Université de Montréal

STUDY OF IMPRINTED GENES IN BOVINE EMBRYOS PRODUCED BY ASSISTED REPRODUCTIVE TECHNOLOGIES

par

JOÃO SUZUKI JUNIOR

Département de biomédecine vétérinaire

Faculté de médecine vétérinaire

Thèse présentée a la Faculté des études supérieures et postdoctorales

en vue de l'obtention du grade

Philosophiae Doctor (Ph.D.)

en sciences vétérinaires

option reproduction

Août, 2008

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée

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présentée par

JOÃO SUZUKI JUNIOR

a été évaluée par un jury composé des personnes suivantes

Bruce D. Murphy, président-rapporteur

Lawrence C. Smith, directeur de recherche

Alan K. Goff, codirecteur

Réjean Lefebvre, membre du jury

Sarah Kimmins, examinateur externe

Jacques G. Lussier, représentant du doyen de la FÉS

RÉSUMÉ

Les techniques de reproduction assistée utilisées chez certains mammifères, y compris les humains, ont été associées à des grossesses et une progéniture anormales. Bien que les causes demeurent incertaines, l'expression anormale de plusieurs gènes possédant une empreinte génétique et impliqués dans la croissance foetale et le développement placentaire laisse sous-entendre une origine épigénétique. La méthylation des résidus cytosine de l'ADN aux dinucléotides CpG est souvent associée avec la répression transcriptionnelle et impliquée dans le maintien de la stabilité génomique. Elle est également impliquée dans la régulation des gènes possédant une empreinte génétique exclusivement exprimée par un seul allèle parental. En utilisant un modèle inter-espèce bovin contenant un polymorphisme allélique, nous avons analysé l'empreinte génétique associée à l'expression du gène H19 et des gènes «insulin-like growth factor-2 receptor» (IGF2R) et « small nuclear ribonucleoproteinN » (SNRPN) associées à l'expression paternelle dans la pré-implantation des embryons bovins dérivés de l'insémination artificielle (IA), de la fertilisation *in vitro* (FIV) et du transfert nucléaire des cellules somatiques (TNCS). L'expression maternelle de H19 et IGF2R a été exprimée de façon bi-allélique lors de la préimplantation au jour 17, alors que l'expression paternelle de SNRPN était mono-allélique, sauf dans le placenta provenant de la FIV et du TNCS. Au jour 40 de la gestation, l'expression de H19 était mono-allélique lors de l'IA et la FIV. L'expression de SNRPN était monoallélique dans les tissus embryonnaires provenant de la FIV et de l'IA, alors qu'une expression bi-allélique était observée dans le placenta provenant de la FIV. Les taux de méthylation de H19

et IGF2R n'étaient pas affectés par la culture *in vitro* dans le groupe FIV, cependant au jour 40 foetal une perte de méthylation a été observée dans la région différentiellement méthylée de SNRPN dans le groupe FIV, comparativement au témoin *in vivo* (IA). De pertes importantes de méthylation et d'expression bi-allélique ont été observées dans tous les échantillons qui provenaient du TNCS chez les embryons en pré- et post-implantation. Ces résultats démontrent que l'empreinte génétique de ces gènes est établie différemment lors du stade de pré-implantation embryonnaire. Les tissus placentaires sont plus susceptibles aux effets nuisibles de la culture *in vitro* dans l'expression allélique et la méthylation de l'expression paternelle de SNRPN, alors que l'expression maternelle de H19 et IGF2R ne sont pas susceptibles à la culture *in vitro*. Les pertes importantes de méthylation et d'expression bi-allélique des gènes possédant une empreinte génétique lors du TNCS ne sont pas rétablies après la reprogrammation de la cellule donneuse.

Nous concluons que le mécanisme d'empreinte génétique de H19, SNRPN et IGF2R est maintenu suite à l'implantation chez les bovins. Toutefois, certains gènes peuvent démontrer un mécanisme particulier qui survient selon un patron développemental spécifique. La méthylation semble être associée à la régulation de l'empreinte génétique de SNRPN plutôt que H19 et IGF2R. La méthylation de SNRPN est spécifique à certains tissus en plus d'être modifié l'affecter lors de la culture *in vitro*.

Mots clés : ADN, méthylation, empreinte génétique, contrôle épigénétique, embryon, bovin, H19, SNRPN, IGF2R.

ABSTRACT

Assisted reproductive technologies (ART) have been associated with abnormal pregnancies and offspring in a number of mammals, including humans. Although the causes remain unclear, abnormal expression of many imprinted genes involved in fetal growth and placental development suggest an epigenetic origin. DNA methylation of cytosine residues within CpG dinucleotides are often associated with transcriptional repression and implicated in maintaining genomic stability and also implicated in the regulation of imprinted genes, which are exclusively expressed from only one parental allele. Using a bovine interspecies model with an exonic polymorphism, we analyzed the imprinting of the maternally expressed H19 and insulin-like growth factor-2 receptor (IGF2R) genes, and the paternally expressed SNRPN gene, in day 17 pre-implantation bovine embryos and day 40 embryonic (brain,heart,liver,muscle) and extra-embryonic (placenta) tissues derived from artificial insemination (AI), *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT). By sequencing cDNA, we found that maternal expression of H19 and IGF2R genes was normally bi-allelic in pre-implantation day 17 embryos, while the paternal expression of the SNRPN gene was mono-allelic , except in IVF placenta and SCNT where expression was abnormally bi-allelic when compared to AI ($p<0.05$). At day 40, H19 gene expression was mono-allelic in AI and IVF. SNRPN gene expression was mono-allelic in IVF and AI embryonic tissues, while bi-allelic expression was observed in IVF placenta and all tissues from SCNT ($p<0.05$). Using bisulfite mutagenesis reaction, we found that methylation levels of

H19 and IGF2R genes were not affected by in vitro culture in the IVF group, however, loss of methylation with no change in allelic expression was observed in the SNRPN DMR of IVF day 40 fetuses, when compared to *in vivo* controls represented by AI. Severe loss of methylation and bi-allelic expression was observed in all samples originated from SCNT in both pre- and post-implantation animals. These results suggest that for maternally expressed genes, H19 and IGF2R, imprinting is established differently from the paternally expressed gene SNRPN in pre-implantation embryos. Placenta tissues are more susceptible to detrimental effects of *in vitro* culture on allelic expression and methylation of the paternally expressed SNRPN gene, while maternally expressed H19 and IGF2R remained unaffected by *in vitro* culture environment. The severe loss of methylation and bi-allelic expression of all imprinted genes in the SCNT group suggests that imprinting marks are not reestablished after reprogramming of the donor cell.

We conclude from this work that the mechanism of imprinting of H19, SNRPN and IGF2R genes are conserved in cattle after implantation, however some genes might have a particular mechanism that is established in a time specific manner. Methylation seems to be associated to imprinting regulation in some specific genes, such as the paternally expressed SNRPN gene, however, the association is tissue-specific and some genes are more affected (SNRPN) to *in vitro* culture than others (H19 and IGF2R).

Key Words: DNA methylation, Imprinted genes, Epigenetic control, Embryo, Cattle, H19, SNRPN, IGF2R.

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INTRODUCTION

In mammals and plants, the expression of some genes is regulated by genomic imprinting, where the two parental alleles are differentially expressed (Killian, Byrd et al. 2000). The expression of a genetic allele in the next generation is dependent upon whether it resided in a male or female allele in the previous generation. In mammals, the functional significance of genomic imprinting has been a subject of dynamic debates and intense research, and to date, it is attributed to the development and contribution of parental alleles in the formation of extra and embryonic tissues (Reik, Dean et al. 2001; Rideout, Eggan et al. 2001; Surani 2001). Dynamic as well, seem to be the debates about the mechanism involved in imprinting control. Epigenetic control systems confer stability of gene expression during mammalian development, where despite of the same genetic background, different cells and tissues, with individual epigenetic marks, end up having different programs of gene expression (Morgan, Santos et al. 2005). These epigenetic marks are lately well represented by heritable, yet flexible, modifications in DNA molecule without any change in the nucleotide sequence. The addition of methyl groups (CH_3) in cytosine residues is associated with transcript repression. Another important role is represented by histone modifications, which can be modified in by many other factors, and any alterations in the epigenetic control can compromise embryo development.

Assisted reproductive technologies (ART) have been associated with abnormal pregnancies and offspring in a number of mammals, including humans. Although the causes remain unclear, abnormal expression of many imprinted genes involved in fetal growth and placental development suggest an epigenetic origin. Due to the nature of such experiments, in many cases, our knowledge of epigenetic control during embryo development, comes from results obtaining with animal models. Thus, the characterization and comprehension of epigenetic control in other animals will provide clues to study epigenesis in mammals and generally improve assisted reproductive technologies.

CHAPTER I

LITERATURE REVIEW

1. Epigenesis

In mammals and plants, the expression of some genes is regulated by a phenomenon called genomic imprinting. Imprinting differs from classical Mendelian principles of inheritance because despite the equal distribution of parental autosomal genetic content to the progeny, the two alleles are differentially expressed (Killian, Byrd et al. 2000). Thus, the expression of a genetic allele in the next generation is dependent upon whether it resided in a male or female allele in the previous generation. Genomic imprinting was first discovered in plants in 1970, following the analysis of the unusual maternal effect of the R gene responsible for pigmentation of the seed endosperm (an embryonic annex involved in the control of nutrient transfer to the developing embryo in the seed) in maize (Kermicle 1970). In mice, imprinting was discovered following the unexpected outcome of nuclear transplantation experiments. Mouse embryos that had only maternal genomes (parthenogenotes) or only paternal genomes (androgenotes), were grossly abnormal and did not develop beyond mid-gestation (McGrath and Solter 1984; Surani, Barton et al. 1984). These experiments established that a maternal and a paternal genome are both required to achieve normal development.

Particularly in mammals, the functional significance of genomic imprinting has been a subject of dynamic debates and intense research. The reptile lineage gave

rise to mammals diverged from other reptiles 310 million years ago (MYA). Monotremes diverged 210 MYA from the therian mammals (marsupials and eutherians), which diverged from each other 180 MYA (Woodburne, Rich et al. 2003). These mammal groups all have different reproductive strategies. Marsupials (such as kangaroos and opossums) give birth to tiny and underdeveloped (altricial) young that complete development attached to a teat, often protected within a pouch (Hore, Rapkins et al. 2007). The placenta is short-lived and less developed than the complex hormone-producing placenta that supports the extended gestation of eutherians (also referred to as 'placental mammals'). The extraordinary monotremes (such as the platypus) lay eggs as reptiles do and, in the absence of teats, the hatchlings suck milk from the mother's abdomen (Hore, Rapkins et al. 2007). Thus, lactation evolved before the three mammalian clades diverged 210 MYA, viviparity arose 180–210 MYA in therians, and the complex eutherian placenta evolved before the eutherian radiation 105 MYA (Woodburne, Rich et al. 2003). Over the years, scientists have developed some hypothesis concerning the importance of genomic imprinting. Here, we present the two most disputed of these hypotheses.

1.1 The genome conflict theory

In mammals and plants, the embryo remains connected to the mother inside the reproductive tract. Maternal support of embryogenesis is linked to differentiation of distinctive structures specialized for the transport of maternal nutrients to the embryo – the fetal placenta connected to maternal decidua in mammals, and the seed

endosperm connected to the maternal seed coat in plants (Feil and Berger 2007). Based on this information, Haig and Westoby (Haig and Westoby 2006) articulated a hypothesis for the evolution of imprinting : the genome conflict hypothesis. This hypothesis proposes that the interest of the father is to drive more maternal resources to his offspring leading to greater fitness. One prerequisite for the parental conflict hypothesis is a mode of reproduction involving several males per female during her reproductive life, leading to litters containing offspring from different fathers (Feil and Berger 2007). By contrast, the maternal interest is to produce as many viable embryos as possible and, therefore, to share resources equally among embryos. Moreover, the mother has to balance resource allocation to the embryo and her own nutritional needs to ensure her survival. As a result, embryo growth would be enhanced by paternal regulators and inhibited by maternal regulators (Wilkins and Haig 2003; Haig 2004). Several imprinted genes clearly act as enhancers or inhibitors of embryo growth (Morison, Ramsay et al. 2005), or even have an effect on the mother/infant relationship. The best example to support the genome conflict theory is provided by the first two genes discovered to be imprinted (Moore and Haig 1991). The paternally expressed insulin like growth factor 2 (IGF2), whose loss-of-function phenotype is a 40% reduction in growth in mice (De chiara 1990) and the maternally expressed insulin like growth factor 2 receptor (IGF2R), which mutations phenotype are oversized (Filson, Louvi et al. 1993; Lau, Stewart et al. 1994; Wang, Fung et al. 1994).

However, the theory does not fit in four major aspects: (1) Some genes affect embryonic growth but are not imprinted (e.g., Insulin like growth factor 1 (IGF1), which can be explained by considering recessive, deleterious mutations on the coding regions, (2) A gene exists that shows the pattern that is a perfect reversal (Mash2), which is needed for placental growth, and yet has an active maternal allele and an inactive paternal allele (Iwasa 1998). This can be explained if the overproduction of this gene causes dose-sensitive abortion to occur in early gestation. (3) Paternal disomies are sometimes smaller than normal individuals. As documented in Angelman syndrome (AS), marked delayed development is a typical phenotype (Williams, Beaudet et al. 2006; Jedele 2007). This is a likely outcome of evolution if imprinted genes control the allocation between placenta and embryo by modifying the cell developmental fate (Iwasa 1998). 4) Genes on X chromosomes do not follow the predictions of the genetic conflict hypothesis. Two additional forces of natural selection (sex differentiation and dosage compensation) cause genomic imprinting, possibly in the opposite direction (Iwasa 1998). Although, the genetic conflict hypothesis is very successful in explaining the observed patterns of imprinting for autosomal genes and probably is the most likely evolutionary explanation for them to date (Iwasa 1998).

1.2 The ovarian time bomb hypothesis

The unequal expression in mammals, demonstrated by imprinted genes, reduces the benefit of having a diploid genome (Perrot, Richerd et al. 1991; Otto and

Goldstein 1992). As a consequence, genomic imprinting confers a disadvantage on any imprinted individual and yet many mammalian loci appear to have evolved from a nonimprinted state to become imprinted (Bartolomei and Tilghman 1997). To explain this paradox, one of the earliest suggestions postulated that imprinting, by requiring genetic input from both genomes, evolved to prevent parthenogenesis (Solter 1988). This hypothesis received some criticisms because only the inactivation of an allele could prevent parthenogenesis, however by destroying or inactivating some parts of its carriers progeny, such allele would actually decrease its frequency relative to a nonimprinting allele that permitted asexual reproduction (Weisstein, Feldman et al. 2002). Thus, selection at the level of the individual would oppose the evolution of imprinting to prevent parthenogenesis (Weisstein, Feldman et al. 2002). By suggesting that parthenogenesis is the end of the line, evolutionarily speaking, the parthenogenesis-preventing hypothesis requires group-level selection, which would be subverted by individual-level selection (Weisstein, Feldman et al. 2002). As a result, this hypothesis has been considered insufficient to account for observed patterns of imprinting (Weisstein, Feldman et al. 2002).

Another version of the previous theory, the ovarian time bomb hypothesis (OTBH), (Varmuza and Mann 1994) explicitly postulates the ovarian trophoblastic disease as an individual-level cost of parthenogenesis. This disease could arise as a consequence of an unfertilized egg spontaneously developing in the ovaries, and a way to prevent it would be inactivating or down-regulating the maternal copy of a growth-enhancing gene in a mother's offspring (Weisstein, Feldman et al. 2002).

Thus, this form of imprinting in a mother would prevent parthenogenesis and confer on her a selective advantage over those genotypes that are not imprinted and make the paternal genetic contribution essential to embryo development (Weisstein, Feldman et al. 2002).

The OTBH presents some flaws when applied to the male genome. According to the OTBH, the imprinting would benefit only females from having such disease, however both sexes profit from imprinted gene characteristics. The OTBH assumes a risk of ovarian cancer and thus cannot easily explain the presence of imprinting in other species like *Drosophila* (Lloyd, Sinclair et al. 1999). The OTBH also does not predict the stably coexistence of imprintable and non-imprintable genes within the same population. Thus, the apparent polymorphic imprinting status of the IGF2R gene (Xu, Goodyer et al. 1993) in humans cannot be explained.

On the other hand, the genome conflict theory can be applied to species other than mammals because it is not restricted to females. The particular case of the human polymorphic IGF2R imprinting would be better explained by the genome conflict theory, because in humans, the majority of pregnancies are singletons, thus the competition between siblings for maternal resources is not a matter of survival. However, from the genome conflict theory's point of view is difficult to predict stable coexistence of imprinting and biallelic expression in a population (Weisstein and Spencer 2003).

Despite all the hypotheses involved, the origin of imprinting remains

unknown in placental mammals. Because imprinting affects both embryo and placenta, it is difficult to determine the origin of imprinting being designated by one or the other. Possibly imprinting evolved independently in the embryo and in the placenta (Feil and Berger 2007).

2. Epigenetic control

Epigenetic control systems confer stability of gene expression during mammalian development, where different cells and tissues end up having different programs of gene expression. Thus, each cell type in the individual has its own epigenetic marks, which reflects the genotype, developmental history and environmental influences, and is ultimately reflected in the phenotype of the cell and organism (Morgan, Santos et al. 2005). These marks are substantially represented by heritable modifications, performed by epigenetic modifications, and occur independently of changes in the primary DNA sequence. Furthermore, these modifications must be reversibly applied to the cell according to its developmental stage. It is know that DNA methylation, histone tail modifications and non-histone proteins that bind to chromatin fit perfectly the conditions needed as epigenetic markers (Bird 2002).

For most cell types in the individual, in a normal situation, these epigenetic markers become established once the cells differentiate or exit the cell cycle. In mammals, genome wide epigenetic reprogramming involving DNA methylation and histone modifications generally occurs at stages when developmental potency of

cells changes (Morgan, Santos et al. 2005). These changes, accompanied by methylation and histones modifications occur mostly at two times: during gametogenesis, when reprogramming takes place in primordial germ cells (PGCs) in which parental imprints are erased and totipotency is restored (Reik, Dean et al. 2001; Rideout, Eggan et al. 2001; Surani 2001) and after fertilization, when gametic marks are erased and replaced with embryonic marks important for early embryonic development of both embryonic and trophoblastic cells (Reik, Dean et al. 2001; Rideout, Eggan et al. 2001; Surani 2001).

Epigenetic reprogramming is also important in some disease situations such as cancer (Feinberg and Tycko 2004); and occurs in the donor cell following somatic cell nuclear transfer (SCNT) (Dean, Santos et al. 2003; Hochedlinger, Rideout et al. 2004). In this section we review some of the most studied epigenetic modifications and their relevance to epigenetic control during mammalian embryo development.

2.1 DNA methylation

It has been known that DNA from various sources contains the methylated bases N6-methyladenine, 5-methylcytosine, and N4-methylcytosine in addition to the four standard nucleobases (Jeltsch 2002). In prokaryotes, all the three types of methylation are observed and its biological roles involve: 1) distinction of self and non self DNA, 2) direction of post-replicative mismatch repair and 3) control of DNA replication (Jeltsch 2002). The first two issues are associated with

restriction/modification systems (RM systems) (Wilson and Murray 1991) functioning as defense against infection by other bacteria. Thus different bacteria have different methyltransferases and different restriction enzymes to protect self DNA and attack non self DNA. The third issue is best understood in the *Escherichia coli* DNA adenine methyltransferase (DAM) system, where DNA is modified at adenine residues in GATC sequences (Messer and Noyer-Weidner 1988; Barras and Marinus 1989). During the short time span between DNA replication and DAM methylation activity, a directed repair of replication errors is possible, because the methylation mark allows the unmethylated daughter strand, which must be repaired, and the methylated original template strand, whose nucleotide sequence is correct, to be distinguished (Barras and Marinus 1989).

In higher eukaryotes, DNA methylation is the only known covalent DNA modification. So far, in mammals only cytosine- C5 methylation has been found in DNA, and it occurs mainly at palindromic CG sequences (Reisenauer, Kahng et al. 1999). About 60 to 90% of CG residues are modified in mammals (corresponding to 3 -8 % of all cytosines residues) (Jeltsch 2002). Since methylation takes place in both DNA strands at palindromic sites, DNA replication transforms the pattern of fully methylated sites into a pattern comprising unmodified and hemimethylated CG sites (Jeltsch 2002). Therefore, after DNA replication the information encoded in the pattern of DNA methylation is still available and the initial pattern of methylation can be reestablished by a maintenance methyltransferase (MTase), referred to as methyltransferase 1 (DNMT1), that specifically modifies hemimethylated but not

unmethylated target sites (Leonhardt, Page et al. 1992). Thus, the DNA methylation pattern is stable through cell divisions and is somatically inherited. Nonetheless, it can be edited, either by *de novo* methylation or by demethylation, which makes DNA methylation a unique way to encode information in a stable but reversible manner (Jeltsch 2002). Given these properties, DNA methylation is ideally suited to control processes like cellular differentiation or development.

2.2 DNA Methyltransferases

As mentioned previously, the only modification of mammalian genomic DNA is methyation at the 5-position of the cytosine residue within the cytosine-guanine dinucleotides (CpG), resulting in the formation of 5-methylcytosine (m^5C). DNA methyltransferases (DNMTs) use S-adenosyl-L-methionine (AdoMet) (Figure 1) as a methyl donor group (Pradhan, Bacolla et al. 1999) and a conservative AdoMet binding site has been observed in all DNMTs from prokaryotes to eukaryotes (Jeltsch 2002). Up to date DNMTs of known functions can be divided in 2 groups: *de novo* methyltransferases that can methylate post-replication unmethylated DNA (DNMT3A, DNMT3B and DNMT3L), and maintenance methyltransferases (DNMT1) that can attach preferentially to hemimethylated DNA during replication (Bestor and Verdine 1994; Bestor 2000; Margot, Ehrenhofer-Murray et al. 2003). DNMT2, another methylatransferase, is the smallest found in the mammalian genome. It is composed solely of the C-terminal domain, and does not possess the regulatory N-terminal region. The DNMT2 catalytic domain does not

exhibit particular *de novo* or maintenance methyltransferase activity in embryonic stem cells (ES) or adult somatic tissue (Siedlecki and Zielenkiewicz 2006). The structure of DNMT2 suggests that this enzyme can be involved in the recognition of DNA damage, DNA recombination and mutation repair (Okano, Xie et al. 1998; Pradhan and Esteve 2003).

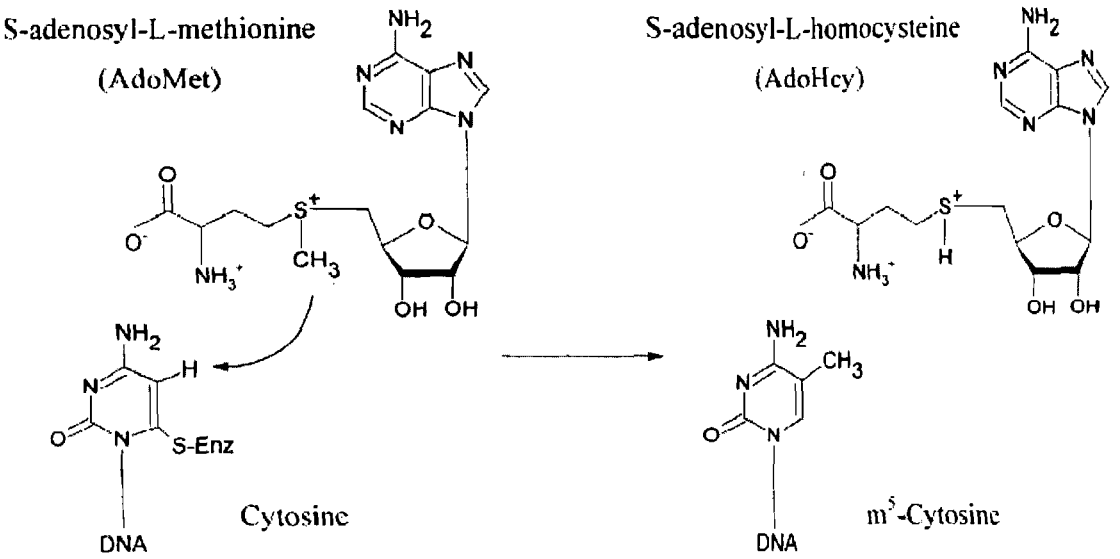

Figure 1. The methylation mechanism of cytosine (C) to 5 methylcytosine (m5C). Methylation of C within CpG dinucleotides is conducted by methyltransferases that use AdoMet as a donor of a methyl group. (Turek-Plewa and Jagodzinski 2005)

2.3 *De novo* Methylation

During proliferation and migration of primordial germ cells (PGCs) genomic methylation is widely erased and is reestablished in a sex-specific manner during

spermatogenesis and oogenesis (Trasler 2006) (Figure 2). The epigenetic patterns are reacquired by a *de novo* methylation wave that is directed to transposons and their remnants, and to clustered repeats (primarily pericentric satellite DNA). Lesser amounts are directed to single-copy sequences and the differentially methylated regions (DMRs) of imprinted loci (Goll and Bestor 2005). The DNMT3A/B and DNMT1 families of DNA cytosine-5 methyltransferases are responsible for the establishment and maintenance of methylation patterns, respectively, and are expressed in most dividing cell types (Goll and Bestor 2005). DNMT3L (DNMT3-like) is related in sequence to DNMT3A and DNMT3B but lacks enzymatic activity. It is expressed only in germ cells and only at the stages where *de novo* methylation occurs (Goll and Bestor 2005), and acts as a regulator of DNMT3A and DNMT3B (Suetake, Shinozaki et al. 2004). *De novo* methylation by DNMT3L occurs in populations of non-dividing cells in both female and male germ lines (Schaefer, Ooi et al. 2007), however the timing of events are different according to the sex. In the male, methylation acquisition is a premeiotic phenomenon, where it occurs in prenatal prospermatogonia, whereas in the female it occurs in growing oocytes that are arrested at the diplotene stage of meiosis I (Goll and Bestor 2005; Trasler 2006). Loss of DNMT3L results in very different phenotypes depending upon the sex examined. Deletion of DNMT3L in female mice prevents establishment of maternal methylation imprints in oocytes without marked effects on retrotransposon methylation (Goll and Bestor 2005). The result is a maternal-effect lethal phenotype in which the heterozygous offspring of homozygous DNMT3L null females (which

are of normal phenotype) show biallelic expression of genes that are normally maternally methylated and repressed. This leads to abnormal development of extraembryonic structures and death of the embryo before mid-gestation (Schaefer, Ooi et al. 2007). Male germ cells that lack DNMT3L show fulminating expression of retrotransposons of the LINE-1 (long interspersed elements) and IAP (intracisternal A particles) classes, severe asynapsis and nonhomologous synapsis at meiotic prophase, and eventual apoptosis of all germ cells before pachytene (Bourc'his and Bestor 2004). Methylation patterns at the small number of paternally methylated DMRs are almost normal, but there is a failure to methylate retrotransposons (Schaefer, Ooi et al. 2007).

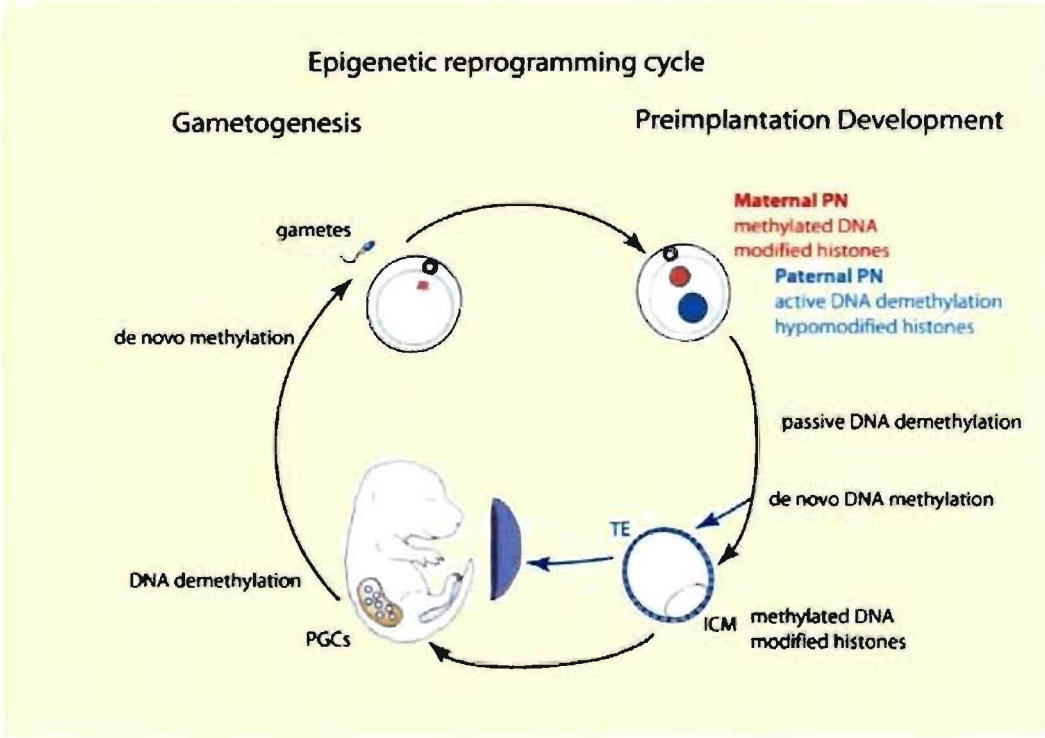

Figure 2. Epigenetic reprogramming cycle. Epigenetic modifications undergo

reprogramming during the life cycle in two phases: during gametogenesis and preimplantation development. PGCs arise from somatic tissue and develop into mature gametes over an extended period of time. Their genome undergoes DNA demethylation in the embryo between E11.5 and E12.5, including at imprinted genes. Following demethylation, the genomes of the gametes are de novo methylated and acquire imprints; this process continues up to E18.5 in males and in maturing oocytes before ovulation in females. Fertilization signals the second round of reprogramming during preimplantation development. The paternal genome is actively demethylated and its histones initially lack some modifications present in the maternal pronucleus (PN). The embryo's genome is passively DNA demethylated during early cell cycles before blastulation. Despite this methylation loss, imprinted genes maintain their methylation through this preimplantation reprogramming. De novo methylation roughly coincides with the differentiation of the first two lineages of the blastocyst stage, and the inner cell mass (ICM) is hypermethylated in comparison to the trophectoderm (TE). These early lineages set-up the DNA methylation status of their somatic and placental derivatives. Histone modifications may also reflect this DNA methylation asymmetry. Particular classes of sequences may not conform to the general genomic pattern of reprogramming shown. (Santos and Dean 2004).

Although the time window of DNMT3L expression in the null phenotypes are different for male and female germ lines, the enzyme appears to be essentially

identical in both lines. This implies that DNMT3L potentially recognizes a preexisting mark that is established by other factors and located at different genomic regions in male premeiotic and female postmeiotic germ cells; the nature of the mark may be a particular posttranslational histone modification or set of modifications (Schaefer, Ooi et al. 2007). This could explain how histone modifications, which have been denominated as transient passive inheritance, might be converted into heritable patterns of DNA methylation that can impose long-term transcriptional silencing on the affected sequences.

De *novo* methylation also occurs during embryo development. When the sperm fuses with the oocyte, its protamines, which help to organize the sperm DNA into a highly compact structure, are rapidly replaced by histones originating from the oocyte cytoplasm (McLay and Clarke 2003; Yanagimachi 2003). At this stage, intensive demethylation of the paternal but not maternal DNA can be observed in mouse, rat and pig (Mayer, Niveleau et al. 2000; Beaujean, Hartshorne et al. 2004; Chen, Zhang et al. 2004; Shi, Dirim et al. 2004; Fulka, Fulka et al. 2006; Zaitseva, Zaitsev et al. 2007). However, some species such as bovine and humans exhibit only partial paternal genome demethylation (Beaujean, Hartshorne et al. 2004; Fulka, Fulka et al. 2006). No demethylation at all has been observed in rabbit and sheep (Beaujean, Hartshorne et al. 2004; Chen, Zhang et al. 2004; Shi, Dirim et al. 2004). Because this phase of demethylation precedes DNA replication of the parental genomes, it is termed ''active demethylation'' and it is supposed that these processes are caused by the action of as yet unidentified demethylase(s) (Fulka, St John et al.

2008). As the embryo udergoes further cell divisions, differences in DNA methylation between the extraembryonic and embryonic lineages are observed (Figure 2). These differences arise as early as the blastocyst stage, when the combinations of active and passive demethylation result in a low basal level of methylation in the TE (Santos, Hendrich et al. 2002; Santos, Zakhartchenko et al. 2003; Santos and Dean 2004). The ICM, in contrast, shows clear signs of extensive de novo methylation, which may begin as early as the late morula stage (Santos, Hendrich et al. 2002); it is likely that this is caused by DNMT3B as this *de novo* methylase is detectable in blastocysts in the ICM but not in the TE (Watanabe, Suetake et al. 2002). DNMT3B levels is profoundly increase in various tumor cell lines, indicating that it plays an important role in tumorigenesis (Robertson, Uzvolgyi et al. 1999).

