



## Epigenetic anomalies associated with prenatal survival and neonatal morbidity in cloned calves

L.C. Smith<sup>1,3,4</sup>, J. Suzuki Jr.<sup>1</sup>, A.K. Goff<sup>1</sup>, F. Filion<sup>1</sup>, J. Therrien<sup>1</sup>, B.D. Murphy<sup>1</sup>, H.R. Kohan-Ghadr<sup>1</sup>, R. Lefebvre<sup>1,2</sup>, A.C. Brisville<sup>2</sup>, S. Buczinski<sup>2</sup>, G. Fecteau<sup>2</sup>, F. Percin<sup>3</sup>, F.V. Meirelles<sup>3</sup>

<sup>1</sup>Centre de Recherche en Reproduction Animale, <sup>2</sup>Departement de Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Canada.

<sup>3</sup>Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brazil.

### Abstract

Many of the developmental anomalies observed in cloned animals are related to fetal and placental overgrowth, a phenomenon known as the “large offspring syndrome” (LOS) in ruminants. It has been hypothesized that the epigenetic control of imprinted genes, *i.e.* genes that are expressed in a parental-specific manner, is at the root of LOS. Our recent research has focused on understanding the epigenetic alterations to imprinted genes that are associated with assisted reproductive technologies (ART), such as early embryo *in vitro* culture (IVC) and somatic cell nuclear transfer (SCNT) in cattle. We have searched and identified single nucleotide polymorphisms in *Bos indicus* DNA useful for analysis of parental-specific alleles and their respective transcripts in tissues from hybrid embryos derived by crossing *Bos indicus* and *Bos taurus* cattle. Due to the frequency of placental anomalies in SCNT and in some IVC gestations, our initial studies focused on genes known to be necessary for trophoblast proliferation (Mammalian Achaete Scute-like Homologue 2; ASCL2) and differentiation (Heart and neural crest cell derivative 1; HAND1). ASCL2 was bi-allelically expressed prior to implantation but paternally silenced after implantation. At day 17, SCNT embryos showed more abundant ASCL2 and less abundant HAND1 transcripts. After implantation, SCNT fetal cotyledons displayed higher ASCL2 and HAND1 than AI and IVC tissues. To further investigate epigenetic anomalies, we analyzed the differentially methylated regions of other imprinted genes in cattle, *i.e.* SNRPN, H19 and the IGF2R. Compared with the patterns observed *in vivo* (AI), we observed a generalized hypomethylation of the imprinted allele and the bi-allelic expression of embryos produced by SCNT. Together, these results indicate that imprinting marks are erased during the reprogramming of the somatic cell nucleus during early development, indicating that such epigenetic anomalies may play a key role in the mortality and morbidity of cloned animals.

**Keywords:** cattle, cloning, embryos, epigenetics, imprinting.

### Introduction

Somatic cell nuclear transfer (SCNT) is one of a few assisted reproductive techniques that has failed to achieve the minimal efficiency required for use in commercial practice. Although many mammalian species have been cloned to date, the percentage of reconstructed oocytes that develop into normal, healthy offspring remains remarkably low, often below 1% (Panarace *et al.*, 2007). The majority of losses often occur very early during development, leading to the absorption of the deceased embryo. However, developmental failure can also occur during more advanced stages of gestation, possibly resulting from abnormal fetal-maternal interaction due to a number of placental malformations (Hill *et al.*, 2000). Finally, full-term progeny are often lost during the first few hours or days after birth due to organ malfunctions, particularly the lungs.

The objective of this review is to highlight some of the morphological and clinical abnormalities identified in SCNT cattle and to discuss the potential role that imprinted genes may play. Particular focus is given to the imprinted gene ASCL2, a transcription factor involved in the formation of the placenta, as a potential cause of the irregular fetal-maternal exchange. Moreover, we have characterized and analyzed the methylation patterns of the regions controlling the parental-specific expression of several imprinted genes in cattle, including the paternally expressed SNRPN and the maternally expressed transcript of H19, a non-coding mRNA that suppresses the expression of IGF2 from the maternal allele.

