Université de Montréal

REGULATION OF TROPHOBLAST DEVELOPMENT IN THE BOVINE EMBRYO

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RÉSUMÉ

Le développement placentaire anormal chez les embryons produits par transfert nucléaire (TN) à partir de cellules somatiques est relié avec une incidence élevée de mortalité embryonnaire en cours de gestation. Plusieurs études ont décrit le développement morphologique du placenta chez le bovin alors que les mécanismes de régulation au niveau moléculaire demeurent en grande partie inconnus. Les objectifs de la présente thèse étaient de ; 1) caractériser, chez le bovin, le gène Ascl2 responsable de la différenciation des trophoblastes ; 2) quantifier l'expression du gène Ascl2 ainsi que d'autres gènes critiques dans le développement des trophoblastes en début de gestation chez des embryons produits par insémination artificielle (IA), fécondation *in vitro* (FIV) et TN; 3) déterminer si les modifications épigénétiques chez les embryons produits par NT sont causées par l'expression de gènes responsables de la détermination du profil épigénétique (Dnmt1, Dnmt3b, HAT1 et HDAC1) et également développer des lignées cellulaires de trophoblastes issus d'embryons produits par FIV et TN comme modèles potentiels du développement des trophoblastes chez le bovin.

L'ARNm du gène Ascl2 chez le bovin présente une forte homologie avec l'humain et la souris particulièrement au niveau du domaine de liaison à l'ADN et dans les régions bHLH. L'expression du gène Ascl2 a été détectée exclusivement dans les tissus du cotyledon avec une abondance supérieure mesurée chez les embryons filamenteux au jour 17 correspondant à la période de prolifération rapide des trophoblastes. Une réduction de

l'abondance de l'ARNm mesurée chez des blastocystes parthénogénétiques au jour 8 suggère une atténuation de l'allèle paternel de ce gène d'expression maternelle. Avant l'implantation (jours 8 et 17), l'ARNm du gène Ascl2 semble présenter une expression biallélique, mais l'expression de l'allèle paternel semble être atténuée après l'implantation (jours 40 et 60). En conclusion, le gène Ascl2 est hautement conservé d'une espèce à l'autre et son expression chez le bovin est exclusive au placenta. Le gène Ascl2 chez le bovin s'avère être exprimé par le biais de l'allèle maternel après implantation quoique le génome paternel tient également un rôle dans la régulation de l'expression de ce gène.

Les niveaux d'ARNm du gène Ascl2 étaient plus élevés chez les embryons résultant de TN comparativement à ceux produits par IA alors que l'inverse a pu être mesuré pour le gène Hand1. Aucune différence n'a été notée au niveau de l'abondance de l'ARNm du gène IFN-τ entre les différents groupes d'embryons. Aucune expression du gène PAG-9 n'a été détectée chez les embryons produits par TN alors que les plus hauts niveaux ont été mesurés chez les embryons produits par IA. Au jour 40, les cotylédons des embryons produits par TN présentaient de plus hauts niveaux d'expression des gènes Ascl2 et Hand1 que tout autre tissu provenant d'embryons produits par IA. Les cotylédons des embryons produits par TN au jour 40 avaient le plus petit nombre de cellules binuclées fonctionnelles suivis des embryons produits par FIV et ensuite par IA. Ainsi, certains gènes critiques pour le développement normal du placenta sont altérés chez les embryons bovins produits par TN et conduisant à une différenciation anormale des trophoblastes avec une incidence directe au niveau de la mortalité embryonnaire. Seul l'ARNm pour le gène Dnmt1

montrait une abondance inférieure chez les embryons produits par TN au stade blastocyste. Aucune différence dans les niveaux d'expression pour l'ensemble des gènes n'a été notée dans les tissus placentaires au jour 40. Les cellules de trophoblaste des embryons produits par TN ont montré une abondance réduite des niveaux d'ARNm du gène Dnmt3b mais une plus forte abondance en ce qui a trait au gène HAT1. De surcroît, les trophoblastes des embryons produits par TN ont montré un taux de croissance plus rapide tout en exprimant davantage le gène Ascl2 comparativement aux cellules de trophoblaste des embryons produits par FIV. Ces résultats suggèrent que l'expression de gènes impliqués dans certains processus épigénétiques peut ne pas être la cause d'un dérangement de la reprogrammation du génome associée au transfert nucléaire. De plus, l'habilité des trophoblastes développés à partir d'embryons produits par TN à maintenir un niveau d'ARNm du gène Ascl2 similaire aux niveaux retrouvés *in vivo* suggère que ces lignées soient un modèle adéquat dans l'étude du développement des trophoblastes en milieu de culture.

La détermination des évènements moléculaires impliqués dans le développement des trophoblastes chez le bovin permet une meilleure compréhension des mécanismes de régulation impliqués dans le développement normal du placenta. Il en ressort également la possibilité d'analyser la façon dont l'ensemble de ces évènements moléculaires peuvent être manipulés afin d'améliorer l'efficacité de certaines techniques de reproduction assistée telles que le transfert nucléaire.

Mots clés: Bovin, développement des cellules de trophoblaste, transfert nucléaire, Placenta, Ascl2

ABSTRACT

Altered placental development in embryos produced by somatic cell nuclear transfer (SCNT) is associated with a high number of pregnancy losses. Several studies have described morphological development of the bovine placenta, whereas the molecular regulation remains unknown. The objectives of this thesis were to; 1) characterize the trophoblast gene Ascl2 in the bovine; 2) determine the expression of Ascl2, as well as other trophoblast genes, in early gestation of embryos produced by artificial insemination, in vitro fertilization and SCNT; and 3) determine if the epigenetic alterations associated with SCNT embryos is due to altered expression of epigenetic modifying genes (Dnmt1, Dnmt3b, HAT1 and HDAC1), as well as development of IVF and SCNT trophoblast cell lines as a potential model for bovine trophoblast development.

Bovine Ascl2 mRNA shares high homology with human and mouse Ascl2, especially in the DNA binding domain and bHLH regions. Expression Ascl2 mRNA was exclusively in the cotyledon tissue with the greatest abundance of Ascl2 mRNA in day 17 filamentous embryos, during the time of rapid trophoblast proliferation. Reduction in Ascl2 mRNA abundance was detected in day 8 parthenogenetic blastocysts which suggests a paternal regulation of the maternally expressed gene. Prior to implantation (days 8 and 17), Ascl2 mRNA appears to have biallelic expression, but is paternally silenced after implantation (days 40 and 60). In conclusion, the Ascl2 is highly conserved across species and is specifically expressed in the bovine placenta. Bovine Ascl2 appears to be maternally

expressed after implantation, but the paternal genome plays a role in regulating bovine Ascl2 expression.

Ascl2 mRNA was greater in NT embryos compared to AI, while Hand1 was greater in AI embryos compared to NT. IFN-τ mRNA abundance did not differ among groups. PAG-9 mRNA was detectable in AI embryos but not in NT embryos. At day 40, NT fetal cotyledons had higher Ascl2 and Hand1 than did AI tissues. Day 40 NT cotyledons had the fewest functional binucleate cells, followed by IVF and AI. Thus, genes critical for normal placental development are altered in NT bovine embryos leading to abnormal trophoblast differentiation and contributing to pregnancy loss.

In day 8 SCNT blastocysts, abundance of Dnmt1 mRNA was less than IVF counterparts. In day 40 placental tissues no difference in mRNA was detected in any of the epigenetic modifying genes studied. In SCNT primary trophoblast stem (TS) cells abundance of Dnmt3b mRNA was less and HAT1 mRNA was more. In addition SCNT TS cells grew at a faster rate and expressed more Ascl2 mRNA, than IVF TS cells. These results suggest that the expression of epigenetic modifying genes may not be the cause of altered reprogramming associated with SCNT. In addition, the ability of TS, developed from SCNT embryos, to maintain elevated levels of Ascl2 mRNA was similar to that found in embryos and cotyledonary tissues, suggesting that this cell line are a model for investigating bovine trophoblast development in culture.

By understanding the underlying molecular events involved in bovine trophoblast development, we can gain insight into the regulatory mechanism involved in successful

placentation and how these events may be manipulated to improve assisted reproductive techniques such as somatic cell nuclear transfer.

Keywords: Bovine, Trophoblast Cell Development, Somatic Cell Nuclear Transfer, Placenta, Ascl2

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To my Family

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LIST OF ABBREVIATIONS

Ascl2	Achaete scute-like homolog 2
bHLH	Basic helix-loop-helix
BT-1	Bovine trophoblast 1
Cdx2	Caudal-related homeobox 2
DMR	Differentially methylated regions
DNA	Deoxyribonucleic acid
Dnmt	De novo methyl transferase
Eomes	Eomesoderm
FBS	Fetal bovine serum
Fgf4	Fibroblast growth factor 4
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Gcm1	Glial cell missing 1
Hand1	Heart and neural crest derivative 1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICM	Inner cell mass
INF-τ	Interferon tau
IVF	In vitro fertilization
LOS	Large offspring syndrome

mRNA	Messenger ribonucleic acid
Oct4	Octamer-binding transcription factor 4
PAG-9	Pregnancy-associated glycoprotein 9
PBS	Phosphate-buffered saline
PpiA	Peptidylprolyl isomerase A
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription-polymerase chain reaction
SCNT	Somatic cell nuclear transfer
TBS	Tris-buffered saline
TS	Trophoblast Stem

INTRODUCTION

Normal placental development is pivotal for successful pregnancy in mammals, such as cattle. The placenta is a unique and complex organ that functions as the sole source of nutrients and protection for the embryo/fetus until birth. In mammals, the form and shape of the placenta is highly variable between species. In ruminants, placentation is of the epithiliealchorial-syndesmochorial or placentomal type. As in all mammals, if the bovine placenta does not form or function properly, pregnancy is lost. In the bovine, survival rates of in vitro produced conceptuses diminish from 93% on day 8 to 55% by day 40 (Diskin and Sreenan, 1980). Many of these losses are due to abnormal embryonic development which do not allow the fetal/maternal interface to form correctly (Thatcher et al., 1995).

Since the development of assisted reproductive techniques in cattle there have been several reports of abnormal placental development. Altered placental development and high pregnancy losses appear to be the norm for somatic cell nuclear transfer (SCNT) rather than the exception. The most common observation is reduced and underdeveloped placentomes early in gestation. Several researchers have reported poor vascular development and absence or reduced number of cotyledons in day 40 to day 60 SCNT placental tissue (Hill et al., 2000a; Stice et al., 1996). The rare SCNT fetuses that are able to survive through pregnancy are usually associated with a fewer number of enlarged placentomes later in gestation (Cibelli et al., 1998; Hill et al., 1999). These results suggest that the nutrient demand of the fetus may influence placental growth.

Although elegant studies have described the cellular morphology of the bovine trophoblast (outer cells layer of the placenta), little is known about the molecular regulation

of these processes. The majority of genetic and developmental information on placental development has been taken from mice knockout experiments where embryonic death occurred. By utilizing the extensive research done in mice, one gene that is pivotal for placental development is achaete scute-like homologue 2 (Ascl2; also known as Mash2). Embryonic death occurs at 10 days postcoitum (d.p.c.) in homozygous mutant Ascl2 mice due to the lack of development of the spongiotrophoblast layer and an increase in giant cell number and size (Guillemot et al., 1994). Homozygous mutant mice did survive when diploid Ascl2-/- embryos (which will give rise to the ICM) were aggregrated with tetraploidy wild-type embryos (which will give rise only to the trophoblast cells) suggesting that Ascl2 is only involved in placental development in the mouse (Guillemot et al., 1995). In addition, Ascl2 appears to be a maternally expressed imprinted gene (Tanaka et al., 1999). Bovine Ascl2 mRNA has been reported in blastocyst embryos (Wrenzycki et al., 2001), but its characteristics and regulation in cattle are not known.

One of the leading causes of SCNT developmental failure is thought to be the incomplete reprogramming of the donor cells to a totipotent state similar to a zygote and consequent genetic and epigenetic alterations that persist through development (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). One epigenetic process that appears to be altered in SCNT embryos is DNA methylation. DNA methylation plays a critical role in imprinting. Genome-wide demethylation that normally occurs in zygotes, appears to fail in cloned embryos with the methylation patterns of these embryos similar to the donor cells utilized (Dean et al., 2001).

Another epigenetic process is modification to the histone proteins bound to DNA. Santos and co-authors (2003) demonstrated that SCNT bovine embryos had altered histone acetylation causing inhibition of transcription. These results provide further evidence towards the incomplete epigenetic reprogramming of SCNT embryos.

By understanding the underlying molecular events involved in bovine trophoblast development, we can gain insight to regulatory mechanism involved in successful placentation and how these events may be manipulated to improve assisted reproductive techniques such as somatic cell nuclear transfer

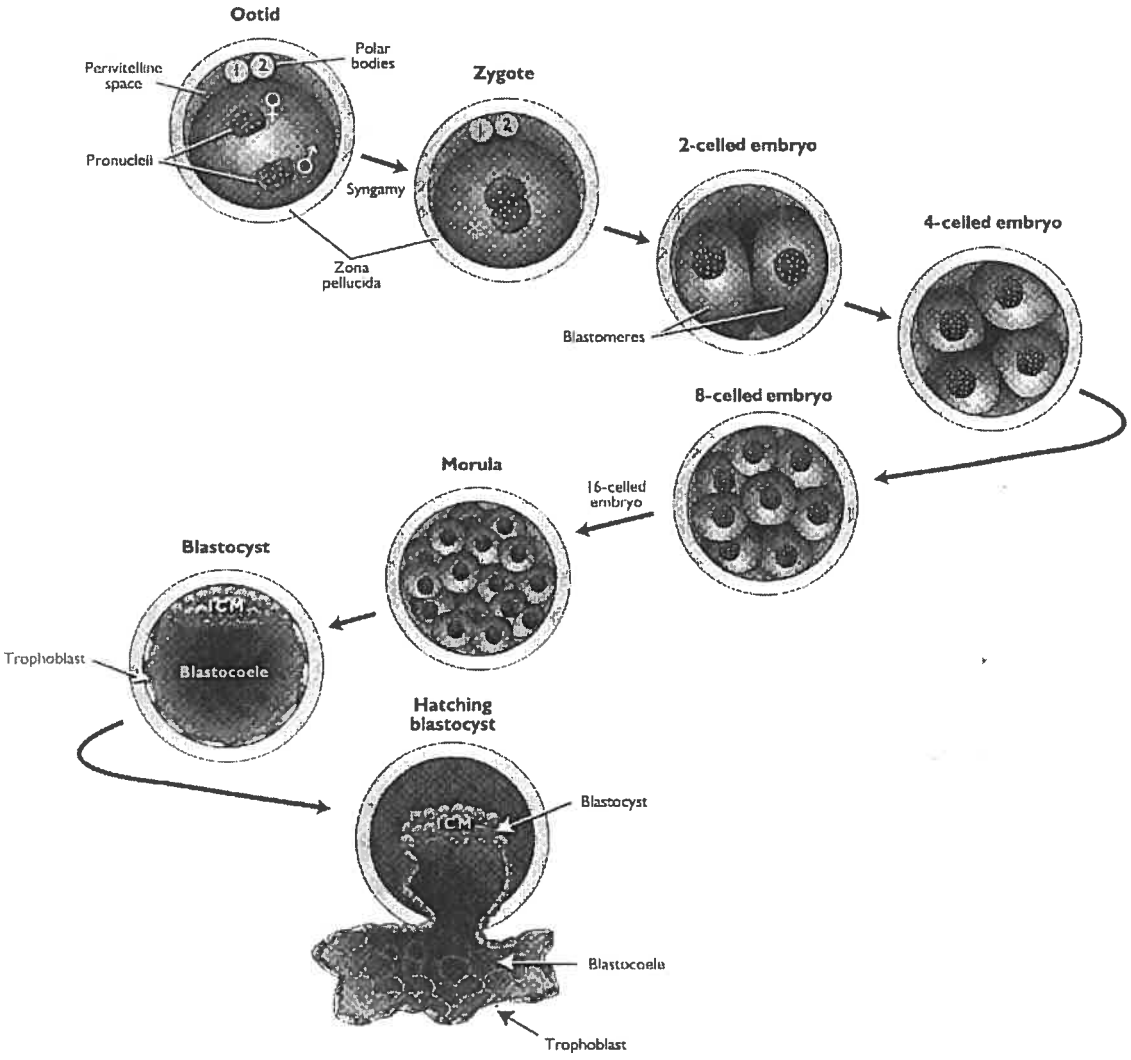
CHAPTER I

LITERATURE REVIEW

Embryonic and Placental Development

Development of the embryo commences with the fertilization of the egg by the sperm. Without increasing in size the zygotes starts to undergo cleavage (Figure 1). Cellular division continues up to the 8-cell stage under the control of the maternal genome (Barnes and First, 1991). After the 8-cell stage the embryonic genome becomes activated. As cleavage proceeds, the totipotent cells compact to form a ball of cells referred to as a morula (Patten, 1964). Compaction of these totipotent cells required the coordination of several factors including gene regulation, cell polarity and the increase in cell number without the increase in embryo diameter (for review see Fleming et al., 2001). These cells will divide further and form a fluid filled central cavity. These cells are commonly referred to as blastomeres and the central cavity is referred to as a blastocoel. This embryonic structure is commonly referred to as a blastocyst (Patten, 1964). In cattle, the blastocyst appears around days 7-8 of gestation (day 0 = fertilization; McLaren, 1990). The single layer of cells that surround the blastocyst are called trophectoderm or trophoblast cells (Figure 1). The Dutch scholar, A.A.W. Hubrecht first used the term "trophoblast" to designate the blastocyst derived cells that are essential for nourishment but do not contribute to the embryo (Boyd and Hamilton, 1960). These trophoblast cells will

Figure 1. Development of a preimplantation embryo (taken from Senger, 1999).