2.4 Maintenance Methylation

DNMT1 is the major enzyme responsible for DNA methylation patterns during cell replication (Hermann, Goyal et al. 2004). During the replication of eukaryotic genomic DNA, approximately 40 million methylated CpG dinucleotides are converted into the hemimethylated state in the newly synthesized DNA strand (Turek-Plewa and Jagodzinski 2005). These hemimethylated CpG sites must be accurately methylated to maintain the original DNA methylation pattern. DNMT1 is located at the replication fork and methylates newly biosynthesized DNA strands directly after the replication round (Hermann, Goyal et al. 2004). DNMT1 displays a

5- to 40-fold higher activity *in vitro* for hemimethylated DNA than for unmethylated DNA (Bestor 2000; Hermann, Goyal et al. 2004). However, this enzyme also exhibits very weak *de novo* methylation activity which is stimulated by DNMT3A (Fatemi, Hermann et al. 2002). Two other isoforms of DNMT1 have been found and although the mechanism underlying their expression is still not known, scientist have observed that the first exon posseses sex-specific promoters. The mRNA of DNMT1 is present at high levels in postmitotic female and male germ cells. However, the biosynthesis and localization of its isoforms during various stages of gametogenesis is controlled by unique sequences of the first gene exon, which are formed during alternative splicing (Turek-Plewa and Jagodzinski 2005). Although the mechanism is not elucidated, the first exon of the DNMT1 sequence is differently spliced according to the transcript isoforms which were found in growing oocytes (DNMT1o), pachytene spermatocyte (DNMT1p) and somatic cells (DNMT1s) (Ko, Nishino et al. 2005). The DNMT1o protein is stored in the cytoplasm of the mature metaphase II oocyte and in the cytoplasm of the pre-implantation cleavage stages of embryos. After implantation of the eight-cell-stage embryo DNMT1o is translocated to the nucleus (Ratnam, Mertineit et al. 2002; Huntriss, Hinkins et al. 2004). This may suggest that DNMT1o is not required for the acquisition of the maternal imprint but seems to be essential for maintaining imprints of the eight-cell blastomere (Lucifero, Mertineit et al. 2002). By contrast, the DNMT1p transcript first exon sequence is designated as 1p and cannot be associated with polyribosomes. The spermatocyte-specific 1p exon sequence interferes with the translation machinery

and prevents DNMT1 biosynthesis (Trasler, Alcivar et al. 1992). Thus, DNMT1 activity is absent in spermatocytes during spermiogenesis.

The expression of DNMT1 is precisely regulated during female and male gametogenesis and the main differences are found in the pre- and perinatal expression of this enzyme. Transcripts of DNMT1o and DNMT1p are at a higher level in the testis and ovary than in other tissues. Neither transcript is found in female and male mature germ cells. The transcriptional mechanism responsible for the suppression of DNMT1o and DNMT1p transcription in somatic cells and in other phases of gametogenesis is still unknown and requires further investigation (Turek-Plewa and Jagodzinski 2005).

The DNMT1p transcript is abundantly present in the pachytene spermatocytes, whereas the same cells lack DNMT1 protein. DNMT1 protein is present at significant levels in mature oocytes and pre-implantation embryos; however, the mRNA content of this enzyme is very low in the same cells (Bestor 2000). The significance of correlation loss between the DNMT1 transcript and the protein levels in germ and early embryonic cells is still not known, however this may suggest that other unidentified DNMTs may be responsible for the imprinting status in zygote and early embryos (Mertineit, Yoder et al. 1998; Turek-Plewa and Jagodzinski 2005).

2.5 Histone modifications

The human genome comprises approximately 2 metres of DNA, which fits

into a cell that is only 10 μmetres in diameter approximately. Such incredible degree of compaction is performed by histones, which wrap up DNA molecule to form chromatin. The basic unit of chromatin is the nucleosome, which contains 146 base pairs of DNA wrapped around the histone core, an octamer consisting of two copies each of H2A, H2B, H3 and H4 (Ooi and Henikoff 2007). Chromatin structure is also important for biological processes, such as transcriptional silencing and mitosis. For example, centromeres are defined as where centromeric H3 (CenH3), a histone H3 variant, assembles (Amor, Bentley et al. 2004; Dawe and Henikoff 2006; Heun, Erhardt et al. 2006). Another histone H3 variant, H3.3, marks active chromatin and is enriched in histone modifications associated with transcriptional activity (Waterborg 1990; Ahmad and Henikoff 2002; McKittrick, Gafken et al. 2004; Mito, Henikoff et al. 2005; Hake, Garcia et al. 2006). Since transcription is different depending on cell type, histones could be candidates for transmission of epigenetic information phenotypes that can be stably inherited via cell division without changes in DNA sequence.

All histones have a tripartite structure, with a central globular domain surrounded by more extended tail domains which are very basic, rich in lysines and arginines (Godde and Ura 2008). These tails are believed to be inherently flexible and largely unstructured in solution (Godde and Ura 2008). The N-terminal tail, especially, has many sites where covalent chemical modifications take place in the cell, namely acetylation, methylation, as well as phosphorylation (Strahl and Allis 2000).The core histones that constitute the nucleosome are subject to more than 100

different posttranslational modifications, including acetylation, methylation, phosphorylation, and ubiquitination (Bernstein, Meissner et al. 2007). These modifications occur primarily at specific positions within the amino-terminal histone tails. Although the majority of these modifications remain poorly understood, the understanding of lysine acetylation and methylation have advanced considerably. While lysine acetylation almost always correlates with chromatin accessibility and transcriptional activity, lysine methylation can have different effects depending on which residue is modified. Methylation of histone H3 lysine 4 (H3K4) and H3 lysine 36 is associated with transcribed chromatin (Bernstein, Meissner et al. 2007). In contrast, methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27), and H4 lysine 20 (H4K20) generally correlate with repression (Bernstein, Meissner et al. 2007) (Figure 3).

Probably the most important event on chromatin remodeling occurs upon fertilization. In mice, cytological observations have revealed that most protamines are removed from sperm chromatin within 30 minutes after fertilization, and active demethylation of the paternal genome begins at about this time (Oswald, Engemann et al. 2000). The time between fertilization and the completion of chromatin remodeling events presents a narrow, but crucial, window of opportunity for the zygote to mark or retain the identity of the parental genome and its associated epigenetic information (Ooi and Henikoff 2007). Understanding chromatin dynamics during this time is crucial for understanding how epigenetic information can be transmitted via the germline and has important implications for epigenetic events

such as genomic imprinting. One example of chromatin action on epigenesis is the placental genomic imprinting and imprinted X inactivation (Ooi and Henikoff 2007), which is the specific inactivation of the paternal X chromosome. Imprinted X inactivation occurs in preimplantation embryos, and is later maintained only in the placenta (Lewis, Mitsuya et al. 2004; Reik and Lewis 2005). In contrast, the choice of which X chromosome to inactivate in the post-implantation embryo is random. Both placental genomic imprinting and imprinted X inactivation utilize non-coding RNAs in cis, such as XIST RNA for imprinted X inactivation and Kcnqot1 RNA for chromosome 7 IC2 locus imprinting. Interestingly, neither process requires the DNA maintenance methyltransferase DNMT1 (Lewis, Mitsuya et al. 2004). Instead, as with imprinted X inactivation, these placental imprinted genes are marked by histone modifications associated with transcriptional repression H3K9me and histone H3 methylated lysine 27 (H3K27me) as well as the Polycomb H3K27 methyltransferase complex, which is involved in the maintenance of transcriptional repression (Wang, Mager et al. 2001; Lewis, Mitsuya et al. 2004). These results suggest that chromatin conformation and histone modifications play important roles during the acquisition and maintenance of epigenetic inheritance. In fact, it has been suggested that histone modification is the more ancient imprinting system, whereas DNA methylation, a more stable mark, would have evolved later to maintain imprinting (Reik and Lewis 2005; Wagschal and Feil 2006).

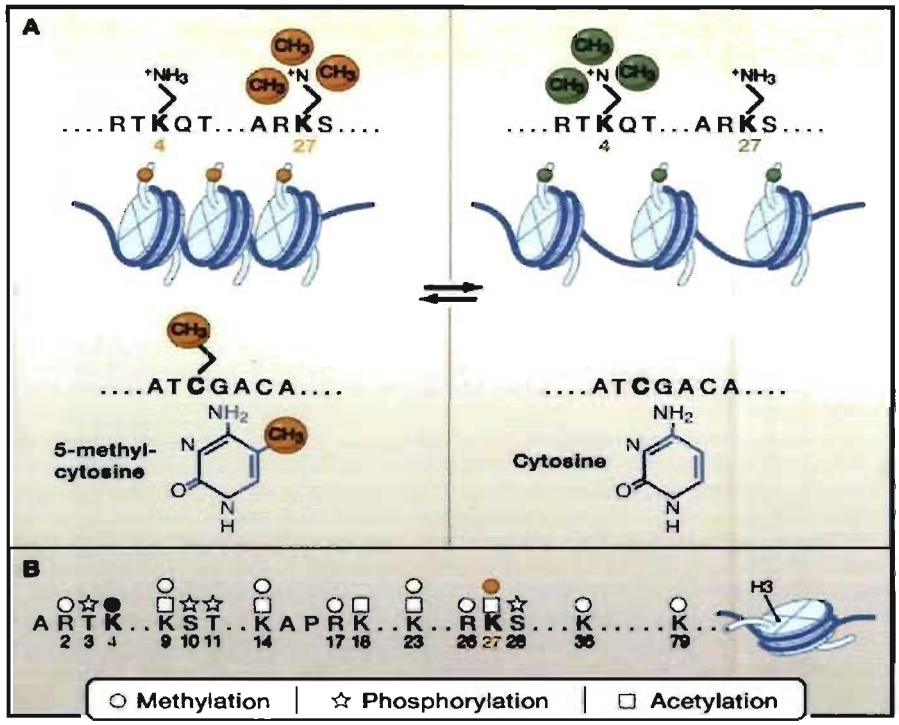

Figure 3. Cytosine methylation is the only known covalent modification of DNA in mammals. In contrast, histones are subject to hundreds of modifications, including acetylation, methylation, phosphorylation, and ubiquitination. (A) illustrates the structures and effects of cytosine methylation (repressive/red) and two histone marks: H3K27 methylation (repressive/red) and H3K4 methylation (activating/green). (B) illustrates the diversity of histone H3 modifications (Bernstein, Meissner et al. 2007).

3. Imprinting during embryo development

The formation of extraembryonic (trophoblast) tissues, and in particular the placenta, is a priority to ensure growth and survival of the embryo during intrauterine

development in mammals. Cells belonging to the extraembryonic lineage are required to confer attachment of the blastocyst to the uterus. Subsequently, specialized trophoblast cell types gain access to the maternal blood supply and establish an intricate feto-maternal circulatory interface within the placenta that ensures adequate nutrition of the growing embryo (Hemberger 2007). At the same time, the placenta is also essential to dispose of waste products from the embryo into the maternal circulation (Cross, Werb et al. 1994; Benirschke 1998).

Extraembryonic development begins at the blastocyst stage with formation of the outer trophectoderm layer (Hemberger 2007). With this differentiation step, the trophoblast cell lineage is set aside. Pluri- or multipotency is maintained in trophoblast stem cells contained within this trophoblast population, but the potential of these cells to differentiate is restricted to the various trophoblast subtypes (Rossant 2007). Secondly, trophoblast giant cells invade the uterine basement membrane and penetrate into the uterine stroma where they erode maternal arteries and adopt pseudoendothelial cell functions (Hemberger 2007). This step is crucial for the conceptus to become connected to the maternal vascular system. Thirdly, chorioallantoic fusion and establishment of a functional labyrinth also represent a key step in placental development, a process for which the interplay of a multitude of factors is required that mediate induction events and a continuous cross-talk between the allantoic mesoderm and the chorionic ectoderm (Rossant and Cross 2001; Watson and Cross 2005).

Evidences suggest that several basic aspects of epigenetic control are

required for extraembryonic differentiation. On the global level, the trophectoderm/trophoblast lineage seems to be characterized by lower epigenetic modification levels than the embryonic lineage. This appears to be the case in particular for silencing modifications and has been shown for overall DNA methylation levels (Chapman, Forrester et al. 1984; Rossant, Sanford et al. 1986; Santos, Hendrich et al. 2002) and for the repressive histone marks H3K27me1, H3K27me2, H3K27me3 at the blastocyst stage (Erhardt, Su et al. 2003). Despite the differences found in time of de novo methylation between mice and other species, there are also lower methylation levels in trophectoderm when compared to the inner cell mass in the bovine (Santos, Zakhartchenko et al. 2003).

In both ICM and trophoblast cells, the expression of imprinted genes are essential for embryo and extra-embryonic tissues. Imprinted genes can act as enhancers or inhibitors of embryo growth or even have an effect on the mother/infant relationship (Morison, Ramsay et al. 2005). The transcriptional activity of each allele is determined by the gender of the parental germ line to which it was most recently exposed. Individual germ-line marks can control the allele-specific silencing or activation of multiple neighboring genes, which leads in many general cases to clusters of imprinted transcripts. Once localized, these clusters represent an attractive opportunity for studying of epigenetic transcriptional regulation, since both the active and silent allele are present in the same cell nucleus, and thus potentially exposed to the same regulatory factors (Wood and Oakey 2006). To date, estimates put the total number of imprinted loci between 100 and 200 (Hayashizaki, Shibata et

al. 1994; Maeda and Hayashizaki 2006). A more recent study based on sequence features in the region of known imprinted promoters identified 600 genes that are potentially imprinted (Luedi, Hartemink et al. 2005). The importance of imprinted genes are represented by the phenotypes ranging from early embryonic lethality to postnatal effects on growth and development, likely resulting from the misexpression of imprinted genes situated within the uniparentally duplicated region (Cattanach and Kirk 1985). In the next topic we discuss the maternally expressed genes H19 and IGF2R and the paternally expressed gene SNRPN.

3.1 The maternally expressed H19 gene

H19 is paternally silenced and the maternal allele is active in a wide array of mesenchymal and epithelial tissues, both in mice and humans (Bartolomei, Zemel et al. 1991; Zhang and Tycko 1992). H19 encodes an abundant spliced and polyadenylated RNA that accumulates in the cytoplasm (Brannan, Dees et al. 1990) and has a conserved exon–intron structure among mammals, but lacks conserved open reading frames. In bovine, the H19 gene, located on chromosome 29, is organized into five exons and four unusually small introns, similar in size to introns in the sheep and pig H19 gene (Zhang, Kubota et al. 2004). It was shown that the bovine gene is 91.8% and 71.2% identical to the sheep and pig genes, respectively (Zhang, Kubota et al. 2004). The exact function of H19 gene has not been elucidated so far. Probably the strongest reason to study the expression of H19 gene is the fact that the IGF2 expression is linked to H19 and net expression of H19 generally

parallels that of IGF2 (Pachnis, Brannan et al. 1988; Ohlsson, Hedborg et al. 1994; Leighton, Ingram et al. 1995; Drewell, Brenton et al. 2000). The H19 and IGF2 genes coordinate expression, with H19 being expressed from the maternally inherited allele whereas IGF2 is paternally transcribed are abundantly expressed during embryonic development in identical tissues (Figure 4a) and is downregulated shortly after birth, except in skeletal muscle. Somatic overgrowth was observed in knockout mice lacking the H19 gene and its immediate upstream sequences (Leighton, Ingram et al. 1995). IGF2 showed biallelic expression in these mice and crossing of H19 females with IGF2 males eliminated the overgrowth of the conceptuses (Leighton, Saam et al. 1995). This implied that the biological function of the H19 locus may be restricted to controlling IGF2 in cis. The cis regulation also uses epigenetic modifications and are well exemplified by so-called insulators, which are DNA elements identified on the basis of their ability to protect a gene from outside influences, avoiding inappropriate activation or silencing of the gene. Insulators have been divided into two classes: enhancer-blocking (EB) insulators, which prevent distal enhancers from activating a promoter when placed between an enhancer and promoter ; and barrier insulators, which block heterochromatinization and consequent silencing of a gene (Wallace and Felsenfeld 2007).

The theory of cis regulators was reinforced with the discovery of an EB boundary/insulator factor known as the CTCF binding site (Chung, Whiteley et al. 1993), located upstream of the H19 gene (between the IGF2 promoter gene and its enhancer) in a region denominated differentially methylated domain (DMD) (Arney

2003) which carries a 'germ line imprint', as the differential methylation is set up in the parental germ cells and then maintained throughout development. These findings allowed the postulation of a model normally referred to as the boundary model (Figure 4b), where the DMD is unmethylated on the maternal allele and acts as a boundary/insulator element. The insulator function is mediated by the zinc finger protein CTCF (Bell and Felsenfeld 2000) which binds to the unmethylated site simultaneously blocking the access to IGF2 from the downstream enhancers, meaning they are targeted to the H19 promoter, resulting in expression of H19 from the maternal chromosome. DMD is methylated on the paternal allele, thus CTCF binding is avoided and no boundary is formed. This leaves the downstream enhancers free to interact with IGF2, resulting in IGF2 expression from the paternal chromosome. It was also proposed that DMD would act as a tissue specific silencer in endodermal tissues, repressing paternal expression of H19 (Drewell, Brenton et al. 2000).

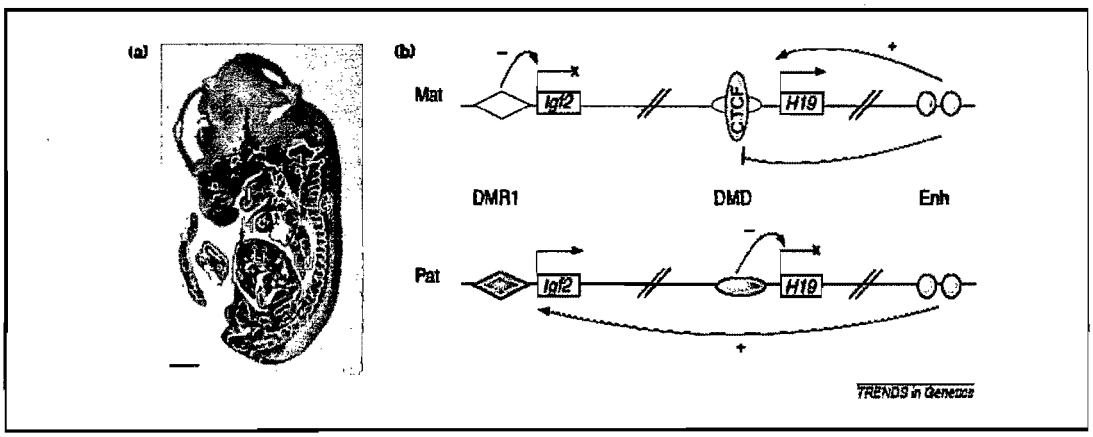

Figure 4. Embryonic expression of H19, and the boundary model of the H19/IGF2

imprinted gene regulation. (a) Expression of H19 at embryonic day 14. Expression of H19 can be seen in a wide range of embryonic tissues, including liver (Li), lung (Lu), tongue (T), heart (H) and spinal cartilage (C) as detected by in situ hybridization of a sagittal section. Scale bar 1/4 1 mm. Courtesy of R. Drewell. (b) Mechanism of imprinting at H19/IGF2. On the maternal allele (Mat), the H19 differentially methylated domain (DMD) is unmethylated (empty red ellipse) allowing the transcription regulatory protein CTCF to bind and form a boundary/insulator. This prevents downstream enhancers (Enh, green circles) from interacting with IGF2, instead directing them to act on H19 and activating maternal transcription (+). In addition, the unmethylated differentially methylated region 1 (DMR1, empty blue diamond) upstream of the IGF2 promoter acts as a silencer to repress maternal transcription (–). On the paternal allele (Pat), the H19 DMD is methylated (filled red ellipse). This prevents the binding of CTCF, abrogating boundary function. The methylated DMD also acts as a silencer to repress transcription from the paternal H19 allele. In the absence of the boundary the downstream enhancers are free to interact with IGF2 (+). Not to scale. (Arney 2003)

However, another result from mice carrying a deletion of the DMR1 (Figure 4b), located upstream of IGF2 (Constancia, Dean et al. 2000) resulted in reactivation of the normally silent IGF2 allele in mesodermal tissues, including heart, kidney and lung (but not skeletal muscle) when maternally inherited, suggesting the region acts as a tissue-specific silencer. However, the boundary element at the H19 DMD is presumably intact, highlighted by a lack of epigenetic changes to the DMD or effects

on H19 transcription. This result led to the conclusion that the mechanism involved was more complex than imagined. When two DMRs, one upstream of the promoter gene 1 (DMR1) and another located in exon 6 (DMR2) of the IGF2 gene, were found to play roles as a methylation-sensitive silencer (Constancia, Dean et al. 2000; Eden, Constancia et al. 2001) and a methylation-dependent activator (Murrell, Heeson et al. 2001) respectively, Murrel et al (Murrell, Heeson et al. 2004) proposed the "chromatin loop model" (Figure 5). According to this model, the interaction between the H19 DMR and DMR1 on the maternal chromosome moves IGF2 into a silent chromatin region, away from the enhancers downstream of H19. By contrast, the interaction between the H19 DMR and DMR2 on the paternal chromosome leaves IGF2 in an active domain, placing the gene in close proximity to the downstream enhancers (Kato and Sasaki 2005). In this model, parent-specific interactions between the DMRs provide an epigenetic switch for IGF2. Also according to the model, a deletion of the H19 DMR would result in no loop formation on either parental chromosome, which could lead to ambiguous placing of IGF2 in an active or a silent domain (Kato and Sasaki 2005). The chromatin loop fits well with the reactivation of maternal IGF2 in DMR1 deletion mice (Constancia, Dean et al. 2000) and the inactivation of paternal IGF2 in DMR2 deletion mice (Murrell, Heeson et al. 2001). The model also explains the reactivation of maternal IGF2 and the partial inactivation of paternal IGF2 in H19DMR deleted in mice (Thorvaldsen, Duran et al. 1998).

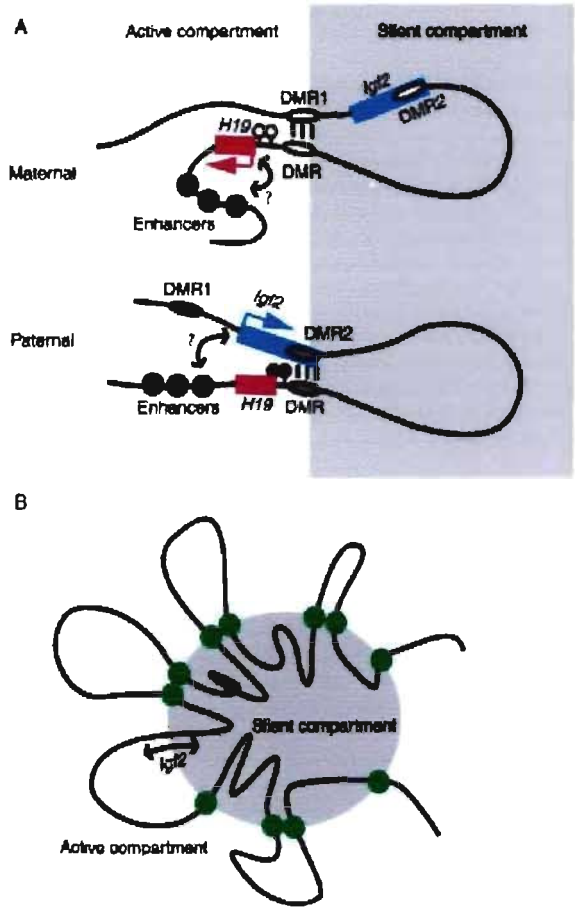

Figure 5. A: A chromatin-loop model for Igf2/H19 imprinting proposed by Murrell et al. The epigenetic status of the DMRs (methylated, filled ovals; unmethylated, open ovals) determines which DMRs interact. The resulting chromatin loop places the IGf2 promoters in either an active or a silent chromatin domain. This provides an epigenetic switch to control IGF2 expression. The hypothetical silent nuclear compartment is shown as shaded area. Activity of H19, which is always in the active compartment, is determined by its own methylation status (methylated, filled lollypops; unmethylated, open lollypops). Physical interactions between the genes and the downstream enhancers (large filled circles) are yet to be tested. B:

Illustration of an active and a silent compartment in the nucleus. Regulatory elements such as insulators (green circles) interact together to form chromatin loops. (4) Highly condensed chromatin is restricted to the silent compartment (shaded area). The epigenetic switch moves IGF2 in and out of the silent compartment (Murrell, Heeson et al. 2004).

Another theory supported by the chromatin model is that such mechanisms as boundary/insulators involve formation of higher order chromatin structures which enable CTCF boundary elements to interact so as to form chromatin loop domains (Chung, Bell et al. 1997). Studies have shown that CTCF can be associated with the nuclear matrix, suggesting that it might be involved in nuclear organization (Dunn, Zhao et al. 2003). These chromatin loops would restrain certain genes (IGF2) in non-active nuclear compartments.

However, in the chromatin model, the interaction of chromatin with DMDs and DMRs in a mechanism involving silencers and activators is predicted. When applied to the model, the loop formed between the H19 DMR and DMR1 (or DMR2) is rendered inactive (5). Unfortunately the answer to why DMR1 or DMR 2 is inactive has yet to be found. Another unsolved mistery is whether the enhancer downstream H19 interacts with IGF2 or not (Figure 5, question mark).

3.2 The maternally expressed IGF2R gene

IGF2R was one of the first genes to be identified as imprinted (Barlow, Stoger et al. 1991), along with IGF2 (DeChiara, Robertson et al. 1991). These

authors found that homozygous IGF2 null mice were 40% smaller than wild-type mice at birth, which was consistent with the growth promoting function of IGF2. It was also noticed that similar developmental rate was present in heterozygous mice, but only when the null allele was paternally inherited, demonstrating that maternal allele was not contributing to expression of IGF2. The opposite was found for M6P/IGF2R, which demonstrated that most of IGF2R expression originated from the maternal allele in mice (Barlow, Stoger et al. 1991; Wang, Fung et al. 1994)

M6P/IGF2R gene encodes for a transmembrane receptor in viviparous mammals that binds to phosphomannosyl glycoproteins and IGF2 through different binding sites (Kornfeld 1992; Dahms, Brzycki-Wessell et al. 1993; Yandell, Dunbar et al. 1999). Rather than mediating cell proliferation and growth through IGF2, its function is related to intracellular trafficking of lysosomal enzymes and the internalization of IGF2 and other extracellular ligands to the lysosomes for degradation (Kornfeld 1992).

In mice, IGF2R paternal repression is dependent on a 3.7 kb imprinting control element (ICE) called Region 2 (Wutz, Smrzka et al. 1997). This Region 2 is located within intron 2 of the IGF2R gene and contains a 1.5 kb fully methylated CpG-island in oocytes and a non-methylated CpG-island in sperm, which is the promoter for an antisense RNA named *Air* that overlaps the IGF2R promoter. The *Air* RNA is specifically expressed from the paternal unmethylated ICE, but not from the maternal methylated ICE. Recently, it has been proposed that the IGF2R-ICE generates a long-range effect that acts in a bidirectional manner to repress upstream

and downstream genes (Zwart, Sleutels et al. 2001). The Region 2 has been also identified in other species such as cattle, located nearer to exon 3 (2.2 kb upstream of exon 3), in humans (the human island is located 3.0 kb upstream of exon 3) and in mice (the mouse island is located 1.1 kb upstream of exon 3), but significantly different from in sheep (the sheep island is nearer to exon 2 and located a further 8.2 kb upstream of exon 3) (Long and Cai 2007). Although the Region 2 homologies have been found in such species, results from IGF2R expression are sometimes confusing and inconclusive. For instance in humans, even though the Region 2 is present, the methylation patterns are correctly inherited, some individuals show biallelic expression of IFG2R. More interesting results were published from studies in human placenta, where the intronic ICR region 2 was the only DMR identified, and this region showed an indistinguishable DNA methylation profile between samples with imprinted or biallelic expression (Monk, Arnaud et al. 2006). Less conclusive were the results found in the marsupial American opossum. The IGF2R gene was found to be paternally imprinted, even though the intron 2 of M6P/IGF2R completely lacked the Region 2 and the differentiated methylation pattern. Moreover, the region comprised only 9 CpGs and neither the parent of origin methylation pattern nor the *Air* RNA was found (Killian, Byrd et al. 2000). Studies performed in dogs also showed that IGF2R is mostly maternally expressed in a variety of tissues, including uterus and umbilical cord, however neither expression of an anti-sense transcript from the paternally derived allele, nor methylation of the repressed IGF2R promoter is required (O'Sullivan, Murphy et al. 2007). These

findings suggest that some imprinted genes either have developing alternatives for imprinting mechanisms between species or they have mechanisms that have yet to be discovered.

Although the imprinting control and expression do not always fit perfectly, effort must be focused on the IGF2R imprinting mechanism. Nonetheless IGF2 and IGF2R have been the columns that support studies and theories about the origin and function of imprinted genes. One theory that benefits the most is the conflict theory, (also called the kinship theory), which so far seems to be the most accepted.

3.3 The paternally expressed SNRPN gene

The maternal allele of SNRPN gene is silent, while the paternal allele active in many fetal and adult tissues, both in mice and humans (Cattanach, Barr et al. 1992; Leff, Brannan et al. 1992; Ozcelik, Leff et al. 1992; Glenn, Porter et al. 1993). The SNRPN gene has a not so common feature observed in mammals, since it had been shown that SNRPN is part of a bicistronic gene called SNURF (SNRPN upstream reading frame)–SNRPN. SNURF encodes a small acidic protein, which is translated from an upstream reading frame of the bicistronic SNURF –SNURF-SNRPN mRNA (Gray, Saitoh et al. 1999). The function of SNURF is not yet known. On the other hand SNRPN has been characterized as a member of an Sm protein, which is a specific factor component in alternative RNA splicing, expressed at highest levels in neurons (Grimaldi, Horn et al. 1993). Like Sm proteins, the product of the SNRPN gene is a component of the U snRNPs, denominated small

ribonucleoprotein particles. Seven small proteins, the Sm proteins form a heptameric ring that functions in the biogenesis of the U snRNPs (Pannone and Wolin 2000).

Many studies have investigated SNRPN imprinting control mainly because evidence suggests that an ICR (imprinting control region) neighboring the SNURF-*SNRPN* locus is responsible for controling the imprinting status of other genes (Rougeulle, Cardoso et al. 1998; Runte, Huttenhofer et al. 2001). Through interaction with the ICR, a second differentially methylated cis element is set up closer to SNRPN and when unmethylated (as it is on the paternally derived allele), this cis element is thought to function as a bidirectional enhancer that activates expression of adjacent genes and SNURF–SNRPN itself (Hore, Rapkins et al. 2007). It has been suggested that a large alternatively spliced non-coding transcript, initiating upstream of SNURF–SNRPN, is responsible for expression of downstream Sm proteins (Runte, Huttenhofer et al. 2001) and extends into the AS sub-domain to produce a transcript antisense to UBE3A (Runte, Huttenhofer et al. 2001; Landers, Bancescu et al. 2004), which confers monoallelic expression of UBE3A by blocking expression from the paternal allele (Rougeulle, Cardoso et al. 1998; Runte, Huttenhofer et al. 2001). UBE3A encodes an enzyme involved in targeting protein degradation. In the brain, the gene is active on the maternal chromosome only, and the loss of function is associated with Angelman Syndrome (AS), a rare neurogenetic syndrome characterized by severe mental retardation, lack of speech, jerky movements and a happy disposition (Horsthemke and Ludwig 2005). The epigenetic mechanism that control expression of UBE3A seems to be quite complex, since

multiple anti-sense transcripts other SNRPN loci are transcribed (Landers, Bancescu et al. 2004). Moreover, the UBE3A multiple alternative splicing and the developmental tissue-specific imprinting makes it difficult to understand the essential role of UBE3A in neuronal development (Yamasaki, Joh et al. 2003). In AS patients with an imprinting defect, the maternal unmethylated allele, maternal expression of SNRPN, and the maternal *UBE3A* allele being silenced are observed (Horsthemke and Ludwig 2005). These findings make the SNRPN ICR an important region in the study of the imprinting mechanisms in this locus.

4. Epigenetic Alterations in Assisted Reproductive Technologies

Fundamental scientific research has become important for advances in farm animal reproduction. The understanding of the physiology of reproduction is no longer enough when human intervention plays a significant role in the process. For instance, in livestock, the use of assisted reproductive technologies (ART) is necessary for improving production. In such case, the study of short and long term consequences of using these techniques is as important as the biological complexity behind it, otherwise no further contribution will be added to the process.

Here, some of the most used techniques, from well-defined standard procedures like super-ovulation, widely used in farm animals and increasingly in humans as well, to elaborate protocols such as somatic cell nuclear transfer are reviewed; and the epigenetic outcome of using them in assisted reproduction is discussed.

4.1 Super-ovulation and Artificial Insemination

Artificial insemination (AI) basically consists on the use of diluted semen from a few males to inseminate a large number of females. This technique has significantly improved reproductive efficiency in farm animals and allowed selection of desired features within a very short period of time. In addition, the ability to freeze, store and transport semen has taken controlled reproduction in farm animals to a whole new level in the industry (Sirard 2007). Along with AI, the use of pituitary extracts or chemical hormone analogs for controlling the ovarian response to hormones and the lifespan of the corpus luteum has dramatically changed the field of animal reproduction. Basically, six hormones (estradiol-17b, progesterone, GnRH, FSH, LH and prostaglandin F2a) have resulted in many treatment protocols that effectively control female reproduction in farm animals, and humans as well, and are considered the foundation of ART as we know it today (Sirard 2007).