### Fetal and placental anomalies observed in cloned cattle

A recent article has reviewed, in detail, the varying clinical outcomes observed during gestation in cloned animals in the *Bos indicus* cattle of South America (Meirelles *et al.*, 2010). In Canada, we have been using a commercial cohort of SCNT gestations produced in Holstein cattle to identify the morphological and functional changes in fetal membranes during pregnancy (Kohan-Ghadr *et al.*, 2008). We found that fetuses were lost in almost 80% of day-30 confirmed pregnancies. Increased echodensity of

<sup>4</sup>Corresponding author: smithl@medvet.umontreal.ca#



uterine fluid, abnormal appearance of the embryo proper and presence of detached fetal membranes in the uterine fluid were good predictors of early embryonic mortality when observed at day-30. Later during pregnancy (>day 80), increased allantoic fluid volume, and irregular, hyperechoic borders and anechoic holes in the placentomes were associated with pregnancy loss. Other abnormal findings, such as the presence of amniotic plaques and a thicker amniotic membrane, were not as good for predicting pregnancy loss. Placentome length increased as gestation progressed but was significantly greater in clones at all stages of pregnancy compared to control group. Umbilical diameter was significantly larger in clones relative to controls at all stages, however the change was not associated with pregnancy loss. The hormonal profile of recipients revealed that mean blood progesterone concentration (P4) was significantly lower in cloned pregnancies than in the control group. Mean P4 levels were particularly low in heifers carrying clones at day 80 and day 120 of gestation. Progesterone level at day 180 was significantly lower in cows bearing clones that later aborted compared to control cows and cows bearing clones that did not abort. Heifers carrying clones secreted more Pregnancy Specific Protein B (PSPB) at day 150 of pregnancy than the controls; after day 150 there was no difference between the two groups of recipients. These variations coincided with the sharp increase in abortions among cloned bearing recipients between days 150 and 180. A higher variation from control levels in cloned pregnancies at days 150 was found in recipients that aborted shortly after the 150 day milestone. Throughout gestation, estrone sulphate increased significantly at a steady rate in both groups (clones and controls) without significant difference between groups. In contrary, estradiol blood concentrations were higher in cloned pregnancies than in control animals during the whole monitoring period (day 80 to 240). However, the statistical significance was only measured from day 120 to 180 of the gestation. There was a significant correlation between estradiol concentration and placentome size. Of all clones, neonatal mortality (>day 240) occurred in cows with a lower estradiol serum concentration at the earlier stage compared to those with higher serum level. Therefore, phenotype (structural changes) assessments by ultrasound and changes in hormonal profiles can be used to improve our understanding of developmental anomalies associated with cloned animals. Such assessments can be used as diagnostic tools for troubled pregnancies and increase the efficiency of veterinary intervention.

Another clinical objective was to assess antenatal well-being (Buczinski *et al.*, 2007, 2009). Holstein cows in the last month of pregnancy were assessed by transabdominal ultrasonographic examination with a 3.5 MHz probe to obtain fetal heart rate (FHR), fetal movements, fetal annexes appearance (maximal fluid depth, allantoamniotic membrane

thickness) as well as placentome size. Survival rates were 9 of 10 calves in controls and 3 of 10 calves in clones. Average heart rate did not differ significantly between groups. There was no correlation between FHR and fetal activity. When fetal inactivity was observed, it was associated with fetal death ( $n = 2$ ). Fetal hyperactivity seems to be a sign of fetal distress since all fetuses that were moving throughout the duration of the ultrasonographic examination had a fatal outcome (born dead or perinatal death). Hyperechoic particles present in amniotic and allantoic fluids were observed in three fatal cloned pregnancies. Estimated placentome area was larger in clones ( $35.5 \text{ cm}^2$ ) than in controls ( $25.9 \text{ cm}^2$ ). Allantoamniotic membrane was measured in 2 cases of hydrallantois (2.0 and 2.2 cm) in clones, which was thicker than in controls (mean = 0.69 cm).