Ootid
Perivitelline space
Polar bodies
Pronuclei
Syngamy
Zona pellucida
Zygote
2-celled embryo
Blastomeres
4-celled embryo
8-celled embryo
16-celled embryo
Morula
Blastocyst
ICM
Trophoblast
Blastocoele
Hatching blastocyst
Blastocyst
ICM
Blastocoele
Trophoblast

causes the blastocyst to expand. Another population of specialized cells can be seen at this time. These cells appear as a flat disc-like cluster of cells at one pole of the blastocyst (Patten, 1964). These cells are referred to as the embryo proper or inner cell mass (ICM). The ICM will eventually give rise to the fetus. As the blastocyst expands, it will break through the zona pellucida (day 8). At this time, another layer of specialized cells (Endoderm) will grow from the ICM. These cells grow inside the trophoblast cell layer and surround the fluid in the blastoceol, forming the yolk sac (Patten, 1964). A third layer of cells (Mesoderm) will grow from the ICM, between the endoderm and the trophoblast layers. The expanded blastocyst will continue to expand, maintaining its round structure. The mesoderm cells will divide to form a two layer thick sheet between the endoderm and trophectoderm. The mesoderm layer closest to the endoderm is referred to as the splanchnic mesoderm (Patten, 1964). The outer layer is referred to as the somatic mesoderm. By day 11-12, the trophoblast cell and somatic mesoderm cell layers combine and begin to grow rapidly forming the chorion (Patten, 1964). The chorion will grow around the embryo and will fuse, forming a fluid-filled space around the embryo called the amnion (Patten, 1964).

Prior to the abdominal wall of the newly formed fetus closing, an outgrowth from the hindgut extends from the fetus into the space between the yolk sac and the chorion. This structure is referred to as the allantois, and along with the splanchnic mesoderm, it will carry with it the blood vessels that will ultimately vascularize the chorion and amnion (Patten, 1964). As the chorion extends out into the uterine lumen, the allantois grows

rapidly inside it. Ultimately, the allantois and chorion fuse to form the chorioallantois (Patten, 1964; Schlafer et al., 2000). This vascular membrane along with the amnion are the extra-embryonic fetal membranes. The chorioallantois will continue to grow into the uterine lumen to fill two-thirds of the entire uterus. At the same time the nutrients in the yolk sac that the embryo was utilizing diminishes, and the embryo is no longer able to absorb enough nutrients from the uterine milk. The chorioallantois must attach to the uterine endometrium to provide a more permanent and direct source of nourishment (Patten, 1964).

In the cow, placentation is of the epithilialchorial-syndesmochorial or placentomal type, which is also found in other ruminant species. Attachment of the fetal membrane primarily occurs in specialized areas of the endometrium called caruncles. There are approximately 100 caruncles evenly spaced throughout the uterus in four rows running the length of each horn (Schlafer et al., 2000). Caruncles can be visually identified as early as birth. The number of caruncles at birth will not increase, but may decrease over time due to injury caused by parturition. The area of fetal tissue that attaches to the caruncles is referred to as the cotyledon.

Relationship between Placental Growth and Function

As shown in many mammals, the weight of the fetus increases exponentially throughout gestation, with the most dramatic change in growth occurring during the last half of gestation (Evans and Sack, 1973). In cattle, placental weight also increases

exponentially throughout gestation, but the absolute rate of increase is much less than that of fetal weight (Ferrell and Ford, 1980; Ferrell et al., 1976; Reynolds et al., 1990). These findings demonstrate a high correlation between fetal and placental weights (Alexander, 1964a; Alexander, 1964b; McKeown and Record, 1953). However, weight is only a gross measure of placental growth, and may not accurately reflect placental function.

As stated above, there is a high correlation between placental and fetal weight across gestation. However, when evaluated separately, caruncular weight was 2.4-fold greater than cotyledonary weight during the last two-thirds of gestation (Reynolds et al., 1990). Interestingly, total cell numbers for caruncular and cotyledonary tissue increase 19-fold from day 100 to 250 of gestation (Reynolds et al., 1990). Researchers have shown that, although placental growth slows, placental transport keeps pace with fetal growth. For example, uterine blood flow increases approximately three- to fourfold from mid– to late gestation in mammalian species, including humans (Ford et al., 1984; Hard and Anderson, 1982; Meschia, 1983; Metcalfe et al., 1988; Reynolds et al., 1986; Rosenfeld et al., 1974). In addition, umbilical blood flow also increases throughout gestation (Reynolds et al., 1986) and umbilical blood flow per kilogram of fetus remains constant throughout the last half of gestation, averaging 0.18 $L \cdot min^{-1} \cdot kg^{-1}$ in cattle (Reynolds and Ferrell, 1987). Increased blood flow allows for an increase in the rate of nutrient extraction throughout gestation (Ferrell, 1989; Meschia, 1983; Metcalfe et al., 1988; Reynolds et al., 1986). Conditions that are associated with reduced fetal and placental growth (e.g. maternal genotype, increased number of fetuses, maternal nutrient deprivation, environmental heat

stress) are also associated with reduced placental blood flow rates and reduced fetal oxygen and nutrient uptake (Christenson and Prior, 1978; Ferrell, 1991; Ferrell and Reynolds, 1992; Ford et al., 1984; Morriss et al., 1980; Reynolds et al., 1985a; Reynolds et al., 1985b). Thus, fetal growth and development, and ultimately neonatal survival, are greatly impacted by factors that regulate placental vascular development and function (Alexander, 1974; Huffman et al., 1985).

Placentomal Region

Attachment of the chorioallantois to the uterus occurs at about 4 weeks of gestation in cattle (Greenstein et al., 1958). The fetal membranes, which are completely smooth until this time, start to develop villous projections above the caruncles that are in close proximity to the embryo (King et al., 1979; Melton et al., 1951). As mentioned earlier, these areas of villous development on the chorioallantois are referred to as cotyledons. Melton et al. (1951) reported that the first placental plates appeared on the surface of the chorion immediately surrounding the embryo 720 hours after ovulation (30 days). In addition, the surface of the caruncles immediately surrounding the embryo changed from a smooth-rounded surface to an undulated surface 31 days after ovulation (Melton et al., 1951). There are conflicting opinions on whether the surface epithelium of the caruncles is lost prior to attachment (Melton et al., 1951) or remains intact (King et al., 1979). As gestation continues the caruncles remodel to form crypts into which the villous projections of the cotyledons invade. The combination of the cotyledonary and caruncular tissue together is

referred to as the placentome. The formation of placentomes is gradual. At 33 days after ovulation the attachment is fragile with three or four placentomes immediately surrounding the embryo (Melton et al., 1951). Thirty-five days after ovulation, attachment of the placentomes is stronger (size of 5 mm in diameter) and there are more placentomes developing farther away from the fetus (Melton et al., 1951). As gestation progresses, the placentomes become dome-shaped ovals and grow in size until mid-gestation. Placentome size ranges from 10 to 12 cm long and 2-3 cm thick (Schlafer et al., 2000), with the largest placentomes immediately surrounding the fetus and the smallest located in the opposite uterine horn to the one the fetus occupies. The extensive interdigitation of the fetal and maternal tissues in the placentome allows for greater surface area contact. This contact is estimated to be 130 m^2, and is involved in the exchange of nutrients between the conceptus and dam by haemotrophic transfer (Schlafer et al., 2000).

Interplacentomal Region

The chorioallantois that contacts the endometrium between the placentomes is generally referred to as the interplacentomal area. The contact is generally smooth with gentle folds. The intercotyledonary tissue utilizes histotrophic transfer of nutrients produced by the endometrial glands (Schlafer et al., 2000). Endometrial uterine glands, located between the caruncles of ruminant placentas, synthesize and secrete a mixture of hormones, enzymes, cytokines, growth factors and other compounds, referred to as histotrophs, required for conceptus survival, implantation and development (Bazer, 1975;

Bazer et al., 1979; Carson et al., 2000; Gray et al., 2001a; Gray et al., 2001b; Roberts and Bazer, 1988; Spencer and Bazer, 2004; Wimsatt, 1950). Histotrophs are secreted throughout gestation in ruminants and play a critical role in pregnancy. Unique placental structures called areolae develop over the endometrial glands as specialized areas of absorption and transport of histotrophs to bathe the conceptus in ruminants and pigs (Spencer and Bazer, 2004). Trophoblast cells in the placental areolaes utilize diffusion, phagocytosis, and active transport via concentration gradients to facilitate movement of histrophs across the placenta (Ehrhardt and Bell, 1997; Jones et al., 1997; Schlafer et al., 2000). In some incidences there have been reports of "adventitious" placentomes (Hammond, 1927). These are areas that appear to form rudimentary placentomes or mild interdigitation between the chorioallantois and the endometrial tissues. These areas are generally seen in late pregnancy. However, this finding suggests that the fetal membranes have the ability to initiate formation of new adventitious caruncular growth (Hammond, 1927).

Trophoblast Development and Function

As stated above, the trophoblast cells make up the epithilial layer of the chorion, however, there are different subpopulations of trophoblast cells. Greenstein et al. (1958) described the presence of three different types of bovine trophoblast cells in early embryos. These researchers referred to these different cell types as; trophoblast stem cells, mononucleate cells and binucleate/giant cells.

Trophoblast "Stem Cells" and Cell Lines

Trophoblast "stem cells" are cuboidal in shape and are first detected at the time of blastocyst formation. These cells are believed to proliferate and differentiate into the other two cell types (Greenstein et al., 1958). However, other researchers believe there is no evidence for a stem cell population in the trophoblast (Wooding, 1992). One possible line of evidence is the development of trophoblast cell lines. This type of cell line is seen in several mammalian species (Desmarais et al., 2004; Miyazaki et al., 2002; Ramsoondar et al., 1993; Steven et al., 1980), and can be isolated, cultured and maintained in vitro for extended periods of time from either blastocyst stage embryos (Shimada et al., 2001; Talbot et al., 2000; Tanaka et al., 1998) or early gestational placental tissue (Quinn et al., 1997). To develop these cell lines, blastocysts or placental tissues are cultured in the presence of conditioned media or on a fibroblast feeder cell layer. Talbot et al. (2000) reported the development of bovine trophectoderm and endoderm cells lines from day 10-11 and day 7-8 in vitro produced blastocysts, respectively. These cell lines were established using STO mouse fibroblast cells as feeder cells (Talbot et al., 2000). The trophectoderm cell line was continuously cultured for 2 years (76 passages) without noticeable morphological changes occurring (Talbot et al., 2000). Overall morphology of the trophectoderm cells was similar to in vivo expanded preimplantation blastocysts (Talbot et al., 2000).

To control for any effects caused by co-culturing trophoblast cells with feeder cells, Shimada et al. (2001) developed a trophoblast cell line (BT-1) utilizing uterine fibroblast-conditioned media. Shimada et al. (2001) was able to continuously culture BT-1 cells for

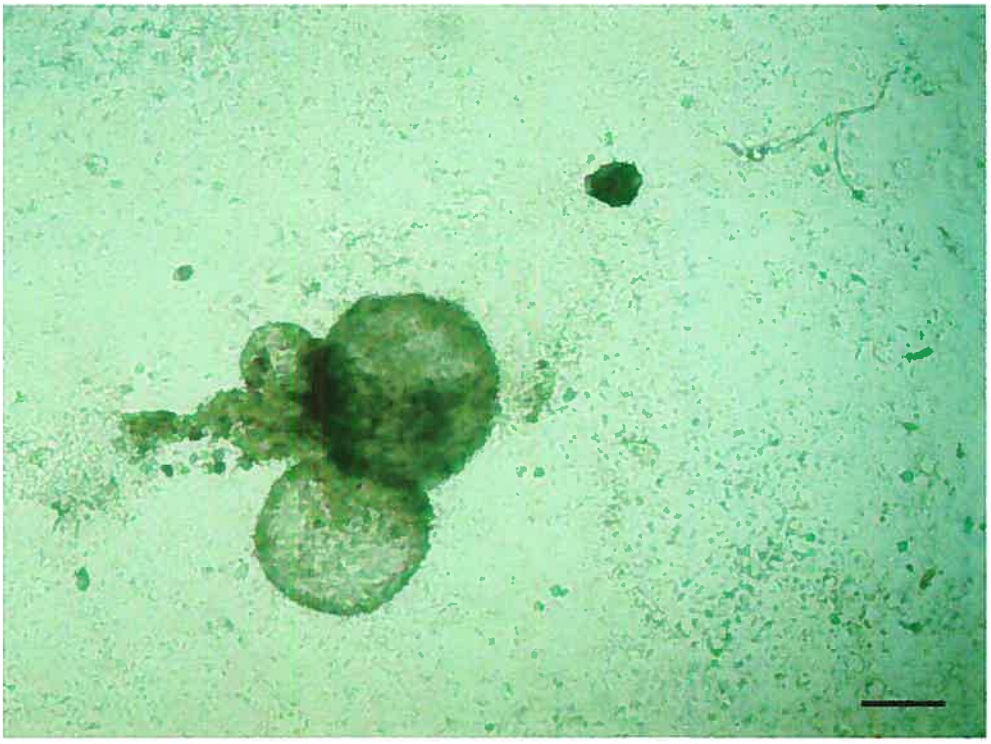
18 months (75 passages) without senescent or morphological changes. Fibroblast-conditioned media stimulated cellular growth better than serum or serum-free media (Shimada et al., 2001). These bovine trophoblast cell lines were able to differentiate into the two other cell types (Shimada et al., 2001; Talbot et al., 2000). Cellular markers for mononucleate and binucleate trophoblast cell functions such as interferon tau (IFN-τ) and placental lactogen (PL), respectively, were detected. These and other trophoblast genes are discussed in detail below.

An interesting observation seen in these three experiments, were the formation of domes of cells within the culture that eventually formed free floating vesicles (Figure 2; Shimada et al., 2001; Talbot et al., 2000). These free floating vesicles, along with the difficultly to disassociate the trophoblast cells, gives some insight to the tight cellular junction that make up the blastocyst.

Mononucleate Cells and Their Function

The mononucleate cells are cuboidal to columnar in shape and make a majority (80%) of the trophoblast cells population (Boshier and Holloway, 1977; Wooding and Wathes, 1980). They hold the typical features characteristic of epithelial cells. Microvillar processes are detected on the apical surface membrane to allow for the interdigitation of the fetal/maternal interface (Bjorkman, 1969). One characteristic of mononucleate cells is the

Figure 2. Formation of a bovine trophoblastic vesicle from bovine trophoblast cells cultured in vitro. (bar = 200 μm)



presents of large lipid vacuoles (Greenstein et al., 1958). The precise function of these lipid droplets is unknown, but it has been suggested to be involved with steroid secretion (Bjorkman, 1954). The mononucleate cells play a major role in producing factors involved in pregnancy recognition.

The trophectoderm cells of the bovine embryo produce interferon tau (IFN-τ), a secretory protein with antiluteolytic effects (Bazer et al., 1994; Helmer et al., 1987; Roberts et al., 1992; Thatcher et al., 1995). Interferon-τ expression is first seen around the time of blastoceol formation (Hernandez-Ledezma et al., 1993; Hernandez-Ledezma et al., 1992), and continues until just prior to attachment (Roberts et al., 1992). The increase in IFN-τ production coincides with elongation of the conceptus (Geisert et al., 1988; Nephew et al., 1989). Additionally, high quality in vitro-produced blastocysts secret more IFN-τ than lower quality blastocysts (Hernandez-Ledezma et al., 1993). These findings may be related to developmental competence, since higher quality in vitro-produced embryos reach the blastocyst stage at a faster rate than lower quality in vitro-produced embryos (Kubisch et al., 1998). IFN-τ binds to receptors in the endometrium and causes a down regulation in the uterine oxytocin receptors, by which blocking the production of the main luteolytic component, prostagladin $F_{2\alpha}$ (Thatcher et al., 1985).