In humans, the use of AI and superovulation protocols has been associated with infertility problems. Intrauterine insemination (IUI) of prepared sperm is a common treatment for subfertility, which is often combined with ovarian stimulation (OS), using either clomiphene citrate or gonadotrophins (Verhulst, Cohlen et al. 2006; Bensdorp, Cohlen et al. 2007). Although IUI has been considered a non-invasive procedure and relatively safe, the ovarian stimulation has been studied more profoundly in terms of the epigenetic outcome. Recently, Sato et al (Sato, Otsu et al. 2007) have found that superovulated GV and MI oocytes from infertile women show a gain of H19 methylation and a loss of PEG1 methylation in a single-cell

methylation assay. According to the authors, it was not possible to distinguish whether the changes in DNA methylation were due to the process of superovulation, the age of the patients or are inherent to the infertility problems suffered by some of these individuals. Natural changes in gene expression of DNMT 3 proteins required in the establishment of the germ line imprint levels occur during ageing (Okano, Xie et al. 1998; Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002; Hamatani, Falco et al. 2004; Kaneda, Okano et al. 2004) which may be due to changes in the endocrine environment. However, it was also demonstrated that DNA methylation was found at the normally unmethylated maternal H19 DMR allele in murine after superovulation treatment, which suggests that the changes seen at the human H19 locus are due, at least in part, to the superovulation procedure (Sato, Otsu et al. 2007). In another study, superovulation followed by embryo transfer at 3.5dpc resulted in biallelic expression of H19 in the placenta (Fortier, Lopes et al. 2008). The expression of IGF2 was increased in the placenta following superovulation with or without embryo transfer (Fortier, Lopes et al. 2008). Authors concluded that both maternally and paternally methylated imprinted genes were affected, suggesting that superovulation compromises oocyte quality and interferes with the maintenance of imprinting during preimplantation development (Fortier, Lopes et al. 2008).

Although results may differ from lab to lab, the screening for maintenance of loss of imprinting in early embryos of patients following ART is strongly advised, as well as the follow up of pregnancies.

4.2 In vitro fertilization and in vitro culture

Embryo culture was initially used to maintain embryo viability between flushing of the donor dams and transfer to the recipient in embryo transfer procedures. This practical approach has expanded the use of maturation and fertilization of oocytes in vitro. The possibility of making embryos from oocytes obtained from the ovaries of slaughtered animals has given rise to several other fields of investigation. The use of artificial insemination (AI), *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and the control of estrous cycle in farm animal reproduction made possible the development of new techniques, such as embryo transfer. These approaches, when combined, increased production and made possible the fine-tuning of desirable genetic features in farm animals (Betteridge 2003; Betteridge 2006). Similarly in humans IVF, *in vitro* culture and embryo transfer have been commonly used in couples with infertility problems. Although the capacity of IVM efficiency can be improved, to date in both humans and farm animals, reduced meiotic maturation rates, fertilization rates, and blastocyst production reveal short-term developmental insufficiency of oocytes when compared with in vivo-matured controls (Banwell and Thompson 2008). In these IVM oocytes abnormal methylation patterns in the maternal non-methylated DMR allele of the H19 was found in human studies (Borghol, Lornage et al. 2006), suggesting that imprinting failures could be associated as well.

Several outcome studies have highlighted increased developmental disorder rates in IVF children compared with the general population (Dhont, De Sutter et al.

1999). Some complications have been attributed to intrauterine growth retardation (Xu, Goodyer et al. 1993) and a higher frequency of multiple births, however studies have also shown that singleton IVF infants have a greater risk of low birthweight (Schieve, Meikle et al. 2002) and birth defects (Hansen, Kurinczuk et al. 2002; Belva, Henriet et al. 2007). Some of these defects include an increased incidence of Beckwith–Wiedemann syndrome and Angelman syndrome in children from IVF. Increased frequency of ART conceptions in BWS cohorts has been reported (4% of BWS cases were ART conceptions, compared with 1.2% of the general population) (Maher, Brueton et al. 2003) and similar results have been found in other two laboratories (DeBaun, Niemitz et al. 2003; Gicquel, Gaston et al. 2003).

Animal data have demonstrated that *in vitro* embryo culture, and related procedures, may be associated with epigenetic changes, disordered genomic imprinting and alterations in intrauterine growth (Maher 2005). However contrary to what is reported in humans, animal models rather show signs of overgrowth and higher birth weight. In some cases, large offspring syndrome (LOS), characterized by increased birth weight and perinatal morbidity, occurs after embryo manipulation and is associated with a loss of methylation at an imprinting control element in the maternally expressed IGF2R in some cases (Young, Fernandes et al. 2001). In a more general situation, embryos, fetuses and placenta differ significantly morphologically and physiologically, making it difficult to associate consistently abnormal expression of a single gene to certain symptoms. However, strong evidence suggests that abnormal regulation of imprinted genes is linked to LOS (Farin,

Piedrahita et al. 2006). Perhaps *in vitro* culture may have negative effects on DNA methylation, as observed in mice, where a loss of methylation in the paternal allele of H19 ICR, and consequently an increase in paternal expression of H19 gene transcript, was observed when Whitten's medium was used during the culture of mouse embryos, but not when cultured with KSOM medium (Doherty, Mann et al. 2000; Mann, Lee et al. 2004). In another study, mouse pre-implantation embryos have shown fetal overgrowth and abnormal gene expression and methylation patterns of the imprinted genes IGF2 and H19 after implantation (Khosla, Dean et al. 2001), suggesting that certain culture components may be detrimental to imprinting regulation. Together, these results indicate that even though symptoms may differ from one species to another, generally *in vitro* culture alters the epigenetic outcome of blastocyst development and puts the pregnancy at risk.

4.3 Somatic Cell Nuclear Transfer

The first attempts of somatic cell nuclear transfer (SCNT), in the early fifties, came from initial reports from Briggs and King on the production of adult frog clones using nuclei from embryonic cells (Briggs and King 1952). Four decades later, with improvement of culture media and finer micromanipulation tools, mammalian SCNT was achieved (Campbell, McWhir et al. 1996). To date, SCNT has been accomplished in a growing list of species. In each case, an enucleated oocyte has successfully reprogrammed the nucleus of a somatic cell in such a way that the embryonic program could be reset and progress to the development of a live

offspring. However, the fact that SCNT works at all, is mostly shadowed by the generally very low frequency of success. Many factors contribute to the low success obtained. One factor affecting SCNT efficiency that is frequently ignored is the frequency of NT practiced by different groups. Groups that perform SCNT on a regular and frequent basis tend to have better and more reliable results (Campbell, Fisher et al. 2007; Keefer 2008). Since SCNT involves in vitro manual manipulation and requires exposure of oocytes and cells to constant fluctuations in light, temperature, atmospheric conditions, and different media; consistency and speedy completion of the SCNT process results in minimal exposure of oocytes to detrimental conditions (Keefer 2008). Other physical challenges involve systems used for enucleation (micromanipulation, chemical, zona-free), transfer of the donor nucleus (electrofusion or direct injection) and activation procedures (electrical, chemical and biological extracts). What may seem as a minor alteration at any one step in the process can have significant effects on the success rates (Ribas, Oback et al. 2006). Furthermore, different species require specific adjustments to the steps listed above (Hinrichs, Choi et al. 2007).

Once all manual obstacles are overcome, the oocyte-donor cell complex faces the new challenges of reprogramming. Dedifferentiation of the differentiated donor somatic cell to a totipotent embryonic state, followed by redifferentiation of cloned embryos to different somatic cell types during later development (Yang, Smith et al. 2007). The first step of nuclear reprogramming involves the erasure of the donor cell epigenetic pattern after nuclear transfer and the re-establishment of embryonic

epigenetic characteristics and gene expression in the cloned embryo. The second step of nuclear reprogramming refers to redifferentiation of cloned embryos from a totipotent status to various differentiated states for tissue generation or organogenesis during post-implantation development (Yang, Smith et al. 2007). In general, mammalian blastocysts derived by SCNT and IVF can develop at a similar rate, for instance in cattle 30 to 50% of oocytes develop to blastocysts (Cibelli, Campbell et al. 2002). SCNT embryos die mostly during post-implantation development and the survival rate to birth for clones is about 1 to 5%, a very low number when compared to 30 to 60% for IVF. Even those clones that manage to survive to term present a variety of defects (Cibelli, Campbell et al. 2002). One of the defects, LOS syndrome, is commonly seen in mice, sheep and cattle and is characterized by large size at birth and severe placental deficiency (Young, Sinclair et al. 1998). Abnormal placentation has been described in several cloned species. Placentas of cloned mice are enlarged without exception (Suemizu, Aiba et al. 2003), and those of cloned calves have fewer but much larger placentomes, probably to compensate for the reduced number of sites of maternal-fetal exchange (Chavatte-Palmer, Heyman et al. 2002; Yang, Smith et al. 2007). Possibly many of the abnormalities observed in cloned fetuses, including LOS, may be secondary to defects in placental function (Constant, Guillomot et al. 2006). Recent evidence supports the hypothesis that the placental lineage is especially vulnerable to problems arising from reprogramming of the imprinted genes in somatic nucleus after nuclear transfer (Yang, Smith et al. 2007). Genomic imprinting, the preferential expression of one parental allele, is regulated

primarily by DNA methylation of CpG islands, as previously discussed. The majority of imprinted genes have roles in fetal growth and development, and both the maternal and paternal genomes are required for normal development (Wilkins and Haig 2003). Studies confirmed that at the blastocyst stage, the methylation status of the ICM is relatively normal whereas abnormal hypermethylation was observed in the trophoblast cells (Dean, Santos et al. 2001). Methylation of histone H3K9 showed a similar pattern, with abnormally high levels of methylation in the trophectoderm of cloned bovine blastocysts (Santos, Zakhartchenko et al. 2003). The studies that have focused on the acetylation of histones in cloned embryos have found aberrancies (Santos, Zakhartchenko et al. 2003; Enright, Sung et al. 2005; Suteevun, Parnpai et al. 2006). Studies performed with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) showed an increase in cloning efficiency (Kishigami, Mizutani et al. 2006; Ding, Wang et al. 2008; Iager, Ragina et al. 2008; Shi, Miao et al. 2008), suggesting that hypoacetylation is also another problem faced by clones. In addition to the hypermethylation observed in the trophectoderm, several imprinted genes have been found to be normally expressed in cloned fetuses but abnormally expressed in the placenta (Yang, Smith et al. 2007). Therefore the study of SCNT, especially concerning the regulation of imprinted genes and their relationship with the placenta, can provide insightful material to understand cell reprogramming and also contribute to progress in therapeutic cloning, which is directed toward stem cell research.

5. Rationale, Hypothesis and Objectives

5.1 Rationale

To date, ART are widely applied in farm animals and humans as well. In farm animals ART is consistently used to increase production, propagate desirable genetic features in the progeny in a shorter period of time. In humans, estimative data reveals that one out of ten individuals develop fertility problems and ART has given sub-fertile couples the opportunity to have children.

Despite the advances accomplished in embryo culture and ART in general, many questions remains unanswered; for example, what is the cause of LOS in ruminant and rodent pregnancies originating from in vitro embryo culture. Is it caused by epigenetic alterations? What we know is that many imprinted genes are abnormally expressed in animals developing such a syndrome, however the epigenetic mechanisms involved are poorly understood in species other than mice. In humans, children conceived by IVF and in vitro culture have a greater risk of low birthweight and increased incidence of Beckwith–Wiedemann syndrome and Angelman syndrome. Although these anomalies are associated with imprinting failures, the exact mechanism behind it and what caused such abnormality remains unsolved. Recently many mice models have been created to study these diseases, however it is important to have as many models as possible that provide clues about imprinting failures during gestation of embryo produced by ART. For instance, we think that by using bovine species as a model, some useful information can be

generated to understand the effects of embryo culture on imprinted genes and pregnancies conceived by them.

5.2 Hypothesis

Abnormalities observed in early development and pregnancies from embryos produced by IVF, in vitro culture and SCNT present aberrant expression of imprinted genes due to alterations in DNA methylation patterns.

5.3 Objectives

The objectives of this work are:

1. To characterize putative DMRs of the imprinted genes SRNPN, H19 and IGF2R, search for *Bos indicus* polymrphisms (SNP), and to verify the allele-specific methylation patterns in sperm and oocytes in cattle.
2. To identify exonic SNP and examine the patterns of parental expression of the maternally imprinted (SNRPN) and paternally imprinted (H19 and IGF2R) genes during pre- (day 17) and post-implantation (day 40) in cattle.
3. To determine whether embryo manipulation, such as *in vitro* culture (IVF) and cloning (SCNT), interfere with the DMR and parental-specific expression patterns of pre- and post-implantation bovine embryos.

CHAPTER II

Monoallelic expression of bovine H19 gene is established after implantation in normal bovine embryos but not in embryos produced by somatic cell nuclear transfer

Joao Suzuki, Jacinthe Therrien, France Filion, Rejean Lefebvre, Alan K. Goff and Lawrence C. Smith*

Centre de Recherche en Reproduction Animale (CRRA), Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, J2S 7C6 Canada

* Corresponding author

ABSTRACT

Assisted reproductive technologies (ART) have been associated with abnormal pregnancies and offspring in a number of mammals, including humans. Although the causes remain unclear, abnormal expression of many imprinted genes involved in fetal growth and placental development suggest an epigenetic origin. Using a bovine interspecies model with an exonic polymorphism, we demonstrate a transition from bi-allelic to mono-allelic expression of the maternally expressed H19 gene between day 17 (pre-implantation) and 40 (post-implantation) of gestation in pregnancies derived from artificial insemination (AI) and *in vitro* fertilization (IVF). However, pregnancies derived by somatic cell nuclear transfer (SCNT) remained bi-allelic at day 40 of gestation, indicating a failure to completely silence expression from the paternal allele during implantation. To complement our findings on allele-specific expression, we analyzed the methylation patterns of a differentially methylated region (DMR) on the H19-IGF2 imprinting control region during pre- and post-implantation development. Although the CTCF binding site in the H19 DMR of both IVF and SCNT embryos was hypomethylated at day 17, after implantation (day 40) the methylation status of IVF pregnancies was not different from AI and hypermethylated compared to the SCNT group. Together, these results indicate that the H19 imprinting is not yet established in preimplantation bovine embryos and that, contrary to SCNT, the demethylation effects of *in vitro* culture on the H19 DMR are reversed by day 40 of gestation, enabling proper silencing of the paternal allele during post-implantation.

INTRODUCTION

In mammals, paternal and maternal genomes are not functionally equivalent and an example of nonequivalence is the expression of imprinted genes, mostly important during embryo development, differentiation and cell growth. Even though copies of imprinted genes are present in both paternal and maternal genomes, they are expressed solely from one parental genome. Imprinted genes are usually located in clusters and normally one or more imprinted gene expression is controlled by activation of imprinting control regions (ICRs) [1]. Both parental genomes have ICRs but only one will be active, thus controlling the expression in an allele specific manner. Expression is controlled by epigenetic modifications in specialized DNA sequences, without changing the sequence itself. One of the best characterized modifications of ICRs studied in mice and humans is DNA methylation [2]. Methyltransferases (DNMTs) catalyse the methylation at the 5-position of the cytosine (C) residue within cytosine-guanine dinucleotides (CpG), resulting in the formation of 5-methylcytosine (m5C) [3]. DNMT1, DNMT3A and DNMT3B are responsible, together with accessory proteins like DNMT3L, for methylation pattern acquisition during gametogenesis, embryogenesis and somatic tissue development [3].

Several imprinted genes play key roles in the regulation of cellular proliferation and growth. Among these are the insulin-like growth factor-2 (IGF2) and H19 genes. IGF2 is expressed only from the paternal allele and encodes a growth factor involved in fetal and postnatal growth [4]. H19 is a non-translated RNA with

no clear role in early development. However, H19 expression is tightly linked to IGF2 and both are controlled by the IGF2-H19 ICR. This ICR is methylated exclusively on the paternal allele. Studies in mice have shown that the unmethylated maternal allele is bound by several copies of a zinc-finger protein called CTCF [5]. This creates a chromatin boundary, insulating the IGF2 gene from enhancers located downstream of H19 and as a consequence IGF2 is not expressed from the maternal allele. On the paternal allele, DNA methylation prevents CTCF binding, allowing access to the enhancers and expression of IGF2 [5]. In mice, studies demonstrated that CTCF binding sites play a critical role on establishing of imprinting at the IGF2-H19 ICR [6]

In mice and humans the IGF2-H19 imprinting domain, located upstream of the H19 promoter region, has been extensively studied due to its important role during development and cell growth [4, 7, 8]. Increased levels of IGF2 expression in the mouse have been shown to cause fetal overgrowth [7], whereas reduced expression is associated with growth deficiency [8]. Although the flanking maternally expressed H19 gene encodes a non-coding RNA of unknown function, its expression, along with IGF2, has been intensively studied in humans and mice as well as the regulation by an ICR located upstream of H19 (the 'H19 ICR') [4]. In bovine, the H19 gene, located on chromosome 29, is organized into five exons and four unusually small introns, similar in size to introns in the sheep and pig H19 genes [9]. It was shown that the bovine gene is 91.8% and 71.2% identical to the sheep and pig genes, respectively [9]. Also, using the single-strand conformation

polymorphism method (SSCP), it was reported that the bovine gene is maternally expressed (the paternal allele is silent) in all tissues analyzed [9, 10]. H19 is also highly expressed in amnion, chorion, and allantois in fetal tissues and in muscle of adult animals [11]. However, little is known about the mechanism that controls H19 expression in cattle and the effect of ART on the H19 imprinting mechanism.

Animals derived from *in vitro* produced (IVP) embryos develop occasionally the Large Offspring Syndrome (LOS) at birth, which is characterized by an increased length of pregnancy, fetal oversize, and increased placentome size [12, 13]. Similarly, LOS involving hydrops of the fetal fluids (hydrallantois, hydramnios) is frequently reported in cloned pregnancies produced by SCNT [12]. In both IVP and SCNT produced animals, the authors suggested that the abnormal expression of imprinted genes is implicated [12, 13]. In support to this hypothesis, studies in mice have shown that certain conditions of *in vitro* culture can alter the DNA methylation levels during embryo development, leading to loss of imprinting and abnormal gene expression [14, 15]. Moreover, there is strong evidence supporting the association of LOS with abnormal methylation patterns of imprinted genes such as IGF2R in sheep [16] and cattle [17]. A recent study has also demonstrated that imprinted genes are abnormally expressed in cattle showing high mortality rate during the perinatal period [18]. However, in cattle, little is known about the regulation of H19 gene transcription and the effects of *in vitro* culture during preimplantation development and early gestation.

In this study our objectives were to characterize putative mechanisms of H19 imprinting regulation (H19 DMR), to analyze the imprinting status of the H19 gene during pre- and post implantation development, as well as the effects of *in vitro* culture and SCNT on H19 transcript expression. The bovine model information on the impact of *in vitro* culture and cell reprogramming on imprinting regulation in this species, but also support its use as a model to study human growth disorders, such as Beckwith-Wiedemann overgrowth syndrome [4] and Silver-Russell Syndrome [19, 20], where the over expression of IGF2 is associated with the deregulation and loss of imprinting at the H19-IGF2 domain. With help of a single nucleotide polymorphism (SNP) present in *Bos indicus* cattle, we produced F1 embryos by breeding *Bos taurus* x *Bos indicus* and analyzed the parental allelic expression of the bovine H19 gene.

MATERIAL AND METHODS

All procedures were performed in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Training, approved by the animal experimentation committee of the Université de Montréal sanctioned by the Canadian Council on Animal Care.

Nuclear donor cells

Fetal fibroblast cell cultures were established from a 60-day-old crossbred fetus produced by artificial insemination of a Holstein (*Bos taurus*) heifer with semen from a Nelore (*Bos indicus*) bull. Fetal tissues (brain, heart, liver, muscle and

placenta) were minced manually and digested with 0.25% trypsin and 0.02% EDTA (Gibco BRL, Burlington, ON, Canada) at 37 °C for 10 min. Isolated cells were washed and cultured for approximately 4 d in Dulbecco modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 0.5% antibiotics (penicillin 10000 U/ml and streptomycin 10 000 μg/ml; Gibco BRL) at 37 °C in 5% CO_2. When the cultures were confluent, primary passage cells were frozen in culture media supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen. Donor cells were thawed at 37 °C for 1 min and cultured to confluence for a maximum of 5 passages before use as donor cells.

Host oocytes

Cattle ovaries were collected from a local abattoir and transported to the laboratory in saline at 30–35 °C within approximately 2 h after slaughter. Follicles with diameters between 2 and 10 mm were punctured with a 18-gauge needle, and cumulus oocyte-complexes (COCs) with approximately 4 to 6 layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL) supplemented with 10% (vol/vol) FBS. Groups of 20 COCs were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 μg/ml LH (Ayerst, London, ON, Canada), 0.5 μg/ml FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 μg/ml estradiol 17-β (Sigma-Aldrich, St. Louis, MO), 22 μg/ml pyruvate (Sigma-Aldrich), and 50 μg/ml gentamicin (Sigma-Aldrich). After 19 to 20 h of *in vitro* maturation, cumulus cells were removed from the COCs by vortexing for 2 min in PBS and 0.2%

hyaluronidase (Sigma-Aldrich). Only oocytes with homogeneous cytoplasm and intact cell membrane were selected for micromanipulation.

In vivo and in vitro-derived embryos

Production of embryos and fetuses for *in vivo* (AI) and *in vitro* (IVP) controls, as well as donor cells were conducted as described previously [21]. Briefly, Holstein heifers were superovulated by intramuscular injection of porcine FSH (Folltropin-V) given every 12 h in decreasing doses starting at Day 9–10 of the estrous cycle. Cows received an injection of 500 μg of cloprostenol (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) and were artificially inseminated at 52 h and 86 h after the initiation of superovulation [21].

In vitro-matured oocytes were fertilized *in vitro* using standard protocols [21]. Briefly, 20–25 COCs were placed in 100 μl drops of Tyrode medium supplemented with 0.6% BSA (fraction V; Sigma-Aldrich), lactate, pyruvate, gentamicin, and heparin (10 μg/ml). Frozen-thawed spermatozoa were washed and centrifuged through a Percoll (Sigma) gradient and diluted to 10^6 live spermatozoa/ml. At 20 h following the start of incubation with spermatozoa, COCs were denuded of cumulus cells by brief shaking, and the putative zygotes were transferred to 25 μl drops of synthetic oviduct fluid (SOF medium) and cultured for 8 d, with additional 25 μl of SOF medium under the same conditions used for the SCNT embryos.

Somatic Cell Nuclear Transfer

The SCNT protocol used was a slight modification from that previously reported [22]. Oocytes were selected in groups of 100 and placed in 1.5 mg/ml pronase in TCM 199 supplemented with FBS 10% for about 4 min. Zona-free oocytes were washed thoroughly in TCM-199 supplemented with FBS 20% for 3 min and cultured in 0.4 μg/ml demecolcine for at least 30 min. Treated oocytes with a visible protruding membrane were placed in medium supplemented with 5 μg/ml cytochalasin and FBS 10% and manually bisected using a micro blade on a stereomicroscope. After bisection, oocytes were stained with 2 μg/ml Hoescht 33342 and checked for the absence of chromatin. Nuclear donor cells were thawed, washed and placed in 50 μl of culture media (DMEM, supplemented with 10% FBS and 0.5% antibiotics). Nuclear transfer was performed using confluent cells that were maintained in culture for 3–5 passages. Cytoplasts were placed individually in a 50 μl drop containing 500 μg/ml of phytohemaglutinin (Sigma) for about 3 sec and then quickly positioned over a single donor cell placed at the bottom of the dish. After attachment of the donor cell, the cytoplast-somatic cell pairs were placed in 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.2 kV electric pulse lasting 70 μsec. After electrical stimulation, couplets were washed and cultured individually in 10 μl drops of 6-dimethylaminopurine (DMAP, Sigma-Aldrich) for 3 h. After DMAP treatment, reconstructed oocytes were washed and cultured in 40 μl drops of SOF modified medium supplemented with 0.8% BSA-V fatty acid free (Sigma-Aldrich) under equilibrated mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. Embryos were cultured in groups of 4

per drop in small individual wells (500 μm diameter) prepared with a sterile needle to avoid separation of blastomeres during development. Reconstructed embryos were cultured for a period of 8 d. After *in vitro* culture, IVF and SCNT showed similar results of blastocyst development for IVF (33.75%) and SCNT (27.9%), indicating that the handling of the oocytes for SCNT was not detrimental to the early stages of development to the blastocyst stage.

Day-17 Elongating Embryos and Day-40 fetuses

The estrous cycle of Holstein heifers was synchronized by an injection of 500 μg of the prostaglandin F2α analogue, cloprostenol (Estrumate, Schering Canada Inc). Six to 8 d after standing heat, Day-8 *in vitro*-produced or SCNT blastocysts were transferred to the uterine horn ipsilateral to the corpus luteum. One recipient was used to obtain three day-17 *in vivo*-derived embryos (AI group). Embryos were washed with TCM-199 Hepes-buffered medium supplemented with 10% of FBS, loaded into a 250 μl straw and transferred to recipient heifers. One group of heifers received between 10 to 15 Day-8 IVP or SCNT embryos and allowed to develop for another 9 d in the uterine horn. 5 (50%) Day-17 elongated embryos were non-surgically recovered from IVF and 6 (45%) from SCNT by flushing the uterus of the recipient heifers with PBS using a Foley catheter. Embryos were removed from the flushing media and inspected to select those that were recovered intact. After selection, embryos were washed three times in PBS and frozen individually at -70 °C in 0.2 ml of distilled water. Only those embryos that were recovered intact were used for the experiments. The second group of heifers, carrying 1 ro 2 blastocysts each,

was allowed to continue gestation to day 40. Recipients carrying fetuses with a normal heart beat were slaughtered at the local slaughterhouse and transported within approximately 1 h. As expected survival rates (presence of a heart beat) were higher in the IVF (50%) than in the SCNT group (30%). For day 40 fetuses, a total of 4 recipients were inseminated and 3 pregnancies were confirmed at day 39 of gestation and slaughtered the following day. Samples from liver, muscle, heart, brain and placenta were collected from each viable gestation, snap-frozen in liquid nitrogen and stored at -70 °C until further analysis.

Search for the Bovine H19 DMR

Genome walking was used to identify the position of the DMR of the bovine H19 gene. Total genomic DNA was extracted from fibroblasts of an adult *Bos indicus* using a DNeasy Tissue Kit (Qiagen). DNA libraries were obtained using the Universal Genome Walker Kit (Clontech), which requires two nested PCR reactions per library. Briefly, the primers for the PCR reaction consisted of adaptor primers provided with the kit and gene-specific primers. The H19 DMR is localized approximately 2.0 kb upstream of the H19 promoter region in most species analyzed to date. Therefore, we designed our gene-specific primers in exon 1 using sequences obtained from GenBank (AF087017, AF049091) and moved in the 5' direction. The protocol produced a series of four fragments (2.1, 2.0, 0.9, and 0.8 kb) that covered 2.2 kb upstream of the promoter region, until the 5' end of the exon 4. Each PCR fragment was cloned (pGEM-T easy) and sequenced for analysis. The cloned

nucleotide sequence matches sequence data now available in the *Bos taurus* chromosome 29 (NW_001494548).

Bisulfite Sequencing

DNA was isolated from Day-17 embryos and Day-40 tissues using Qiagen DNeasy extraction kit, according to the manufacture's instructions. Approximately 200 ηg of total genomic DNA was used for a bisulfite treatment reaction using the EZ DNA methylation Kit™ (Zymo Research), following the manufacturer's instructions. Primers specific for bisulfite-converted DNA were designed according to a previous publication [21]. Nested PCR amplification primers were designed according to bisulfite standards (no CpG sites within primers and at least 2 cytosines within primer sequence to select for converted sequences). For the outside nested PCR, the primer sequences were: forward 5'-ACACCTTAAAAAACTCAAATAAATACC - 3'; reverse 5'-TATTTTAGATAGGGTTGAGAGGTTG - 3'. After the first PCR, a nested was carried out with the following primers: forward 5'-AGTGAGGTTTATATATTATTATAAAGG - 3'; reverse 5'-TGTTCCAAGTCCCAGCATGA -3'. Each 50 µl PCR reaction contained 4 µl of bisulfite-treated DNA, 1 µl of each primer (10 µM), 2.5 µl (100 µM) deoxynucleotide triphosphates (Invitrogen), 5 µl 5X PCR buffer (300 mM Tris-HCl, 7.5 mM ammonium sulfate, 12.5 mM $MgCl_2$) (Invitrogen), and 1.25 U of DNA Taq polymerase (Invitrogen). First-round PCR was performed under the following conditions: 94 °C (4 min), 50 °C (2 min), and 72 °C (2 min) for two cycles, followed

by 35 cycles of PCR at 94 °C (1 min), 50 °C (2 min), and 72 °C (2 min). For the second round of PCR, 4 μl of the first-round sample were used, and the conditions for the PCR were the same as the first-round conditions, except that the first two cycles were omitted.

Fragments were resolved in 1.2 % agarose gels, followed by purification using agarose purification kit from Qiagen. Purified fragments were subcloned in pGEM T easy Vector (Promega), and the cell transfection protocol was performed using competent *Escherichia coli* DH5α cell. Clones containing the appropriate inserts were sequenced using an automated sequencer. Since bisulfite converts all unmethylated cytosines, whether or not they are in CpG dinucleotides, to guanines, only sequences with greater than 95% bisulfite conversion efficiency were used for analysis (i.e., to avoid false overestimation of methylated CpGs). Sequence mutations or any type of modifications (polymorphisms) between cloned sequences with similar CpG methylation profiles were verified to ensure that unique clones were represented. We examined 28 CpG sites in a 550-bp fragment of H19 DMR. Absence of strain-specific single nucleotide polymorphisms prevented the parental origin of the sequenced strands from being determined.

Allele-specific polymorphism in cDNA

RNA was extracted using the RNeasy Extraction kit (Qiagen) following manufacturer's instructions. Reverse transcription and polymerase chain reaction (RT PCR) was performed using Omniscript RT-PCR kit (Qiagen). cDNA was used as a template for the next PCR using primers Forward (5'-

AGTGGGAGGGGCATTGGACTT) and Reverse (5'-TGTTCCAAGTCCCAGGCATGA). A 50 µl reaction was performed consisting of 5 µl 10X PCR buffer (Promega), 4 µl 25 mM $MgCl_2$, 1.25 µl 10 mM dNTPs, 2.5 µl 3 M forward primer, 2.5 ml 3 M reverse primer, 2 µl DNA, and 1 ml Taq (Promega). PCR reactions were performed for 35 cycles at 94 °C (2 min), 94 °C (30 sec), 64 °C (30 sec), 72 °C (35 sec), 72 °C (3 min), and finally held at 10 °C. Fragments were resolved in 1.2 % agarose gels, purified and subcloned in sequencing vectors pGEM T easy Vector (Promega) and transformed in competent *Escherichia coli* cells. Sequence analysis indicated the presence of a single nucleotide polymorphism (SNP) between the *Bos indicus* and *Bos taurus* genomes.

Fifteen to 20 colonies were picked and subjected to plasmid purification, according to Qiagen's protocol, sequence analysis. Plasmid DNA sequence results were examined individually for the presence of the paternally expressed *Bos indicus* genome (adenine) or maternally expressed *Bos taurus* genome (guanine) SNP. Results are expressed in percentages of individual cloned sequences possessing either an A or a G SNP. To validate our colony picking procedure, we designed an experiment where *Bos indicus* sequences (A) were mixed in equal (50% each) proportions with *Bos taurus* sequences (G) for PCR, subcloning and sequencing and demonstrated that the A/G ratio remained at approximately 1:1.

Statistical Analyses

Statistical analysis was performed using the Chi-square test. For methylation analysis, data was analyzed by computing frequency of methylated sites over the

number of unmethylated CpG islands. For gene expression, data was analyzed using Bioedit software aligning program and frequency of paternal computed over maternal allele SNP. For both cases the level of significance was set at $P<0.05$.

RESULTS

Allele-specific expression of H19 gene

Sequencing of genomic DNA and cDNA samples obtained from animals of the *Bos taurus* and *Bos indicus* subspecies enabled the identification of a single nucleotide polymorphisms (SNP) for use in allele specific expression profiles from F1 samples. Genome walking sequences stretching from exon 1 to 4 enabled the identification of a guanine to adenine (**G/A**; *Bos taurus/Bos indicus*) SNP at position +30 downstream of exon 3 of the H19 gene. The SNP was confirmed by sequencing of PCR fragments obtained with H19 primers using genomic DNA samples obtained from a number of *Bos taurus* (maternal control), *Bos indicus* (paternal control) (Figure 1). Preliminary experiments using predetermined mixture of *Bos indicus* and *Bos taurus* cDNA were performed to validate the quantitative protocol of parental-specific transcript analysis (data not shown). Once the reliability of the SNP and the cDNA quantification protocol was validated, H19 allele-specific expression was assessed in the mRNA samples from the pre- and post-implantation groups.