To investigate the anomalies during the perinatal period in clones, we analyzed 26 offspring originating from 10 different genotypes (8 males and 2 females). The birth weight varied from 35 to 80 kg (mean = 56 kg, SD = 8.9 kg). Only three calves were considered completely normal and did not need perinatal support. Three calves were euthanized because of severe arthrogryposis. The remaining calves ( $n = 20$ ) suffered from some degree of respiratory dysfunction, enlarged umbilical vessels, poor suckling reflex, and generalized weakness. Two broad categories of respiratory dysfunction were recognized. One category consisted of 13 calves suffering from hypoxemia without significant hypercapnia. They were treated with intranasal oxygen supplementation. They responded well and were discharged after 38 to 288 h of oxygen supplementation (median = 70 h). The other category consisted of 7 calves suffering from severe hypoxia associated with hypercapnia. They needed mechanical ventilation support and two calves died despite treatment. The other 5 calves were ventilated for 17 to 48 h (median = 32 h) and subsequently treated with intranasal oxygen supplementation. They were discharged after 89 to 288 h of oxygen supplementation (median = 140 h). All calves had enlarged umbilical vessels and postnatal bleeding was a frequent complication. Among them, 11/21 needed umbilical surgery later in life. Furthermore, when a calf suffered with respiratory problems it also had a poor suckling reflex and the colostrum and milk were administered with an esophageal feeder. Although the incidence of perinatal diseases (20/23, 87%) was higher, compared to calves produced by artificial insemination or *in vitro* fertilization, an intensive monitoring associated with an aggressive management limited the lethality rate (2/20, 10%) and the mortality rate (2/23, 9%). The precise etiology of the respiratory dysfunction and enlarged umbilical vessels remains to be identified.

#### Anomalies with imprinted gene expression

Gamete genomic DNA is epigenetically reprogrammed during the period preceding and



following fertilization. Paternal DNA is actively and rapidly demethylated after fertilization, whereas the maternal DNA undergoes passive demethylation during the initial cleavages (Oswald *et al.*, 2000), and remethylation of both alleles occurs simultaneously during development to the blastocyst stage (Dean *et al.*, 2001). Anomalies in global and repetitive element DNA methylation have been well characterized in SCNT (Bourc'his *et al.*, 2001). However, imprinted gene epigenetic marks are expected to escape the demethylation and remethylation waves that occur throughout the genome. In mice, previous studies have identified aberrant expression of several imprinted genes and have shown parental allele-specific loss of DNA methylation at the *Snrpn* and *H19* differentially methylated regions, or DMRs (Mann *et al.*, 2003). The authors were able to undertake allele-specific expression and DMR studies using the B6(CAST) substrain that carries *Mus musculus castaneus* (CAST) chromosome 7 on a C57BL/6J genetic background. Unfortunately, such backcrossing experiments to generate animal models carrying allele-specific markers cannot be obtained with random-bred populations, particularly cattle.

#### *A model for studying imprinted genes during development in cattle*

It is well known that imprinted genes play an important role in the epigenetic anomalies observed in LOS produced by *in vitro* culture (Young *et al.*, 2001) and cloning (Yang *et al.*, 2005) in ruminants. Nonetheless, the lack of appropriate animal models has prohibited a detailed investigation of the molecular

mechanisms implicated. Since little information is known about imprinted genes in domestic animals, including cattle, it was necessary to identify parental-specific single nucleotide polymorphisms (SNP) to enable parental-specific analysis of transcripts and genomic sequences. Therefore, we developed a F1 hybrid model in which the paternal and maternal genomes were either from the *Bos taurus taurus* (BT) or *Bos taurus indicus* (BI) subspecies of cattle (Fig. 1). For *in vivo* samples, purebred Holstein and Nellore animals were artificially inseminated (AI) with frozen semen from the reciprocal subspecies to obtain BT x BI F1 hybrids. For IVF samples, host oocytes were derived from slaughterhouses that process either exclusively BT (Holstein cows in Canada) or BI (Nellore cows in Brazil) cattle and the *in vitro* matured oocytes were inseminated *in vitro* using semen from the reciprocal sub-species. In the case of SCNT, enucleated oocytes were fused to fibroblasts obtained from the F1 fetuses as described above. IVF and SCNT embryos were cultured *in vitro* for 8 days and transferred at the blastocyst stage to synchronized recipient cows. Pre-implantation embryos were recovered non-surgically on day 17. Post-implantation fetal and extra embryonic tissue samples were recovered for analysis during early (day 30 to day 50), mid (day 90), late (day 150) or term gestation. This access to tissues from different stages in development provides a unique means to study the dynamic epigenetic mechanisms controlling the parental-specific expression of imprinted genes in cattle; and the epigenetic risks or consequences of using different artificial reproductive techniques.