Binucleate Cell Formation and Function

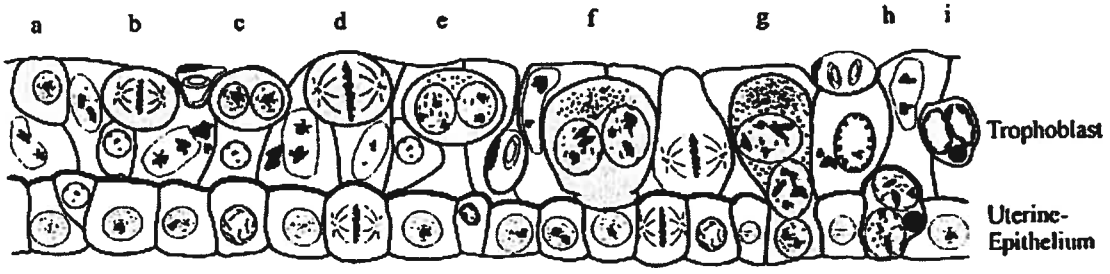
Most notable of the trophoblast cells are the binucleated cells or giant cells. Binucleated cells are seen as early as day 17 of gestation (Wooding and Wathes, 1980).

These cells have larger nuclei, are round in shape and account for 20% of the total trophoblast cell population throughout gestation (King et al., 1979). Early in development, the binucleate cells are scattered within the trophectoderm layer, away from the trophoblast apical border (Wango et al., 1990; Wooding, 1984). As gestation progresses, the binucleate cells tend to group into small clusters (Wooding, 1984). Either from trophoblast stem cells (Greenstein et al., 1958) or mononucleate cells (Wooding, 1992), formation of binucleate cells is through acytokinetic mitoses (figure 3; Klisch et al., 1999). Klisch and co-authors (1999) also observed trinuclear cells formed by either undergoing another acytokinetic mitoses or by fusion with uterine epithelial cells. Giant cells are also seen on the maternal side. These cells are thought to undergo migration from the chorion into the maternal epithelium (Wooding and Wathes, 1980). As these cells migrate, they appear to fuse with single endometrial cells and discharge cytoplasmic granules (Schlafer et al., 2000). These fused cells temporarily form cells with three nuclei. The migration of these binucleated cells has been a cause for debate as to whether bovine placentation is epithiliealchorial or syndesmochorial.

The binucleate cells in the placentomal and interplacentomal area appear similar, but differences in antigen expression have been observed (Lee et al., 1986; Lee et al., 1985). By utilizing the monoclonal antibody, SBU-3, these researchers demonstrated that SBU-3 antigen was expressed only from binucleate cells in the placentomal region.

One of the main functions of bovine binucleate cells is to migrate and fuse to the uterine epithelial cells, allowing for the release of several types of factors into the

Figure 3. Schematic drawing of the bovine trophoblast and uterine epithelium (a, uninuclear trophoblast cell without contact to the uterine epithelium; b, 1st acytokinetic mitosis; c, binuclear cell with two diploid nuclei; d, second acytokinetic mitosis; e, binuclear cell with two tetraploid nuclei; f, formation of the pseudopodium; g, fusion with an uterine epithelial cell and formation of a trinuclear feto-maternal hybrid cell; h, degeneration after exocytosis of PAS-positive granules; i, phagocytosis by trophoblastic cells (taken from Klisch et al., 1999).


a
b
c
d
e
f
g
h
i
Trophoblast
Uterine-
Epithelium

maternal circulation. One such factor is placental lactogen. Placental lactogen (PL) is a nonglycosylated, single–chain 23-kDa protein (Anthony et al., 1995). In the ovine uterus PL binds to receptors and causes endometrial gland morphogenesis and increased production of uterine milk protein (Johnson et al., 2003; Spencer and Bazer, 2004). Other hormones produced by the binucleate cells are estradiol (Matamoros et al., 1994), progesterone (Reimers et al., 1985), prolactin-related protein-1 (Anthony et al., 1995) and pregnancy-associated glycoproteins (Green et al., 2000; Zoli et al., 1992). The monoclonal antibody SBU-3 recognizes pregnancy-associated glycoproteins (Lee et al., 1986). These pregnancy proteins can be detected in the maternal serum during gestation (Zoli et al., 1992).

Binucleate cells in culture have been also shown to produce these factors as well. Placental lactogen protein was localized to binucleated cells, suggesting that fibroblast-conditioned media did not inhibit trophoblast differentiation (Shimada et al., 2001). To investigate the formation of binucleate cells, Nakano et al. (2002) grew BT-1 cells on collagen substrata, instead of plastic culture dishes coated with a collagen film. They reported that BT-1 cells grown on collagen substrata formed clusters of binucleate cells that produced PL (Nakano et al., 2002). These researchers concluded that the physical conditions (development on a pliable surface) determined the cell's fate to become binucleate (Nakano et al., 2002).

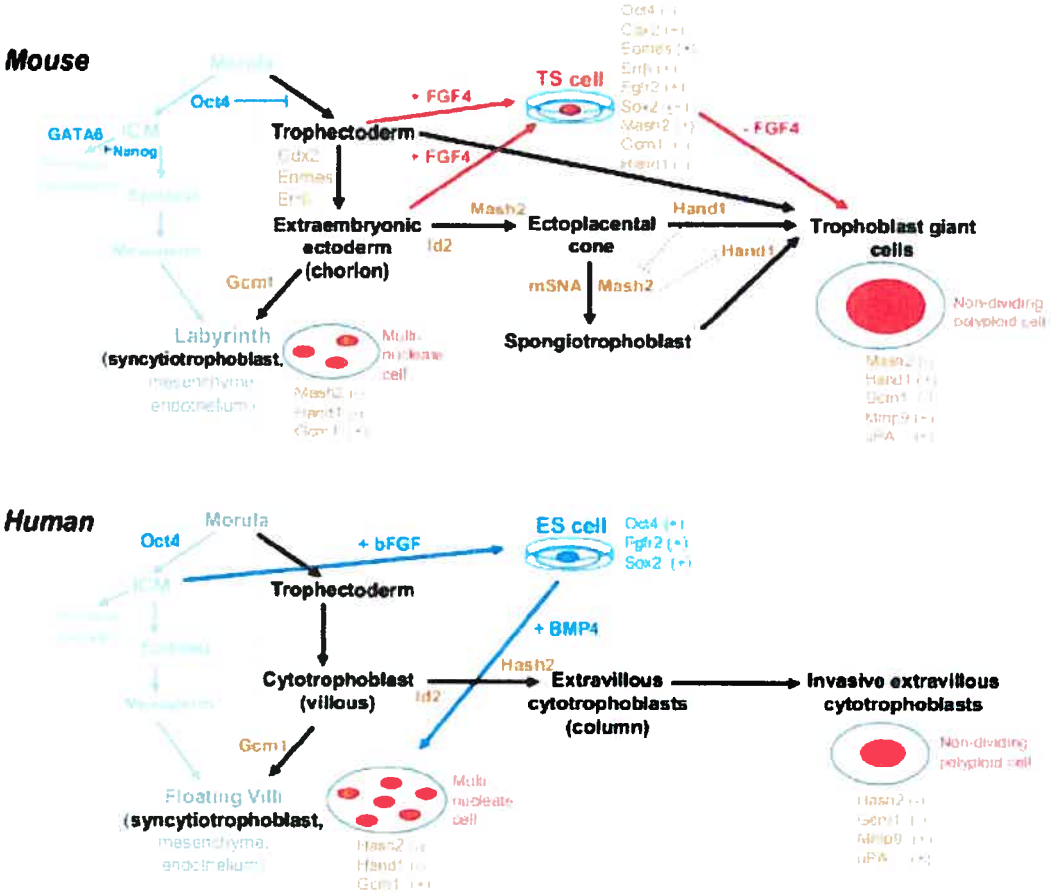
Molecular Regulation of Placental Development

Although elegant studies have described the cellular morphology of the bovine trophoblast, the molecular regulation of these processes still remains elusive. The majority of genetic and developmental information on placental development has been taken from mice, where most of the information exists (figure 4). Many of the genes involved in placental development have been discovered by knockout experiments that have caused embryonic lethality. A bulk of these genes were discovered by chance when researchers were hoping to observe abnormal phenotypes in homozygous mutant adults but encountered embryonic death after implantation. Researchers in human development commonly compared similar phenotypes of knockout mice with those of genetic diseases that have placental abnormalities to identify genes of interest. Other studies have utilized gene screening to identify candidate genes localized to placental tissues. By employing the extensive research done in mice, these genes can be investigated in other species for homology and common characteristics. This section will focus primarily on the genes involved in mouse placental development with reference to other species, particularly bovine, when available.

Gene Regulation of Trophoblast Stem Cells

At the morula stage, all cells have the potential to develop into either the inner cell mass (ICM) or trophoblast cells. When the first cellular differentiation occurs, the two

Figure 4. Schematic of the gene involved in trophoblast lineage derivation (black lines) and inner cells mass lineage derviation (grey lines) for mouse and human (taken from Roberts et al., 2004).


Mouse
Morula
Oct4
GATA6
ICM
Nanog
Trophectoderm
Cdx2
Eomes
Erfb
+ FGF4
TS cell
Oct4 (+)
Cdx2 (+)
Eomes (+)
Erfb (+)
Fgfr2 (+)
Sox2 (+)
Mash2 (+)
Gcm1 (+)
Hand1 (+)
+ FGF4
- FGF4
Extraembryonic ectoderm (chorion)
Mash2
Id2
Ectoplacental cone
Hand1
Trophoblast giant cells
Gcm1
mSNA
Mash2
Hand1
Labyrinth (syncytiotrophoblast,
mesenchyme
endothelium)
Multi-nucleate cell
Mash2 (+)
Hand1 (+)
Gcm1 (+)
Spongiotrophoblast
Non-dividing polyploid cell
Mash2 (+)
Hand1 (+)
Gcm1 (+)
Mmp9 (+)
uPA (+)
Human
Morula
Oct4
ICM
+ bFGF
ES cell
Oct4 (+)
Fgfr2 (+)
Sox2 (+)
Trophectoderm
+ BMP4
Hash2
Cytotrophoblast (villous)
Id2
Extravillous cytotrophoblasts (column)
Invasive extravillous cytotrophoblasts
Gcm1
Floating Villi
(syncytiotrophoblast,
mesenchyme,
endothelium)
Multi-nucleate cell
Hash2 (+)
Hand1 (+)
Gcm1 (+)
Non-dividing polyploid cell
Hash2 (+)
Gcm1 (+)
Mmp9 (+)
uPA (+)

factors influencing the cellular fate are blastomere position and expression of octamer-binding transcription factor 4 (Oct4). The blastomere on the outside of the compacting morula embryo will become trophoblast, while the blastomeres on the inside of the ball will become the ICM (Pedersen et al., 1986). The transcription factor Oct4 is expressed in all blastomeres early in development but then becomes localized only the cells of the ICM at the time of blastocyst formation (Palmieri et al., 1994). In Oct4 knockout mice, only trophoblast cells develop suggesting that Oct4 is vital for ICM development (Nichols et al., 1998). However, this may be a unique feature to the mouse since Oct4 expression is detected in trophoblast cells of human and bovine 2 to 3 days after blastocyst formation, albeit at lower levels than the ICM (Kirchhof et al., 2000; van Eijk et al., 1999).

The development of the trophoblast is not entirely controlled by the down-regulation of Oct4. Two other genes that have been shown to have expression at the blastocyst stage are caudal-related homeobox 2 (Cdx2; Beck et al., 1995) and Eomesoderm (Eomes; Hancock et al., 1999). These two genes are expressed only in the trophoblast cells. Knockout mice for these genes develop to the early blastocyst stage, but fail to implant (Chawengsaksophak et al., 1997; Russ et al., 2000). When cultured, these mutant blastocysts do not form trophoblast outgrowths and cannot be induced to produce trophoblast stem cells (Rossant, 2001). These genes appear not to be involved in the early stage of trophoblast cell development but in the proliferation of the trophoblast stem cells. Homologues to Cdx2 (Ponsuksili et al., 2001) and Eomes (Hall et al., 2005) have been

detected in the bovine embryo, but similarity in function to the mouse homologues remains unknown.

The development of the trophoblast and the ICM are not mutually exclusive. The ICM produced factors that stimulate trophoblast cell proliferation. One of these growth signals is fibroblast growth factor 4 (Fgf4). Expression of Fgf4 from the ICM is regulated by Oct4 and another ICM transcription factor Sox2 (Avilion et al., 2003; Feldman et al., 1995). The Fgf receptor R2 (FgfR2) is localized to the trophectoderm and mutants for both Fgf4 and FgfR2 are embryonic lethal (Arman et al., 1998; Feldman et al., 1995). In addition, mouse trophoblast stem cells are maintained in culture when Fgf4 or fibroblast conditioned media are supplemented (Tanaka et al., 1998). These trophoblast stem cells express Cdx2 and Eomes and are responsive to Fgf4 (Tanaka et al., 1998). Bovine trophoblast cells in culture require fibroblast conditioned media, but Fgf4 alone appears to have no effect (Rexroad and Powell, 1997).

Basic Helix-Loop-Helix (bHLH) Transcription Factors

Several members of the bHLH transcription factor family are involved in trophoblast differentiation. In this groups of transcription factors, the HLH domain will dimerize while the basic domains bind to DNA (Voronova and Baltimore, 1990). The bHLH E-factors, which are widely express, will form heterodimers with bHLH proteins that are tissue specific (Cross et al., 1995; Johnson et al., 1992). Two of the more

characterized bHLH's are achaete scute-like homologue 2 (Ascl2; also known as Mash2) and heart and neural crest cell derivative 1 (Hand1).

In mice, Ascl2 is expressed in the ectoplacental cone (EPC), the chorion and the spongiotrophoblast cell layers during placental development and is absent in primary and secondary giant cells (Guillemot et al., 1994). Homozygous mutant Ascl2 mice are embryonic lethal with death at 10 days postcoitum (d.p.c.) due to the lack of development of the spongiotrophoblast layer and an increase in giant cell number and size (Guillemot et al., 1994). Homozygous mutant mice did survive when diploid Ascl2-/- embryos (which will give rise to the ICM) were aggregrated with tetraploidy wild-type embryos (which will give rise only to the trophoblast cells) suggesting that Ascl2 is only involved in mouse placental development (Guillemot et al., 1995). The mouse Ascl2 gene is located on the distal portion of chromosome 7, near a cluster of imprinted genes (H19, Igf2 and Ins2; Guillemot et al., 1995). The mouse Ascl2 gene appears to be imprinted with the paternal Ascl2 allele being expressed in the early postimplantation conceptus (5.5 d.p.c.), then undergoing a progressive decline until silenced by 9.5 d.p.c. (Tanaka et al., 1999). Analogous to the mouse, human Ascl2 is only expressed in the extravillus trophoblast and has been mapped to the imprinted region of chromosome 15 along with Igf2 and H19 (Alders et al., 1997). The human Ascl2 gene, however, contains two promoters that encode two proteins, Hash2 and Human Acheate Scute Associated Protein (HASAP). Unlike the Hash2, HASAP is expressed in several tissues and lacks the bHLH domain (Westerman et

al., 2001). Bovine Ascl2 mRNA has been reported in blastocyst embryos (Wrenzycki et al., 2001), but its characteristics and regulation are not known.

Hand1, another gene critical for trophoblast differentiation, is predominately expressed in mouse trophoblast giant cells but has been detected in the extraplacental cone and areas of the spongiotrophoblast cell layer that also express Ascl2 (Scott et al., 2000). Development is arrested in mouse embryos carrying a mutated Hand1 gene around 7.5 d.p.c in association with a significantly reduced number of trophoblast giant cells (Riley et al., 1998). Cross et al. (1995) reported that the Rcho-1 trophoblast cell line over-expressing Hand1 differentiated into giant cells. Giant cell development was inhibited when $Hand1^{+/+}$ Rcho-1 cells co-expressed Ascl2 (Cross et al., 1995). Unlike Ascl2, the Hand1 factors are involved in the regulation of several types of tissues (for review see Firulli, 2003). Hall et al. (2005) were not able to detect Hand1 expression from day 8 bovine blastocysts, suggesting that either at this stage there is no expression or there is no homologous gene in the bovine.

These two genes appear to have opposing effects on trophoblast differentiation. Since both Ascl2 and Hand1 form dimers with E-factors, they could also compete for DNA-binding sites. The Ascl2-E-factor dimers bind to and activate transcription from E-box sequences (CANNTG), whereas HAND1-E-factor complexes bind to a different consensus sequence (NNTCTG) (Hollenberg et al., 1995; Johnson et al., 1992). The overlap with E-box sequences may allow for the competition between dimers.