Our findings indicated that 11.8% of the H19 transcripts present in day 17 embryos obtained by AI were of paternal (*Bos indicus*) origin (Figure 2), indicating leaky biallelic expression during preimplantation. Previous reports on allele-specific

expression of H19 in mouse preimplantation embryos have considered paternal transcript levels below 10% to be monoallelic [15], indicating that the levels of H19 paternal transcripts observed in our AI group were at the lower limits of biallelic expression. Similarly to AI, day-17 embryos obtained by IVF showed a low biallelical expression of H19 (13.3% *Bos indicus* transcripts). The levels of paternal transcripts in day 17 embryos derived by SCNT showed slightly higher levels of biallelic expression (20% Bos indicus transcripts) than observed in AI and IVF embryos (P=0.06), indicating a slightly increased leakage of transcripts from the paternal allele. Together, these results indicate that, during preimplantation, low levels of bi-allelic expression is observed from H19 and that the protocols used for SCNT seem to induce slightly more leakage from the paternal allele.

Our next step was to verify whether the patterns of allelic H19 expression observed during preimplantation development are maintained after implantation in both embryonic and extra-embryonic tissues. Samples from liver, muscle, brain, heart and placenta were taken from fetuses at day 40 and analyzed quantitatively to determine the ratios of maternal and paternal transcripts (Figure 3). Although a minute (3%) presence of paternal transcripts was observed in the placenta of the AI group, all samples obtained from AI fetuses along with every embryonic and placental sample obtained from the IVF group showed exclusive monoallelic (maternal) expression of the H19 gene. However, allelic expression in SCNT samples ranged from leaky (<10%) to biallelic (>10%) in every tissue examined. Leaky paternal expression was observed in the heart (8.3%) and brain (10%) whereas

muscle (13%), liver (17%) and placenta (20%) showed higher levels of paternal expression (Figure 3). Similar to results found at day 17 in SCNT embryos (Figure 2), placental tissue from SCNT group was the most affected ($P<0.05$). These results indicate that H19 gene expression, although leaky during preimplantantion, is monoallelic in bovine embryonic tissues produced by AI and IVF. However, H19 imprinting seems to be perturbed in SCNT embryos leading to increased expression from the paternal allele.

Characterization of the Bovine Putative H19 DMR

Previous studies in mice and humans have indicated that H19 imprinting is controlled by epigenetic alteration to a differentially methylated region (DMR) located upstream of its promoter [5, 15, 23]. To verify whether the bovine H19 gene is controlled in a similar manner, a bovine genomic library was created and used to amplify and obtain the 5' H19 sequences for analysis. Our results indicate that, similar to other known H19 promoters, the bovine upstream sequence obtained by genome walking contains a large CpG rich region, confirming sequences currently available on the NCBI site of the *Bos taurus* chromosome 29 (NW_001494548.1). Our primer design allowed the identification of a methylated region of 28 CpG sites located inside a 550 bp fragment and positioned approximately 2.0 kb upstream of the H19 gene (Figure 1a). The next step was to determine by bisulfite sequencing the CpG-rich region parent of origin methylation in somatic tissues. Analysis of the methylation profile by bisulfite sequencing of genomic DNA recovered from fetal liver and in vitro cultured skin fibroblast cells indicated a methylation pattern

approaching the expected 1:1 paternal/maternal ratio, indicating that the CpG region could indeed be a DMR (Figure 4). Interestingly, the CpG island was not uniformly methylated throughout the analyzed region, indicating a rather patchy methylation of the DMR in the somatic tissues analyzed. To further characterize this putative DMR in cattle, we performed a sequence analysis using the Clustal W (1.81) software and a site for CTCF binding was found, which shared homology with similar CTCF sites identified in the H19 DMR of other species (Figure 1b). Although the CpG island configuration differed between species, three CpG islands were found inside the putative bovine CTCF binding site. A thorough comparison between *Bos indicus* and *Bos taurus* sequences was performed within the putative DMR region resulting in no reliable SNP, which prevented the verification of parental origin of the allele.

DMR methylation patterns in gametes and at pre- and post-implantation stages

In contrast to what was found in mice [15] and primates [23], which show complete methylation of the whole H19 DMR CpG island on the paternal allele, a recent ovine study also identified a patchy methylation pattern [24], suggesting that the degree of methylation of the H19 DMR varies among mammals. To further confirm the differentially methylated profile of the bovine putative H19 DMR, we examined the methylation patterns in immature GV-stage oocytes and in ejaculated spermatozoa. Oocytes were completely denuded of cumulus cells and the zona pellucida was removed using pronase digestion to exclude any source of contamination with somatic DNA. Similarly to mouse and humans, alleles amplified from bovine sperm were heavily methylated (100%, defined as percentage strands

with more than 50% CpGs methylated) (Figure 4). In contrast, oocyte alleles were almost completely unmethylated, confirming that the region analyzed was within a putative DMR.

Following the analysis of gametes, somatic cells and tissues, we examined the methylation profile of the putative H19 DMR in preimplantation embryos produced by AI, IVF and SCNT. A representative methylation pattern obtained from embryos in each group is shown in Figure 4. As observed in somatic tissues, a patchy methylation pattern was observed in all embryos analyzed, regardless of treatment group. All three experimental groups showed similar percentages of DNA methylation and, although H19 imprinting was observed in AI and IVF groups, overall methylation levels were not statistically different from SCNT (Figure 5a). Interestingly, a more consistent methylation pattern was observed in three CpG islands (19, 20 and 21) located within the CTCF binding site, where most of the methylated islands were concentrated (Figure 4). Based on this observation we decided to focus the analysis in this region separately. Figure 5b shows the results of the methylation levels at the CTCF binding site, where the higher level of methylated CpG islands (roughly 30%) were found in AI ($P<0.001$). In contrast, methylation profiles of the CTCF binding site within the putative H19 DMR were lower and did not differ among IVF (14%) and SCNT (15%), this regardless of the fact that only the SCNT group showed biallelic expression.

Methylation patterns in day 40 fetal (liver, brain, muscle and heart) and placental tissues showed a similar pattern to those found in day 17 embryos (Figure

6a), i.e. mostly patchy with focal methylation of CpGs within the CTCF site of the putative H19 DMR (Figure 4). The AI group showed general methylation levels varying from 6% (brain) to 12% (placenta), which were generally higher ($P<0.05$) than those observed in the IVF and SCNT groups (Figure 6a). Surprisingly, SCNT methylation levels were as low as or even slightly higher than IVF in a number of tissues, which contrasts with the allelic expression that showed exclusively mono-allelic expression in IVF and mostly bi-allelic expression in SCNT tissues. In an attempt to further verify the relationship of H19 DMR methylation and imprintinted expression after implantation, we also analyzed the methylation level at the CTCF binding site of the H19 DMR sequence. In contrast to the entire region analyzed, IVF methylation levels within the CTCF binding site were similar to AI in tissues such as brain (27 vs. 22%), heart (27 vs. 23%) and placenta (40 vs. 32%), and only slightly lower in liver (23 vs. 30%) and muscle (28 vs. 38%) (Figure 6b). On the other hand, most SCNT-derived tissues showed significantly lower methylation levels within the CTCF binding site (range from 7 to 18%) when compared to the AI and IVF groups. Placentae derived from the SCNT group showed the lowest methylation levels (7%), which is in accordance with the higher levels of paternal H19 expression (20%) in this tissue (Figure 3). Together, these results indicate that the methylation status within the CTCF binding site of the H19 DMR is more closely related to the parental-specific expression of the H19 gene in cattle than the flanking CpGs .

DISCUSSION

In this study we have characterized the putative H19 DMR, as well as the methylation and imprinting status of H19 during pre- and postimplantation development in cattle. We have also shown that SCNT dramatically alters the imprinting status and control mechanisms of the H19 gene. These findings were made possible by the identification of *Bos indicus/Bos taurus* polymorphisms, which enabled the parental-specific analysis of transcripts in tissues recovered from interspecies crosses during a critical window of early development in cattle.

Much of what is known about H19 imprinted genes and epigenetic control mechanisms has been stablished in mice and humans. In cattle, as previously published, H19 was found to be mostly maternally expressed in bovine tissues [9, 10]. Our results from early gestation concur with monoallelic expression. However, due to our detailed study on early development we also found that H19 expression is relaxed in preimplantation embryos, demonstrating a more bi-allelic status. Similar results were found in a recent study where bi-allelic expression of IGF2R (paternally imprinted gene) was observed in pre-implantation ovine embryos and became monoallelic after implantation at day 21 of gestation [24]. In another study, all imprinted genes analyzed such as Peg3, SNRPN, Ube3a and Zac1 showed bi-allelic expression in bovine blastocysts, with the exception of the Xist gene [25]. This results suggest that, particularly in ruminants monoallelic expression may not be required for most imprinted genes during preimplantation development, where monoallelic expression may develop in a gene- and time-dependent manner [25].

Interestingly, methylation levels of pre-implantation embryos were similar to somatic tissues and fetal tissues (Figure 4), where expression is monoallelic. It is known that H19 DMR in other species presents more than only one CTCF binding site (6 in sheep [26], 7 in humans, 6 in mice, and 3 in pigs [27]) and they could be acting in a time and/or tissue specific manner to regulate H19 gene expression. Only one CTCF binding site was identified within the putative DMR analyzed herein, which does not exclude that DNA methylation exerts control from neighboring regions. On the other hand, histone modification has been suggested to be a more ancient imprinting system, whereas DNA methylation, a more stable mark, would have evolved later to maintain imprinting [28, 29], suggesting that histone modification could account for the transition of H19 from bi-allelic to monoallelic status in cattle.

Some *in vitro* culture systems seem to perturb H19 imprinting in contrasting manners. Embryos cultured in human tubal fluid (HTF) (Quinn's advantage) media and the embryonic stem cells derived from such embryos displayed a high frequency of aberrant *H19* imprinting [30]. Bisulfite results revealed increased DNA methylation at a CTCF-binding factor site in the imprinting control region (ICR), as the normally unmethylated maternal allele acquired a paternal methylation [30]. However, studies using Whitten's medium showed loss of methylation of the paternal allele of H19 ICR, and consequently an increase in paternal expression of H19 gene transcript [14, 15]. Nonetheless, as reported with KSOM cultures in mice [14, 15], our results indicate that, when compared to the AI (*in vivo*) controls, exposure to *in*

vitro environments for the initial 10 d of development, i.e. throughout oocyte maturation (1 d), fertilization (1 d) and embryo culture (8 d), did not alter the patterns of parental-specific expression of H19 at the elongated (bi-allelic at day 17) and early fetal stages of development (monoallelic at day 40). However, a loss of methylation was found at the putative H19 DMR after *in vitro* culture at day 17, more precisely at the CTCF binding site (Figure 4), which was restored to AI levels by day 40 of gestation. Parent of origin methylation patterns associated to imprinted gene regulation are maintained from zygote to blastocyst, not being affected by demethylation waves during development [2]. Detrimental effects of some ingredients present in culture, i.e serum, may destabilize the maintenance of methylation in IVF embryos, which could affect their developmental outcome accounting for the lower pregnancy rates when compared to AI. In our study, serum was removed during fertilization and culture *in vitro* and perhaps, as cited above, imprinting failures were marginal at day 17 and reversed by day 40 of gestation.

Loss of methylation and imprinting failures are more severe in SCNT than IVF embryos [21, 31]. In such case, reconstructed embryos face not only potentially detrimental effects of *in vitro* culture and handling but also donor cell reprogramming. Dedifferentiation of the differentiated donor somatic cell to a totipotent embryonic state, followed by redifferentiation of cloned embryos to different somatic cell types during later development is essential for embryo development [32]. Thus, when studying nuclear reprogramming it is important to correlate aberrant methylation with abnormal expression of imprinted genes in the

cloned embryos. In our study we were able to analyze both allelic expression and methylation at the H19 DMR. Although paternal expression was only marginally higher at day 17, a generalized bi-allelic expression was observed in fetal tissues and placentae at day 40 compared to a strictly maternal in the AI and IVF groups. Therefore, transition from bi-allelic to monoallelic was not completed in cloned embryos, suggesting a disruption of imprinting after SCNT. Bi-allelic expression has been reported in bovine deceased clones [9] and, in mice [33], only 4% of cloned embryos had normal expression patterns of H19 along with other imprinted genes, which may explain the low success of cloning. As reported in mice [33], our SCNT methylation results at the CTCF binding site showed severe loss of methylation, suggesting that imprinting regulation of H19 is controlled by methylation of CpG at the CTCF position. Maintenance and *de novo* DNA methyltransferases have been found to be expressed in bovine preimplantation embryos [34], and any alteration in their reprogramming caused by *in vitro* culture and/or SCNT procedure may cause hypomethylation [35]. Finally, further characterization of other imprinted genes in and their regulation mechanisms in bovine models will provide additional information to understand imprinting in mammals and consequently, improve the success ART.

ACKNOWLEDGEMENTS

Supported by the Canada Research Chair (CRC) program and the Natural Sciences and Engineering Research Council (NSERC) of Canada. This work was part of the Program on Oocyte Health (http://www.ohri.ca/oocyte) funded by grant HGG62293 under the Healthy Gametes and Great Embryos Strategic Initiative of the Canadian Institutes of Health Research (CIHR) Institute of Human Development, Child and Youth Health. JS was supported by a studentship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil.

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FIGURE 1. Schematic structure of the bovine H19 gene and putative DMR with its CTCF binding site. (**a**) Genomic organization of the bovine H19 gene based on the *Bos taurus* sequence [9]. Numbered boxes indicate exons and lines indicate intron distribution. Region (550 bp) within the putative differentially methylated region (DMR; grey box) is amplified to indicate the 28 CpG (circles) and the CTCF binding site (box) containing 3 CpG. (**b**) Alignment of the bovine CTCF binding site region analyzed with homologous regions in mouse and human was performed using ClustalW. Asterisks indicate aligned bases. (**c**) Sequence chromatograms of the H19 locus amplified from cDNA obtained from *Bos taurus* and *Bos indicus* genomic DNA indicating the "G" and "A" SNP utilized to identify transcripts of maternal and paternal origin, respectively.