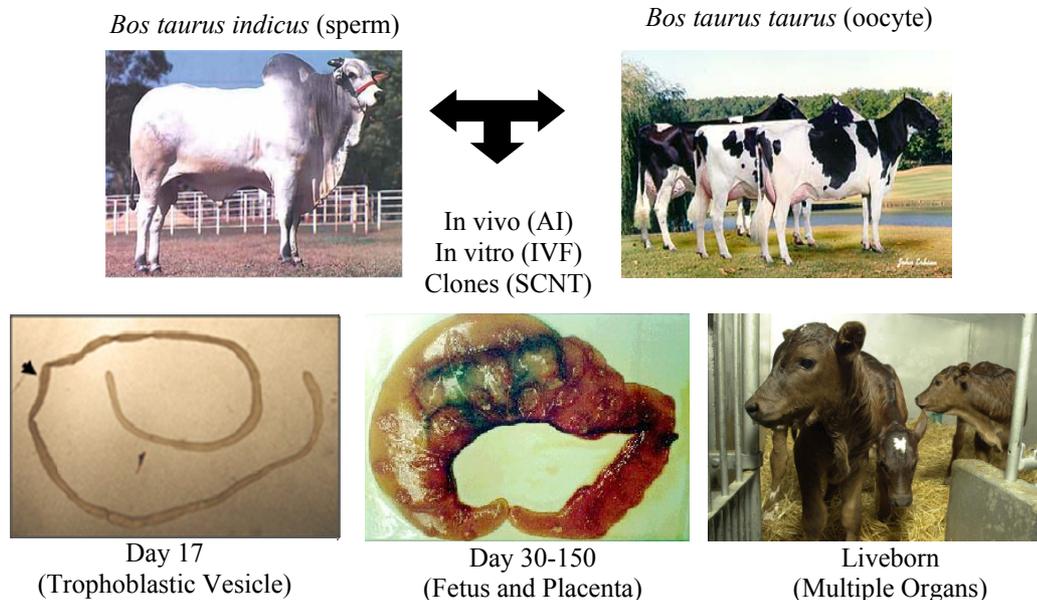


Figure 1. Animal model for studying the control of imprinted gene expression during development in cattle. The model benefits from the evolutionary distance between *Bos indicus* and *Bos taurus* cattle which, regardless of the robust reproduction of hybrids, carry numerous single nucleotide polymorphisms (SNP) that can be used to identify the parental origin of alleles and transcripts during analysis. Tissues were recovered during different stages of development, including pre-implantation (day 17), post-implantation (days 30 to 150) and after birth.

### Trophoblast and placental gene expression is affected by SCNT in cattle

Much of what is known of the mechanisms controlling placental development in mammals has been obtained from the mouse model, which can differ from cattle in many aspects. Particularities during early differentiation of trophoblast cells in ruminants include the derivation of mononucleate cells that produce interferon-tau (IFN- $\tau$ ), the pregnancy recognition signal, and binucleate cells that produce pregnancy-related proteins such as pregnancy-associated glycoproteins (PAG's), placental lactogen (PL), and prolactin-related proteins. We set out to study the expression of a set of genes involved in the function and differentiation of the placenta in cloned bovine embryos (Arnold *et al.*, 2006a). We focused initially on analyzing the abundance of transcripts of ASCL2, HAND1, IFN- $\tau$  and PAG-9 mRNA as indicators of trophoblast differentiation and function. Comparisons in the expression of these genes were made between *in vivo* (AI), *in vitro* (IVF), and SCNT produced bovine