Other bHLH transcription factors shown to be expressed during trophoblast development are Stra13 (Boudjelal et al., 1997) and the inhibitors of differentiation proteins Id1 and Id2 (Jen et al., 1997). Expression of Stra13 occurs in the giant cells as Ascl2 and the E-factors are down-regulated (Boudjelal et al., 1997; Scott et al., 2000). The Id transcription factors, which are missing the DNA binding domain, inhibit E-factor function by forming heterodimers that will not bind to DNA (Hasskarl and Munger, 2002). Trophoblast stem cells express both Id1 and Id2 (Jen et al., 1997).

Other Regulatory Factors

Another important transcription factor involved in placental development is glial cells missing-1 (Gcm1). Gcm1 protein is necessary for differentiation of glial cells in the nervous system and scavenger cells in the immune system of *Drosophila melanogaster* (Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995). Gcm1 has also been reported in the placenta of mice, rats and humans (Akiyama et al., 1996; Altshuller et al., 1996; Basyuk et al., 1999; Janatpour et al., 1999; Kim et al., 1998). Interestingly, expression of Gcm1 in trophoblast cells depends on the allantoic mesoderm fusing with the chorion (Hunter et al., 1999). Gcm1 mRNA expression has been detected in clusters of trophoblast cells located at the chorionic plate, which gives rise to the labyrinth of the mouse placenta (Anson-Cartwright et al., 2000). The role of Gcm1 is in differentiation and morphogensis of the choroinic trophoblast to form villi. A mutation in the Gcm1 gene causes a complete failure in villous branching of the chorioallantois (Anson-Cartwright et

al., 2000). In these Gcm1 knockout mice, chorioallantois fusion occurs normally, but the chorionic plate remains flat, resulting in no villous development and loss of pregnancy by day 8.5 d.p.c. (Anson-Cartwright et al., 2000). In the bovine, Gcm1 mRNA has been detected but further characterization is needed (Arnold and Smith, unpublished data)

Several other regulatory factors have been identified in the mouse (for review see Hemberger and Cross, 2001), but their role in the bovine remains unknown.

Epigenetic Regulation

In recent years the research area of epigenetic regulation has added a new dimension to understanding gene regulation. Several definitions have been used for epigenetic regulation recently, but the most common among developmental biologists is the inheritable regulation of gene expression, mitotically and meiotically, without alterations in the DNA sequence (Levenson and Sweatt, 2005). Gene expression is controlled by physically affecting the DNA, either directly or indirectly through proteins associated with the DNA. By regulating the DNA without change in its sequence, genetically identical cells (such as cells in one organism) are able to be phenotypically distinct depending on their location (i.e. a skin cell is distinct from a bone marrow cell in the same organism) (Levenson and Sweatt, 2005). In addition, these changes may be reversible. The control of gene regulation by epigenetics can either be a short-term effect (expression of a gene only during a specific development stage) or a long-term effect (imprinting of a paternal allele).

Imprinting and DNA Methylation

As briefly discussed above, imprinting is in most cases the silencing of expression of a gene from either the paternal or maternal allele. This unique epigenetic process was first observed in mice (DeChiara et al., 1991). These observations help explain the failure of parthenogenetic (two copies of the maternal genome only) and androgenetic (two alleles of the paternal genome only) embryos to develop to term (McGrath and Solter, 1984; Surani et al., 1984). It is estimated that 100-200 genes of the total mouse and human genome are imprinted (Lucifero et al., 2004). It is commonly thought that maternally expressed imprinted genes are growth repressors, whereas paternally expressed imprinted genes promote growth. Methylation of the 5-carbon position of the cytosine nucleotide in the CpG dyad is the most studied form of imprinting. The methylated nucleotide inhibits the binding of transcriptional factors and enhancers to inhibit gene expression. These CpG islands (a series of CpG dyads in a row) are common throughout the genome, but methylation of these islands is not. Non-imprinted housekeeping genes that contain CpG islands in their DNA sequence are usually hypomethylated (Bird, 1986). Imprinted genes usually contain large regions of CpG islands that are referred to as differentially methylated regions (DMRs) (Jones, 1999).

The methylation of CpG islands is through the DNA methyltransferase (Dnmt) enzymes (Bestor, 2000). Three Dnmt's have been identified to play critical roles in methylation patterns. Dnmt1 is involved in maintenance of DNA methylase activity (Yoder et al., 1997). Dnmt3a and Dnmt3b have poor maintenance activity, but have a high

affinity for causing de novo methylation (Okano et al., 1998a). A fourth Dnmt, Dnmt2, has been identified but appears to have weak DNA methylation activity and its biological function is unknown (Okano et al., 1998b). A Dnmt3 like protein (Dnmt3L) has been shown to be involved in establishing methylation at specific regions (Aapola et al., 2000; Bourc'his et al., 2001). As further studies are conducted, other factors appear to be acting as enhancers to Dnmt's actions. Expression of Dnmt's 1, 3a and 3b have been detected in bovine embryos and fetal and adult tissues (Golding and Westhusin, 2003).

Interestingly, many of the genes associated with placental development are imprinted and are usually clustered together on the same chromosome (Coan et al., 2005; Wutz et al., 1997; Zemel et al., 1992). A good example is the cluster of H19, IGF2, Ins2 and Ascl2 on mouse chromosome 7. Even though these genes are clustered together, their parental expression differs. For instance, H19 and Ascl2 are maternally expressed genes, whereas IGF2 is paternally expressed. The H19 gene is an untranslated RNA (Brannan et al., 1990). The imprinting of H19 and IGF2 is controlled by the same DMR (Arney, 2003). Briefly, when the DMR is methylated, H19 is inhibited, allowing downstream enhancers to stimulate IGF2 transcription (on the paternal allele). On the contrary, the DMR on the maternal allele is unmethylated allowing H19 to be expressed and IGF2 to be silenced.

Even though the Ascl2 gene is within the same imprinting cluster, its imprinting appears to be regulated by something other than methylation. The paternal allele was not restored in mice deficient for the gene DNA-methyltransferase I (Dnmt1), which is involved in maintenance of the methylated state (Tanaka et al., 1999).

Chromatin Modification

Another epigenetic process is chromatin modification. Chromatin is a highly conserved structural polymer that contains genetic information bound by proteins. Nucleosomes are the fundamental repeating units of chromatin. A nucleosome consists of 146 base pairs of DNA wrapped around a core of histone proteins. The histone proteins that make up this core are 2 copies each of H2A, H2B, H3 and H4. A linker histone, H1, is bound to the DNA between nucleosome, allowing for the solenoid helical fiber structure of DNA in the nucleus. These core histones are highly conserved across species. A histone has a globular carboxy-terminal domain that binds the DNA and a flexible amino-terminal tail that extends out of the nucleosome structure. Modification of histones occur on amino residues of the amino-terminal tail (for review see Fischle et al., 2003). Depending on the type of modification that occurs, the nucleosome will either open up to allow for transcriptional factors to bind or remain tightly wound. Histone acetylation is commonly associated with activated transcription, whereas deacetylation is associated with transcriptional repression (Fischle et al., 2003; Struhl, 1998; Tazi and Bird, 1990). Histone amino groups can also be methylated, phosphorylated and ubiquitinated (Fischle et al., 2003). Methylation of histone is commonly associated with transcriptional repression. The region of DNA on the chromosome that is constitutively silenced (telomere, centromeres and heterochromatin) is hypoacetylated (Berger, 1999; Kuo and Allis, 1998). In addition, these regions are highly methylated (Brown et al., 1997; Cortes et al., 1999; Nan et al., 1998).

Several enzymes have been reported to control histone modification. Two proteins involved with controlling the acetyl groups are histone acetyltransferase (HAT; adds acetyl groups) and histone deacetylase (HDAC; removes acetyls groups). Several of these enzymes (HDAC's 1, 2, 3, 7 and HAT1) have been detected in bovine embryos (McGraw et al., 2003). As more research is conducted, it becomes apparent that crosstalk between enzymes is a common feature. For example in *Schizsaccharomyces pombe,* HDAC is required for deacetylating the histone 3 lysine 9 residue in order for histone methyltransferase to act on that particular residue (Nakayama et al., 2001). Interactions also occur between HDAC's and Dnmt's (Fuks et al., 2000; Fuks et al., 2001; Robertson et al., 2000).

Imprinting of several gene involved in mouse placental development are linked to histone modification independent of DNA methylation. Lewis and co-authors (2004) demonstrated that the imprinting center 2 on mouse chromosome 7 recruits histone methylation to repress the paternal alleles of the placenta specific genes in the region (including Ascl2). These findings suggest that imprinting involves histone modification as well as DNA methylation.

Somatic Cell Nuclear Transfer

To investigate placental development in cattle, researchers have gleaned valuable data from mouse and human studies. The use of somatic cell nuclear transfer (SCNT) in bovine provides a unique model for investigating placental development. Since the

development of assist reproductive techniques in bovine, like SCNT, there have been several reports of abnormal placental development. Many of these observations carry similar phenotypes to those of knockout mice or genetic diseases of humans.

History of Nuclear Transfer

The theory of nuclear transfer has a long history. In 1905, Spemann demonstrated that newt salamander embryonic cells up to the 16-cell stage were pluripotent (Foote, 1999). From his work, Spemann (1938) proposed that transferring isolated nuclei into an enucleated oocyte would develop into an offspring genetically identical to the donor nuclei. However, technology at the time was inadequate to perform such experiments. It was not until 14 years later when Briggs and King (1952) working with Rana frogs, demonstrated that transferring a differentiated cell nuclei into oocyte cytoplasm would differentiate and develop into a tadpole. However, these researchers were unable to produce an adult from these tadpoles. The first adult frog was cloned by Gurdon (1962) by utilizing serial transplantation of intestinal endoderm cells of tadpoles as donor cells. Research in embryonic development and oocyte manipulation were conducted in frogs and other amphibians due to the large size of the oocytes and the rapid rate of development. It was not until 15 years later that Illmensee and Hoppe (1981) were able to successfully transfer embryonic nuclei in mice to produce blastocysts. Five years later, Willadsen et al. (1986) successfully cloned a sheep using embryonic cell nuclei. These reports were quickly followed by the production of cloned cattle (Bondioli et al., 1990; First, 1990; Foote and

Yang, 1992; Heyman and Renard, 1996; Marx, 1988; Prather et al., 1987; Robl and Stice, 1989; Willadsen, 1989; Yang and Anderson, 1992; Yang et al., 1993). As these experiments demonstrated, production of cloned calves derived from nuclei of early embryos were successful, but highly inefficient. Other species were quickly cloned using similar techniques and early embryonic nuclei as donor cells (pigs, Prather et al., 1989; rabbit, Stice and Robl, 1988; rhesus monkey, Meng et al., 1997; mice, Cheong et al., 1993).

However successful the use of early embryonic nuclei as donor cells may have been, they were not a suitable cell type for genetic modification. To genetically modify a cell requires ample culturing time and all research to date was not able to produce viable offspring. The ideal donor cells would be cell lines that could be maintained in culture for long periods of time and easily collected. Several researchers had tried to use adult cell nuclei as donor, with no success believing that the differentiated cells were irreversibly programmed (Collas and Barnes, 1994; Moor et al., 1992; Stice et al., 1994). Other researchers believed it was a lack of knowledge of the control of gene expression (McKinnell, 1981). Campbell and co-authors (1996) were the first to produce viable sheep from nuclear transfer of cultured embryonic cells. These researchers were able to accomplish this feat by causing the donor cells to enter into quiescence. By inducing the donor cells to exit the growth phase and synchronizing the cell cycle of the host oocyte, they caused the nuclei to reprogram the gene expression, allowing for the nuclear transferred oocyte to stimulate embryonic growth. The notion of controlling the cell cycle of the donor nuclei, allowed the researchers to have an ideal donor cell for NT. This also

meant that cultured cells could be used for NT. These findings led to one of the most historical scientific breakthroughs.

One year later Dolly was introduced (Wilmut et al., 1997). Dolly was the first sheep born from nuclear transfer using donor cells derived from adult tissue. This breakthrough revolutionized the nuclear transfer field. Dolly meant that cells from adult tissues could be collected, cultured, genetically manipulated, and transferred into enucleated oocytes to produce viable offspring. After Dolly, several researchers reported the production of live calves (Chan, 1999; Hill et al., 2000b; Kato et al., 1998; Kubota et al., 2000; Stice et al., 1998), sheep (Wells et al., 1997), goats (Baguisi et al., 1999; Keefer et al., 2001), pigs (Onishi et al., 2000; Polejaeva et al., 2000), mice (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999), cats (Shin et al., 2002), horses (Galli et al., 2003), dogs (Lee et al., 2005) as well as transgenic sheep (Schnieke et al., 1997) and cattle (Bordignon et al., 2000; Cibelli et al., 1998a; Cibelli et al., 1998b).

General Problems of Somatic Cell Nuclear Transfer

Poor Efficiency

Although somatic cell nuclear transfer has been shown to be successful, there are still problems with cloning that are inhibiting its use in industry. One problem is the poor efficiency of reconstructed embryos to develop into live offspring. Wilmut et al., (1997) reported that the percent of live sheep born from reconstructed embryos using embryonic, fetal and adult donor nuclei were 1.0%, 1.7%, and 0.04%, respectively. These percentages

vary widely across studies with live births of calves per SCNT embryos transferred ranging from 0% to 83% (Westhusin et al., 2001).

However, several factors are involved to progress from a donor cell and host oocyte to a healthy offspring. Sources of variation may include donor cell type, genetic makeup of donor cells, treatment of donor cells prior to SCNT, source and manipulation of recipient oocytes, as well as technique used and the laboratories conducting the research. For instance, the development of SCNT embryos to blastocyst is highly variable among laboratories ranging from 5% to greater than 65% (Westhusin et al., 2001). Fusion of the donor cell to the cytoplasm of the enucleated cell is also variable, with a range of 28-89% (Hill et al., 2000b; Kato et al., 1998; Westhusin et al., 2001). Another factor effecting SCNT embryonic development is chromosomal abnormalities. Chromosomal anomalies have been documented to be as high 43% in SCNT embryos, with most anomalies due to and nuclear fragmentation and aneuploidy (Bureau et al., 2003). Interestingly, these researchers demonstrated a high correlation between chromosomal anomalies of the SCNT embryos with the donor cell utilized, suggesting proper screening of donor cells is vital for successful SCNT embryo production (Bureau et al., 2003). As stated previously, blastocyst formation in vitro is not the only factor that causes poor efficiency in nuclear transfer.

Fetal Loss

Fetal loss of SCNT embryos has been reported to occur at any time between embryo transfer to parturition, with most losses occurring during the early stage of gestation.

Early fetal loss usually consists of fetal resorption. Hill et al. (2000a) reported 35% loss of cloned fetuses between days 30 to 40 of gestation and additional loss of 32% between days 40 to 60. Similarly, Yamada et al. (2001) reported a loss of 59% between embryo transfer (day 8) and day 40 of gestation, whereas Cibelli et al. (1998a) reported a 78% loss during the same time. Several researchers have reported placental abnormalities, which may be the reason for the high percentages of fetal loss.

In cloned sheep, De Sousa et al. (2001) reported a pregnancy loss of 50-55% by day 35 of gestation (gestation length =145). Similarly, of 15 cloned fetuses collected at day 35, five (67%) of the fetuses were developmentally retarded, compared to naturally bred sheep. In addition, four fetuses had dermal hemorrhages, six had enlarged livers, and two had fourth ventricle swelling (De Sousa et al., 2001). The cloned fetuses also displayed abnormal placentas. Most notably was the reduced number of cotyledons, with a range of 8-22 compared to the mean of mated purebred, crossbred, or IVP being 32 ± 3.4, 25 ± 4.5, and 25 ± 2.2, respectively (De Sousa et al., 2001). Similar placental abnormalities have been demonstrated in cloned bovine fetuses (Hill et al., 2000a; Stice et al., 1996; Wells et al., 1999).

Another notable problem with SCNT fetuses of sheep and cattle is the high incidence of large offspring syndrome (LOS). This syndrome is usually defined as birth weights greater than 30-40% of normal, however twice the normal weight is not uncommon (Cibelli et al., 1998a; Wilson et al., 1995). Large offspring syndrome is not only seen in SCNT offspring. It has been documented that culturing in vivo produced embryos for as

little as three days can cause LOS in sheep (Walker et al., 1992). Walker et al. (1996) reported a lamb that was five times the mean birthweight for their breed. In sheep, fetal overgrowth has been detected as early as day 21 of gestation (Young et al., 1996). Other in vitro culture studies in cattle and sheep have reported similar effects (Farin and Farin, 1995; Walker et al., 1992). Studies involving asynchronous embryo transfer (Wilmut and Sales, 1981; Young et al., 1995) or administration of exogenous progesterone to recently ovulated dams (Kleemann et al., 1994) also produced abnormally large offspring. McEvoy et al. (1998) reported pregnant sheep that receive high levels of non-protein nitrogen in their diet produced larger offspring. In addition to LOS, there is a higher incidence of physical abnormalities, increased gestation length, increased rate of abortions, and increased mortality and morbidity rates (Walker et al., 1996). Developmental alteration have been detected early in SCNT as well as IVF produced embryos. Cell allocation in the blastocysts seem to favor the trophoblastic cells rather than the inner cell mass, compared to in vivo produced embryos (Du et al., 1996). Given that the placenta is derived from the trophoectoderm, the alteration in cell number may be expected to influence placental size and/or function.