Fig. 1a

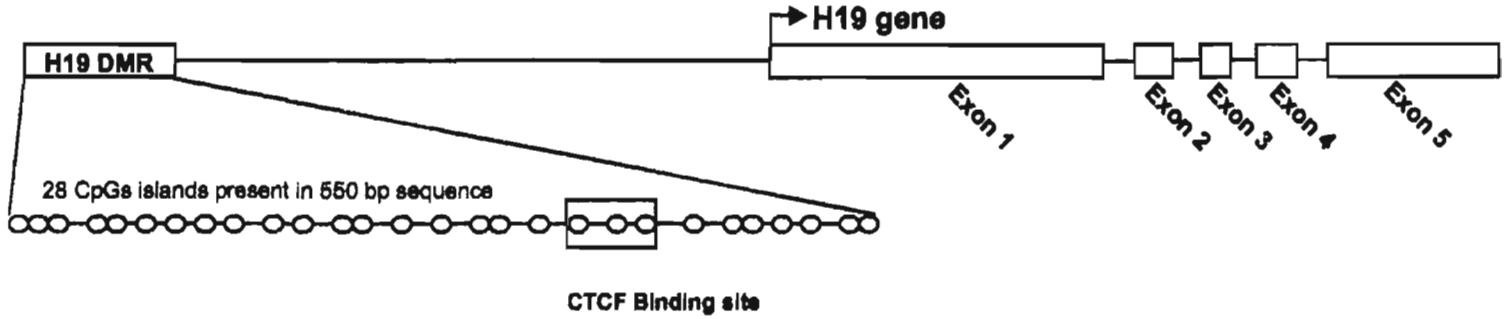

Fig. 1b

CTCF binding site sequence homology
Using CLUSTAL W (1.81) multiple sequence alignment

```
Mouse     -TGCC GCACGGCGGCAGTGAAGTCTCGTACATCGCAGTCCTA
Rat       -TGCC GC GTGCCGGCAGTGAAGTCGCGTACATCGCATCCCTG
Human     TGGCCGCGCGGCGGCAGTGCAGGCTCACACATCACAGCCCGA
Bovine     C GGCTGCGAGGTGGCAGTGCAGGCTCACACATCAGCGGTGGTG
              ** **  *  ******* ** * *  *****
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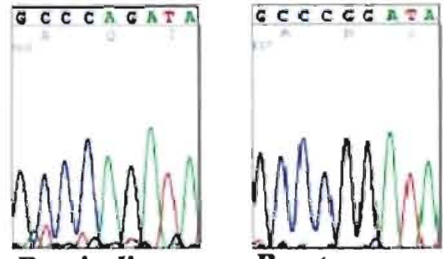
Fig 1c



Bos indicus *Bos taurus*

FIGURE 2. Allele-specific expression analysis at day 17 of gestation in F1 embryos produced by AI (*in vivo* – black bars), IVF (*in vitro* culture – white bars) and SCNT (gray bars). After RNA extraction, H19 cDNA fragments were amplified by PCR, cloned into a plasmid vector and sequenced to identify the parental-specific SNP. Ratios were based on the total number of clones with paternal allele (*Bos indicus*) found over the total number of clones sequenced.

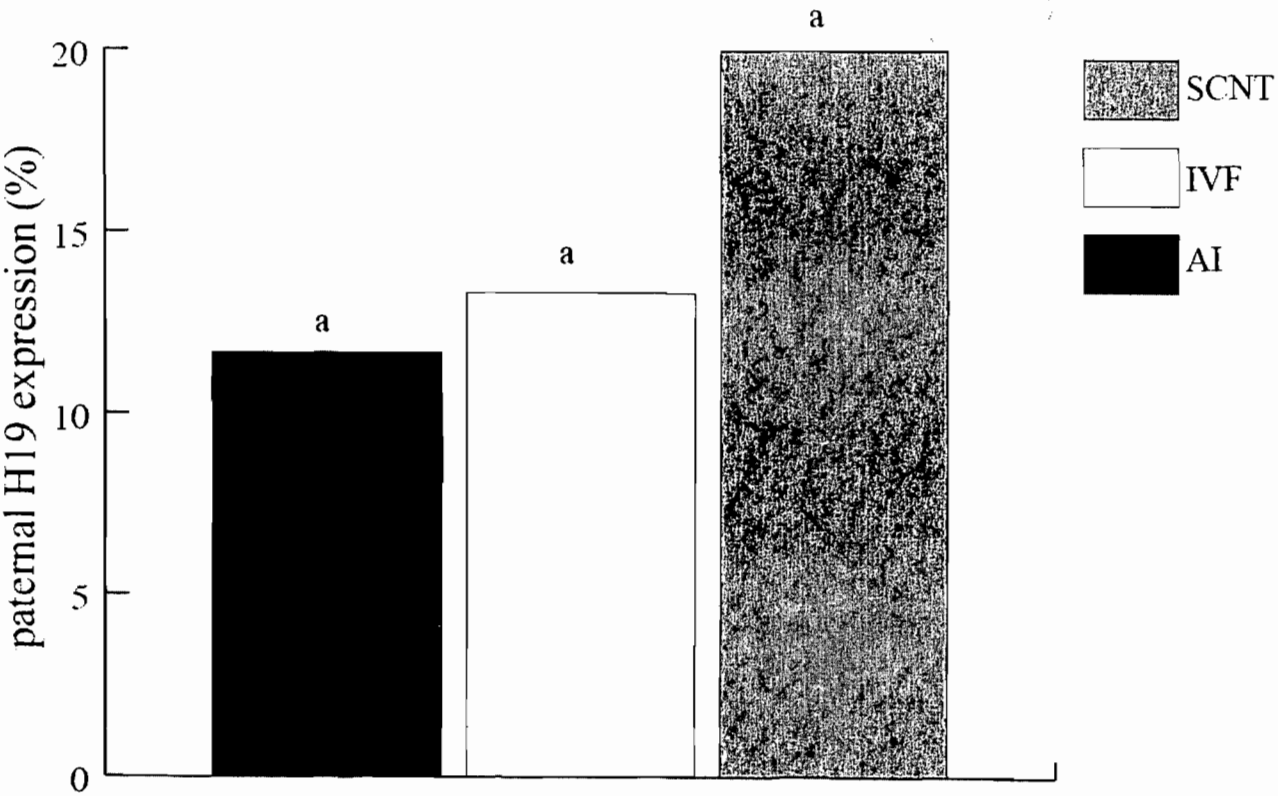

a
a
a
20
15
10
5
0
paternal H19 expression (%)
SCNT
IVF
AI

FIGURE 3. Allele-specific expression analysis at day 40 of gestation in F1 tissues (brain, heart, liver, muscle and placenta) produced by AI (*in vivo* – black bars), IVF (*in vitro* culture – white bars) and SCNT (gray bars). After RNA extraction, H19 cDNA fragments were amplified by PCR, cloned into a plasmid vector and sequenced to identify the parental-specific SNP. Ratios were based on the total number of clones with paternal SNP (*Bos indicus*) over the total number of clones sequenced. Letters describe group differences within each tissue analyzed ($P<0.05$).

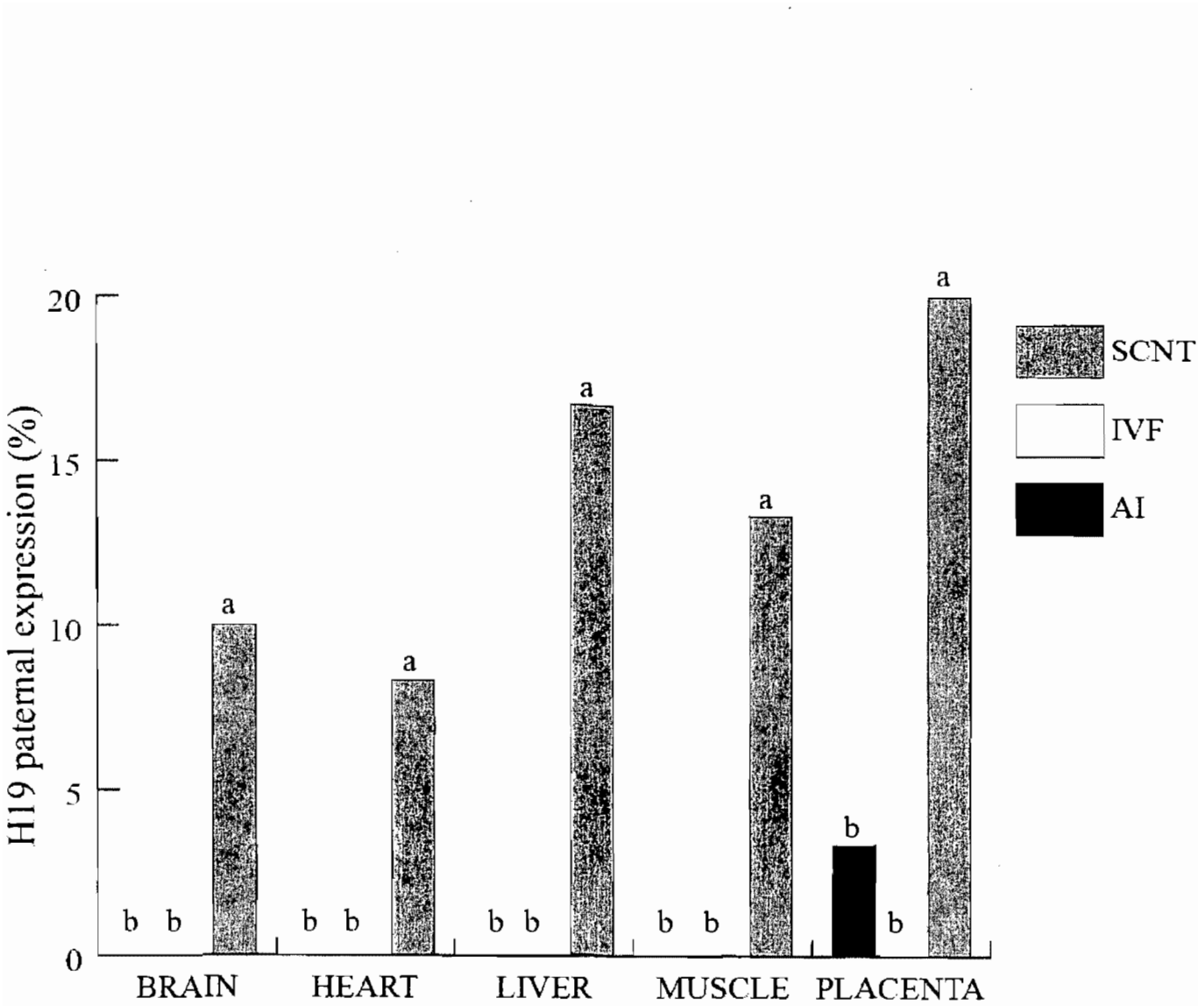

H19 paternal expression (%)
20
15
10
5
0
b b
a
b b
a
b b
a
b b
a
b
b
a
BRAIN
HEART
LIVER
MUSCLE
PLACENTA
SCNT
IVF
AI

FIGURE 4. Methylation status of the bovine putative H19 DMR with location of the CTCF binding site. Representative bisulfite methylation analysis of **(a)** GV-stage oocytes; **(b)** spermatozoa; **(c)** *in vitro* cultured adult skin fibroblasts used for SCNT; (d, e, f) elongated day 17 embryos produced by **(d)** AI, **(e)** IVF, **(f)** SCNT; and (g, h, i) placenta from day 40 gestations derived by **(g)** AI, **(h)** IVF, **(i)** and SCNT. Circles represent either methylated (filled) or unmethylated (open) CpG sites and rectangles indicate the 3 CpG found within the CTCF binding site.

Fig 4

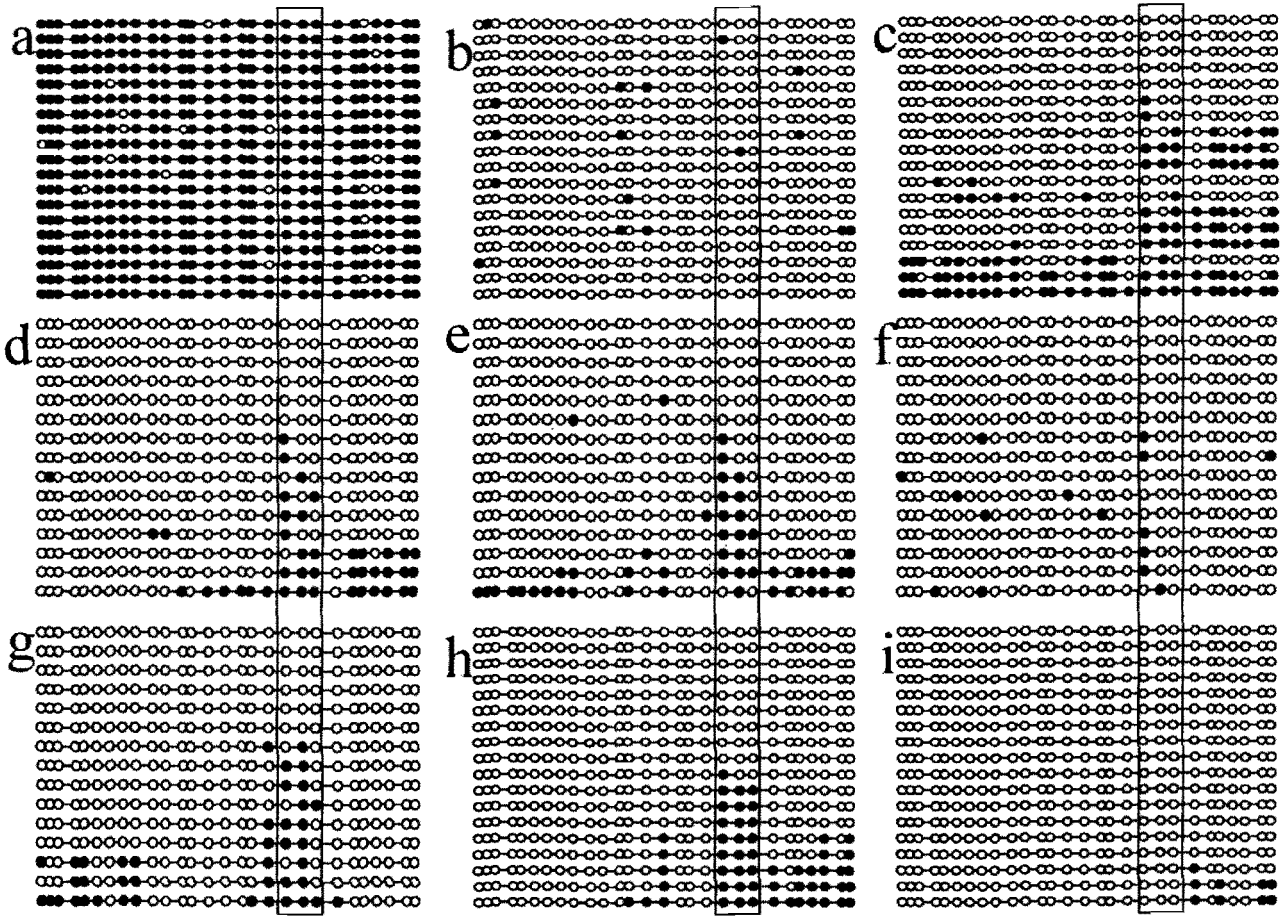


FIGURE 5. Methylation profile of putative H19 DMR in day 17 embryos. Percentages are calculated from **(a)** the whole 55 bp DMR region analyzed and **(b)** exclusively within the CTCF binding site (CpGs 19,20 and 21) of day 17 embryos derived by AI (*in vivo* – black bars), IVF (*in vitro* culture – white bars) and SCNT (gray bars). The frequencies of methylated sites in groups were analysed using Chi-Square test and letters indicate significant differences ($P<0.05$).

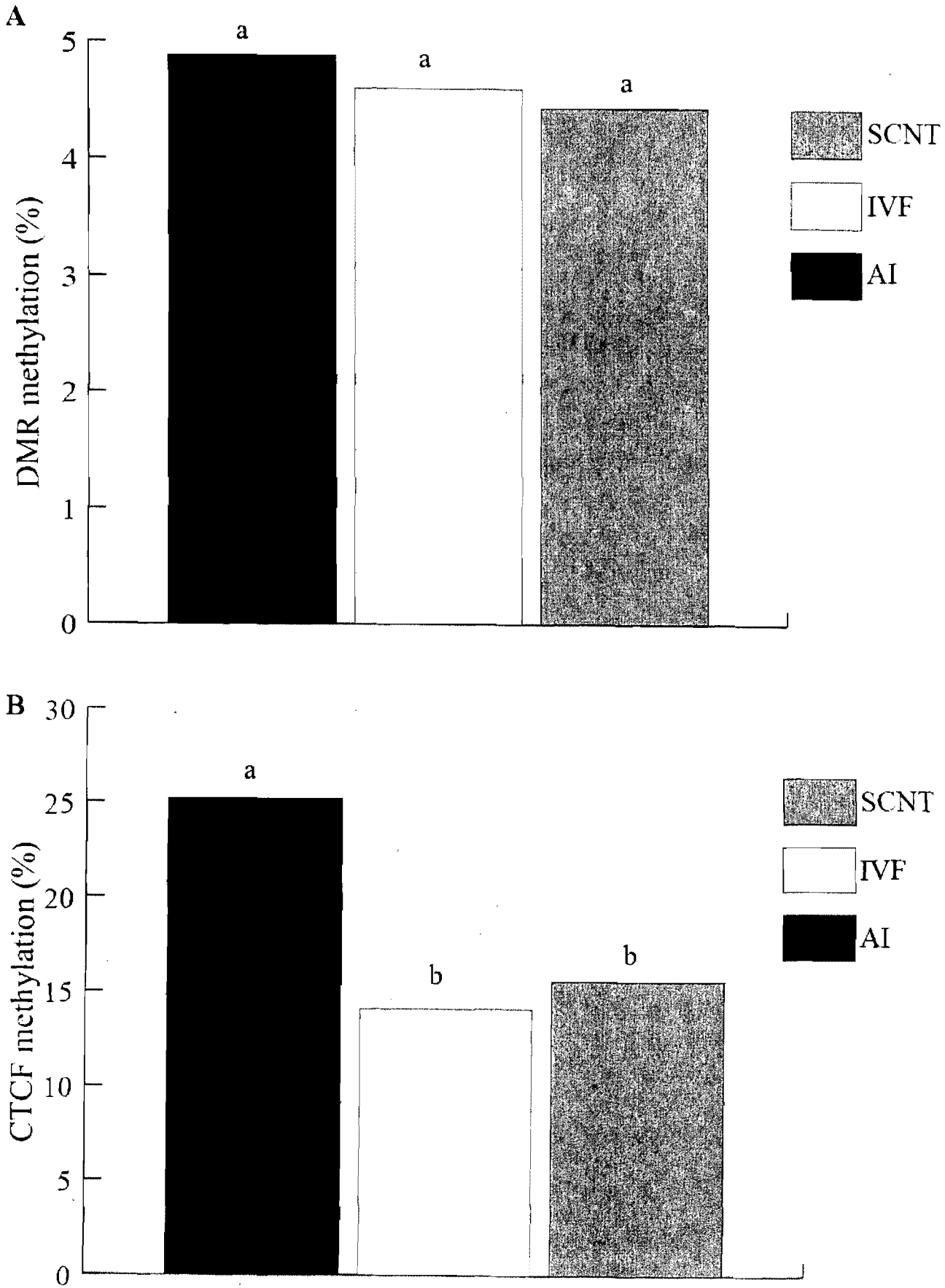
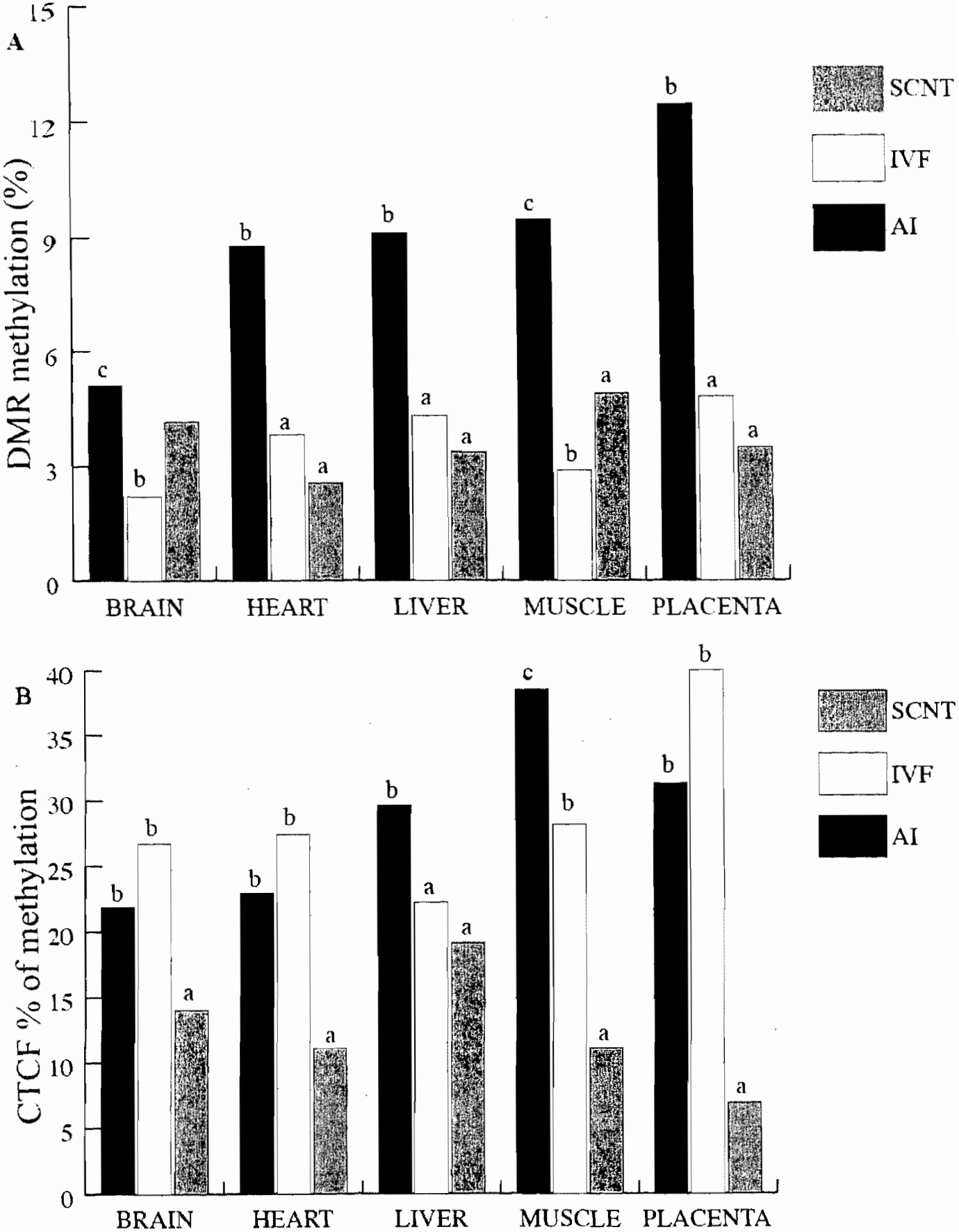

A
DMR methylation (%)
0
1
2
3
4
5
a
a
a
SCNT
IVF
AI
B
CTCF methylation (%)
0
5
10
15
20
25
30
a
b
b
SCNT
IVF
AI

FIGURE 6. Methylation profile of putative H19 DMR in tissues obtained at day 40 of gestation derived by AI (*in vivo* – black bars), IVF (*in vitro* culture – white bars) and SCNT (gray bars). Percentage obtained from **(a)** the whole 55 bp DMR region analyzed and **(b)** within the CTCF binding site (CpGs 19, 20 and 21) of day 40 tissues (brain, heart, liver, muscle and placenta). The frequencies of methylated sites were analysed using Chi-Square test and letters indicate significant differences ($P<0.05$).


A
DMR methylation (%)
15
12
9
6
3
0
c
b
b
a
a
b
a
a
c
b
a
b
a
a
BRAIN
HEART
LIVER
MUSCLE
PLACENTA
SCNT
IVF
AI
B
CTCF % of methylation
40
35
30
25
20
15
10
5
0
b
b
a
b
b
a
b
a
a
c
b
a
b
b
a
BRAIN
HEART
LIVER
MUSCLE
PLACENTA
SCNT
IVF
AI

CHAPTER III

In vitro culture and somatic cell nuclear transfer (SCNT) affect imprinting of SNRPN gene in pre-implantation embryos and placenta of cattle

Joao Suzuki, Jacinthe Therrien, France Filion, Rejean Lefebvre, Alan K. Goff and Lawrence C. Smith*

Centre de Recherche en Reproduction Animale (CRRA), Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, J2S 7C6 Canada

* Corresponding author

Email addresses:

[redacted]

ABSTRACT

Background

Embryo *in vitro* manipulations during early development are thought to increase mortality by altering the epigenetic regulation of some imprinted genes. Using a bovine interspecies model with a single nucleotide polymorphism, we assessed the imprinting status of the SNRPN bovine gene in embryos produced by artificial insemination (AI), *in vitro* culture (IVF) and somatic cell nuclear transfer (SCNT) and correlated allelic expression with the DNA methylation patterns of a differentially methylated region (DMR) located on the SNRPN promoter.

Results

In the AI group, SNRPN is maternally imprinted at day 17 and 40 of development and a third of the alleles analyzed are methylated in the DMR. In the IVF group, maternal transcripts were identified at day 17 but methylation levels were similar to the AI group. However, day 40 fetuses in the IVF group showed significantly less methylation when compared to the AI group and SNRPN expression was mostly paternal in all fetal tissues studied, except in placenta. Finally, the nuclear transfer group presented severe loss of methylation patterns in both day 17 embryos and 40 fetuses and biallelic expression was observed in all stages and tissues analyzed.

Conclusions

Together these results suggest that, in combination with *in vitro* culture, SCNT leads to abnormal reprogramming of imprinting of SNRPN gene by altering methylation levels at this locus.

Background

The procedure of SCNT in mammals results in pregnancy rates much lower than those obtained *in vivo* after insemination and from transfer of embryos derived *in vitro* [1-4]. Furthermore, cloned fetuses that survive to term often have disorders such as oversized organs, increased or decreased overall growth, respiratory failure and limb malformations. In cattle and other ruminants, these abnormal phenotypes are known as the large offspring syndrome, or LOS [5, 6]. Detailed examination of the extra embryonic membranes of SCNT pregnancies often highlights numerous placental abnormalities, including a reduction in the number of cotyledons, and a decrease in chorio-allantoic blood vessels. These observations are also consistent with other reports where no placentomes were observed in the placenta in pregnancies that were lost between days 30 and 60 of gestation [7, 8]. Together, these results suggest that improper development of the placenta may play a major role in the fetal abnormalities and low pregnancy rates in cattle SCNT. It has been suggested that the pathological phenotypes in the placental and fetal development of clones are associated with abnormal reprogramming by the host ooplasm of the donor cell used for nuclear transfer [9]. These abnormalities often disturb the epigenetic regulation mechanisms inherited from the differentiated donor cell, by altering the dynamic nature of DNA methylation and chromatin modification patterns during embryo development [10]

One of the most studied epigenetic modifications is DNA methylation of cytosine residues within CpG dinucleotides; these are often associated with transcriptional

repression and implicated in maintaining genomic stability, as well as silencing repetitive elements. DNA methylation is also implicated in the regulation of genomic imprinting, genes that are exclusively expressed from only one parental allele [10]. To date, only a few imprinted genes have been characterized in cattle [11-15] and most play essential roles in fetal development and placental function. The bicistronic gene SNURF-SNRPN, referred here as SNRPN, has been extensively studied in mice and humans due to the correlation between disorders within the SNRPN differentially methylated region (DMR) and the pathogenesis of neurodevelopmental disorders known as Prader-Willi Angelman and Beckwith Wiedemann syndromes. Interestingly, decreased levels of the maternal allele methylation in the SNRPN DMR has been observed in children conceived by assisted reproductive technologies (ART), suggesting that the SNRPN methylation pattern is affected by *in vitro* culture systems [16, 17]. As demonstrated previously in cattle [18], the SNRPN gene is also maternally imprinted in preimplatantion bovine embryos, with a characterized DMR. However, little is known about the effect of altered DNA methylation patterns on allelic expression of the SNRPN gene. By using a bovine interspecies model [*Bos indicus* (paternal genome) x *Bos taurus* (maternal genome)] to assess genomic imprinting, our objective was to characterize the imprinted status of SNRPN before (day 17) and after (day 40) implantation, to determine whether the pattern of gene expression is associated with DNA methylation levels, and finally to examine short and mid term effects of in vitro culture on imprinting status of SNRPN gene in

embryos produced by IVF and SCNT, we also evaluated pre- and post-implantation embryos derived IVF and SCNT.

RESULTS

Development during early gestation

To characterize the imprinted status of SNRPN gene in bovine embryos, we artificially inseminated superovulated cows to obtain *in vivo* controls (AI group). Embryos were allowed to develop until 17 days after insemination and were collected by flushing the uterine horns. Due to low RNA and DNA yield, only whole embryos were used in this experiment. A total of 3 intact embryos were collected (Table 1). Our next goal was to examine the development *in vivo* of SCNT and IVF blastocysts during the first week after transfer. *In vitro* development of IVF and SCNT was assessed at day 8 of *in vitro* culture and developmental rates were similar for both groups (Table 1), indicating that the prolonged handling of the oocytes during SCNT was not detrimental to the early stages of development *in vitro*, and that the SCNT protocol used is suitable for embryo development and comparable to standard protocols. Groups of 10 blastocysts were transferred non-surgically to synchronous recipients and recovered at day 17 after estrus. Fewer day 17 embryos were collected from the SCNT than the IVF group, however no significant difference was observed (Table 1). Table 1 shows the results of blastocyst development and day 17 recovery for all experimental groups.

To obtain day 40 fetuses in the IVF and SCNT groups, 1-2 embryos were transferred per recipient and viability was checked the day before slaughter. As expected survival rates were higher for IVF than for SCNT (Table 1). For the control *in vivo* (AI group) day 40 fetuses, a total of 4 recipients were inseminated and 3 pregnancies were confirmed at day 39 of gestation and slaughtered the following day.

Allelic expression profiles of SNRPN gene

DNA and RNA were extracted, purified and used as a template for sequencing and searching for single nucleotides polymorphisms (SNPs) between the two *Bos taurus* and *Bos indicus* subspecies. Based on a published sequence (AF101040 access number), we detected a G/A *Bos indicus/Bos taurus* SNP at position +151 located at exon 2 of the SNRPN upstream reading frame protein [19], encompassing exons 1-3, and corresponding to the 71 amino acid protein SNURF (Figure 1). PCR was performed using SNRPN primers set on genomic DNA samples obtained from *Bos taurus* (maternal control), *Bos indicus* (paternal control). According to Figure 1, "G" mutations was specific to *Bos indicus* demonstrating that the interspecies breeding used in this experiment is suitable for gene expression analysis.

SNRPN expression was observed in all groups at day 17 and the G/A SNP was detected in the fragment amplified from genomic DNA. In the AI group, expression of the SNRPN gene was totally paternal (Figure 2), thus demonstrating the imprinted status of SNRPN gene locus. In the IVF group, bi-allelic expression was observed at

day 17 (Figure 2). Although the paternal expression was higher in IVF animals, average values were not different from SCNT (Figure 2). However, in SCNT embryos, more than 30% of total expression was maternal ($P<0.001$) and when individual values for percentage of methylation and percentage of paternal expression of SNRPN gene were assessed, only SCNT embryos showed exactly 50% of paternal expression.

Samples of liver, muscle, brain, heart and placenta collected from day 40 fetuses were analyzed qualitatively for mRNA expression (Figure 3). All AI samples showed monoallelic expression, except heart and placenta, where a leaky maternal expression was observed, although not enough to be considered bi-allelic, demonstrating that imprinting was maintained after embryo implantation. IVF embryonic tissues in general showed mostly paternal expression of the SNRPN gene. Leaky expression was observed in the IVF group in liver and muscle. Interestingly, bi-allelic expression was found in placenta, suggesting that imprinting was not properly reestablished after *in vitro* culture, particularly in this tissue. In the SCNT group all tissues showed bi-allelic expression and maternal expression levels that were higher than 15%. Heart, liver and placenta were the most affected, where more than 20% was maternally expressed. Together, the results of allelic expression indicate that SNRPN gene is maternally imprinted at preimplantantion stages and this status is maintained throughout development until day 40 in all embryonic tissues analyzed in our control group (AI). However, the placenta seemed to be

affected by *in vitro* culture, since bi-allelic expression mode continued even after implantation in the IVF group.

Methylation analysis of the SNRPN DMR

Once imprinting status was characterized, we assessed the methylation of the SNRPN DMR. Genomic DNA was extracted from the samples and after bisulfite reaction, the ratio of methylated CpG sites over the total number of CpG sites was evaluated. In bovine preimplantation embryos, parent of origin methylation was represented by roughly 40 to 50% of methylated versus non methylated sites [18]. To validate the method we used for colony picking, we mixed equal proportions (50% of each) of bisulfite treated DNA extracted from germinal vesicle (GV) oocytes (fully methylated CpG sites) with frozen sperm (non methylated sites) (data not shown). Figure 4 shows representative CpGs levels at the SNRPN DMR obtained from the bisulfite treatment. The percentage of paternal alleles was approximately 38%, which is roughly consistent with the percentage results of methylated sequences found in the AI control groups from previous publications [18]. Once the method was validated we assessed the SNRPN DMR methylation levels in the AI control group. In bovine preimplantation embryos, parent of origin methylation is often represented by roughly 50% of methylated versus non methylated sites [18]. We confirmed that AI day 17 embryos maintained differentiated methylation patterns inherited from gametes, as roughly 40% of SNRPN DMR was found to be methylated (Figure 5). IVF group methylation ratio was not different from the AI

group with a percentage of approximately 30% of methylated sites (Figure 5), suggesting that *in vitro* culture effects were detrimental to methylation maintenance. Severe loss of methylation was observed in the SCNT group, where less than 5% of methylated sites were observed. Similar results were found in clones produced by traditional methods of zona intact enucleation [18] and we could confirm that, in terms of methylation outcome, hand made cloning [20] has comparable results. Interestingly, we observed that embryos with lower methylation levels also showed lower levels of paternal expression, particularly from the SCNT group (Table 2).

Similar methylation patterns to those seen in AI day 17 embryos were also observed in AI day 40 fetuses (Figure 6). Almost all tissues showed 40% of overall methylation, supporting the hypothesis that parent of origin methylation is maintained throughout embryo development. Surprisingly, heart samples showed very low levels of methylation, although gene expression was mostly paternal. In the IVF group significantly lower methylation levels were found in all tissues except heart, where methylation ratio was comparable to the AI control. As in day 17 embryos SCNT, loss of methylation levels was observed in day 40 fetuses, suggesting that abnormal methylation levels are not corrected by in vivo environment and are rather maintained through gestation. These results suggest that methylation failures acquired during early stages persist throughout the development of embryo.

Interestingly, when results from allelic expression and methylation ratio were combined, particular patterns were observed in different tissues. For instance, during

pre-implantation development, methylation and paternal SNRPN expression had a positive association (Table 2), where AI embryos showed highest methylation ratio and mostly paternal expression, IVF embryos showed intermediate, yet not different from AI, and mild bi-allelic expression and finally SCNT embryos developed bi-allelic expression and had the lowest methylation ratio observed. After implantation certain tissues such as brain and placenta developed some particularities within the same group analyzed. For example, although similar methylation levels were found in AI brain and placenta (Table 3), allelic expression was different, being monoallelic in brain and leaky maternal expression in placenta. Particularities were also found among the 3 groups; for instance, in heart tissue, even though a difference in methylation ratio was found between IVF and SCNT animals, both groups showed an overall methylation ratio below 10% (Figure 6). In an attempt to correlate methylation patterns with expression a bivariate analysis was performed on data from day 17 embryos and from the separate tissues from day 40 fetuses (Figure 7). A positive correlation was found between expression and methylation in day 17 embryos ($P<0.0006$), and brain of day 40 fetuses ($P<0.0003$). Day 40 liver, muscle and heart tended to show some correlation with $P<0.04$; $P<0.030$; $P<0.040$ respectively. Placenta tissues showed no correlation between expression and methylation patterns ($P<0.10$). Together, these results suggests that in general methylation of SNRPN DMR is positively associated with allelic expression, however the association seems to be stronger in particular tissues.

DISCUSSION

The use of a *Bos indicus/Bos taurus* interspecies model enabled us to provide additional information on imprinting regulation of the SNRPN gene in cattle by analyzing simultaneously the previously published methylation status of SNRPN gene in preimplantation embryos [18] and the allelic expression. Furthermore, we characterized SNRPN imprinting in post-implanation embryos and the effect of *in vitro* culture and SCNT at the transitional period between elongated pre-implantation embryos to early gestation.

Imprinted gene profiles have been previously reported in ruminants [11, 13, 14], and the interest in these genes arises from their implication in embryo and fetal development. In many clinical cases in humans or livestock animals, the association of abnormalities found in pregnancies resulting from ART with abnormal expression of imprinted genes is often found. A better knowledge of imprinted genes could provide clues to understand and improve *in vitro* culture conditions. Ultimately, in SCNT, imprinting analysis is essential to define is the ability of the oocyte to reprogram the epigenetic memory of somatic donor cells.

The maternally imprinted SNRPN gene has been extensively studied in mice and humans [21] due to its association to Angelman and Prader-Willi syndromes; it has now also been putatively linked to ART and infertility [16]. Although ART has been extensively used in the bovine species, little is known about SNRPN imprinting status. Although the methylation ratio of IVF embryos was not different from the AI group, we have found the SNRPN gene to be bi-allelic expressed in preimplantation

IVF embryos at day 17 subjected to *in vitro* culture. Recently, bi-allelic of SNRPN gene was found in bovine blastocyst, suggesting that monoallelic expression may not be required for most imprinted genes during preimplantation development, where monoallelic expression may develop in a gene- and time-dependent manner [22]. However, results from our AI control showed no sign of bi-allelic expression, whereas bi-allelic expression found in IVF embryos perpetuated to post-implantation stages, suggesting that placenta tissues might be more susceptible to the effects of *in vitro* embryo culture. In our study, bi-allelic expression extended to post-implantation development in day 40 placenta, and since pre-implantantion embryos are mostly composed of extraembryonic tissue, this indicates that imprinting was already perturbed during earlier development. In the placenta, imprinting is probably regulated by other mechanisms than DNA methylation to establish imprinting. In fact, mice studies revealed that imprinting establishment of *Xist* gene does not require the DNA maintenance methyltransferase DNMT1 [23]. Instead, the process of X chromosome inactivation is rather dependent on histone modifications associated with transcriptional repression by H3K9me and histone H3 methylated lysine 27 (H3K27me) as well as the Polycomb H3K27 methyltransferase complex, which is involved in the maintenance of transcriptional repression [23, 24]. In support of these results we found the lowest association between methylation and allelic expression in placenta (Figure 7). However, methylation ratio was diminished in embryonic tissues and we do not exclude the possibility of further complications due to loss of methylation. Nonetheless we consider that a wide range of genes could

be affected by *in vitro* embryo culture, as reported for preimplantation mouse embryos [25]. These results provide evidence for the hypothesis that placental tissue is more affected by *in vitro* culture of embryos than embryonic tissues [6, 25, 26], and such abnormalities could be related the problems observed in later pregnancies, when the placenta becomes more important to fetal development.

As previously published, SNRPN expression in SCNT embryos and fetuses showed severe loss of methylation [12]. Maternal expression of around 30% was observed in day 17 embryos (Figure 2). Bi-allelic expression also persisted in day 40 extra-embryonic and embryonic tissues, although maternal expression was less pronounced. It is likely, therefore, that problems with pregnancies are initially due to defects at the level of the placenta, rather than the embryo. In support to this theory, some underdeveloped blastocysts (vesicle state) were found in SCNT day 17, but not in IVF, no pathology was found in day 40 embryonic tissues, and the embryo sizes were normal and pregnancy went normally until collection. However, at day 40, the placenta in the SCNT group had no placentomes and no visible signs of vascularization of the choriallantoic membrane. Similar observations have been previously reported in ruminants by Dindot *et al* [7]. These results found in pre-and post-implantation trophoblastic tissue raise the question of whether abnormal expression observed in embryonic tissues is a consequence of donor cell reprogramming failures, or if early anomalies found in trophoblasts would eventually result in abnormal expression of imprinted genes observed in day 40 fetal tissues. Another possibility would be that defects caused by SCNT, other than those related

