes or preimplantation embryos, being possibly present only at more advanced stages of development.

(f) It was possible to establish a positive relationship between promoter-related *IGF2* expression with embryo physiological landmarks such as maturation, fertilization, embryo genome activation compaction, and cavitation.

(g) The P1-driven *IGF2* expression was temporally relevant after global EGA, but disappeared at cavitation, whereas P2-related *IGF2* expression was more representative from oocyte maturation to early cleavages and after compaction. The P4-derived *IGF2* transcripts were very consistent and robust, detected at all analyzed stages, from immature oocytes through expanded blastocysts.

6. PERSPECTIVES

The new optic shown in *in vitro* embryo biology by this work was an small but assertive step towards a better understanding of the embryo developmental processes. The structures from fertilization to cavitation were elected to allow an overview of the changes over time in the pattern of *IGF2* expression, also providing ampler possibilities for future micromanipulation of embryos at distinct developmental stages during *in vitro* embryo production.

Embryonic mortality is a multifactorial problem, intensified in IVP bovine embryos. The use of techniques that stimulate embryonic development in the pre- and postimplantation stages of gestation can favor the establishment and the physiological maintenance of pregnancy. The fourth ART generation brought forward molecular tools, allowing, for example, an overexpression or inhibition of physiological growth factors, such as *IGF2,* for studies either on positive or negative pleiotropic effects on embryo development. In that view, additional future focus on the IGF2R may also provide novel insights in such early control of development.

Early embryo development presents an significative genomic vulnerability. Therefore, less precise genetic modifications become a liability to the offspring, as certain changes in the embryo genome can have unpredictable impacts in development. The spatial and temporal promoter patterns described in the present study offer opportunities for genetic manipulation, seeking an increase in genetic engineering efficiency, specially in the stages that may lead to a reduction in embryo mortality in early pregnancy, such as expanded blastocysts, as such structures represent the most common stage used for micromanipulation, freezing, and transfer to female recipients.

Future genetic manipulation procedures may focus in increasing the *IGF2* effect on embryo development, with particular attention to the P4 genetic region and its

mRNA to provide conditions for more physiological embryo growth and development in studies in developmental biology and prenatal physiology. Promoters P1 and P2 apparently play a secondary role in preimplantation embryos, whereas promoter P3, if manipulated, could bring changes only latter in the embryonic-fetal life. Those are opportunities and insights that may bring about novel strategies for the better understanding of embryo and conceptus development, especially after the use of IVP procedures.

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