Other abnormalities related to SCNT offspring include; hypoxia, acidosis, pulmonary hypertension, hyperthermia, enlarged right ventricle, greater susceptibility to infection diseases, pulmonary artery greater in size than the aorta, and umbilical vessels 2-3 time the normal size (Cibelli et al., 1998a; Garry et al., 1996; Hill et al., 1999; Hill et al.,

2000b; Schmidt et al., 1996). In addition to the fetal abnormalities, several abnormalities are also seen in the placentas of these fetuses.

Specific Placental Problems

As indicated above, several abnormalities are seen, not only in the fetus, but also in the placental tissue of SCNT offspring. Incidences of placental abnormalities have been reported early in pregnancy. Stice et al. (1996) observed the absence of cotyledonary tissue in SCNT bovine fetuses at day 38 of gestation. The caruncular tissue of these SCNT pregnancies had a hemorrhagic response on the surface and no crypt development (Stice et al., 1996). Examining placental and fetal development during the first trimester, Hill et al. (2000a) reported a link between fetal and placental abnormalities. Four of the six SCNT fetuses collect between days 40–60 of gestation were small for their stage and had abnormal placentas (Hill et al., 2000a). Gross examination discovered poor vascular development and reduced number of cotyledons. The placentas of the two normal sized SCNT fetuses appeared to have normal vascularity but possessed approximately half the number of cotyledons as age-matched controls (Hill et al., 2000a). Histologically, the chorionic epithelium of the SCNT pregnancies had small cuboidal epithelia and subepthilelial hemorrhages, similar to what was reported by Stice et al. (1996). Studying placentome formation in SCNT pregnancies, Hashizume et al. (2002) reported similar gross and histological defects. By day 60, the number of placentomes were dramatically less in SCNT pregnancy compared to pregnancies via artificial insemination (23 ± 11.1 versus 80

± 5.4, respectively; Hashizume et al., 2002). These reseachers concluded early fetal loss in SCNT pregnancies during the first half of gestation was due to placental malformation.

Placental abnormalities are also linked to late gestational abortions and peri-natal defects. The most striking of these placental defects is the reduced number and enlarged size of the placentomes (Cibelli et al., 1998a; Hill et al., 1999). These are similar to reports of early stage fetuses that appear normal but have few placentomes (De Sousa et al., 2001; Hill et al., 2000a; Hill et al., 1999; Stice et al., 1996; Yamada et al., 2001). The early normal fetuses that have reduced cotyledons and peri-natal offspring that had few but larger placentomes, suggests that the normal fetuses are able to generate sufficient placentome numbers have a greater chance of surviving until birth. In connection with these abnormal placenta is the enlarged umbilical vessels and enlarged right ventricle of the fetus (Cibelli et al., 1998b). These may be secondary characteristics that the fetus requires to maintain sufficient nutrient supply from fewer placentomes.

Trophoblast Function and Development in Nuclear Transfer Embryos

In IVF and cloned embryos IFN-τ production may alter pregnancy rates. Secretion of IFN-τ has been shown to be similar for in vitro derived blastocysts and age matched in vivo derived blastocysts (Larson and Kubisch, 1999). Wrenzycki et al. (2001) detected elevated levels of IFN-τ mRNA in cloned blastocysts compared to in vitro produced blastocysts. Stojkovic et al. (1999) reported that in vivo produced, in vitro produced and SCNT cloned bovine embryos produce IFN-τ in a linear manner from day 11 to day 15.

However, after day 15, IFN-τ production from cloned embryos levels off whereas in vivo and in vitro embryos still produce IFN-τ in a linear manner out until day 23 (Stojkovic et al., 1999). Interestingly, production of IFN-τ in cloned bovine embryos appears to be affected by the type of donor cells used. Expression of IFN-τ mRNA from day 8 embryos reconstructed with fetal and adult fibroblast cells was less than IVF controls (Pontes, Bordignon and Smith, personal communication). Nuclear transfer using granulosa donor cells had higher levels of IFN-τ mRNA at day 8 than IVF controls. Interestingly, the donor cells that produced the greater number of pregnancies was the fetal fibroblast cells, with the SCNT embryos from granulosa cell donor not surviving past day 60 of gestation. However, the granulosa cell line used in this experiment expressed greater amounts of IFN-τ mRNA when compared to two other granulosa cell line (Pontes, Bordignon and Smith, personal communication).

Several of the genes involved in bovine placental and fetal development appears to be altered in SCNT embryos. Wrenzycki et al. (2001) detected altered expression of Ascl2 and IGF2 receptor in blastocyst stage SCNT embryos compared to their in vitro produced counterpart. The expression of FGF4 was reported to be absent in SCNT blastocyst compared to in vitro blastocysts (Daniels et al., 2000). The expression of imprinted genes IGF2, IGF2R and H19 were relatively normal in SCNT animals that survived to adulthood, whereas expression levels in SCNT animals that died prior to birth were highly variable (Yang et al., 2005). Alteration in these genes may give insight into the abnormal development of the SCNT placenta.

Incomplete reprogramming of the donor cells, resulting in failure to form totipotent nuclei and consequent genetic and epigenetic alterations that persist through development is thought to be a leading cause of SCNT developmental failure (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). Genome-wide demethylation occurs in mouse, bovine and pig zygotes followed by remethylation at later stages (Dean et al., 2001). However, in cloned embryos at the one cell stage demethylation started to occur but then stopped as development continued regardless of species (Dean et al., 2001). In addition, these cloned embryos started to undergo remethylation so at the morula stage, methylation in the blastomeres resembled that of the donor cells (Dean et al., 2001). Santos and co-authors (2003) demonstrated that SCNT bovine embryos had hypermethylation histone H3-K9 associated with genome-wide hypermethylation. Donor cell type also contributed to the extent of epigenetic reprogramming, and there was a correlation between the proportion of embryos developing to blastocyst and the proportion of embryos with normal epigenotypes. Analysis of bovine H19 demonstrated that SCNT animals that died shortly after birth had biallelic expression (Zhang et al., 2004). These results provide further evidence towards the incomplete epigenetic reprogramming of SCNT embryos.

Problem:

The success of somatic cell nuclear transfer has not come without complications. Even though several species have been cloned, this technique is inefficient. One of the

major problems described with the loss of these reconstructed embryos is poor placental development. These placental defects are considered to be one of the main factors for early embryo loss. Some of these defects include reduced cotyledon number, poor vascular development and hydrallantois.

During embryo development the first cellular differentiation occurs between the morula and blastocyst stage, giving rise to the inner cell mass (ICM) and trophoblast cells. The ICM will develop into the embryo proper, whereas the trophoblast cells will develop into the extra-embryonic tissues (i.e. placenta). As pregnancy continues the trophoblast cells proliferate and differentiate into mononucleated and binucleated cells. The mononucleated cells make up a majority of the trophoblast cells of the placenta and are involved in the production of interferon-tau, the pregnancy recognition signal in cattle. The binucleated cells make up about 20% of the trophoblast cells and migrate across the feto-maternal junction and fuse with uterine epithelial cells. These cells produce pregnancy-associated glycoproteins, placental lactogen, and prolactin related proteins, which are detectable in maternal serum as pregnancy progresses.

In cattle, the mechanism of differentiation of the trophoblast cells is still unclear. In mice several genes have been shown to play a role in trophoblast proliferation and differentiation. One of the more studied genes is achaete scute-like homologue 2 (Ascl2), a basic helix-loop-helix transcription factor. Ascl2 is involved in stimulating cell proliferation and inhibiting giant cell development.

A leading cause of inadequate development of cloned embryos is believed to be the incomplete reprogramming of the donor cells to a totipotent form, similar to a zygote, causing alterations in genetic and epigenetic processes that persist throughout development (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). Interestingly, Ascl2 is an imprinted gene in mice with the paternal allele silenced. In addition, abundance of Ascl2 mRNA appears to be altered in blastocysts produced by nuclear transfer.

Given morphological problems reported in placental development of nuclear transfer embryos, it was of considerable interest to explore the expression of factors involved with trophoblast development in the bovine and determine how these factors are expressed and regulated in embryos produced by nuclear transfer.

Hypothesis:

Embryos produced by somatic cell nuclear transfer display alterations in the expression of genes involved in trophoblast differentiation and function early in gestation .

Objectives:

1) Clone and characterize the trophoblast gene, Ascl2 in cattle and determine the function of this gene in regards to bovine trophoblast differentiation.

2) To evaluate expression of genes involved in trophoblast proliferation (Ascl2) and differentiation (Hand1) early in gestation (days 17 and 40) of embryos produced by somatic cell nuclear transfer and determine how these factors affect placental cell population.

3) To develop primary trophoblast stem (TS) cell lines from in vitro produced and nuclear transfer embryos and to analyze the expression of genes regulating histone and DNA modifications in somatic cell nuclear transfer and in vitro produced embryos, day 40 cotyledonary tissue and TS cell lines.

CHAPTER II

CHARACTERIZATION OF THE PLACENTA SPECIFIC ACHAETE SCUTE-LIKE HOMOLOGUE 2 (ASCL2) GENE IN THE BOVINE

Short Title: Ascl2 expression in the bovine placenta

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Abstract

Ascl2, a basic helix-loop-helix transcription factor, stimulates mononucleate trophoblast cell proliferation and inhibits giant/binucleate cell formation. In mice, Ascl2 is a maternally expressed imprinted gene. Regulation of Ascl2 in the bovine is still unclear due to the limited knowledge of the gene. Our objectives were to clone and characterize bovine Ascl2 and evaluate its imprinting status. Bovine Ascl2 mRNA shares 78% and 70% homology with human and mouse, respectively. The DNA binding domain and bHLH region among human, mouse and bovine share 88% and 95% homology, respectively. Expression Ascl2 mRNA was exclusively in the cotyledon tissue of a d60 fetus. The greatest abundance Ascl2 mRNA was in day 17 filamentous embryos, during the time of rapid trophoblast proliferation. Reduction in Ascl2 mRNA abundance was detected in day 8 parthenogenetic blastocysts suggests a paternal regulation of the maternally expressed gene. Prior to implantation (days 8 and 17), Ascl2 mRNA appears to have biallelic expression, but is paternally silenced after implantation (days 40 and 60). In conclusion, the Ascl2 is highly conserved across species and is specifically expressed in the bovine placenta. Bovine Ascl2 appears to be maternally expressed after implantation, but the paternal genome plays a role in regulating bovine Ascl2 expression.

Introduction

Normal placental development is a vital component for successful pregnancies in mammals, such as cattle. Numerous factors must interact in a precise manner to regulate the formation of the mammalian placenta, and one vital step is the formation of the trophoblast cells.

As in other species of mammals, the undifferentiated bovine morula embryo gives rise to the inner cell mass (ICM) and trophoblast cells at the blastocyst stage [1]. The ICM develops into the embryo proper, whereas the trophoblast cells are the progenitors of the epithelial cells of the placenta [1]. As pregnancy continues, ruminant trophoblast cells proliferate and differentiate into mononucleate and binucleate cells [2]. Similar cell types are seen in other mammals such as rodents and humans but are referred to as giant cells and extravillous cytotrophoblast cells, respectively [3]. In bovine, the mononucleate population comprises the majority of the trophoblast contribution to the placenta, and these cells are involved in the production of interferon-tau (IFNτ), the pregnancy recognition signal in cattle [4]. The binucleate cells, approximately 20% of the trophoblast population, migrate across the feto-maternal junction and fuse with uterine epithelial cells [2]. These cells produce pregnancy-associated glycoproteins (PAG's), as well as placental lactogen, and prolactin-related proteins, all of which are detectable in the maternal serum as pregnancy progresses [5-7]. Although eloquent studies have been conducted on the morphological

changes that occur in the bovine trophoblast [for review see [2, 8], little is known on the molecular regulation of these changes.

In mice and humans, a critical gene in trophoblast proliferation is achaete scute-like homologue 2 (Ascl2; also known as Mash2). The Ascl2 gene, a mammalian homologue of the Drosophilia achaete-scute genes, belongs to the basic helix-loop-helix transcription factor family [9]. In mice, Ascl2 stimulates cell proliferation and inhibits progression of trophoblast to their terminally differentiated giant cell form [10-12]. Using homozygous mutant mice, Guillemot et al. [13] demonstrated that inactivation of Ascl2 was embryonic lethal, and where a reduced number of spongiotrophoblast cells along with an excessive number of trophoblast giant cells were observed. Expression of Ascl2 was detected during oogenesis throughout preimplantation development in the mouse [10]. Interestingly, Ascl2 is an imprinted gene in mice with the paternal allele only being expressed until day 7.5 postcoitum (p.c) then silenced [14]. The mouse Ascl2 gene has been localized to the distal portion of chromosome 7, near a cluster of imprinted genes (H19, Igf2 and Ins2)[14]. Analogous to the mouse, human Ascl2 is only expressed in the extravillus trophoblast and has been mapped to the imprinted region of chromosome 15 along with Igf2 and H19 [15]. Unlike the mouse, the human Ascl2 gene contains two promoters that encode two proteins, Hash2 and Human Acheate Scute Associated Protein (HASAP). Unlike the Hash2, HASAP lacks the bHLH domain and is expressed in several tissues [16].

Expression of Ascl2 mRNA has been reported in bovine day 8 blastocysts [17], but characteristics and regulation of the Ascl2 gene in bovine is not known. Therefore the objective of the present study was to clone and characterize the Ascl2 gene in regards to temporal/spatial expression, and imprinting regulation in the bovine.

Materials and Methods

Animals, Embryos and Fetuses

All treatment protocols involving the use of animals were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Unless stated, all investigations were performed using F1 interspecies crossings of *Bos taurus* (holstein) and *Bos indicus* (Nelore) species for the maternal and paternal genome, respectively.

For day 8 in vivo (AI) embryos, non-lactating holstein cows were superovulated as previously described [18]. Briefly cows received intramuscular injections (i.m.) of FOLLTROPIN-V (Bioniche Animal Health, St.-Laurent, PQ, Canada) given every 12 h in decreasing doses for 4 days with an injection of 500 μg Cloprostenol (Estrumate, Schering-Plough Animal health, Pointe-Claire, QC) on the third day. Cows were artificially inseminated at detected estrus. On day 8 of gestation (gestation length = 285 days), blastocyst stage embryos were collected by non-surgical flushing of the uterus with sterile

phosphate buffered saline (PBS). Embryos were pooled in groups of 5 (n=3), snap-frozen in liquid N_2 and stored at -70°C until further processing.

In vitro fertilized (IVF) and parthenogenetic (P) blastocysts were produced from oocytes obtained from slaughterhouse. Oocytes were matured in vitro as previously described [19]. Briefly, cumulus-oocyte complexes (COC) were aspirated from 2 to 7 mm follicles and washed in Hepes-buffered TCM199 (Gibco BRL, Burlington, ON, Canada) supplemented with 10% FBS. Only COCs with several layers of cumulus cells and with homogenous oocyte cytoplasm were selected. Groups of 25 COCs were cultured in 100 µl drops of IVM media [bicarbonate-buffered TCM-199 supplement with 10% FBS, 50 µl/ml of LH (Ayerst, London, ON, Canada), 0.5 µg/ml of FSH (Follitropin-V), 1µg/ml of estradiol-17ß (Sigma, St. Louis, MO), 22 µg/ml pyruvate (Sigma), and 50 µg/ml of gentamicin (Sigma)]. After 20-22 h of IVM, matured COCs were randomly assigned to either IVF or P groups.

For IVF embryos, matured COCs were fertilized in vitro as previously described [20] and cultured in 50 µl drops of modified synthetic oviductal fluid medium (mSOF;108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM NaHCO3, 1.7 mM CaCl2-H2O, 0.5 mM glucose 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 µg/ml gentamicin, and 0.01% phenol red) plus amino acids (1.4 mM glycine, 0.4 mM alanine, 1 mM glutamine, 2% essential amino acids, 1% non-essential amino acids [21]) at 39°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days. Embryos were pooled in groups of 5 (n=3), snap-frozen in liquid N_2 and stored at -70°C until further processing.

To produce parthenogenetic embryos, oocytes were cleared of cumulus cells by washing with a 0.2% hyaluronidase (Sigma) solution, and only oocytes with the first polar body present were selected. Matured oocytes were exposed to 5 μM ionomycin (Sigma) for 4 min then treated with 2 mM 6-dimethylaminopurine in mSOF for 4 hours to induce parthenogenetic activation. Activated oocytes were cultured in mSOF at 39°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days. Embryos were pooled in groups of 5 (n=3), snap-frozen in liquid N_2 and stored at -70°C until further processing.