to genomic imprinting, affect cell fate choices in early development and that cells with more anomalies (competence, cell division, polyploidy) are preferentially incorporated into the trophectoderm rather than the inner cell mass (ICM), as seen in tetraploid complementation [27]. However, loss of methylation in embryonic tissues seems associated with reprogramming failures of the donor cell [28]. Studies indicate that the methylation of imprinted genes is maintained throughout embryo development and determines either the repression or expression of these genes while the rest of the genome becomes demethylated [10]. Probably failures in donor cell reprogramming and detrimental effects of in vitro culture could together account for the severe abnormalities found in the placentas of SCNT.

To our knowledge, this study is the first to compare methylation directly with imprinting status in different tissues during pre- and post-implantation stages of development. More studies are needed to determine whether, in cattle, there are other DMRs acting on the same locus, or if another imprinting mechanisms, i.e. histone acetylation, play a role as important as DNA methylation in the control of SNRPN expression.

CONCLUSIONS

Bi-allelic SNURF-SNRPN gene expression was found in IVF and SCNT preimplantation embryos subjected to in vitro culture, which extended only in fetal

tissues of cloned cattle. Loss of methylation was also found in extra and embryonic tissues of pregnancies derived by IVF embryos cultured *in vitro*. Furthermore, bi-allelic expression was observed, in placenta, but not fetal tissues. Thus, we postulate that the detrimental effects of *in vitro* culture on pre- and post-implantation, play an important role in the establishment of SNRPN, particularly in placenta tissues, and in SCNT, is aggravated by failures in donor cell reprogramming.

METHODS

All procedures were performed in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Training, approved by the animal experimentation committee of the Université de Montréal sanctioned by the Canadian Council on Animal Care.

Nuclear donor cells

Fetal fibroblast cell cultures were established from a 60-day-old crossbred fetus produced by AI of a Holstein (*Bos taurus*) heifer with semen from a Nelore (*Bos indicus*) bull. Fetal tissues (brain, heart, liver, muscle and placenta) were minced manually and digested with 0.25% trypsin and 0.02% EDTA (Gibco BRL, Burlington, ON, Canada) at 37 °C for 10 min. Isolated cells were washed and cultured for approximately 4 d in Dulbecco modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 0.5% antibiotics (penicillin 10000 U/ml and streptomycin 10 000 µg/ml; Gibco BRL) at 37

°C in 5% CO_2. When the cultures were confluent, primary passage cells were frozen in culture media supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen. Donor cells were thawed at 37 °C for 1 min and cultured to confluence for a maximum of 5 passages before use as donor cells.

Host oocytes

Cattle ovaries were collected from a local abattoir and transported to the laboratory in saline at 30–35 °C within approximately 2 h after slaughter. Follicles with diameters between 2 and 10 mm were punctured with a 18-gauge needle, and cumulus oocyte-complexes (COCs) with approximately 4 to 6 layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL) supplemented with 10% (vol/vol) FBS. Groups of 20 COCs were placed in 100 µl of bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 µg/ml LH (Ayerst, London, ON, Canada), 0.5 µg ml/ml FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 µg ml/ml estradiol 17-β (Sigma-Aldrich, St. Louis, MO), 22 µg ml/ml pyruvate (Sigma-Aldrich), and 50 µg/ml gentamicin (Sigma-Aldrich). After 19 to 20 h of *in vitro* maturation, cumulus cells were removed from the COCs by vortexing for 2 min in PBS and 0.2% hyaluronidase (Sigma-Aldrich). Only oocytes with homogeneous cytoplasm and intact cell membrane were selected for micromanipulation.

***In vivo* and *in vitro*-derived embryos**

Production of embryos and fetuses for *in vivo* and *in vitro* controls, as well as donor cells were conducted as described previously [18]. Briefly, *in vivo*-derived embryos were obtained from Holstein heifers that were superovulated by intramuscular injection of porcine FSH (Folltropin-V) given every 12 h in decreasing doses beginning with 60 mg at day 9, 50 mg at day 10, 30 mg at day 11 and finally 20 mg at day 12 of the estrous cycle. At day 13, cows received an intramuscular injection of 500 μg of cloprostenol (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) and were artificially inseminated (AI) 48 h later.

In vitro-produced (IVP) embryos were derived using standard protocols [12]. Briefly, bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 4 h in saline at 32 °C. Cumulus-oocyte complexes (COC) were aspirated from ovarian follicles using a 17-gauge needle and selected for the procedure. For *in vitro* maturation (IVM), groups of 20–25 COCs were cultured in 100 μl drops of Tyrode medium supplemented with 0.6% BSA (fraction V; Sigma-Aldrich), lactate, pyruvate, gentamicin, and heparin (10 μg/ml). For *in vitro* fertilization (IVF), frozen-thawed spermatozoa were washed and centrifuged through a Percoll (Sigma) gradient and diluted to 10^6 live spermatozoa/ml. After 24 of IVM, COCs were added to fertilization drops for 20 h, with spermatozoa. Oocytes were then denuded of cumulus cells by brief shaking. For *in vitro* culture (IVC), putative IVF zygotes were transferred to 25 μl drops of synthetic oviduct fluid (SOF medium)

and cultured for 8 d, with additional 25 µl of SOF medium. The same IVC conditions were used for the oocytes reconstructed by SCNT.

Nuclear Transfer

The SCNT protocol used was a slight modification from a previous report of hand made cloning (HMC) [20]. Oocytes were selected in groups of 100 and placed in 1.5 mg/ml pronase in TCM 199 supplemented with FBS 10% for about 4 min. Zona-free oocytes were washed thoroughly in TCM supplemented with FBS 20% for 3 min and cultured in 0.4 µg/ml demecolcine for at least 30 min. Treated oocytes with a visible protruding membrane were placed in medium supplemented with 5.0 µg/ml cytochalasin and 10% FBS and manually bisected using a micro blade on a stereomicroscope. After bisection, oocytes were stained with 2 µg/ml Hoescht 33342 and checked for the absence of chromatin. Nuclear donor cells were thawed, washed and placed in 50 µl of culture media (DMEM, supplemented with 10% FBS and 0.5% antibiotics). Nuclear transfer was performed using confluent cells that were maintained in culture for 3–5 passages. Cytoplasts were placed individually in a 50 µl drop containing 500 µg/ml of phytohemagglutinin (Sigma) for about 3 sec and then quickly positioned over a single donor cell placed at the bottom of the dish. After attachment of the donor cell, the cytoplast-somatic cell pairs were placed in 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.2 kV electric pulse lasting 70 µsec. After electrical stimulation, couplets were washed and cultured individually in 10 µl drops of 6-dimethylaminopurine (DMAP,

Sigma-Aldrich) for 3 h. After DMAP treatment, reconstructed oocytes were washed and cultured in 40 µl drops of SOF modified medium supplemented with 0.8% BSA-V fatty acid free (Sigma-Aldrich) under equilibrated mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. Embryos were cultured in groups of 4 per drop in small individual wells (500 µm diameter) prepared with a sterile needle to avoid separation of blastomeres during development. Reconstructed embryos were cultured *in vitro* for a period of 8 d.

Day-17 Elongating Embryos and Day-40 fetuses

The estrous cycle of Holstein heifers was synchronized by an injection of 500 µg of the prostaglandin F2α analogue, cloprostenol (Estrumate, Schering Canada Inc). Six to 8 d after the standing heat, day-8 *in vitro*-produced or SCNT blastocysts were transferred to the uterine horn ipsilaterally to the corpus luteum. Embryos were washed with TCM-199 Hepes-buffered medium supplemented with 10% of FBS, loaded into a 250 µl straw and transferred to recipient heifers. One group of heifers received between 10 to 15 day-8 IVF or SCNT embryos and allowed to develop for another 9 d in the uterine horn. Day-17 elongated embryos were non-surgically recovered by flushing the uterus of the recipient heifers with PBS using a Foley catheter. Embryos were removed from the flushing media and inspected to select those that were recovered intact. After selection, embryos were washed three times in PBS and frozen individually at -70 °C in 0.2 ml of distilled water. Only those embryos that were recovered intact were used for the experiments. The second group

of heifers was allowed to continue gestation to day 40 after SCNT or IVF. Recipients carrying fetuses with a normal beat heart were slaughtered at the local slaughterhouse and transported within approximately 1 h. Samples from liver, muscle, heart, brain and placenta were collected from each viable gestation, snap-frozen in liquid nitrogen and stored at -70 °C until further analysis.

Bisulfite Sequencing

DNA was isolated from day-17 embryos and day-40 tissues using Qiagen DNAeasy extraction kit, according to the manufacture's instructions. Approximately 200 ng of total genomic DNA was used for a bisulfite treatment reaction using the EZ DNA methylation kit supplied by Zymo Research®, according to the manufacturer's instructions. Primers specific for bisulfite-converted DNA for SNRPN were designed according to previous publication [18]. Nested PCR amplifications were necessary due to the limited amounts of DNA (approximately 200 ng) available for analysis. Primers were designed according to bisulfite standards (no CpG sites within primers and at least 2 cytosines within primer sequence to select for converted sequences). For the outside nested PCR, the primer sequences were as follows: Forward 5'GGAAAGTTTGAGGAAATTTGAATAAGG-3'; Reverse 5'-CAAATACCCCCAAAACCTAACAAAAC-3'. The primers used for the inside nested reaction were as follows: Forward 5'-TTGGGAGGTATTATTTTGGGTTGAAG-3'; Reverse 5'-AAAAAATCAATCCAACCCCAAACCTC-3'. Each 50 µl PCR reaction contained

4 µl of bisulfite-treated DNA, 1 µl of each primer (10 µM), 2.5 µl (100 µM) deoxynucleotide triphosphates (Invitrogen), 5 µl 5X PCR buffer (300 mM Tris-HCl, 7.5 mM ammonium sulfate, 12.5 mM $MgCl_2$) (Invitrogen), and 1.25 U of DNA Taq polymerase (Invitrogen). First-round PCR was performed under the following conditions: 4 min at 94 °C, 2 min at 55 °C, and 2 min at 72 °C for two cycles, followed by 35 cycles of PCR consisting of 1 min at 94 °C, 2 min at 55 °C, and 2 min at 72 °C. For the second round of PCR, 4 µl of the first-round sample were used, and the conditions for the PCR were the same as the first-round conditions, except that the first two cycles were omitted. Fragments were resolved in 1.2 % agarose gels, followed by purification using agarose purification kit from Qiagen. Purified fragments were subcloned in pGEM T easy Vector (Promega), and cell transfection protocol was performed using competent *Escherichia coli* cells.

Clones containing the appropriate inserts were sequenced using an automated sequencer. Since bisulfite converts all unmethylated cytosines, whether or not they are in CpG dinucleotides, to guanines, only sequences with greater than 95% bisulfite conversion efficiency were used for analysis (i.e., to avoid false overestimation of methylated CpGs). Nucleotide mutations or any difference within the sequence (polymorphisms) between clones with similar CpG methylation profiles were verified to ensure that unique clones were represented. We examined 39 CpG sites in a 548-bp fragment of SNRPN. Absence of strain-specific single nucleotide polymorphisms prevented the parental origin of the sequenced strands from being determined.

Allele-specific polymorphism in cDNA

RNA was extracted using the RNAeasy Extraction kit (Qiagen) following manufacturer's instructions. Reverse transcription and polymerase chain reaction (RT PCR) was performed using Omniscript RT-PCR kit (Qiagen). cDNA was used as a template for the next PCR using primers SNRPN Forward (5'-GGAGATGCGTGACGTTGTGT) and Reverse (5'-GGTGTTCCAATACTGCTTTAACC). A 50 µl reaction was performed consisting of 5 µl 10X PCR buffer (Promega), 4 µl 25 mM $MgCl_2$, 1.25 µl 10 mM dNTPs, 2.5 µl 3 M forward primer, 2.5 ml 3 M reverse primer, 2 µl DNA, and 1 ml Taq (Promega). PCR reactions were performed for 35 cycles at 94 °C (2 min), 94 °C (30 sec), 65 °C (30 sec), 72 °C (35 sec), 72 °C (3 min), and held at 10 °C. Fragments were resolved on 1.2 % agarose gels, purified and subcloned in sequencing vectors pGEM T easy Vector (Promega) and transformed in competent *Escherichia coli* cells. Sequence analysis indicated the presence of a SNP between the *Bos indicus* and *Bos taurus* genomes.

Plasmids were purified, according to the Qiagen protocol, and results examined individually for the presence or absence of the paternally expressed *Bos indicus* genome (guanine) or maternally expressed *Bos taurus* genome (adenine) SNP. Results are expressed in percentages of individual cloned sequences possessing either and G or a A SNP.

Statistical Analyses

Statistical analysis was performed using the Chi-square test. For methylation analysis, data was analyzed by computing frequency of methylated sites over the number of unmethylated CpGs islands. For gene expression, data was analyzed using Bioedit software aligning program and frequency of paternal computed over maternal allele SNP. For both cases the level of significance was set at $P<0.05$.

AUTHORS' CONTRIBUTIONS

Joao Suzuki carried out the allelic expression studies, IVM, IVF and SCNT, participated in data statistical analysis and drafted the manuscript. Jacinthe Therrien and France Fillion carried out DNA methylation analysis and participated on sample collection. Alan K. Goff carried out the data statistical analysis and helped to draft the manuscript. Rejean Lefebvre carried out superovulation, synchronization of recipients, embryo transfer and sample collection. Lawrence C. Smith conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We acknowledge the help of Drs. Bruce Murphy and Jacquetta M. Trasler with the discussion of these data. Supported by the Canada Research Chair (CRC) program and the Natural Sciences and Engineering Research Council (NSERC) of Canada. This work was part of the Program on Oocyte Health (http://www.ohri.ca/oocyte) funded by grant HGG62293 under the Healthy Gametes and Great Embryos Strategic Initiative of

the Canadian Institutes of Health Research (CIHR) Institute of Human Development, Child and Youth Health. JS was supported by a studentship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil.

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FIGURE 1. Sequence chromatograms of the SNRPN locus amplified from day 17 AI, IVF and SCNT embryos. A) Chromatogram sequenced from a pure breed *Bos indicus* showing the "G" mutated paternal allele. **B)** Sequence obtained form *Bos taurus* genomic DNA demonstrating the presence of maternal "A" mutation. Arrows indicate the site of the mutation.

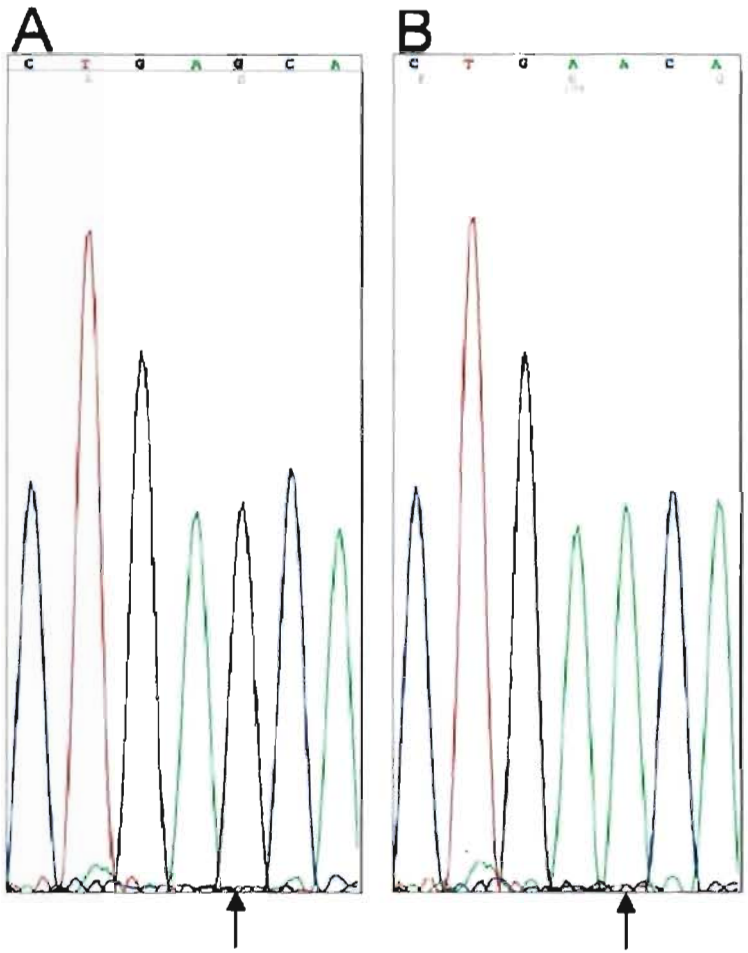
A
B

FIGURE 2. Maternal expression analysis of SNRPN gene at day 17. Embryos produced by AI (n=3), IVF (n=5) followed by in vitro culture SCNT (n=6). SNRPN reverse transcription-polymerase chain reaction fragments were cloned into a plasmid vector and sequenced for the parental SNP. Ratios were based on the total number of paternal alleles (G SNP) found over the total number individual clones sequenced. *Subscripts represent significant differences within groups ($P<0.05$).

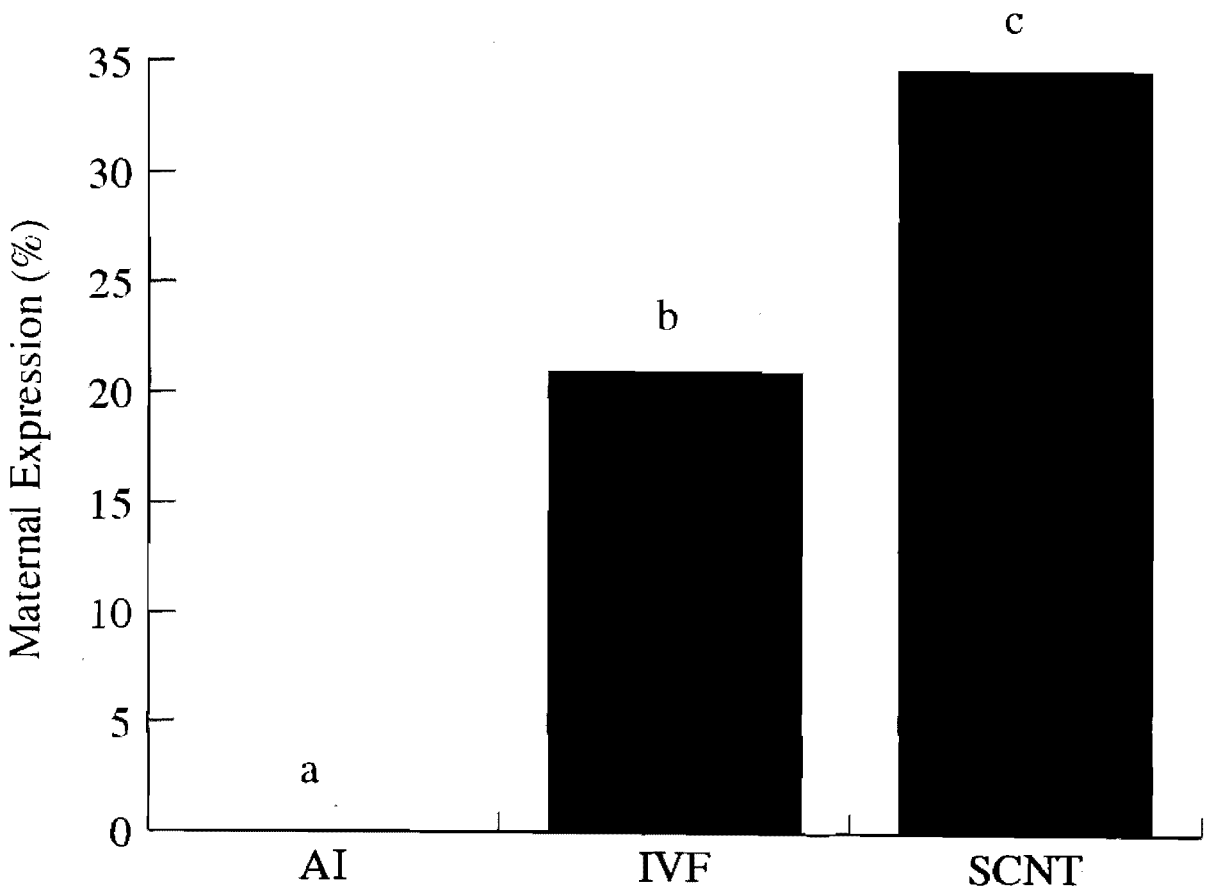
Maternal Expression (%)
35
30
25
20
15
10
5
0
a
b
c
AI
IVF
SCNT

FIGURE 3. Maternal expression analysis of SNRPN gene at day 40. Samples of placenta, brain, heart, liver and muscle of fetuses were produced by AI, IVF and SCNT. SNRPN reverse transcription-polymerase chain reaction fragments were cloned into a plasmid vector and sequenced for the parental SNP. Ratios were based on the total number of paternal alleles (G SNP) found over the total number individual clones sequenced. AI (black bars), IVF (white bars), SCNT (gray bars). a, b superscripts represent significant differences within tissues ($P<0.05$).

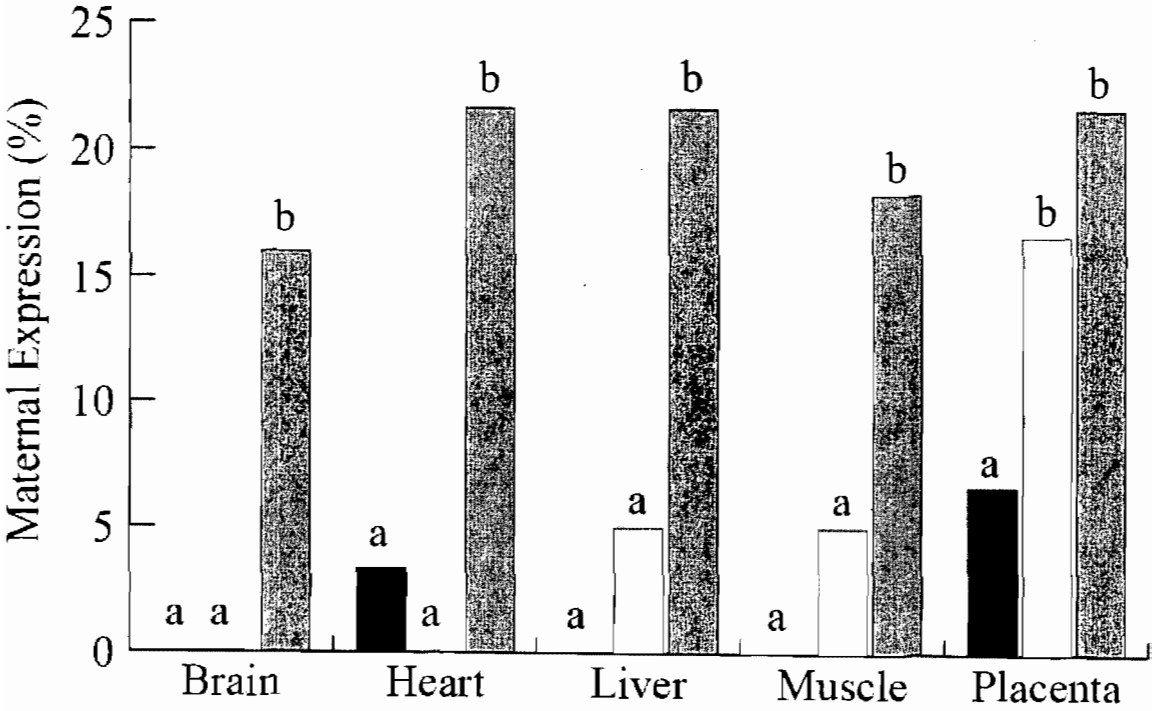
Maternal Expression (%)
25
20
15
10
5
0
a a
b
a
a
b
a
a
b
a
a
b
a
b
b
Brain
Heart
Liver
Muscle
Placenta

FIGURE 4. SNRPN DNA methylation in day 40 placenta and brain derived by AI, IVF and SCNT. Representative samples of bisulfite methylation analysis of **a)** AI day 40 placenta, **b)** AI day 40 brain, **c)** SCNT day 40 placenta, **d)** SCNT day 40 brain, **e)** IVF day 40 placenta, **f)** IVF day 40 brain, **g)** control fibroblast donor cell derived from a day 60 in vivo produced control and **h)** control paternal (unmethylated)/maternal (methylated) 50/50% ratio. Each line represents an individual clone that was sequenced. Black filled circles represents methylated CpG islands, and open circles indicate unmethylated CpG sites.

Fig 4

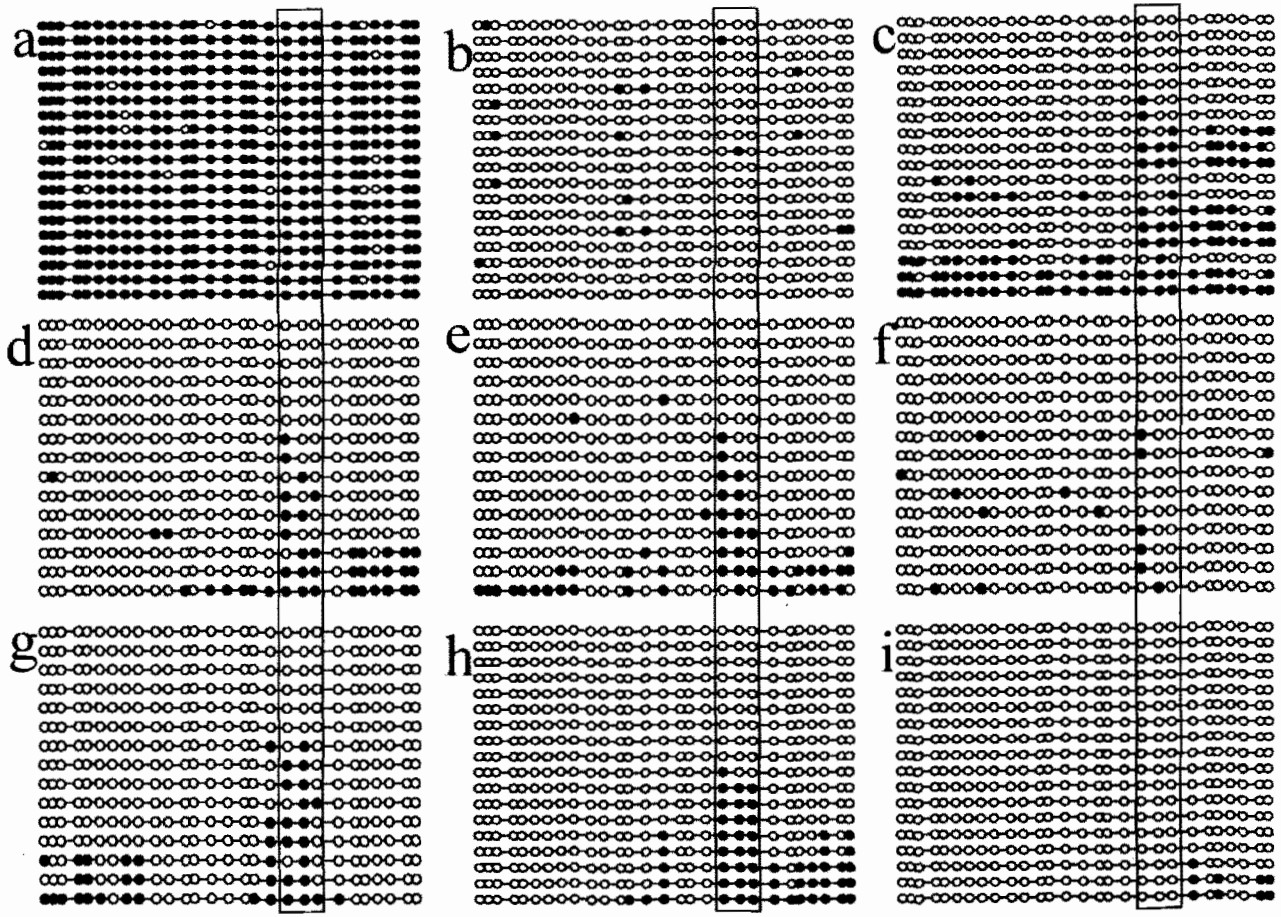

FIGURE 5. Percent SNRPN DNA methylation analysis of day 17 samples. Details of methylation analysis are detailed in Figure 4. For each sample, the bisulfite methylation data was analyzed by computing the number of methylated CpG sites over the total number of CpG sites. * represents significant differences within groups ($P<0.05$).

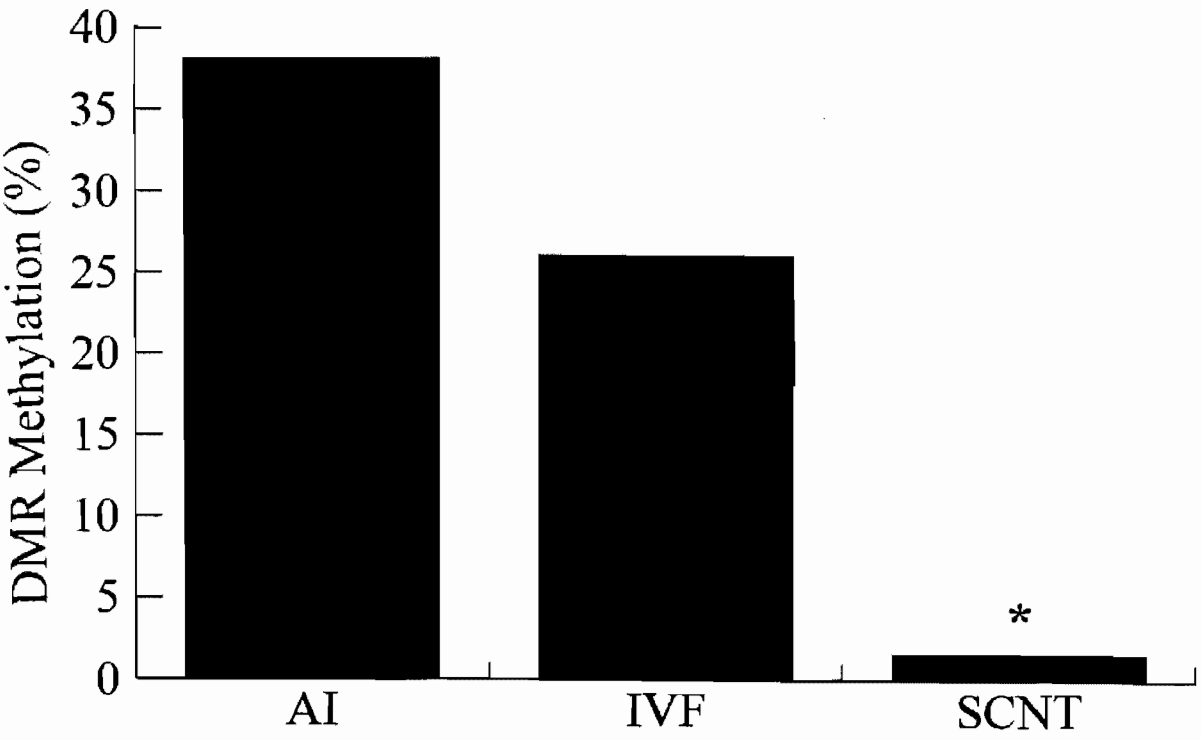
DMR Methylation (%)
40
35
30
25
20
15
10
5
0
*
AI
IVF
SCNT

FIGURE 6. Percent methylation analysis of day 40 placenta and fetus samples. Details of methylation analysis are detailed in Figure 4. For each sample, the bisulfite methylation data was analyzed by computing the number of methylated CpG sites over the total number of CpG sites. AI (black bars), IVF (white bars), SCNT (gray bars). a, b, c superscripts represent significant differences within tissues ($P<0.05$).

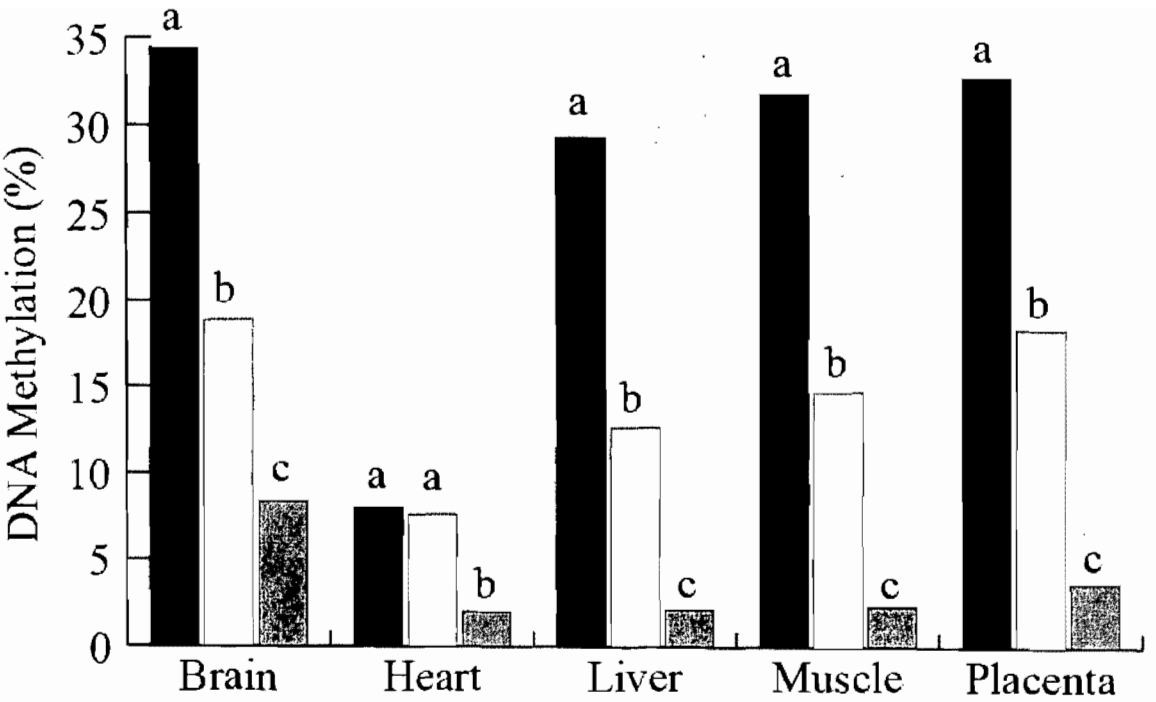
DNA Methylation (%)
35
30
25
20
15
10
5
0
a
b
c
a
a
b
a
b
c
a
b
c
a
b
c
Brain
Heart
Liver
Muscle
Placenta

FIGURE 7. Bivariate analysis of methylation and SNRPN gene expression percentages. Methylation values were based on overall number of methylated sites over unmethylated CpGs. Expression profile was based on number of paternal sequences over total of sequences per tissue. **a)** day 17 embryos, **b)** day 40 liver, **c)** muscle, **d)** heart, **e)** brain and **f)** placenta. **(.)** IVF, **(+)** *in vivo*, **(x)** SCNT.

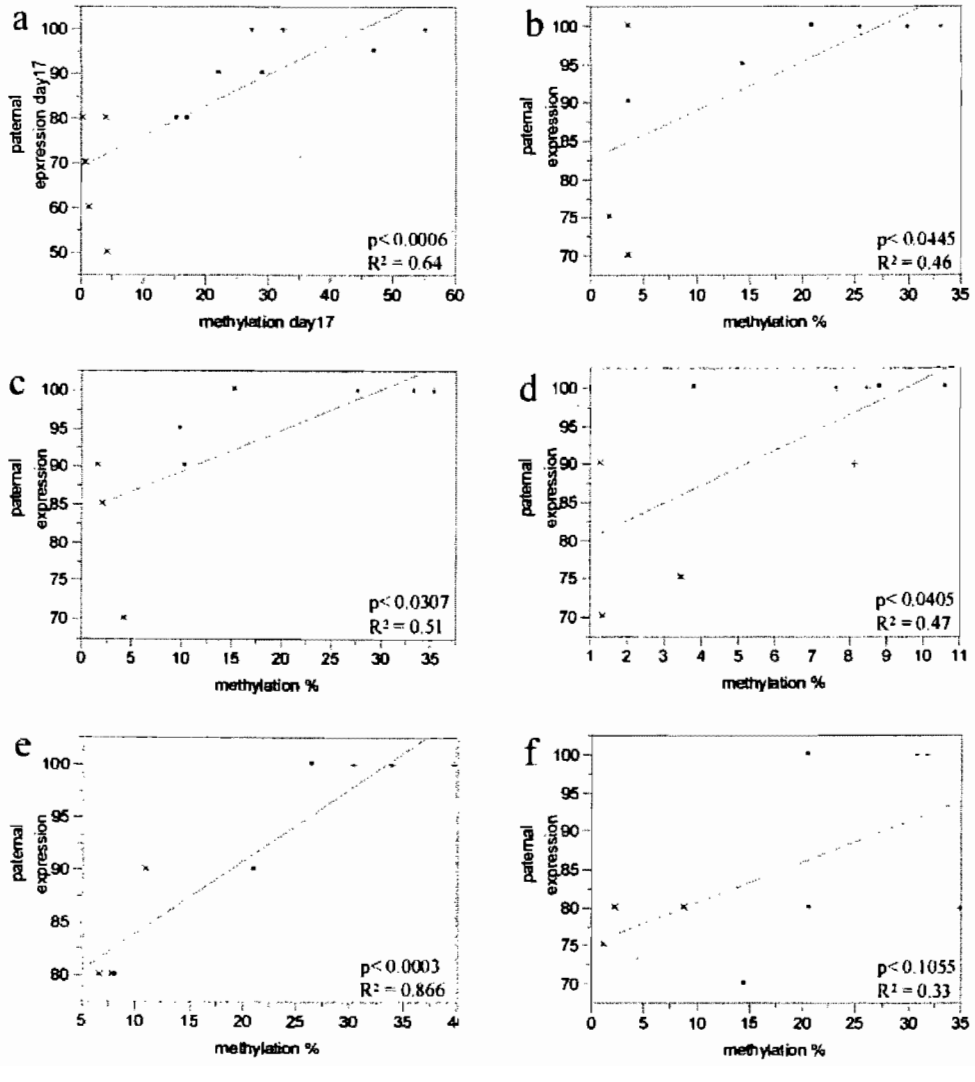
a
paternal epression day17
methylation day17
p< 0.0006
R² = 0.64
b
paternal expression
methylation %
p< 0.0445
R² = 0.46
c
paternal expression
methylation %
p< 0.0307
R² = 0.51
d
paternal expression
methylation %
p< 0.0405
R² = 0.47
e
paternal expression
methylation %
p< 0.0003
R² = 0.866
f
paternal expression
methylation %
p< 0.1055
R² = 0.33

Table 1. *In vitro* development of bovine oocytes subjected to SCNT, IVF and recovering of embryos from AI.

Group	Blastocyst development to day 7.5*	Embryos recovered from uterus	
		day 17 *	day 40 *
SCNT	26 (27.9%)	6 (45%)	3 (30%)
IVF	27 (33.75%)	5 (50%)	3 (50%)
AI			3 (75%) **

* percentage of embryos were calculated from the total number of transferred embryos.

** percentage of pregnancy was calculated from the total number of AI.

Table 2. Percentage of paternal expression of SNRPN gene and methylated CpGs islands on SNRPN DMR of AI, IVF and SCNT day 17 embryos.

	AI			**IVF**					**SCNT**					
Embryos	1	2	3	1	2	3	4	5	1	2	3	4	5	6
Paternal Expression	100	100	100	90	90	95	80	80	60	70	50	70	80	80
Methylation	27.2	32.2	55.0	29.1	22.0	47.1	17.0	15.3	0.9	0.5	4.0	0.4	0.1	3.8

Table 3. Percentage of paternal expression of SNRPN gene and methylated CpGs islands on SNRPN DMR of AI, IVF and SCNT tissues of day 40 fetuses and placenta.

		Paternal Expression (%)					Methylation (%)				
Groups	fetuses	liver	muscle	heart	brain	placenta	liver	muscle	heart	brain	placenta
AI	1	100	100	90	100	100	25.3	35.23	8.1	33.7	31.6
	2	100	100	100	100	80	33	33.19	7.6	30.2	34.7
	3	100	100	100	100	100	29.7	27.45	8.4	39.6	30.6
IVF	1	90	90	100	90	100	3.46	10.33	3.8	21.1	20.4
	2	95	95	100	80	70	14.3	9.8	8.8	8.1	14.5
	3	100	100	100	100	80	20.9	15.39	10.6	26.5	20.6
SCNT	1	75	70	90	80	80	1.63	4	1.2	7.6	2
	2	100	90	70	90	80	3.35	1.4	1.3	10.9	8.6
	3	70	85	75	80	75	3.36	1.8	3.4	6.5	1

CHAPTER IV

Imprinting of IGF2R in pre-implantation bovine embryos is not dependent on IGF2R DMR2 DNA methylation

Joao Suzuki, Jacinthe Therrien, France Filion, Partick Vincent, Rejean Lefebvre, Alan K. Goff and Lawrence C. Smith*

Centre de Recherche en Reproduction Animale (CRRA), Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC, J2S 7C6 Canada

* Corresponding author

ABSTRACT

Genomic imprinting is a form of gene regulation that results in a parent of origin-dependent expression manner, which is in turn normally regulated by epigenetic marks such DNA methylation. In somatic cells, it is stably inherited during cell division. However, when transmitted to individuals of opposite sex, the inheritance is reversibly acquired. Using a bovine interspecies model with an exonic polymorphism, we demonstrate a bi-allelic expression mode of the maternally expressed IGF2R gene in day 17 pre-implantation embryos derived from artificial insemination (AI) and *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT). Using and intronic polymorphism located within the IGF2R DMR CpG reach region, we also demonstrate that methylation levels on maternal allele of embryos subjected to in vitro culture were not different from AI *in vivo* controls. SCNT embryos, however, showed severe loss of methylation on maternal allele. Finally, we demonstrate that TSA treatment restored biallelic expression of IGF2R in fibroblast cells in dose dependent manner without any alteration within the IGF2R DMR 2. Together, these results suggest that imprinting regulation of IGF2R gene is established by methylation in pre-implantation embryos, and acethylation play an important role on IGF2R imprinting in fibroblast cells.

INTRODUCTION