embryos prior to implantation. The abundance of ASCL2 mRNA was greatest in SCNT embryos compared to AI embryos whereas the abundance of HAND1 and PAG-9 mRNA were lower in SCNT embryos. No differences in IFN- $\tau$  mRNA were detected among these groups (Fig. 2). To perform bi-allelic expression analysis, ASCL2 cDNA from day 60 BT and BI placental tissues were sequenced and analyzed for the presence of single nucleotide polymorphisms (Arnold *et al.*, 2006b). Digestion of cDNA using *Sfi* I indicated that ASCL2 was biallelically expressed in all groups, indicating that overexpression of ASCL2 in SCNT embryos and fetuses was not due to alteration in imprinting. Placental samples collected on day 40 were labelled with a monoclonal antibody that recognizes PAGs. Results indicated that AI fetuses contained 16% binucleate cells whereas the IVF and SCNT placentas had 12 and 8.6% binucleate cells, respectively. Therefore, these results suggested that abnormal development of the binucleate cells of the placenta could be involved, at least in part, with the problems in implantation and placental development associated with nuclear transfer.

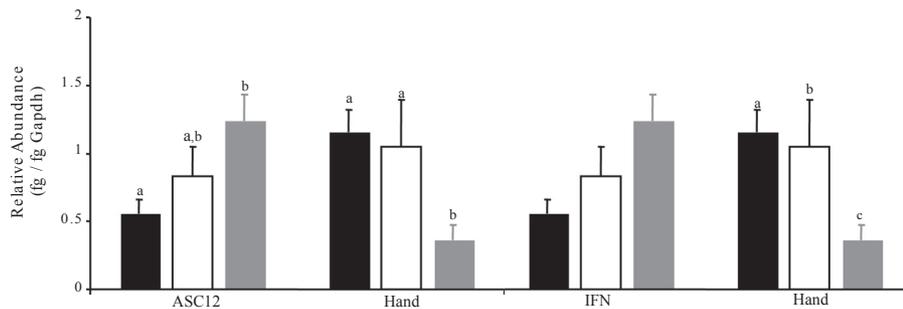


Figure 2. Transcript abundance of placental genes in day 17 bovine embryos derived *in vivo* (black), *in vitro* (white) and by somatic cell nuclear transfer (grey). ASCL2, HAND1 and PAG-9 expression was significantly altered in SCNT embryos when compared to *in vivo* controls.

### Imprinted genes are affected by SCNT in cattle

The abnormal expression of the imprinted gene, ASCL2, in SCNT placentas instigated further studies to examine the epigenetic alterations of other imprinted genes that might have been caused by SCNT in cattle. We set out to identify the DMRs of a series of imprinted genes that could potentially be implicated with placental development and the LOS phenotype in cattle. Using the bovine F1 hybrid model described above (section "A model for studying imprinted genes during development in cattle"), we were able to identify SNPs in proximity to the DMR and exonic polymorphisms that enabled us to analyze the imprinting status of several genes, including the maternally expressed H19 and insulin-like growth factor 2 receptor (IGF2R) genes, and the paternally expressed SNRPN gene (Fig. 3). Analysis was performed on day 17 pre-implantation embryos, and is currently being performed on post-implantation embryonic and extra-embryonic (placenta) tissues at different stages of development. Methylation patterns of the SNRPN DMR are affected in SCNT, whereas no

differences were observed between *in vivo*- and *in vitro*-derived day 17 embryos (Lucifero *et al.*, 2006), suggesting that this epigenetic anomaly was caused by an erroneous reprogramming of the somatic cell chromatin by the oocyte cytoplasm and not by the culture conditions employed during initial development to the blastocyst stage. In follow-up experiments we have confirmed that the demethylation of SCNT is also observed after implantation, in both embryonic (brain, heart, liver and skeletal muscle) and extra-embryonic (placenta) tissues (Suzuki *et al.*, 2009). Surprisingly, a less prominent hypomethylation of the SNRPN DMR was also observed in *in vitro*-derived embryonic and extraembryonic tissues, indicating that *in vitro* culture during early development can induce epigenetic errors that persist beyond implantation. Indeed, having identified a *Bos indicus* SNP within exon 2 of SNURF, we demonstrated that whereas *in vivo*-derived day 17 are mono-allelic, *i.e.* expressing exclusively from paternal allele, both *in vitro* and SCNT embryos showed significant levels of maternal expression. However, *in vitro*-derived bi-allelic expression was no longer



observed in embryonic tissues after implantation (day 40), suggesting that the hypomethylation patterns observed in these tissues are insufficient to induce maternal expression. Nonetheless, placental tissues remained bi-allelic beyond implantation, suggesting that the extraembryonic tissue may sustain higher levels of

hypomethylation. In contrast, SCNT-derived embryonic and extra-embryonic tissues showed substantial bi-allelic expression and DMR hypomethylation, indicating that even those embryos carrying severe epigenetic anomalies in this imprinted gene can survive beyond implantation.