Day 17 embryos were produced and collected by utilizing superovulated cows as described above. At day 17 of pregnancy, filamentous embryos were collected by non-surgical flushing of the uterus with sterile PBS + 0.4% BSA. Individual whole embryos or partial embryos with inner cell mass were snap-frozen in liquid N_2 and stored at -70°C until further processing.

For days 40 and 60 samples, non-lactating cows were injected with 500 μg cloprostenol and were artificially inseminated at estrus. At days 40 and 60 of gestation, pregnant uteri were collected at the abattoir, and transported on ice to the laboratory. Fetal and placental tissues were snap-frozen in liquid N_2 and stored at –70°C until further processing.

Cloning and Sequencing of the Bovine Ascl2 5'UTR and Promoter Regions

The 5'-flanking region, open reading frame and 3'- untranslated region of the bovine Ascl2 gene was cloned by PCR using the Universal Genome Walker Kit (CLONTECH Laboratories, Inc., Palo Alto, CA) on a *Bos taurus* genomic DNA library constructed according to the manufacturer. The Expand High Fidelity kit (Roche Diagnostics, Laval, QC, Canada) served for amplification. The PCR products were cloned into a pGEM-T vector (Qiagen, Mississauga, ON, Canada) for sequencing, which was performed by automated DNA sequencing (Service d'Analyze et de Synthèse d'Acides Nucléiques de Université Laval, Québec, Canada). Total RNA from a day 17 *Bos taurus* filamentous embryo was utilized to obtain the whole bovine Ascl2 mRNA using a 5'/3' Rapid amplification of cDNA ends kit (RACE, Roche) according to the instructions from the manufacturer. Primers used for Genome Walk and 5'/3' RACE are described in Table 1. To predict the transcriptional start, 5' RACE was repeated on two separate day 17 samples with two sets of primers. In addition, the DNA sequence was analysed with the promoter prediction program of the Berkeley Drosophilia Genome Project (http://www.fruitfly.org/seq_tools/promoter.html). In silico analysis of the predicted amino acid sequence was performed with Gene Inspector Analysis Software (Textco BioSoftware Inc., West Lebanon, NH).

RNA Extraction, Purification and Reverse Transcriptase (RT) Reaction

Day 8 embryo pools were homogenized in buffer RLT (Qiagen) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Micro kit (Qiagen), as recommended by the manufacturer. Total RNA was eluted in 10 μl and used for RT using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation, Madison, WI) according to the instructions from the manufacturer. For PCR, 1 embryo equivalent was used.

Individual day 17 embryos, day 40 placental tissues and day 60 fetal and placental tissues were homogenized in buffer RLT (Qiagen) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Protect Mini kit (Qiagen), as recommended by the manufacturer. Total RNA was measured by spectrophotometry at 260 nm and 1.0 μg/sample of total RNA was used for the RT reaction with M-MLV RT.

Quantitative and Semi-quantitative RT-PCR

To analyze steady state amounts of Ascl2 and peptidylprolyl isomerase A (PpiA) mRNA, specific quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were performed by utilizing a modified technique previously described [22]. Briefly, PCR products using gene specific primers (Ascl2 Primer set 1, forward: 5'-GAG CTG CTC GAC TTC TCC AG-3', reverse: 5'-TGG AAG GTC TCT GCG GAC AG-3'; PpiA, forward: 5'-ACC GTC TTC TTC GAC ATC GC-3', reverse: 5'-CTT GCT GGT CTT GCC ATT CC-3') were gel extracted and pooled using Qiaquick Gel Extraction and

QIAquick Purification kits, respectively (Qiagen) to generate standard curve ranging from 0.01 fg/μl to 100 fg/μl. Annealing temperatures for Ascl2 and PpiA were 60°C and 62°C, respectively. Five known standard concentrations and samples were subjected to PCR amplification consisting of an initial denaturing (95°C/5 min), amplification cycles of denaturing (94°C/30 s) annealing (60 or 62°C /30 s) and elongation (72°C/30 s) and a final elongation (72°C/4 min). Optimal cycle number for amplification during the exponential phase was determined for each gene. Quantitative PCR products for Ascl2 and PpiA were amplified for 35 and 23 cycles, respectively. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Densities of the amplified fragments were calculated with a computer imaging system and analyzed with Macintosh NIH image software (National Institute of Health, Bethesda, MD). Results were expressed as fg of Ascl2/fg of PpiA.

For abundance of Ascl2 in day 60 fetal and placental tissues, semi-quantitative reactions were carried at the chosen number of cycles in a final volume of 50 μl and using Taq DNA polymerase (Amersham Biosciences Corp., Baie d'Urfe, Canada). Bovine specific primers for Ascl2 (Primer set 2: 5' primer, 5'-CGC TGC GCT CGG CGG TGG AGT A-3', 3' primer, 5'-GGG ACC CGG GCT CCG AGC TGT G-3'; [17]) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh, forward: 5'-TGT TCC AGT ATG ATT CCA CCC-3', reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3'; GenBank Accession No. AF077815) were utilized. Amplifications were carried out in 50 μl with annealing conditions of 35 cycles of 67.5°C/30 s and 20 cycles of 59°C/30 s for Ascl2 and

Gapdh, respectively. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

Determination of Parental allele Expression

To determine parental allele expression of Ascl2, total RNA from day 60 placental samples of *Bos taurus* (BT) and *Bos indicus* (BI) species were extracted and reverse transcribed as described above. PCR products, using Ascl2 primer set 2, from BT and BI cotyledonary tissues of the expected size (210 bp sequence within the coding region) were excised and purified using a Gel Extraction kit (Qiagen). Purified cDNA was then ligated into a pGEM-T Easy Vector System I (Promega Corp., Nepean, Canada) according to the instructions of the manufacturer, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the use of a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA), and at least 3 independent samples were sequenced for verification of authenticity. A single polymorphism was detected at the 71 bp position (T/C) between the BT and BI 210 bp PCR sequences. The polymorphism was within the recognition site of the restriction enzyme *Sfi* I, which allowed the paternal allele (BI) to be digested whereas the maternal allele (BT) remained intact (Fig. 5A). PCR products from days 8, 17, 40 and 60 samples were digested with *Sfi* I at 50°C for 15 hrs.

Statistical Analysis

Relative expression of Ascl2/PpiA (fg/fg) were used as a value for each sample and data were analyzed using the least square analysis of variance by the General Linear Model procedures of SAS. When significant differences were found, comparisons of means were further analyzed by Duncan's Multiple Range Test. A probability level of $P<0.05$ was defined as significant.

Results

Characterization of the Bovine Ascl2 Gene.

Cloning of the *Bos taurus* DNA and RNA was conducted to obtain the genetic structure of the bovine Ascl2 gene. A 3,279 bp sequence of bovine Ascl2 containing a promoter region of 1,404 bps, 5'-untranslated region of 274 bps, coding region of 591 bps and a 3'-untranslated region of 418 bps spanning an intron of 592 bps was obtained (Fig 1A). Computational analysis of the DNA sequence also detected only one transcriptional start site. The coding region of bovine Ascl2 is contained within one exon while the mRNA spans 2 exons. In silico analysis of the predicted amino acid sequence demonstrated the DNA binding domain and basic helix-loop-helix region characteristic of the Ascl2 gene (Fig. 1B). The amino acid sequence shared 78 and 70% homology with the human Hash2 (GenBank Accession no. AF442769) and mouse Mash2 (GenBank Accession no. AF139595), respectively. However, the homology of the DNA binding

domain to that of human Hash2 and mouse Mash2 was 85%, while the bHLH region was 95% homologous (Fig. 1B), demonstrating evolutionary conservation of the Ascl2 gene among different species.

Spatial and Temporal Expression of Bovine Ascl2 mRNA

To determine the expression of bovine Ascl2 mRNA from different tissues, various organs of a day 60 fetus were analyzed by RT-PCR. Expression of a housekeeping gene Gapdh was detected in all tissues investigated (Fig. 2). Expression of Ascl2 mRNA was detected in the cotyledonary tissue of the placenta, whereas no Ascl2 expression was detected in brain, heart, intestine, kidney, liver, lung, muscle, skin, stomach, testis and thymus of the fetus or in the caruncular tissue of the uterus (Fig. 2). No expression of Ascl2 mRNA was measured in the other placental tissues (umbilical cord and inter-cotyledonary tissue; Fig. 2). These results indicate that bovine Ascl2 expression is located specifically in the placental tissues involved in implantation.

To examine the temporal abundance of bovine Ascl2 mRNA, day 8 blastocyst stage IVF embryos, day 17 filamentous embryos, day 40 and day 60 cotyledonary tissues were evaluated by qRT-PCR. Ascl2 was detected in day 8 blastocysts (1 embryo equivalent) increasing at day 17, then diminishing by days 40 and 60 ($P < 0.05$, Fig. 3). These results suggest Ascl2 mRNA is greatest during rapid trophoblast proliferation prior to implantation, declining as the trophoblast cells mature and differentiate.

Abundance of Ascl2 mRNA in Day 8 In Vivo, In Vitro and Parthenogenetic Blastocysts

To determine the effects of in vitro culture on abundance of Ascl2, day 8 in vivo (AI), IVF and parthenogenetic (P) embryos were analyzed. The parthenogenetic embryos (blastocysts with two copies of the maternal genome and no paternal genome) would also allow for the evaluation of Ascl2 expression from only the maternal allele. Given that in other species Ascl2 is a maternally expressed imprinted gene, the parthenogenetic embryos carrying two maternal alleles would in theory have elevated Ascl2 expression if at this stage bovine Ascl2 is imprinted. Similar Ascl2 mRNA abundance was detected between AI and IVF blastocysts (Fig. 4). Parthenogenetic day 8 blastocysts had less compared to AI and IVF blastocysts ($P < 0.05$, Fig. 4). In vitro production and culture of bovine embryos did not affect the abundance of Ascl2 compared to in vivo blastocysts. However, the abundance of Ascl2 mRNA from parthenogenetic blastocysts suggests the requirement of the paternal allele for normal expression.

Parental Allele Expression of Ascl2

To further analyze the imprinting status of the bovine Ascl2 gene, digestion of PCR products from *Bos taurus* (maternal allele) and *Bos indicus* (paternal allele) cotyledonary tissue, day 8, 17, 40 and 60 samples were evaluated. At days 8 and 17 expression of Ascl2 were found to be from both paternal and maternal origin (Fig 5B and C). However, the maternal allele appears to produce more Ascl2 mRNA than the paternal allele (Fig 5B and C). At days 40 and 60, the paternal Ascl2 allele appears to be silenced, with a few embryos

still expressing from both alleles (Fig 5D and E). These results indicate that the Ascl2 gene is parentally regulated in cattle, with expression primarily from the maternal allele particularly after implantation.

Discussion

In the present study we demonstrate that the Ascl2 gene is expressed in bovine placental tissue and maintains the gene's characteristics shown in the mouse and human, where it plays a critical role in trophoblast development. By cloning and analyzing the bovine Ascl2 gene, we provide evidence into the evolutionary conservation of this gene in mammals. In addition, the temporal and spatial expression of Ascl2 mRNA in bovine embryos mimics that seen in other species, with expression exclusively in the placental tissues and greatest during rapid trophoblast proliferation. The expression of bovine Ascl2 appears to be imprinted with a silencing of the paternal allele after implantation, as seen in mice [14]. By utilizing a parthenogenetic model, we also provide the first evidence that the paternal genome is required for normal expression of bovine Ascl2 mRNA from the maternal allele.

To date, research in gene regulation of placental development has focused primarily on the mouse and human models with limited studies done in cattle. Although gestation length and placental morphology of these species differ, several characteristics are shared. Bovine trophoblast cells undergoes acytokinetic mitosis to become binucleate cells [8],

similar to mouse spongiotrophoblast cells undergoing endoreduplication to develop into giant cells [23] and the human trophoblast cells developing into extravillous cytotrophoblast cells [24]. Another commonality is the expression of genes involved in embryo development, such as Cdx-2, Eomes [25], Oct-4 [26] and the IGF2 family [27], to name a few (for review see [28]).

In regards to trophoblast development, a gene highly investigated in mice is the basic helix-loop-helix (HLH) transcription factors Ascl2. In mice, Ascl2 is expressed in the ectoplacental cone (EPC), the chorion and the spongiotrophoblast cell layers during placental development and is absent in primary and secondary giant cells [13]. Homozygous mutant Ascl2 mice die at 10 days postcoitum (d.p.c.) due to the lack of development of the spongiotrophoblast layer [13]. An increase in giant cell number and size was also observed in their study. In humans, the homologue gene Hash2, is expressed in the corresponding extravillous cytotrophoblast cells [15]. In the present study, we demonstrate the expression of the bovine Ascl2 mRNA only in the cotyledonary tissue of placenta and the trophoblast cells of the bovine embryos. The temporal expression of Ascl2 corresponds to the developmental pattern of bovine trophoblast cells. Expression was observed in the day 8 blastocyst at the time the trophoblast cells first appear. The greatest expression is during the time the trophoblast cells are undergoing rapid cell proliferation (day 17) to elongate into the uterine lumen prior to attachment. After implantation, which occurs around day 28-30 of gestation (gestation = to 285 days), Ascl2 expression starts to decline. By day 60 when the placentomes (area of fetal/maternal

nutrient exchange in ruminants) are clearly visible, expression of bovine Ascl2 is minimal. The decrease in Ascl2 mRNA at this time would correspond to trophoblast maturation and differentiation.

Analysis of the bovine Ascl2 gene and amino acid structure also provides evidence of the similarities between bovine, mouse and human species. Like the mouse and human, the bovine Ascl2 mRNA spans two exons. However, the bovine sequence resembles the human in that the coding region is located on one exon, whereas the mouse Ascl2 mRNA coding region spans two exons [29]. Interestingly, Westerman et al. [16] reported the human Ascl2 gene had two promoter regions giving rise to separate proteins, Hash2 and HASAP. The HASAP mRNA was expressed in numerous tissues and did not have the bHLH region associated with Hash2 [16]. In the bovine, an alternate start site was not detected. In the mouse, Stepan et al. [30] identified three different Mash2 transcripts, all expressed solely in the mouse placenta. Analysis of the bovine mRNA with 5' RACE only revealed one transcript. In silico analysis of the predicted amino acid sequence demonstrated the characteristic DNA binding domain and bHLH region of the Ascl2 gene. The homology was greatest with human Hash2 than mouse Mash2. These findings concur with the hypothesis that the bovine and human genomes are evolutionary closer than the bovine and mouse [31].

Parthenogenetic embryos and inter-species cross embryos and placental tissues were utilized to evaluate the imprinting status of bovine Ascl2. Day 8 parthenogenetic blastocysts expressed much less Ascl2 mRNA than their in vivo and IVF counterparts.

Altered trophoblast development and abnormal gene expression has been reported in parthenogenetic mice embryos. Barton et al. [32] reported developmental failure of the trophectoderm and inner cell mass (ICM) of parthenogenetic mouse embryos. The failure in trophectoderm development was not rescued when normal ICM were introduced into the parthenogenetic trophoblastic vesicle [33]. Altered expression of other imprinted genes such as Igf2, H19, Snrpn, Peg3 and Peg1/Mest have been documented in parthenogenetic mouse embryos [34]. Genes like E-cadherin, Hxt, placental lactogen-1 and MMP-9 are expressed normally in primary trophoblast cell lines from parthenogenetic mouse embryos [35]. However, these cell lines had reduced viability and less DNA replication than zygotic embryos when left in culture [35]. The present results suggest paternal factors may play a role in the regulation of Ascl2 expression either directly or indirectly via repression of Ascl2 inhibitors. Direct interaction of paternal and maternal imprinted genes regulating growth has been demonstrated for P57kip2:Igf2 [36] and Igf2: Igf2R [37]. Further studies are needed to investigate any paternally expressed gene that could regulate Ascl2.

Bi-allelic expression of Ascl2 was observed in days 8 blastocyst and day 17 filamentous bovine embryos prior to implantation. By days 40 and 60, the paternal allele of Ascl2 appears to be silenced in the bovine embryo, even though a low level paternal expression was detected in a few samples. The results support those found in mouse where paternal expression was detected during pre-implantation stages and then disappeared following implantation [29]. The delayed imprinting suggests the importance of Ascl2 during the time of rapid trophoblast proliferation, but the process of silencing the paternal

allele remains elusive. The fact that expression from the paternal allele was not restored in mice deficient for the gene DNA-methyltransferase I (Dnmt1), maintenance of DNA methylation may not appear to be controlling the imprinting of Ascl2 [29]. Another epigenetic mechanism that may be regulating ascl2 expression is histone modification. Lewis et al. [38] demonstrated that in the mouse, histone H3 located on the paternal promoter region of the Ascl2 gene were highly dimethylated at the lysine 9 amino acid. Dimethylated H3-lysine 9 is commonly associated with chromatin inactivation (silencing). However, the maternal ascl2 allele was enriched with acetylated H3-lysine 4, a characteristic of transcriptionally active chromatin [38].