Genomic imprinting is a form of gene regulation that results in a parent of origin-dependent expression manner. In somatic cells, it is stably inherited during cell division. However, when transmitted to individuals of opposite sex, the inheritance is reversibly acquired. During gamete development, the epigenetic marks are erased and established differently in sperm and oocytes. Although the distribution of parental genetic content is equal, the expression of an allele is dependent upon whether it resides in a male or female inheritance (Reik, Dean et al. 2001). The mechanism of inheritance is possible because epigenetic modifications are reversible and do not alter DNA sequence. DNA methylation has been defined as one of the most studied epigenetic modifications (Reik, Dean et al. 2001). Methyltransferases (DNMTs) catalyses the methylation at the 5-position of the cytosine (C) residue within cytosine-guanine dinucleotides (CpG), forming 5-methylcytosine (m5C) (Turek-Plewa and Jagodzinski 2005). DNMT1, DNMT3A and DNMT3B, which together with accessory proteins, like DNMT3L, are responsible for methylation pattern acquisition during gametogenesis, embryogenesis and somatic tissue development (Turek-Plewa and Jagodzinski 2005). In vertebrates, most CpGs in the genome are methylated (Bird 2002; Goll and Bestor 2005), but during gamete development the CpG methylation marks are reset according to the germ line sex and persist even after fertilization, when the embryo undergoes active

(male pronucleus) and passive (embryo) demethylation waves (Reik, Dean et al. 2001).

The first two genes identified as imprinted were insulin-like growth factor-2 (IGF2) (DeChiara, Robertson et al. 1991) and insulin-like growth factor-2 receptor (IGF2R) (Barlow, Stoger et al. 1991). Homozygous IGF2 null mice were 40% smaller than wild-type mice at birth, which was consistent with the growth promoting function of IGF2. They also noticed similar developmental rate was present in heterozygous mice, but only when the null allele was paternally inherited demonstrating that maternal allele was not contributing to expression of IGF2. The opposite was found for M6P/IGF2R, which is expressed mostly from maternal allele in mice (Barlow, Stoger et al. 1991; Wang, Fung et al. 1994)

M6P/IGF2R encodes for a transmembrane receptor in viviparous mammals that binds to both phosphomannosyl glycoproteins and IGF2 through different binding sites (Kornfeld 1992; Dahms, Brzycki-Wessell et al. 1993; Yandell, Dunbar et al. 1999). To date, evidence suggests that M6P/IGF2R does not mediate cell proliferation and growth through of IGF2 (Korner, Nurnberg et al. 1995), this function is attributed to insulin-like growth factor-1 receptor (IGF1R) and the insulin form receptor A (Kornfeld 1992; Frasca, Pandini et al. 1999). The function of M6P/IGF2R is related to intracellular trafficking of lysosomal enzymes and the internalization of IGF2 and other extracellular ligands to the lysosomes for degradation (Kornfeld 1992). M6P/IGF2R deficiency during mammalian development is associated to cardiac abnormalities, fetal overgrowth and perinatal

lethality (Lau, Stewart et al. 1994; Wang, Fung et al. 1994; Melnick, Chen et al. 1998) and such symptoms are also present in large offspring syndrome (LOS) eventually reported in pregnancies originated from *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) in mice (Rideout, Eggan et al. 2001) and ruminants (Young, Sinclair et al. 1998).

In mice, IGF2R paternal repression is dependent on a 3.7 kb imprinting control element (ICE) called Region2 (Wutz, Smrzka et al. 1997). Region2 is located within intron 2 of the IGF2R gene and contains a 1.5 kb maternally methylated CpG-island that is the promoter for an antisense RNA named *Air* that overlaps the IGF2R promoter. The *Air* RNA is specifically expressed from the paternal unmethylated ICE, but not from the maternal methylated ICE. Recently, it has been proposed that the IGF2R-ICE generates a long-range effect that acts in a bidirectional manner to repress upstream and downstream genes (Zwart, Sleutels et al. 2001).

Even though the imprinting mechanism has been elucidated in mice, other species seem no to fit the theory perfectly. For instance, in the marsupial American opossum, the IGF2R gene is paternally imprinted, even though the intron 2 of M6P/IGF2R completely lacks the DMD CpG islands (Weidman, Dolinoy et al. 2006). In fact, the region comprises only 9 CpGs and neither the parent of origin methylation pattern was found nor the *Air* RNA (Killian, Nolan et al. 2001; Weidman, Dolinoy et al. 2006). Studies performed in dogs also showed that IGF2R is imprinted in a variety of tissues, including uterus and umbilical cord, but neither expression of an anti-sense transcript from the paternally derived allele, nor

methylation of the repressed IGF2R promoter is required (O'Sullivan, Murphy et al. 2007). In humans, the M6P/IGF2R has been considered polymorphic imprinted, as some individuals show imprinted expression and others do not, and recent studies showed absence of *Air* RNA and although the parent of origin methylation is present at the second intronic region, there is no association with maintenance of imprinting (Monk, Arnaud et al. 2006).

In bovine M6P/IGF2R is imprinted in cDNA sequences originated from two fetal livers samples (Monk, Arnaud et al. 2006) and the intron 2 is differentially methylated in sperm and fetal tissues (Long and Cai 2007). However, little is known about the imprinting mechanism of IGF2R. The objective of this study was to characterize the parent of origin methylation status of the IGF2R DMR 2 in pre-implantation embryos produced by AI, IVF and SCNT, as well as associate methylation with parental expression.

MATERIAL AND METHODS

All protocols used here were performed in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Training, approved by the animal experimentation committee of the Université de Montréal sanctioned by the Canadian Council on Animal Care.

Manipulation of host oocytes

Cattle ovaries were collected from a local slaughterhouse and transported to the laboratory in saline at 30–35 °C within approximately 2 h after slaughter. Follicles with a diameter of 2 to 10 mm were punctured with an 18-gauge needle. Cumulus oocyte-complexes (COCs) with approximately 4 to 6 layers of cumulus cells and homogeneous oocyte cytoplasm were washed in Hepes-buffered tissue culture medium (TCM-199; Gibco BRL) supplemented with 10% (vol/vol) FBS. Groups of 20 COCs were placed in 100 μl of bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 μg/ml LH (Ayerst, London, ON, Canada), 0.5 μg ml/ml FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 μg ml/ml estradiol 17-β (Sigma-Aldrich, St. Louis, MO), 22 μg ml/ml pyruvate (Sigma-Aldrich), and 50 μg ml/ml gentamicin (Sigma-Aldrich). After 19 to 20 h of *in vitro* maturation (IVM), cumulus cells were removed from the COCs by vortexing for 2 min in PBS and 0.2% hyaluronidase (Sigma-Aldrich). Only oocytes with homogeneous cytoplasm and intact cell membrane were selected for micromanipulation.

***In vivo* and *in vitro*-derived embryos**

Production of embryos and fetuses for *in vivo* (AI) and *in vitro* (IVF) controls, as well as donor cells were conducted according to protocols published elsewhere (Lucifero, Suzuki et al. 2006). Briefly, Holstein heifers were superovulated by intramuscular injection of porcine FSH (Folltropin-V) given every 12 h in decreasing doses starting at Day- 9–10 of the estrous cycle. Cows received an

injection of 500 μg of cloprostenol (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) and were artificially inseminated (AI) at 52 h and 86 h after the initiation of superovulation (Lucifero, Suzuki et al. 2006).

In vitro-matured oocytes were fertilized *in vitro* using standard protocols (Lucifero, Suzuki et al. 2006). Briefly, 20–25 COCs were placed in 100 μl drops of Tyrode's medium supplemented with 0.6% BSA (fraction V; Sigma-Aldrich), lactate, pyruvate, gentamicin, and heparin (10 μg/ml). Frozen-thawed spermatozoa were washed and centrifuged through a Percoll (Sigma) gradient and diluted to 10^6 live spermatozoa/ml. At 20 h following the start of incubation with spermatozoa, COCs were denuded of cumulus cells by brief shaking, and the putative zygotes were transferred to 25 μl drops of synthetic oviduct fluid (SOF medium) and cultured for 8 days with additional 25 μl of SOF medium under the same conditions used for the SCNT embryos.

Somatic Cell Nuclear Transfer

The SCNT protocol used was a slight modification from that previously reported (Vajta, Lewis et al. 2003). Oocytes were selected in groups of 100 and placed in 1.5 mg/ml pronase in TCM 199 supplemented with FBS 10% for about 4 min. Zona-free oocytes were washed thoroughly in TCM supplemented with FBS 20% for 3 min and cultured in 0.4 μg/ml demecolcine for at least 30 min. Treated oocytes, with a visible protruding membrane, were placed in medium supplemented with 5 μg/ml cytochalasin and FBS 10% and manually bisected using a micro blade

on a stereomicroscope. After bisection, oocytes were stained with 2 μg/ml Hoescht 33342 to stablish the absence of chromatin. Nuclear donor cells were thawed, washed and placed in 50 μl of culture media (DMEM, supplemented with 10% FBS and 0.5% antibiotics). Nuclear transfer was performed using confluent cells that were maintained in culture for 3–5 passages. Cytoplasts were placed individually in a 50 μl drop containing 500 μg/ml of phytohemagglutinin (Sigma) for about 3 sec and then quickly positioned over a single donor cell placed at the bottom of the dish. After attachment of the donor cell, the cytoplast-somatic cell pairs were placed in 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.2-kV electric pulse lasting 70 μsec. After electrical stimulation, couplets were washed and cultured individually in 10 μl drops of 6 dimethylaminopurine (DMAP, Sigma-Aldrich) for 3 h. After 6-DMAP treatment, reconstructed oocytes were washed and cultured in 40 μl drops of SOF modified medium supplemented with 0.8% BSA-V fatty acid free (Sigma-Aldrich) under equilibrated mineral oil at 39 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. Embryos were cultured in groups of 4 per drop in small individual wells (500 μm diameter) prepared with a sterile needle to avoid separation of blastomeres during development. Reconstructed embryos were cultured for a period of 8 days.

Day-17 Elongating Embryos

The estrous cycle of Holstein heifers was synchronized by an injection of 500 μg of the prostaglandin F2α analogue, cloprostenol (Estrumate, Schering Canada

Inc). Six to 8 days after the standing heat, Day-8 *in vitro*-produced or SCNT blastocysts was transferred into the uterine horn ipsilaterally to the corpus luteum. Embryos were washed with TCM-199 Hepes-buffered medium supplemented with 10% of FBS, loaded into a 250 µl straw and transferred to recipient heifers. Two heifers received 5 Day-8 IVF embryos each and another two heifers received between 8 to 10 SCNT embryos and allowed to develop for another 9 days in the uterine horn. Day-17 elongated embryos were non-surgically recovered by flushing the uterus of the recipient heifers with PBS using a Foley catheter. Embryos were removed from the flushing media and inspected to select those that were recovered intact. After selection, embryos were washed three times in PBS and frozen individually at -70 °C in 0.2 ml of distilled water. Only the embryos that were recovered intact were used for the experiments.

Search for the Bovine IGF2R DMR2

Genome walking was used to identify the sequence of the DMR2 within the bovine *IGF2R* gene. Genomic DNA was extracted from fibroblasts of an adult *Bos taurus* using DNAeasy Tissue Kit (Qiagen). DNA libraries were obtained using the Universal GenomeWalker Kit (Clontech). Briefly, PCR amplification primers consisted of adaptors primers provided by the kit and gene-specific primers. The IGF2R DMR2 is located in the intron 2 in most of the species analyzed to date. Thus, gene-specific primers were designed in exon 2 (upstream IGF2R DMR2 and moved

in the 3' direction) and in exon 3 (downstream IGF2R DMR2 and moved in the 5' direction) based on sequences obtained from GenBank (accession # J03527).

Semi-quantitative and allelic IGF2R gene expression

Total RNA was extracted using the Qiagen RNAeasy Tissue kit, following manufacturer's instructions. Reverse transcription and polymerase chain reaction (RT PCR) was performed using Omniscript RT-PCR kit (Qiagen). 200 ng of total RNA was mixed with 2 μl with reverse primer (10mM) G3PDH-R- 5'- TGT TCC AGT ATG ATT CCA CCC, 2 μl of RT buffer, 1 μl of Omniscript Reverse transcriptase (4 units/μl), 1 μl of Rnase inhibitor (Invitrogen, 10 units/μl) and the reaction was carried out in a 20 μl volume for two hours at 37 °C for housekeeping gene GAPDH as an internal control. The same reaction was carried out for the IGF2R gene using 1 μl of primer (10mM) IGF2R-R1 -5'- ACG TAA CTC AGG ACG AGC CT -3'. Synthesized cDNA was purified using the Qiagen MinElute reaction cleanup kit, following manufacturer's instructions and final product was resuspended in 60 μl of Qiagen EB buffer. Primers for semi-quantitative reaction were IGF2Rhyb-F-5'-TAT GCA TGA CTT GAA GAC AGA C-3'; and IGF2R-RE-5'-CAG GGC ACC TCT TTA TTC GCT-3'. For housekeeping gene GAPDH, primer sequences were BOSGPDH222-F-5'-CTC CCA ACG TGT CTG TTG TG-3' and BOSGPDH222-R-5'-TGA GCT TGA CAA AGT GGT CG-3'. Semi-quantitative PCR was carried out using LightCycler® FastStart DNA Master SYBR Green I, following the manufacturer's instructions using 6 μl of purified RT as

template. Reactions consisted of initial denaturation at 94 °C for 6 min, 40 cycles of 0.1 sec at 95 °C, 4 sec at 60 °C, 10 sec at 72 °C, 1 cycle of 0.1 sec at 95 °C, 10 sec at 70 °C, 0.1 sec at 95 °C and a final step of 30 sec at 40 °C. After finishing the cycles, temperature was held at 10 °C. Results were analyzed using the LightCycler Relative Quantification Software. IGF2R gene transcript abundance was normalized by expression of internal control GAPDH gene.

For allele-specific quantification, labeled primers: *acceptor*IGF2R 5' AAA+CGCA+AGC+AGA-Fluorescein-3', and *anchor*IGF2R -5' TCTTCTGG+AATTTA+AATTTA+ACAC+AAC+AGTG+AAC-Phosphate-3' were designed. PCR was carried out following manufacturer's instructions and 6 μl of purified cDNA (described above) was used as template. PCR reaction consisted of initial denaturation at 94 °C for 6 min, 44 cycles of 2 sec at 95 °C, 10 sec at 58 °C, 15 sec at 72 °C, 1 cycle of 0.1 sec at 95 °C, 10 sec at 70 °C, 0.1 sec at 95 °C and a final step of 30 sec at 40 °C. Final temperature was held at 10 °C.

Bisulfite treatment and sequencing

DNA was isolated from Day-17 embryos and using Qiagen DNAeasy extraction kit, according to the manufacture's instructions. Approximately 400 to 500 ng of total genomic DNA was used for a bisulfite treatment reaction using the Qiagen EpiTect Bisulfite kit. Two sets of primers were designed to amplify a fragment of IGF2R-DMR2 from the modified DNA (final PCR product of 515 pb). The first PCR was carried out using the following primer sequences: U- IGF2R-F1:

5'-TT TGG TTT GGT GGA TTT GGT TTG GAG-3'and U- IGF2R-R1: 5'-ACC CCC CAA CCT TAA AAA CCC TCC C-3'. The nested PCR was carried out with the following primers: U- IGF2R-261: 5'-ACC CTA TAC CCA AAA CTC CC-3' and U- IGF2R-773: 5'- TTA GTG TGG TTT GGT TTG G-3'. The nested PCR resulted in a 515 bp fragment.

Each PCR reaction was performed in triplicate. The PCR reaction was carried out in a final 50 µl volume containing 3 µl of bisulfite-treated DNA, 1 µl of each primer (2 µM), 1.5 µl of mixed dNTP (2.5 mM each), 5 µl of 10X PCR buffer and 0.5 µl of DNA Taq polymerase (Amersham). The first PCR reactions was performed using an initial step at 94 °C for 2 min followed by 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C for 35 cycles with a final step at 72 °C for 3 min. Using 4 µl of the first PCR, the second-round PCR was performed with an initial step at 94 °C for 2 min followed by 40 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 50 sec at 72 °C with a final step at 72 °C for 3 min. The nested PCR products were resolved in 1,2 % agarose gels, followed by purification using agarose purification kit from Qiagen. Purified fragments were subcloned in pGEM-T easy Vector (Promega) and cell transformation protocol was performed using competent *Escherichia coli* DH5α cells. To ensure that reliable data was collected, after transformation a total of 12 to 24 clones (at least four clones of each allele) for each group were picked and sequenced.

Cell collection, culture and TSA treatment

Fetal fibroblast cell cultures were established from a 60-day-old crossbred fetus produced by artificial insemination of a Holstein (*Bos taurus*) heifer with semen from a Nelore (*Bos indicus*) bull. A skin biopsy from the donor animal was cut into small pieces (2-3 mm), minced manually and digested with 0.25% trypsin and 0.02% EDTA (Gibco BRL, Burlington, ON, Canada) at 37 °C for 10 min for cell desaggregation. Isolated cells were washed and cultured for approximately 4 d in Dulbecco modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 0.5% antibiotics (penicillin 10000 U/ml and streptomycin 10 000 μg/ml; Gibco BRL) at 38°C in 5% CO_2. When the cultures were confluent, primary passage cells were frozen in culture media supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen. Donor cells were thawed at 37°C for 1 min and cultured to confluence for a maximum of 5 passages before use as donor cells. For TSA treatment, each cell passage was equivalent to approximately two cell-doublings and the exposure to TSA was sustained for 4 d of cell culture. Cells were grown to confluence at passage 5 and subsequently seeded into DMEM plus 10% FBS containing 0, 0.08, 0.3, and 1.25 μM, of TSA. These levels were selected according to previous publication, according to the dose-response effects of these drugs tested in bovine fibroblasts (Enright, Kubota et al. 2003). After TSA treatment, cells were harvested and immediately subjected to RNA and DNA extraction using RNA/DNAeasy Tissues kit (Qiagen) for further analysis

of parental expression of IGF2R gene transcript and IGF2R DMR 2 bisulfite analysis.

RESULTS

Characterization of the IGF2R DMR 2 in cattle

A *Bos taurus* genomic library was created to obtain the IGF2R-DMR2 sequence. The protocol produced a series of 6 fragments covering more then 4.8 kb upstream of the exon 2, and 4 fragments covering about 4 kb from the exon 3. Each PCR fragment was cloned and sequenced for analysis. By overlapping all the sequences generated from the genome walk, the final contig was compared to ovine DMR2 (Young, L.E. et al., 2003), and bovine (Long and Cai 2007). Similarly to previously published (Long and Cai 2007), we also found that the bovine IGF2R DMR2 comprises a fragment of approximately 2.3 kb, stretching from 4.5 kb upstream the exon 2 and to 2.2kb downstream the exon 3 (Figure 1). However, contrary to 26 CpG sites (spaning from 4439 to 4785 downstream the intron 2) previously found in bovine DMR2 (Long and Cai 2007), we found 70 CpG in a 515 bp fragment, spanning from 6154 to 6666 downstream the intron 2. The entire sequence is now available at genebank Nw_0011495620.

In mice, IGF2R paternal repression is dependent on a 3.7 kb imprinting control element (ICE) called Region 2, which is located within intron 2 of the IGF2R gene and contains a 1.5 kb maternally methylated CpG-island (Wutz, Smrzka et al. 1997) (Zwart, Sleutels et al. 2001). To address the question whether the IGF2R

DMR had a parent of origin methylation in bovine, we first investigated the methylation patterns of the IGF2R intronic CpG island in GV oocytes and sperm. To exclude any source of contamination, oocytes were denuded from cumulus cells and zona pellucida was removed by pronase digestion. IGF2R DMR 2 CpG island sequence amplified from bovine GV oocytes showed a high methylation profile, whereas CpG island sequence from sperm showed almost complete unmethylated sequences (Figure 1). Similar results were observed in mice, where IGF2R was completely methylated within intronic differentially methylated region 2 in oocytes and unmethylated in sperm (Lucifero, Mertineit et al. 2002). These results thus indicate a conservative parent of origin methylation profile for bovine gamete.

Once the IGF2R DMR 2 parent of origin methylation was characterized, we searched for *Bos indicus*-specific single nucleotide polymorphisms (SNP). Due to the difficulty of analyzing CG-rich regions, we searched for an eventual SNP present in the same 515 bp PCR product designed for bisulfite treatment. Sequencing of genomic DNA samples obtained from animals of the *Bos taurus* and *Bos indicus* subspecies enabled the identification of a guanine to cytosine (**G/C**; *Bos indicus/Bos taurus*) SNP at 67th CpG site located within the DMR 2 sequence. The SNP was confirmed by sequencing of PCR fragments obtained with IGF2R bisulfite primers using genomic DNA samples obtained from *Bos taurus* (maternal control), *Bos indicus* (paternal control), which fortunately could be identified even after bisulfite treatment. To further characterize this putative IGF2R DMR 2 in cattle, genomic DNA obtained from F1 skin fibroblast cells was analyzed after bisulfite treatment.

Interestingly, from 9 sequences analyzed, 4 sequences carrying the paternal SNP showed demethylated CpG sites, whereas 5 clones with maternal SNP were almost completely methylated (Figure 1). Similar results were found in mice, where differentiated neuron and glia cells showed heavy methylated maternal allele and demethylated paternal alleles (Yamasaki, Kayashima et al. 2005).

DMR methylation patterns in day 17 pre-implantation stages

In mice, sites in an IGF2R intronic DMR 2 had allele-specific modification patterns which were established either in the gametes or shortly after fertilization and were preserved throughout pre-implantation embryogenesis (Brandeis, Kafri et al. 1993). Our results of in vivo preimplantation development provided by AI showed similar methylation patterns of IGF2R DMR 2 (Figure 2). The paternal allele was demethylated as expected and maternal allele showed more methylated sites. Since our approach to the methylation analysis was not site directed we provided a more specific pattern. To test the effects of *in vitro* culture on methylation levels at the IGF2R DMR 2 we also assessed methylation profile in IVF *in vitro* cultured embryos. Figure 2 shows a representative IVF methylation profile similar to AI, the maternal allele showed roughly 50% of methylated sequences, while paternal allele was completely demethylated, suggesting that *in vitro* culture did not significantly affect the methylation levels at this region. In contrast, a severe loss of methylation was observed in embryos produced by SCNT. A representative figure shows that both maternal and paternal alleles showed complete demethylation levels (Figure 2).

Statistical analysis confirmed that methylation levels in the AI control (n=3) were not different from IVF (n=7), however significantly lower methylation levels were found in SCNT pre-implantation embryos (n=18) ($P<0.05$) (Figure 3). Similar loss of methylation was also found in tongue tissue dissected from ovine clones sacrificed after birth. On average, cloned animals had significantly lower methylation ratios when compared to AI produced animals (Young, Schnieke et al. 2003).

Semi-quantitative and allelic analysis of IGF2R gene expression