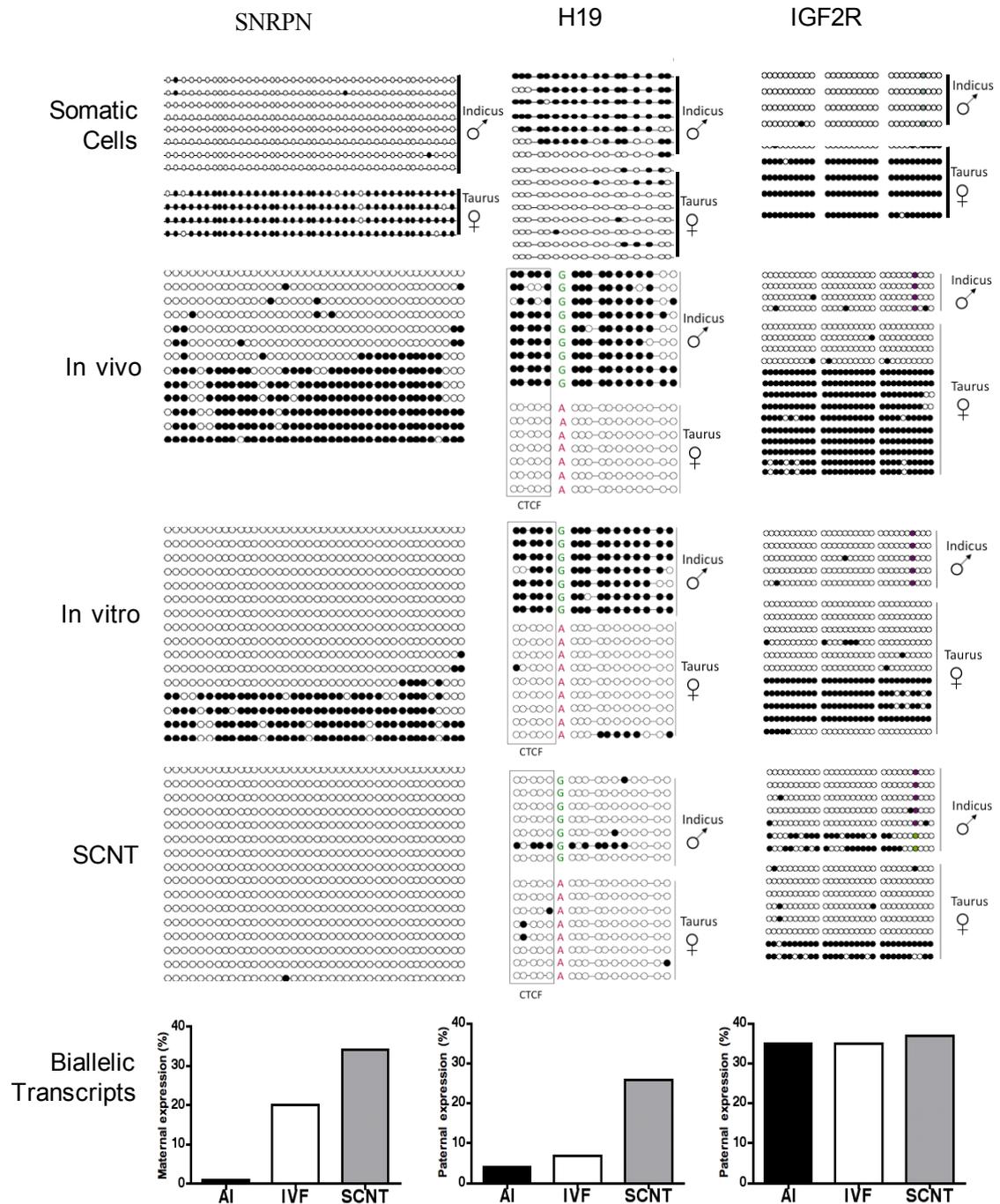


Figure 3. DNA methylation patterns of differentially methylated regions (DMR) in imprinted genes at day 17 of development in cattle. Allele-specific analysis of the DMR of different imprinted genes is possible through the identification of *Bos indicus* SNP that enable parental-specific analysis of methylation patterns by bisulfite sequencing. When compared to somatic cells, and embryos of *in vivo* and *in vitro* origin, SCNT embryos show remarkable hypomethylation of the DMR of SNRPN, H19 and IGF2R genes. Levels of biallelic expression vary for each imprinted gene (bar graphs). With the exception of IGF2R, biallelic expression levels of SNRPN and H19 are strongly associated with parental-specific DMR hypomethylation.



To extend our finding to other imprinted bovine genes, we have identified SNPs in the DMRs and the transcripts (cDNA) of H19 and IGF2R in cattle. Recent analysis of the DMR of the maternally expressed H19 gene indicated a hypermethylation of the paternal allele both within and surrounding a conserved CTCF binding site. Similar to SNRPN, SCNT caused the hypomethylation of the paternal H19 DMR in day 17 pre-implantation embryos, which correlates significantly with increased expression of H19 from the paternal allele, *i.e.* bi-allelic expression. Interestingly, compared to the *in vivo* group, the overall transcript abundance level of both H19 and IGF2 was reduced in SCNT and *in vitro*-derived embryos.

During post-implantation (day 30 to 50) the methylation of the H19 CTCF was not affected in placenta, but was reduced in SCNT embryonic muscle when compared to *in vivo* and *in vitro* samples. However, and in contrast to the unaffected SNRPN DMR during post-implantation, SCNT led to a partial demethylation of the paternal H19 DMR and the respective CTCF binding site on the paternally imprinted allele. Nonetheless, exclusive mono-allelic maternal expression was observed in both embryonic (muscle) and extra-embryonic (placenta) post-implantation tissues, indicating that the hypomethylation levels were insufficient to enable expression from the paternal allele. Interestingly, overall transcript