In summary, our data provide evidence of evolutionary conservation of the structure and function of the Ascl2 gene. In cattle, as in other species, Ascl2 is vital for trophoblast development and is not expressed in other tissue of the developing fetus. The expression of bovine Ascl2 mimics the proliferative/differentiative status of the trophoblast cells. By utilizing parthenogenetic embryos, we provide evidence suggesting that the paternal genome is required for normal Ascl2 expression. In addition, the parental expression of bovine Ascl2 is similar to the mouse with paternal silencing occurring after implantation. By understanding the underlying molecular events involved in bovine trophoblast development, and how these events are conserved across species, we can gain insight to regulatory mechanisms involved in successful placentation.

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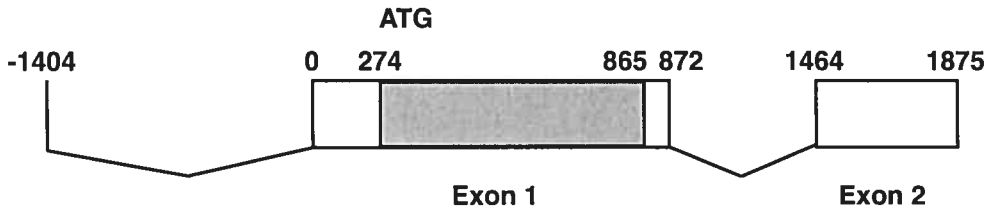
Table 1. Primer sequences used for 5'/3' Genome Walk and 5'/3' RACE.

	Primer Name	Primer (5'-3')	
5' GW	GSP-1	ACC AGG GGA CGA CGA GGC GCA GGA GG	1st PCR
	GSP-2	GCT GCA AGG CGC GGA TGT ACT CCA CC	Nested of 1st
	GSP-3	AGG TCC GGG GAC AGC AAG GCG CAG GA	2nd PCR
	GSP-4	TGC TGT CAG ACG CTC GCC AGG TCT TC	Nested of 2nd
3' GW	GSP3-1	GGT GGA GTA CAT CCG CGC CTT GCA GC	1st PCR
	GSP3-2	CCT CCT GCG CCT CGT CGT CCC CTG GT	Nested of 1st
5' RACE	SP-1	ACG ACC AGG GGA CGA CGA G	cDNA Synthesis
	SP-2	GCA GCC CGC ACA GCA TCG T	1st PCR
	SP-3	ATG TAC TCC ACC GCC GAG C	Nested of 1st
	SP-4	CCA CCT TGC TCA ACT TCT	2nd PCR
	SP-5	AAG GTG GAG GGC AAG GAA	Nested of 2nd
3' RACE	SP3-1	ACG ATG CTG TGC GGG CTG	1st PCR
	SP3-2	TCG TCG TCC CCT GGT CGT	Nested of 1st

Figure 1. (A) Structural organization of the bovine Ascl2 gene and mRNA coding region (grey box) and (B) the predicted amino acid sequence homology among bovine Ascl2, human Hash2 and mouse Mash2. Indicated are: the bipartite nuclear target sequence (light grey), the basic region (medium grey), the helix region (grey), and the loop region (dark grey). Genbank accession numbers: Hash2, AF442769; Mash2, AF139595.