Transcripts of IGF2R and internal control GAPDH were analyzed in individual day 17 elongated blastocysts derived from AI (in vivo development), IVF and SCNT subsequently subjected to standard in vitro culture. GAPDH and IGF2R transcripts were detected in all embryos analyzed. In sheep, downregulation of IGF2R was associated with LOS in animals produced by IVF and SCNT subjected to in vitro culture. To test whether the IGF2R transcript abundance was affected by in vitro culture, we performed semi-quantitative and allelic specific expression in control animals (AI, n=3), IVF (n=7) and SCNT (n=18). IGF2R values obtained from real time PCR were normalized by GAPDH. The results are summarized in Figure 4. As expected, SCNT embryos showed a significant downregulation of IGF2R gene transcript when compared to AI and IVF (Figure 4) ($P<0.01$). The highest relative levels were attributed to IVF embryos, followed by AI (Figure 4). Many reports have demonstrated wide range of varitation of IGF2R in SCNT and, such variations are often associated with particular type (Daniels, Hall et al. 2001;

Wrenzycki, Wells et al. 2001) origin (Kato, Tani et al. 1998) cycle (Tani, Kato et al. 2001) and passage number (Kubota, Yamakuchi et al. 2000) of donor cell. To test the effect of reprogramming in SCNT, we analyzed the relative transcript abundance in the same donor cells we used for embryo reconstruction. Interestingly, relative levels of IGF2R found in donor cell were approximately 11 fold higher than pre-implantation embryos (data not shown). This result suggested that the effect of IGF2R in SCNT embryos was not reflecting from donor cell state prior to cloning.

In vitro culture and SCNT can affect allelic expression of imprinted genes (Doherty, Mann et al. 2000; Mann, Chung et al. 2003; Mann, Lee et al. 2004). To distinguish paternal and maternal allele we used a *Bos indicus/Bos taurus* C/A single nucleotide polymorphism (SNP) located 332 bp downstream the ATG start codon of Exon 1. Based on that SNP, we designed FRET probes to analyze the IGF2R gene allelic by real time PCR. To validate our results, maternal (*Bos taurus*) DNA was mixed with inverse proportions of paternal (*Bos indicus*) DNA ranging from 0 to 100% and used as templates for FRET analysis. Figure 5 shows the standard curve ($R^2 = 0.9793$) generated. Embryos were analyzed individually in AI, IVF and SCNT. Surprisingly, all groups showed bi-allelic expression ranging approximately from 35 to 40% of paternal IGF2R transcripts with no significant difference (Figure 6). Although methylation ratio was low in SCNT when compared to AI and IVF, allelic expression did not differ. In support of our results, IGF2R bi-allelic expression was also found in ovine pre-implantation embryos (Thurston, Taylor et al. 2008). As a control for allelic expression, we used differentiated bovine fetal fibroblast and

imprinting was confirmed in differentiated cells, as more than 80% of maternal transcripts were found (Figure 6). This result suggests once more that reprogramming of donor cell was achieved by SCNT.

TSA treatment

TSA effects have been associated with transcriptional activation and chromatin opening through inhibition of deacethylation of histones (Long and Cai 2007). To test whether TSA treatment would cause chromatin opening and relaxation of IGF2R without any physical changes in DNA methylation, we treated bovine fetal fibroblasts with different TSA concentrations. In vitro cultured cells were allowed to replicate 2 to 3 times before reaching confluent state in the presence of the TSA. The FRET analysis system enabled us to quantify the dose responsiveness of IGF2R allelic expression in 0.08; 0.31; 1.15 μM of TSA. Untreated control cells showed mostly maternal, as expected (Figure 7A). Interestingly, allelic expression was positively associated with TSA doses. Figure 7a summarizes the effects of TSA in fetal fibroblast, where increasing doses of TSA augmented the levels of paternal expression, suggesting that histone modifications plays an important role in IFG2R in somatic cells. Subsequently, we analyzed IGF2R DMR 2 methylation levels, to check if TSA treatment somehow caused alterations in DNA CpG sites and surprisingly, the parent of origin methylation of IGF2R DMR 2 was unaffected, regardless the TSA dose (Figure 7B). Paternal allele kept non methylated CpGs, whereas maternal allele showed fully methylated CpG patterns. This result suggest

that during preimplantation development, histones modifications might be acting on relaxed expression of IGF2R.

Discussion

In this study, we have characterized the parent of origin methylation of IGF2R DMR 2 for the first time in cattle. The use of *Bos indicus/Bos taurus* model enabled us to study the IGF2R imprinting in pre-implantation embryos by assessing the IGF2R DMR 2 parent of origin methylation and the allelic expression of IGF2R transcripts. Finally, we showed with TSA cell treatment that histone modifications cause imprinting relaxation without any change in DNA methylation.

Methylation ratio of IGF2R DMR 2 has been previously studied in mice (Brandeis, Kafri et al. 1993; Yamasaki, Kayashima et al. 2005) and recently in cattle (Long and Cai 2007). Although homology between mice and cattle has been reported for IGF2R DMR 2, sometimes results can be misleading and not conclusive. Unlike mice, most of the times it is difficult to characterize DMR analysis in large animals, since single polymorphism are not available. In such situation we are obligated to characterize DMR as putative. In our study, the parent of origin could be compared to what has been reported in mouse models and we could demonstrate that methylation is inherited differently from gamete and maintained during development. However, when compared to somatic cells, the methylation pattern in preimplantation embryos was not fully established in AI controls. A possible explanation for such phenomenon is that IGF2R DMR 2 is approximately 2.3 kb in

length, and parent of origin methylation could be located in separate regions as marks for adjacent methylation as development progresses. In support of this hypothesis, we showed bi-allelic expression of IGF2R in cattle AI preimplantation embryos in the present study, and recently in sheep (Thurston, Taylor et al. 2008), thus suggesting that if methylation is important for IGF2R imprinting, it might be necessary after implantation. Methylation levels of maternal allele in IVF embryos was not affected by in vitro culture (Figure 3). The attribution of detrimental effects of *in vitro* culture perhaps is linked to the particular conditions and media used. In mice, bi-allelic expression of imprinted genes and loss of methylation has been reported in embryos cultured *in vitro* using Whitten's medium but not with KSOM (Doherty, Mann et al. 2000; Mann, Lee et al. 2004). In another study, downregulation of IGF2R transcript was associated with fetal overgrowth and LOS (Young, Fernandes et al. 2001), in embryos cultured *in vitro* for five days with co-cultured granulosa cells and/or serum before transfer into recipient ewes. The addition of fetal serum *in vitro* culture is probably one of the factors contributing to the abnormalities found. In our experiment, serum was removed from *in vitro* culture system during embryo development and this may account for the normal allelic expression and methylation levels found. However we do not exclude the possibility that failure in IGF2R may arise further on during gestation, since IGF2R transcripts were upregulated in IVF embryos cultured *in vitro* (Figure 4).

SCNT disrupts imprinting in a variety of animals (Young, Fernandes et al. 2001; Mann, Chung et al. 2003; Chen, Jiang et al. 2005; Lucifero, Suzuki et al.

2006). Overall loss of methylation has been previously reported in bovine IGF2R locus when compared to *in vivo* controls (Long and Cai 2007). We found within the same locus, specific loss of methylation at IGF2R DMR 2 on normally methylated maternal allele. Interestingly, such abnormal pattern was not associated with bi-allelic expression in pre-implantation embryos since all groups showed the same expression mode (Figure 5). However we do not exclude the occurrence of further complications in SCNT pregnancies. In ovine, IGF2R transcripts are up-regulated simultaneously with mono-allelic expression after implantation (Thurston, Taylor et al. 2008) and at that time methylation may be effective on switching off paternal allele transcription. If methylation levels are low in clones, imprinting may not be established and IGF2R levels would still be at basal threshold.

Finally we demonstrated that TSA treatment simulates the situation found in pre-implantation embryos, where bi-allelic expression of IGF2R was observed, even though maternal allele was methylated. In fetal fibroblast cells, TSA concentrations positively associated with paternal IGF2R without any changes in DNA methylation (Figure 7). This results suggest that histone modifications play important roles in imprinting regulation, possibly at pre-implantation embryos where bi-allelic expression seems to be a transitory state between pre and pos-implantation. In such case, histone acethylation would be more easily removed and DNA methylation, a more stable and definitive mark would resume its function.

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FIGURE 1. Schematic structure of the bovine IGF2R DMR 2. (**a**) A 515 bp CpG rich region located on intron 2 of IGF2R gene. Black filled circles represent methylated CpG sites and white circles represent unmethylated CpG sites in **a)** gamete and **b)** fibroblasts. Dark gray filled circles represent the "GG" allele present in *Bos indicus* paternal (P) and absent on *Bos taurus* maternal (M) allele.

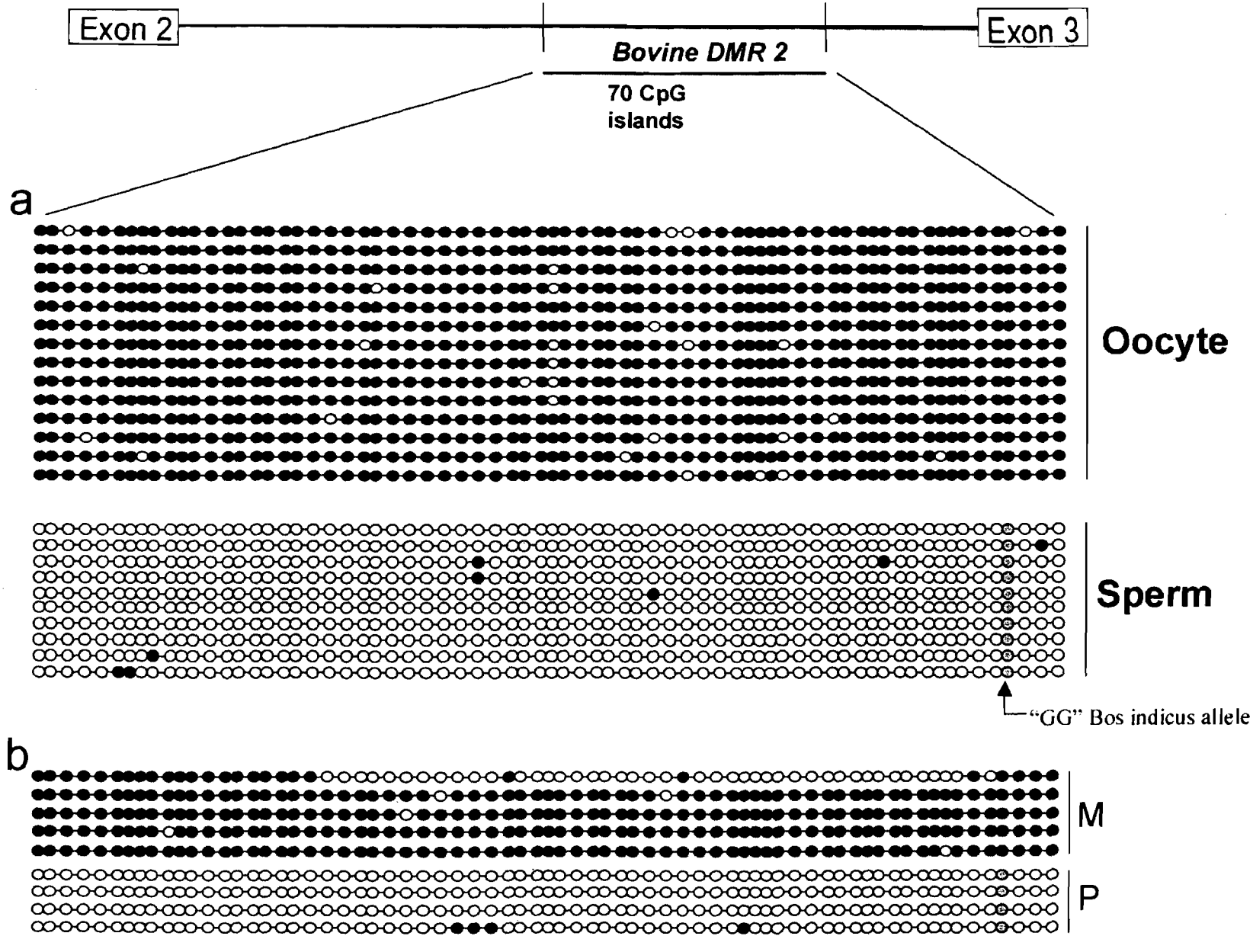

Exon 2
Exon 3
Bovine DMR 2
70 CpG islands
a
Oocyte
Sperm
"GG" Bos indicus allele
b
M
P

FIGURE 2. Methylation status of the bovine IGf2R DMR2. Representative bisulfite methylation analysis of **(a)** SCNT; **(b)** AI; and **(c)** IVF day 17 pre-implantation embryos. Black filled circles represent methylated CpG sites and white circles represent unmethylated CpG sites. Dark gray filled circles represent the "GG" allele present in *Bos indicus* paternal (P) and absent on *Bos taurus* maternal (M) allele.

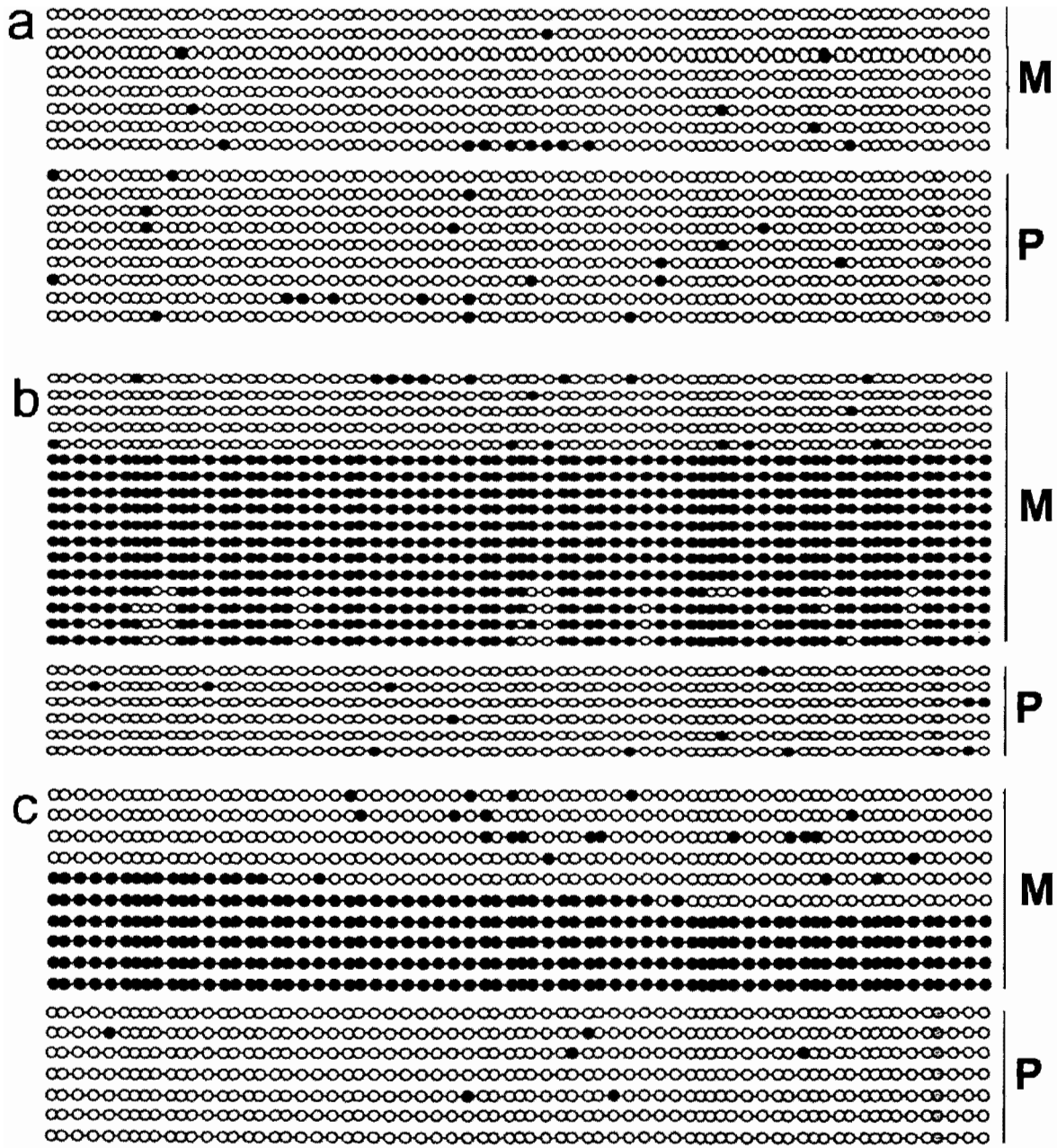

a
M
P
b
M
P
c
M
P

FIGURE 3. Methylation profile of IGF2R DMR2 in day 17 pre-implantation embryos produced by SCNT, IVF, *in vivo* (AI), and fibroblast donor cells. Percentage obtained from methylated over the unmethylated sequences. The frequencies of methylated sites were analysed using Chi-Square test and letters indicate significant differences ($P<0.05$).

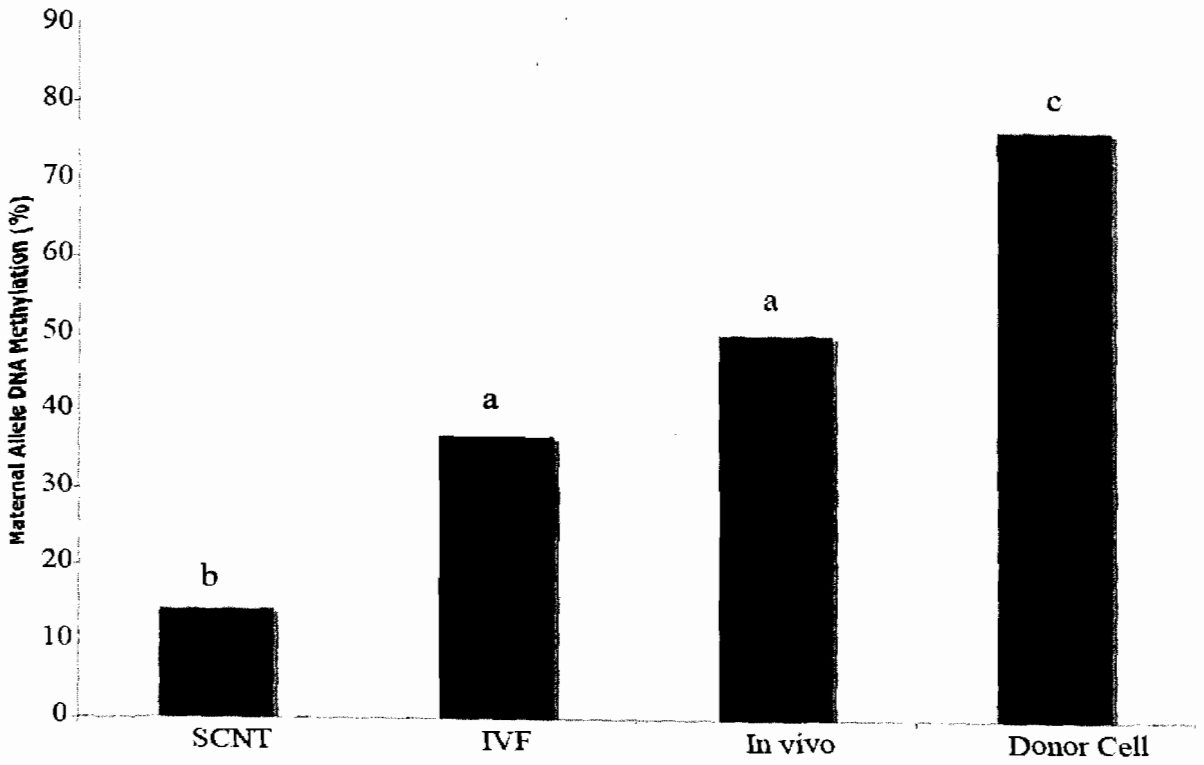
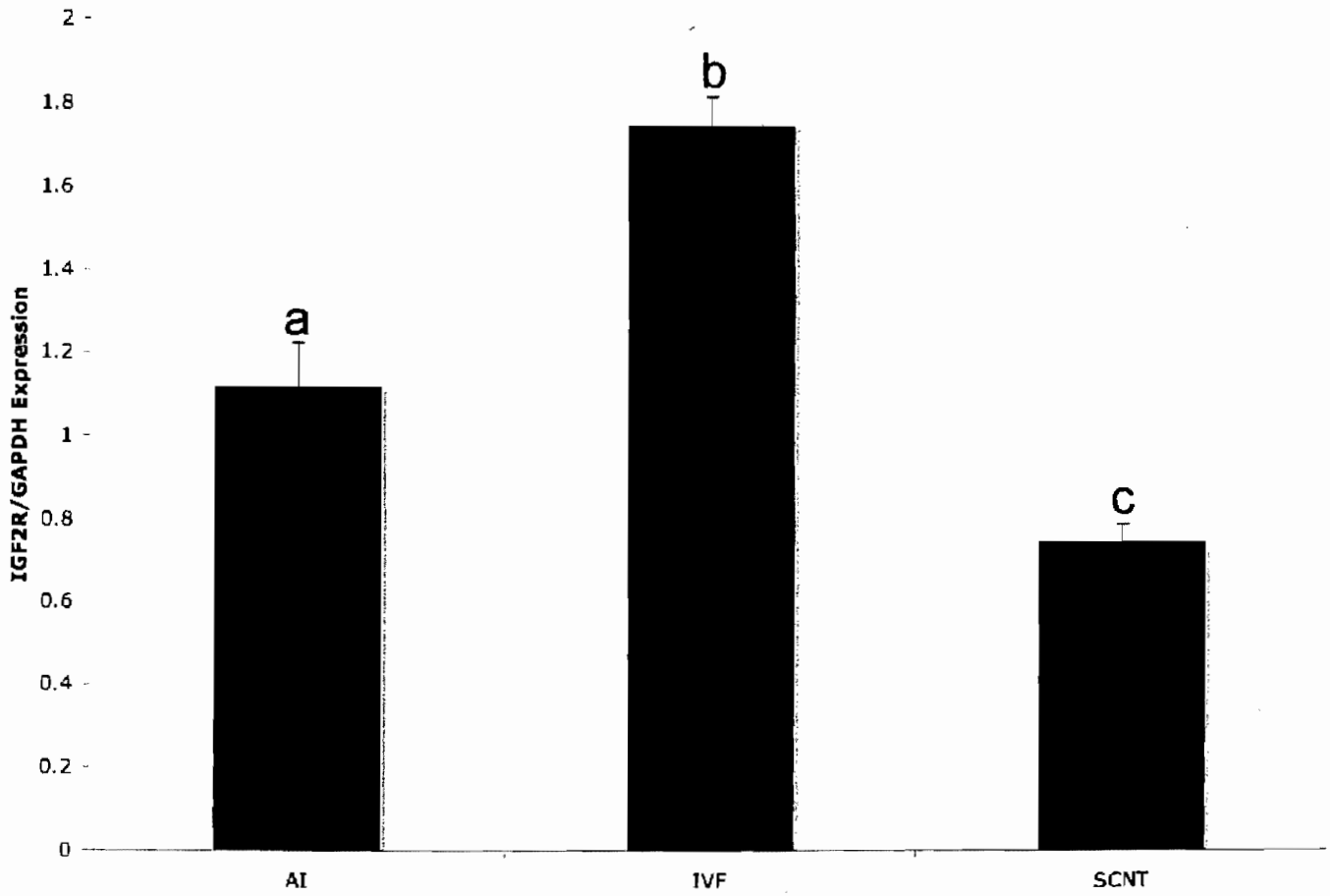

Maternal Allele DNA Methylation (%)
90
80
70
60
50
40
30
20
10
0
b
a
a
c
SCNT
IVF
In vivo
Donor Cell

FIGURE 4. Relative abundance of IGF2R transcripts in day 17 pre-implantation embryos produced by AI, IVF and SCNT. Values were normalized by transcript abundance of internal control GAPDH. Statistical analysis was performed using ANOVA test and letters indicate significant differences ($P<0.05$).


IGF2R/GAPDH Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
AI
IVF
SCNT

P<0.001

FIGURE 5. Standard curve (R^2 = 0.9793) of *Bos indicus* specific allele quantification. Maternal (*Bos taurus*) DNA was mixed with inverse proportions of paternal (*Bos indicus*) DNA ranging from 0 to 100% and used as templates for FRET analysis.

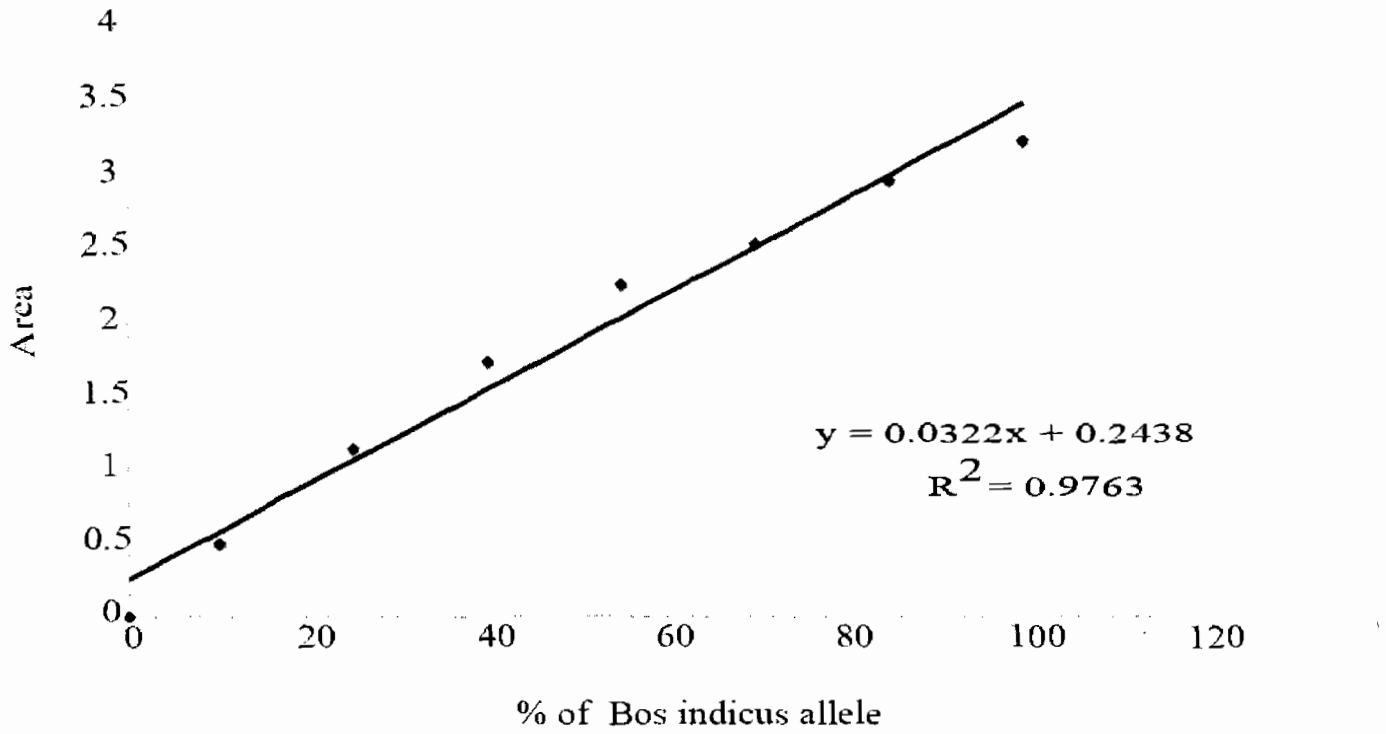

4
3.5
3
2.5
2
1.5
1
0.5
0
Area
0
20
40
60
80
100
120
% of Bos indicus allele
y = 0.0322x + 0.2438
R2 = 0.9763

FIGURE 6. Allele-specific expression analysis in day 17 embryos produced by AI (*in vivo*), IVF (*in vitro* culture) and SCNT and fibroblast donor cells. Values were obtained using the LightCycler Relative Quantification Software. Statistical analysis was perfomed using ANOVA. *= value significantly different ($P<0.05$).

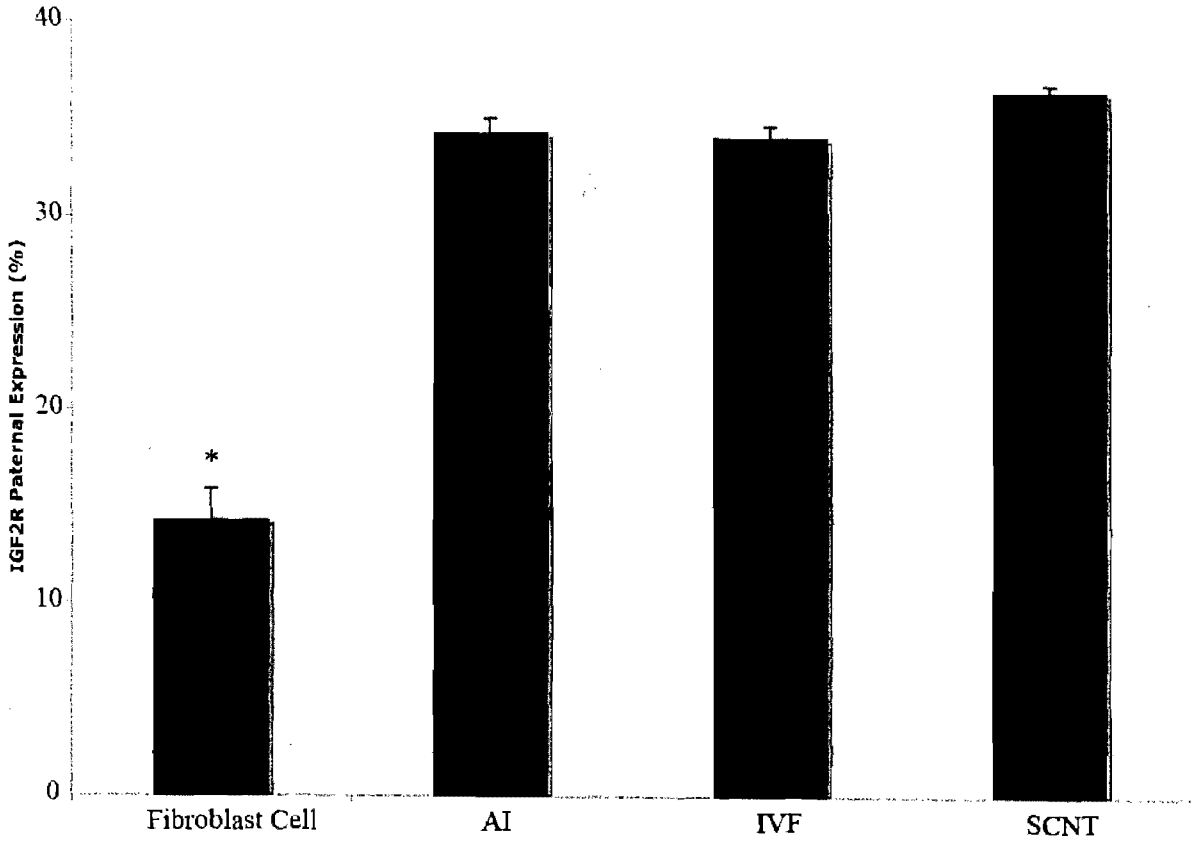
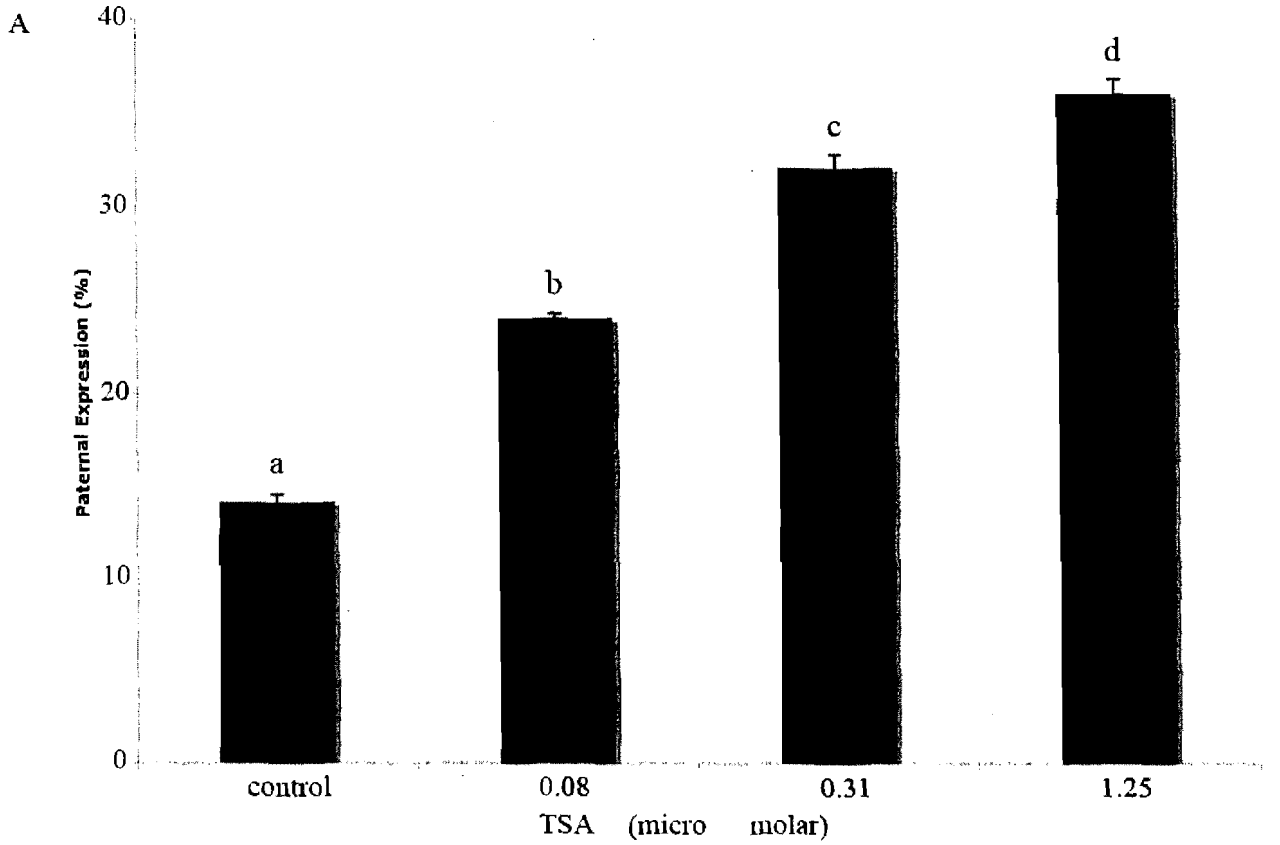
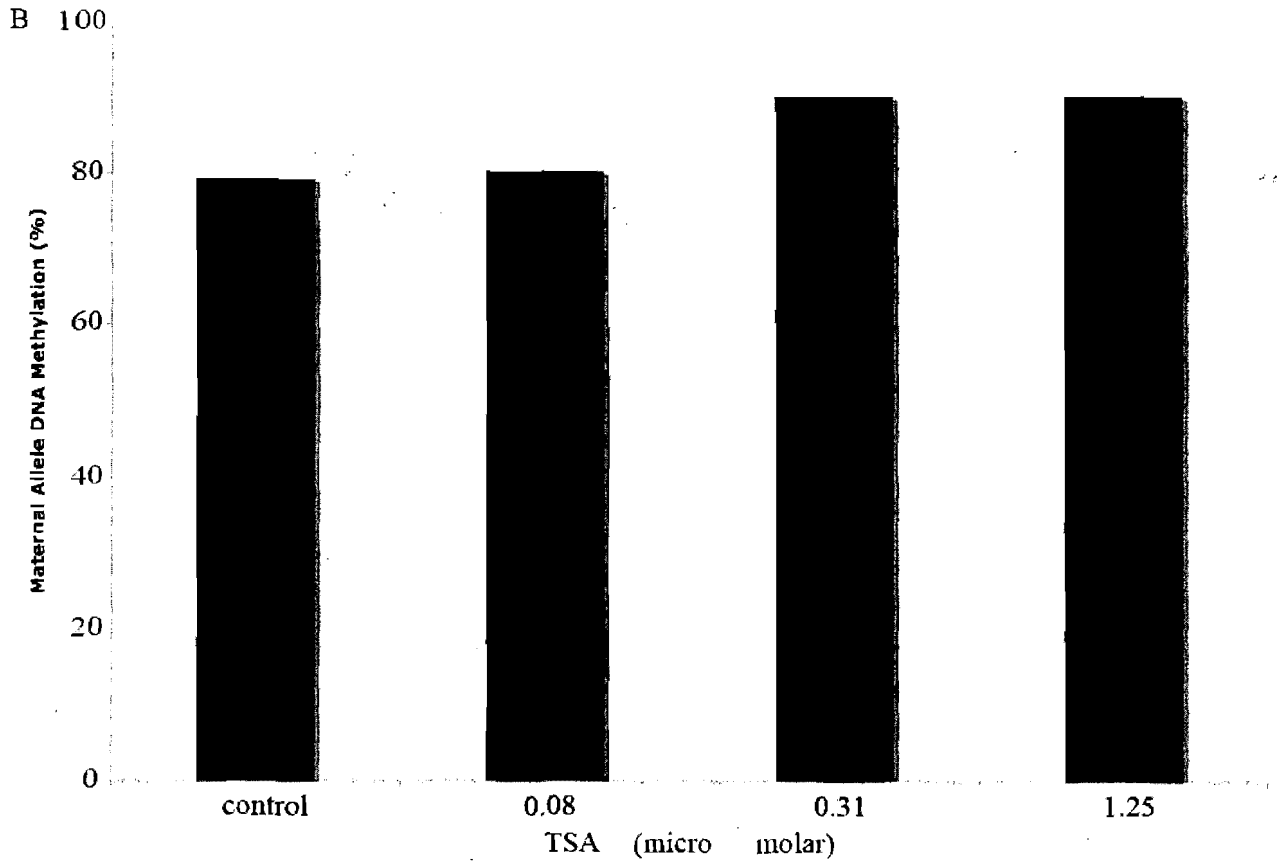

40
30
20
10
0
IGF2R Paternal Expression (%)
*
Fibroblast Cell
AI
IVF
SCNT

FIGURE 7. Allelic expression of IGF2R after TSA treatment. **A)** Allelic expression of *Bos indicus* in control fibroblast cells and cells exposed to different doses of TSA. obtained using the LightCycler Relative Quantification Software . Statistical analysis was perfomed using ANOVA. **B)** Methylation profile of IGF2R DMR2 of fibroblast cells after TSA treatment. The frequencies of methylated sites were analysed using Chi-Square test and letters indicate significant differences ($P<0.05$).


A
40
30
20
10
0
Paternal Expression (%)
a
b
c
d
control
0.08
0.31
1.25
TSA (micro molar)


B
100
80
60
40
20
0
Maternal Allele DNA Methylation (%)
control
0.08
0.31
1.25
TSA (micro molar)

General Discussion

Characterization of putative SNRPN, H19 and IGF2R and parent of origin methylation

In the present study, we have characterized de H19 DMR in cattle and confirmed the differentially methylated pattern of SNRPN as previously published (Lucifero, Suzuki et al. 2006). With IGF2R we were able to confirm that IGF2R DMR 2 is differentially methylated and conserved in cattle (Long and Cai 2007). We provided additional information on IGF2R imprinting by distinguishing parental allele and reporting that methylation is inherited and maintained only on the maternal allele.

From what is known in other species such as mice, methylation marks are erased during proliferation and migration of primordial germ cells (PGCs) genomic methylation is widely erased and is reestablished in a sex-specific manner during spermatogenesis and oogenesis (Trasler 2006). After fertilization, passive demethylation waves reprogram the zygote genome for embryo development (Santos and Dean 2004). However methylation marks of imprinted genes are maintained throughout embryo development and determine either the repression or expression of these genes while the rest of the genome becomes demethylated (Rideout, Eggan et al. 2001). Our results confirmed this hypothesis, since all imprinted analyzed showed conserved methylation patterns in day 17 preimplantation embryos. In cattle

preimplantation embryos, parent of origin methylation were represented by roughly 40 to 50% of methylated versus non methylated sites (Lucifero, Suzuki et al. 2006), which is maintained after implantation according to our results observed in day 40 bovine fetuses. H19 region, denominated differentially methylated domain (DMD) (Arney 2003) which carries a 'germ line imprint' was concurrent with the results from our study. Finally, IGF2R DMR 2 parent of origin methylation results proved to be conserved also in cattle. Together, these results suggest that parent of origin DNA methylation patterns are present also in the bovine species, are conserved during development and can be studied as a mechanism of imprinting regulation that is shared among other mammals as well.

Parental expression of the maternally (SNRPN) and paternally imprinted (H19 and IGF2R) genes

In this study, along with methylation patterns, we simultaneously analyzed the parental expression of the SNRPN, H19 and IGF2R genes. These findings were made possible by the identification of *Bos indicus/Bos taurus* polymorphisms, which enabled the parental-specific analysis of transcripts in tissues recovered from interspecies crosses during a window of early development in cattle, where not much information is available. In this study, mono-allelic maternal expression of SNRPN was characterized in cattle in pre and post-implantation development. This pattern of expression was also observed in mice and humans (Cattanach, Barr et al. 1992; Leff, Brannan et al. 1992; Ozcelik, Leff et al. 1992; Glenn, Porter et al. 1993).

Interestingly, H19 and IGF2R expression was bi-allelic in pre-implantation development and became paternally imprinted after implantation. Although in mice these two genes show monoallelic expression, evidences suggest that in cattle imprinted genes are generally expressed in a bi-allelic state in pre-implantation state, thus suggesting a particular specie specific imprinting regulation. However a study of imprinted genes (SNRPN, IGF2, H19 and IGF2R) performed in mice reported that the expression of paternally imprinted genes (H19 and IGF2R) is bi-allelic in pre-implantation embryos, whereas maternally imprinted genes are monoallelic (IGF2 and SNRPN) (Szabo and Mann 1995). It has been postulated that some aspects of germ line chromatin structure bypasses imprint-dependent regulatory elements, such as enhancers, resulting in the persistent biallelic expression of imprinted genes throughout germ-cell development (Szabo and Mann 1995). In that case, demethylation observed in zygotes after fertilization would cause chromatin opening and bypass of imprinting mechanisms more sensitive to methylation loss. In many instances the stage of establishment of monoallelic expression essentially coincides with the stage of genome-wide remethylation in mice, and this provides additional evidence that imprint-dependent methylation does help to promote or stabilize monoallelic expression. That might be true for IGF2R, where methylation levels of day 17 preimplantation embryos were lower when compared to fetal fibroblasts in our findings. Another possibility is that at least some of the additional paternal-specific methylation observed at later stages (Bartolomei et al. 1993; Ferguson-Smith et al 1993), and which is possibly acquired in response to the inherited methylation

(Tremblay et al. 1995), is also necessary for the establishment of monoallelic expression after implantation. That might explain the patchy methylation pattern found in bovine H19 DMR. However, we cannot exclude the fact that another long-range *cis-acting* mechanisms (probably more CTCF binding sites), localized away from those we analyzed, might be acting at the same time. Nonetheless, additional imprinted genes must be examined to establish if these correlations can be applied generally for parental imprinting.

The chromatin conformation can be another factor playing an important role in gene expression. It has been proposed that structural modification of histones by acetylation plays a role in the regulation of gene expression. TSA, a histone deacetylase inhibitor, causes growth arrest, differentiation or cell death of a variety of hematologic and solid tumor cells in culture. In our studies TSA converted IGF2R gene expression from mono-allelic to bi-allelic in cultured fibroblasts without altering DNA methylation levels, mimicking the situation found in pre-implantation embryos. These results confirm the hypothesis that imprinting can be establishment by histone modifications without DNA methylation (Lewis, Mitsuya et al. 2004). These results suggest that along with methylation studies, more acethylation and histone essays need to be performed with other imprinted genes to accurately analyze the role of chromatin modifications on imprinting control.

Effect of in vitro culture and somatic cell nuclear transfer on imprinting

In vitro culture has been associated with enlarged fetus and placenta tissues and this phenomenon is manifested by wide deregulation of imprited genes, especially in placenta (Young, Sinclair et al. 1998; Mann, Lee et al. 2004; Farin, Piedrahita et al. 2006). Generally, deregulation caused by in vitro culture is associated with loss of DNA methylation and relaxation of imprinted gene expression, which is important for fetal development, and for regulating and promoting growth. To test this postulate, we studied the imprinting status of H19, IGF2R and SNRPN in embryos subjected to in vitro culture, e.g. IVF group. Although in vitro cultured animals showed more individual cases of loss of methylation, statistical analysis showed no difference of DNA methylation ratio when compared to in vivo counterparts. For H19, SNRPN and IGF2R genes, methylation levels were similar to the in vivo group during pre-implantation development. However, up-regulation of IGF2R was found in IVF embryos when compared to AI. Perhaps this result might associated with the type of medium used in our experiment and we do not know if gene expression was normalized after implantation, when IGF2R becomes up regulated in in vivo animals. Interestingly, in vitro culture did cause bi-allelic expression of SNRPN gene in pre-implantation embryos, which extended to placenta samples at day 40. Fetal tissues were not affected until day 40, although methylation levels became evidently lower for day 40 tissues from in vitro culture. The maternally imprinted SNRPN gene has been extensively studied in mice and humans (Shemer, Birger et al. 1997) due to its association to Angelman and Prader-Willi syndromes, which has been putatively

linked to ART and infertility (Gosden, Trasler et al. 2003). As cited previously, in Angelman syndrome (AS) patients with an imprinting defect, loss of methylation on the maternal allele, maternal expression of SNRPN, and the maternal *UBE3A* allele being silenced are observed (Horsthemke and Ludwig 2005). Interestingly, loss of methylation and expression of maternal allele was observed in placenta of our in vitro cultured embryos. Expression of SNRPN in brain tissues was normal, however the methylation ratio than AI brain samples, and this loss could account for mutations in this locus in further stages of pregnancy

The most drastic effects of loss of demethylation and bi-allelic expression are observed in SCNT embryos. Possibly the detrimental effects of in vitro culture on DNA methylation is not the only problem to be solved in nuclear transfer. As mentioned previously, dedifferentiation of the differentiated donor somatic cell to a totipotent embryonic state, followed by redifferentiation of cloned embryos to different somatic cell types during later development seems to be a major issue (Yang, Smith et al. 2007). Nonetheless, results from SCNT can provide insightful information for testing and improving new culture conditions.

GENERAL CONCLUSION

We conclude from this work that parent of origin methylation is present in the bovine as in other species and imprinting mechanism of H19, SNRPN and IGF2R genes are conserved in cattle as well. However some genes might have particular mechanisms, which are established in a time specific manner, since parent of origin methylation did not determine H19 and IGF2R gene expression in pre-implantation stage embryos. For instance, methylation seems to be associated to imprinting regulation in some specific genes, such as SNRPN, however, the association is tissue-specific also. In other genes (H19 and IGF2R), methylation is not associated at all with imprinting control during the pre-implanation period. We also conclude that in vitro culture has detrimental effects on imprinting of the paternally expressed SNRPN gene, however, no effect was observed in maternally expressed H19 and IGf2R genes in the developmental stage analyzed.

Finally, we conclude that the study of imprinting control cannot be based solely on methylation patterns, since histone modifications play an important role in the expression of the IGF2R gene and should definitely be considered as an epigenetic control mechanism.

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