abundance levels of IGF2, but not H19, were slightly increased in the placenta of SCNT-derived gestations. Since SCNT embryos are usually smaller, it is possible that increased IGF2 expression adversely affects the growth rate of the embryo itself. Preliminary analysis of the paternally imprinted IGF2R gene, indicates that, although the methylation patterns of the DMR are reduced in the SCNT day 17 vesicles, expression is consistently bi-allelic, regardless of whether the embryos were derived *in vivo*, *in vitro* or by SCNT. Therefore, IGF2R may not be strongly imprinted at this early stage in development in cattle, and an epigenetic mechanism other than the methylation of the DMR may be implicated in controlling allele-specific expression.

### Conclusions and perspectives

Together, analysis of the methylation patterns in DMRs and allele-specific expression of bovine imprinted genes clearly indicates that the procedure of SCNT leads to significant epigenetic disturbances. The levels of allele-specific transcriptional disturbance vary according to the role DNA methylation plays in controlling mono-allelic expression of each imprinted gene. However, the severe loss of methylation and bi-allelic expression of most imprinted genes in the SCNT group suggests that at least some imprinting marks are erased during the reprogramming of the somatic cell nucleus by the oocyte cytoplasm and/or during early

development. Exposure of donor cells and reconstructed embryos to epigenetic modifiers, such as inhibitors of DNA methyltransferase (5-aza-2'-deoxycytidine) and histone deacetylase (trichostatin A), have been used alone and together to improve development after SCNT (Enright *et al.*, 2005; Ding *et al.*, 2008). However, since such agents act globally on the whole genome it is unlikely that they will be useful for acting specifically and exclusively on the DMRs of imprinted genes. Further investigations are required to devise strategies that inhibit the demethylation of imprinted gene DMRs of donor cells without interfering with the widespread chromatin reprogramming that is required to enable normal development and differentiation of healthy offspring after SCNT.

### Acknowledgments

The authors would like to thank the support of the Canada Research Chair (CRC) Program, the Strategic Grant Program (STPGP 269864-03), and the Discovery Grants Program (RGPIN 106964-05) of the Natural Sciences and Engineering Research Council (NSERC) of Canada. This work was also funded by grant HGG62293 under the Healthy Gametes and Great Embryos Strategic Initiative of the Canadian Institutes of Health Research (CIHR) an Institute of Human Development, Child and Youth Health.

### References

- Arnold DR, Bordignon V, Lefebvre R, Murphy BD, Smith LC.** 2006a. Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. *Reproduction*, 132:279-290.
- Arnold DR, Lefebvre R, Smith LC.** 2006b. Characterization of the placenta specific bovine mammalian achaete scute-like homologue 2 (Mash2) gene. *Placenta*, 27:1124-1131.
- Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E.** 2001. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr Biol*, 11:1542-1546.
- Buczinski SM, Fecteau G, Lefebvre RC, Smith LC.** 2007. Fetal well-being assessment in bovine near-term gestations: current knowledge and future perspectives arising from comparative medicine. *Can Vet J*, 48:178-183.
- Buczinski SM, Fecteau G, Comeau G, Boysen SR, Lefebvre RC, Smith LC.** 2009. Ultrasonographic fetal well-being assessment, neonatal and postpartum findings of cloned pregnancies in cattle: a preliminary study on 10 fetuses and calves. *Can Vet J*, 50:261-269.
- Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W.** 2001. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned



- embryos. *Proc Natl Acad Sci USA*, 98:13734-13738.
- Ding X, Wang Y, Zhang D, Guo Z, Zhang Y.** 2008. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. *Theriogenology*, 70:622-630.
- Enright BP, Sung LY, Chang CC, Yang X, Tian XC.** 2005. Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2'-deoxycytidine. *Biol Reprod*, 72:944-948.
- Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME.** 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol Reprod*, 63:1787-1794.
- Kohan-Ghadr HR, Lefebvre RC, Fecteau G, Smith LC, Murphy BD, Suzuki Junior J, Girard C, Helie P.** 2008. Ultrasonographic and histological characterization of the placenta of somatic nuclear transfer-derived pregnancies in dairy cattle. *Theriogenology*, 69:218-230.
- Lucifero D, Suzuki J, Bordignon V, Martel J, Vigneault C, Therrien J, Filion F, Smith LC, Trasler JM.** 2006. Bovine SNRPN methylation imprint in oocytes and day 17 in vitro-produced and somatic cell nuclear transfer embryos. *Biol Reprod*, 75:531-538.
- Mann MR, Chung YG, Nolen LD, Verona RI, Latham KE, Bartolomei MS.** 2003. Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol Reprod*, 69:902-914.
- Meirelles FV, Birgel EH, Perecin F, Bertolini M, Traldi AS, Pimentel JR, Komninou ER, Sangalli JR, Neto PF, Nunes MT, Pogliani FC, Meirelles FD, Kubrusly FS, Vannucchi CI, Silva LC.** 2010. Delivery of cloned offspring: experience in Zebu cattle (*Bos indicus*). *Reprod Fertil Dev*, 22:88-97.
- Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J.** 2000. Active demethylation of the paternal genome in the mouse zygote. *Curr Biol*, 10:475-478.
- Panarace M, Aguero JI, Garrote M, Jauregui G, Segovia A, Cane L, Gutierrez J, Marfil M, Rigali F, Pugliese M, Young S, Lagioia J, Garnil C, Forte Pontes JE, Ereno Junio JC, Mower S, Medina M.** 2007. How healthy are clones and their progeny: 5 years of field experience. *Theriogenology*, 67:142-151.
- Suzuki J Jr, Therrien J, Filion F, Lefebvre R, Goff AK, Smith LC.** 2009. In vitro culture and somatic cell nuclear transfer affect imprinting of SNRPN gene in pre- and post-implantation stages of development in cattle. *BMC Dev Biol*, 9:9. (abstract).
- Yang L, Chavatte-Palmer P, Kubota C, O'Neill M, Hoagland T, Renard JP, Taneja M, Yang X, Tian XC.** 2005. Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. *Mol Reprod Dev*, 71:431-438.
- Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD.** 2001. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet*, 27:153-154.
-