A



B

```
Bovine:
Human:
Mouse:                          MEA HLDWYGVPGL QEASDACPRE SCSSALPEAR    33

Bovine:                                          MDSRA LPRPAPPAPG    15
Human:                                           ••GGT •••S•••••P    15
Mouse:    EGANVHFPPH PVPREHFSCA APELVAGAQG LNASL••GG• •••LM•TSS•    83

Bovine:   VPGCSAARRR PESPELLRCS RRRRPGAVDP GSGAAAVARR NERERNRVKL    65
Human:    ••VGC••••• •A•••••••• ••••ATAET •G•••••••• •••••••••••    65
Mouse:    •A•AC••••• QA•••••••• ••••S••TEA S•SS•S•••• ••••••••••    133

Bovine:   VNLGFQALRQ HVPHGGASKK LSKVETLRSA VEYIRALQRL LAEHDAVRAA    115
Human:    •••••••••• •••••••••• •••••••••• •••••••••• ••••••••N•    115
Mouse:    •••••••••• •••••••N•• •••••••••• •••••••••• ••••••••••    183

Bovine:   LAGGLLAPAV RHPLPRAPSG TPATAASPSC ASSSPG--RG HSSEPGSPRS    163
Human:    •••••RPQ•• •PSA••G•P• •TPV••••SR •••••••--•• G•••••••••    163
Mouse:    ••••••T••T PPSDEC•QPS ASPAS••L•• ••T••SPD•L GC•••T••••    233

Bovine:   AYSSDDSGCE GALSPAEREL LDFSSWLGGY                          193
Human:    •••••••••• •••••••••• ••••••••••                          193
Mouse:    ••••EE•S•• ••••••••Q•• ••••••••••                          263
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Figure 2. Expression of bovine Ascl2 in day 60 fetal and placental tissues. The housekeeping gene Gapdh served as an internal control.

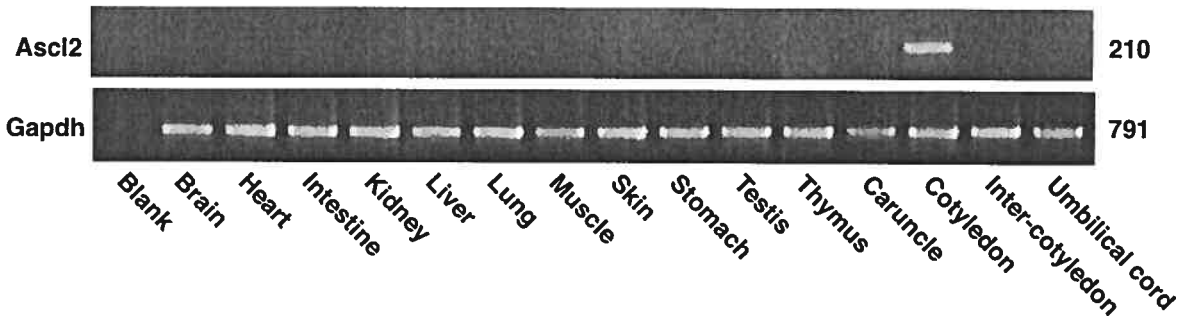

Ascl2
210
Gapdh
791
Blank
Brain
Heart
Intestine
Kidney
Liver
Lung
Muscle
Skin
Stomach
Testis
Thymus
Caruncle
Cotyledon
Inter-cotyledon
Umbilical cord

Figure 3. Quantification of Ascl2 expression across early gestation of day 8 blastocysts, day 17 filamentous embryo, day 40 and day 60 cotyledonary tissue. For day 8, one blastocyst equivalent was used per sample. Graph represent ratio fg Ascl2:fg PpiA. The quantification represents means ± SEM of individual samples (n=3 per group). Different superscripts represent significant differences in means ($P < 0.05$).

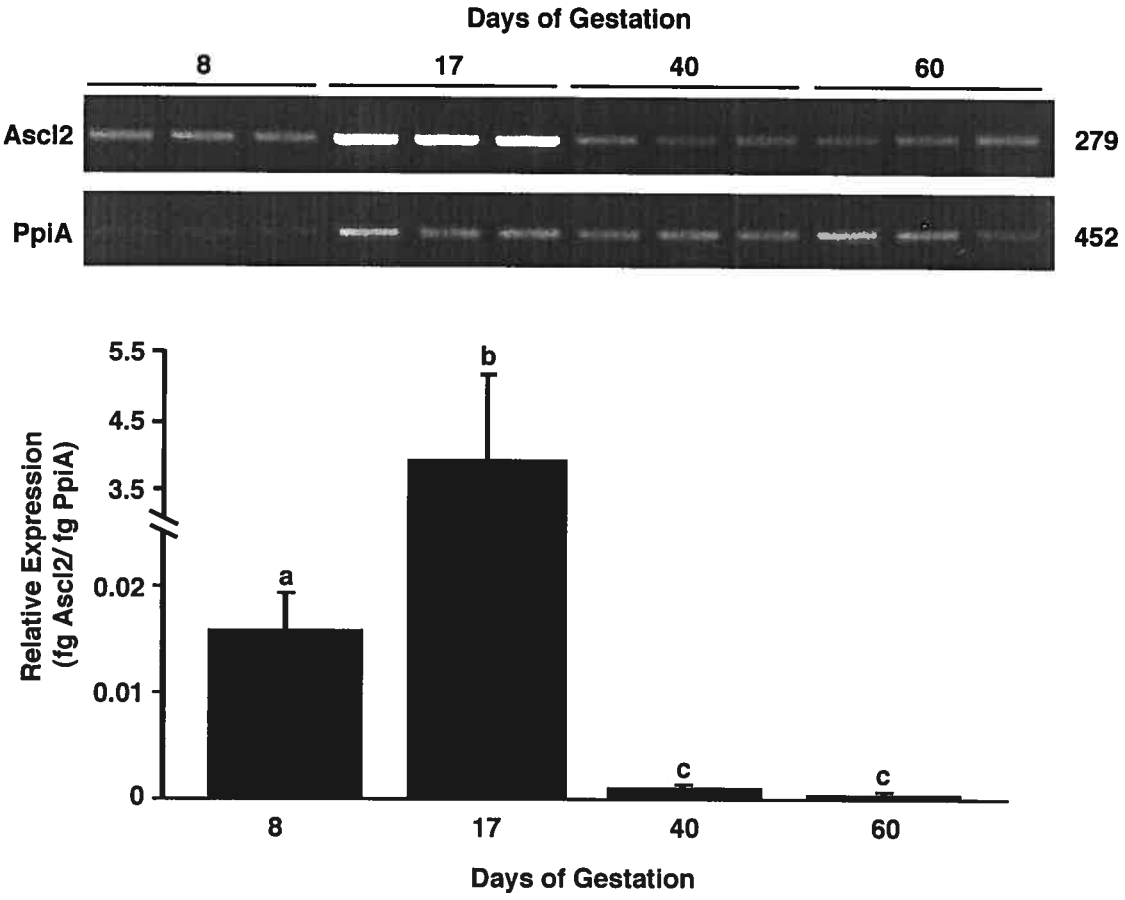

Days of Gestation
8
17
40
60
Ascl2
279
PpiA
452
Relative Expression
(fg Ascl2/ fg PpiA)
5.5
4.5
3.5
0.02
0.01
0
a
b
c
c
8
17
40
60
Days of Gestation

Figure 4. Quantification of Ascl2 expression between day 8 in vivo, in vitro and parthenogenetic produced blastocysts embryos. One blastocyst equivalent was used per sample. Graph represent ratio fg Ascl2:fg PpiA. The quantification represents means ± SEM of individual samples (n=3 per group). Different superscripts represent significant differences in means ($P < 0.05$).

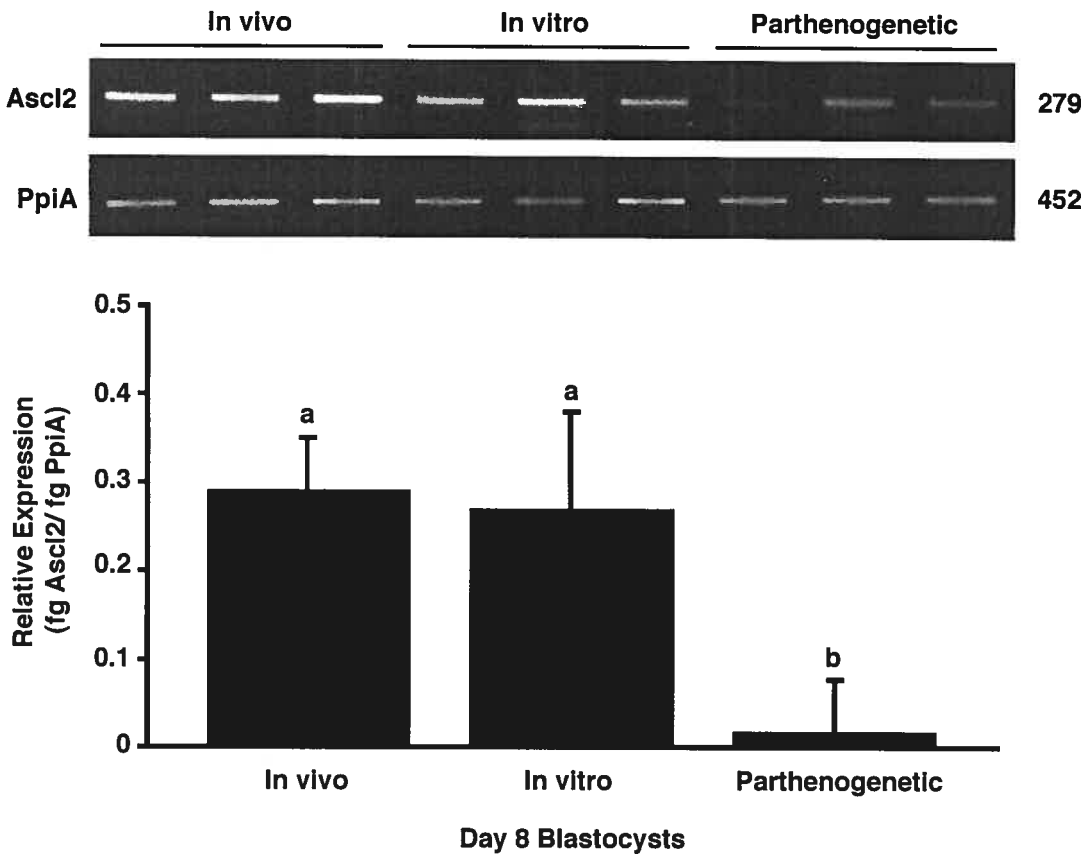
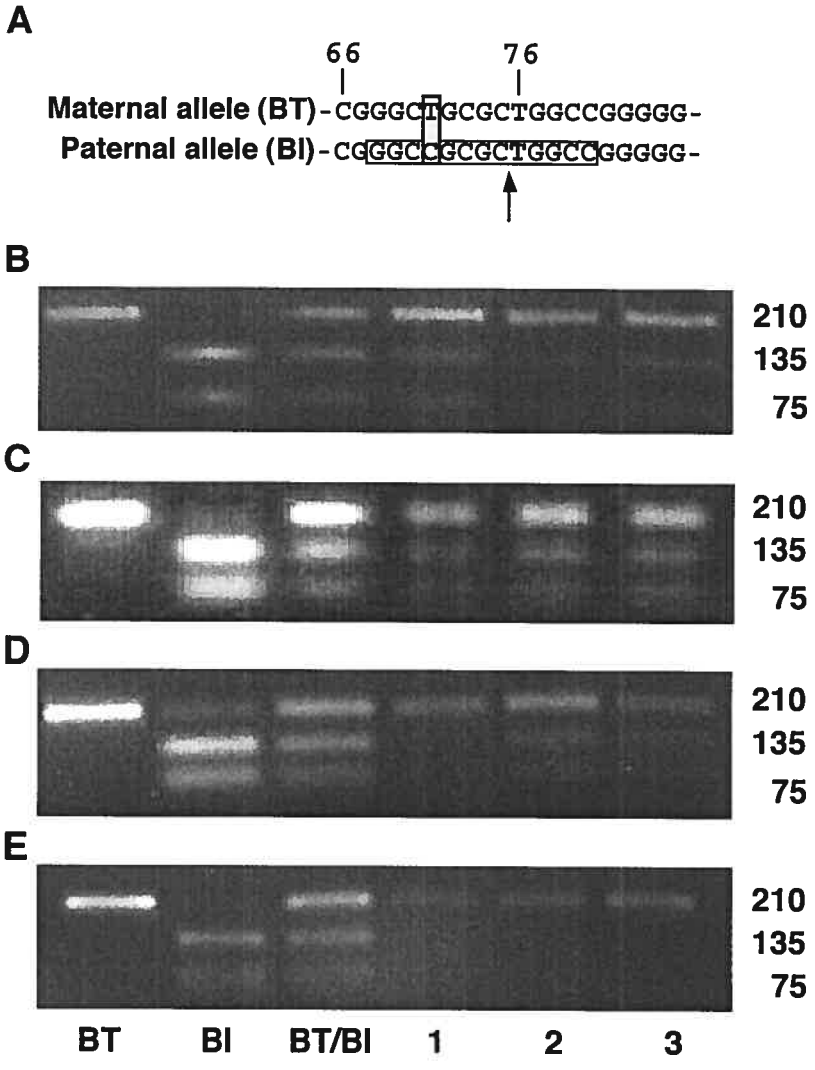

In vivo
In vitro
Parthenogenetic
Ascl2
279
PpiA
452
0.5
0.4
0.3
0.2
0.1
0
Relative Expression
(fg Ascl2/ fg PpiA)
a
a
b
In vivo
In vitro
Parthenogenetic
Day 8 Blastocysts

Figure 5. Analysis of parental expression of bovine Ascl2 in embryos and cotyledonary tissue. (A) A diagram of the single polymorphism difference (grey box) between the maternal (*Bos taurus*) and paternal (*Bos indicus*) Ascl2 PCR products as well as the recognition sequence (white box) and cleavage position (arrow) for the restriction enzyme *Sfi* I. Digestion of Ascl2 PCR products from day 60 *Bos taurus* (BT), *Bos indicus* (BI), mixed (BT/BI) cotyledonary tissues, and representative samples (lanes 1, 2 and 3) of day 8 blastocysts embryos (B), day 17 embryos (C), and days 40 (D) and 60 (E) cotyledonary tissues.


A
66
76
Maternal allele (BT) - CGGGCTGCGCTGGCCGGGGG-
Paternal allele (BI) - CGGGCCGCGCTGGCCGGGGG-
B
C
D
E
210
135
75
BT
BI
BT/BI
1
2
3

CHAPTER III

SOMATIC CELL NUCLEAR TRANSFER ALTERS PERI-IMPLANTATION TROPHOBLAST DIFFERENTIATION IN BOVINE EMBRYOS

Short Title: Trophoblast development in nuclear transfer bovine embryos

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Abstract

Abnormal placental development limits success in ruminant pregnancies derived from nuclear transfer (NT) due to reduction in placentome number and consequently, maternal/fetal exchange. While primarily an epithelial-chorial association, this interface is characterized by progressive endometrial invasion by specialized trophoblast binucleate cells. We compared binucleate cell number and expression of genes known to be necessary for trophoblast proliferation (Ascl2), differentiation (Hand1) and function (IFN-τ and PAG-9) in bovine embryos from pregnancies from artificial insemination (AI), in vitro fertilization (IVF) and NT at days 17 and 40 of gestation. Significant variation was documented. Ascl2 mRNA was greatest in NT embryos compared to AI, while Hand1 was greatest in AI embryos compared to NT. IFN-τ mRNA abundance did not differ among groups. PAG-9 mRNA was undetectable in NT embryos, but greatest in AI embryos. At day 40, NT fetal cotyledons had higher Ascl2 and Hand1 than did AI tissues. Day 40 NT cotyledons had the fewest functional binucleate cells, followed by IVF and AI. Thus, genes critical for normal placental development are altered in NT bovine embryos leading to abnormal trophoblast differentiation and contributing to pregnancy loss.

Keywords: Bovine, Trophoblast proliferation, Binucleate cell differentiation, Somatic cell nuclear transfer

Introduction

The success of cloning by nuclear transfer has not come without complication. Although several mammalian species have been cloned (Chesne et al., 2002; Galli et al., 2003; Kato et al., 1998; Polejaeva et al., 2000; Shin et al., 2002; Wakayama et al., 1998; Woods et al., 2003), the procedures remain inefficient. A leading cause of inappropriate embryogenesis following nuclear transfer (NT) is believed to be the incomplete reprogramming of the donor cells, resulting in failure to form totipotent nuclei, with consequent genetic and epigenetic alterations that persist through development (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004).

A major problem associated with mortality of these reconstructed embryos is poor placental development (De Sousa et al., 2001; Hill et al., 2000; Ogura et al., 2002; Ono et al., 2001a; Ono et al., 2001b). The ruminant placentas associated with pregnancy in NT embryos have been reported to have reduced number of fetal cotyledons, poor vascular development, hydrallantois, and, later in gestation, enlarged placentomes (Hashizume et al., 2002; Hill et al., 2000; Ono et al., 2001a; Stice et al., 1996; Wells et al., 1999). These placental defects are believed to factor into early embryo loss by unknown mechanisms.

As with other species of mammals, the cellular differentiation of the bovine embryo that occurs between the morula and blastocyst stage of development gives rise to the inner cell mass (ICM) and trophoblast cells (McLaren, 1990). The ICM develops into the

embryo proper, whereas the trophoblast cells are the progenitors of the extra-embryonic tissues including the fetal component of the placenta (McLaren, 1990). As pregnancy continues, ruminant trophoblast cells proliferate and differentiate into mononucleate and binucleate cells (Wooding and Wathes, 1980). The mononucleate population comprises the majority of the trophoblast contribution to the placenta, and these cells are involved in the production of interferon-tau, (IFNτ), the pregnancy recognition signal in cattle (Roberts et al., 1992). The binucleate cells, approximately 25% of the trophoblast population, migrate across the feto-maternal junction and fuse with uterine epithelial cells (Wooding and Wathes, 1980). These cells produce pregnancy-associated glycoproteins (PAG's), as well as placental lactogen, and prolactin-related proteins, all of which are detectable in the maternal serum as pregnancy progresses (Kessler and Schuler, 1991; Wooding, 1981; Zoli et al., 1992).

In mice, several genes play a role in trophoblast proliferation and differentiation, including mammalian achaete-scute complex homologue-like 2 (Ascl2; also known as Mash2) and heart and neural crest cell derivative 1 (Hand1), both basic helix-loop-helix transcription factors, which appear to have opposing activities (Cross et al., 1995). The Ascl2 gene stimulates cell proliferation and inhibits progression of trophoblast to their terminally differentiated giant cell form (Cross et al., 1995; Rossant et al., 1998; Tanaka et al., 1997), whereas Hand1 provokes this change (Riley et al., 1998). Using homozygous mutant mice, Guillemot et al. (1994) demonstrated that embryos with inactivated Ascl2 failed to implant and had a reduced number of spongiotrophoblast cells along with an

excessive number of trophoblast giant cells. Mice with mutated Hand1 also failed to implant, but the placenta of these fetuses lacked giant cells (Riley et al., 1998). Interestingly, Ascl2 is an imprinted gene in mice with the paternal allele silenced (Guillemot et al., 1995). Abundance of Ascl2 mRNA has been reported to be altered in blastocysts produced by nuclear transfer (Wrenzycki et al., 2001).

Hashizume et al. (2002) reported the presence of fewer binucleate cells at day 60 in placenta of somatic NT bovine conceptuses compared to AI controls. Given the morphological variation and consequent dysfunction in placentas from NT embryos, it was of considerable interest to explore the expression of factors involved in bovine trophoblast development during early stages of placental formation. Our goal was to examine genes of known significance to successful placental development in other mammals and to determine how these factors affect placental cell population of embryos produced by nuclear transfer.

Materials and Methods

Animals, Embryos and Fetuses

All treatment protocols involving the use of animals were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. All investigations were performed

using F1 interspecies crossings of *Bos taurus* (holstein) and *Bos indicus* (Nelore) species for the maternal and paternal genome, respectively.

Oocyte Collection and In Vitro Maturation (IVM)

Oocytes obtained from slaughterhouse ovaries were matured in vitro as previously described (Bordignon et al., 2003). Briefly, cumulus-oocyte complexes (COC) were aspirated from 2 to 7 mm follicles and washed in Hepes-buffered TCM199 (Gibco BRL, Burlington, ON, Canada) supplemented with 10% FBS. Only COCs with several layers of cumulus cells and with homogenous oocyte cytoplasm were selected. Groups of 25 COCs were cultured in 100 μl drops of IVM media [bicarbonate-buffered TCM-199 supplemented with 10% FBS, 50 μl/ml LH (Ayerst, London, ON, Canada), 0.5 μg/ml FSH (Follitropin-V; Bioniche Animal Health, St.-Laurent, QC, Canada), 1μg/ml estradiol-17ß (Sigma, St. Louis, MO), 22 μg/ml pyruvate (Sigma), and 50 μg/ml gentamicin (Sigma)]. After 20-22 h of IVM, matured COCs were randomly assigned to either IVF or NT groups.

Production of Nuclear Transfer Embryos (NT)

Cumulus cells were removed from oocytes with a 0.2% hyaluronidase (Sigma) solution, and oocytes with the first polar body present were selected for nuclear transfer as previously described (Bordignon et al., 2003). Briefly, cumulus-denuded oocytes were placed in PBS containing 7.5 μg/ml cytochalasin B (Sigma), and approximately 30% of the

host cytoplasm adjacent to the first polar body was removed. To remove completely the chromatin, host oocytes were placed into medium containing 5 μg/ml of Hoechst 33342 (Sigma) for 15 minutes and then subjected briefly to ultraviolet irradiation.

Donor cells were from a primary fibroblast cell line from a *Bos taurus* X *Bos indicus* fetus collected at 60 days of gestation cultured in Dulbecco's Modified Eagles Medium supplemented with 10% FBS and 50 units/ml Penicillin-Streptomycin (GibcoBRL) at 39°C in humidified atmosphere of 5% CO_2. Donor cultures between 2 to 5 passages were allowed to progress to confluency and maintained for two days prior to use so that the cells would be in the G1/G0 stage of the cell cycle. A single fetal fibroblast cell was introduced into the perivitelline space of the enucleated oocyte. The resulting couplet was placed in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and subjected to a 1.5-kV electric pulse lasting 70 μsec. Couplets were then washed and placed into 50 μl drops of modified synthetic oviductal fluid media (mSOF;108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM NaHCO3, 1.7 mM CaCl2-H2O, 0.5 mM glucose 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 μg/ml gentamicin, and 0.01% phenol red) plus amino acids (1.4 mM glycine, 0.4 mM alanine, 1 mM glutamine, 2% essential amino acids, 1% non-essential amino acids; Gardner et al., 1994). After 1-2 h in culture, couplets were examined to determine fusion and then exposed to 5 mM ionomycin (Sigma) for 4 min to induce parthenogenetic activation. Reconstructed oocytes were cultured in mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days.

On day 8 of culture, blastocyst stage embryos were non-surgically transferred to synchronized non-lactating Holstein cows. For day 17 embryo collections, 10-12 embryos were transferred per recipient. For day 40 samples, 1-2 embryos were transferred per recipient.

Production of In Vitro Embryos (IVF)

Matured COCs were fertilized in vitro as previously described (Parrish et al., 1986) and cultured in mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days.

On day 8 of culture, blastocyst stage embryos were non-surgically transferred to estrus synchronized, non-lactating Holstein cows as described above.

Production of In Vivo Embryos by Artificial Insemination (AI)

Non-lactating holstein cows were superovulated by intramuscular injections (i.m.) of FOLLTROPIN-V (Vetrepharm) given every 12 h in decreasing doses starting at day 9-10 of the estrous cycle (day 0= estrus). Cows received an injection of 500 μg Cloprostenol (Estrumate, Schering-Plough Animal health, Pointe-Claire, QC) and were artificially inseminated at 52 h and 86 h after the initiation of superovulation, respectively (Price et al., 1999). For day 40 samples, non-lactating cows were injected with 500 μg cloprostenol and were artificially inseminated at estrus.

Day 17 Embryo and Day 40 Tissue Collection

At day 17 of pregnancy, filamentous embryos were collected by non-surgical flushing of the uterus with sterile PBS + 0.4% BSA. A 0.5 cm section from individual whole embryos or partial embryos with inner cell mass was fixed with 10% buffered formalin for immunocytochemistry. The remainder of the embryo was snap-frozen at -70C until further processing.

Animals at day 40 of pregnancy were slaughtered and pregnant uteri were collected and transported on ice to the laboratory. Cotyledonary and inter-cotyledonary tissues were collected and fixed with 10% buffered formalin or snap-frozen until further processed.

RNA Extraction, Purification and Reverse transcriptase (RT) Reaction

Individual day 17 embryos were homogenized and RNA was purified using TRIzol LS Reagent (GibcoBRL) as recommended by the manufacturer. Day 40 tissues were homogenized in buffer RLT (Qiagen, Mississauga, ON, Canada) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Protect Mini kit (Qiagen), as recommended by the manufacturer. Total RNA was measured by spectrophotometry at 260 nm and 1.0 μg/sample of total RNA was used for the RT reaction using the Omniscript RT kit (Qiagen) according to the instructions from the manufacturer.

Bovine Specific cDNA Cloning

Bovine specific primers for PAG-9 (GenBank Accession No. AF020511), IFN-τ (GenBank Accession No. AF270471) and Ascl2 (Wrenzycki et al., 2001) were employed (Table 1).

Hand1 primers were designed based on homologous sequences between human (GenBank Accession No. NM004821) and mouse (GenBank Accession No. NM008213; Table 1). PCR products of the expected size, obtained from the primers described above were excised and purified using a Gel Extraction kit (Qiagen). Purified cDNA was then ligated into a pGEM-T Easy Vector System I (Promega Corp., Nepean, Canada) according to the instructions of the manufacturer, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the use of a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA), and at least 3 independent samples were sequenced for verification of authenticity.

Bovine specific primers for glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were used (GenBank Accession No. AF077815) as a control (Table 1).

Semi-quantitative RT-PCR

Relative abundance of the Ascl2, Hand1, IFN-τ and PAG-9 were determined by semi-quantitative PCR using Gapdh as a control for RNA quantity and RT efficiency. For each individual product analyzed, the number of cycles was chosen by subjecting the RT

products to PCR reactions of 13 to 45 cycles, and quantification was achieved by amplification in the exponential phase. The number of cycles chosen for Gapdh, Ascl2, Hand1, IFN-τ and PAG-9 were 19, 27, 27, 15 and 40, respectively. For day 40 cotyledonary tissue, 20, 35 and 26 cycles were used for Gapdh, Ascl2 and Hand1, respectively.

The semi-quantitative reactions were conducted in a final volume of 50 μl and using Taq DNA polymerase (Amersham Biosciences Corp., Baie d'Urfe, Canada). Amplifications were carried out with annealing conditions described in Table 1. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Densities of the amplified fragments were analysed using the Collage software (Photodyne, New Berlin, WI). Results were expressed as a density ratio of the target gene to the control (Gapdh).

Determination of Parental Allele Expression of Ascl2

To determine parental allele expression of Ascl2, total RNA from day 60 placental samples of *Bos taurus* (BT) and *Bos indicus* (BI) species were extracted and reverse transcribed as described above.

A 210 bp Ascl2 PCR product from BT and BI cotyledonary tissues, obtained from the primers mentioned above, was excised, purified and sequenced as described above. As before, sequences were verified by in at least 3 independent samples. A single polymorphism was detected (T/C) at the 86 bp position between the BT and BI sequences.

The polymorphism was within the recognition site of the restriction enzyme *Sfi* I, which allowed the paternal allele (BI) to be digested whereas the maternal allele (BT) remained intact (Fig. 5A). PCR products from day 17 and day 40 AI, IVF and NT samples were digested with *Sfi* I at 50° C for 15 hrs.

Immunohistochemistry of Binucleate Cells

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Deparaffinized and hydrated sections were washed in phosphate buffered saline (PBS). For general morphology, sections were stained with hematoxylin and periodic acid-Schiff (H/PAS). Sections were then incubated with 5% bovine serum albumin (BSA) in PBS at room temperature for 45 min. A monoclonal antibody, SBU-3 (kindly provided by Garry Barcham, University of Melbourne; Victoria, Australia) raised against sheep trophoblast microvillous preparation that recognizes ruminant binucleate cells (Lee et al., 1985) was diluted 1:5 in 5% BSA in PBS and incubated overnight at 4° C. The same antibody has been previously used to characterize binucleate cell distribution in bovine placentomes (Lee et al., 1986). After washes in PBS, sections were incubated with a second antibody labelled with the fluorochrome CY3 (Jackson ImmunoResearch, West Grove, PA) 1 hr. Nuclei were detected with DAPI (4',6-diamidino-2-phenylindole) diluted 1:1000. Control sections were subjected to the same procedure expect the SBU-3 antibody was replaced with 5% BSA in PBS. For day 40 samples, 2500-4000 cells from 3-4 cotyledons/cow/group were counted for positive SBU-3 staining.

Statistical Analysis

The ratio of target gene/Gapdh was used as a value for each sample and data were analysed using the least square analysis of variance by the General Linear Model procedures of SAS. The percentage of positive SBU-3 cells per group at day 40 was analysed using the least square analysis of variance by the General Linear Model procedures of SAS. When significant differences in groups were found, comparisons of means were further analysed by Duncan's Multiple Range Test. A probability level of $P<0.05$ was defined as significant.

Results

The results of this investigation demonstrate the occurrence of differences in the gene expression and in frequency of functional binucleate cells in pregnancies derived from NT.

Semiquantification of Ascl2, Hand1, INF-τ and PAG-9 mRNA at Day 17

To determine if genes involved in trophoblast development in other species play a role in the bovine embryo, the abundance of Ascl2 and Hand1 mRNA was compared among AI, IVF and NT day 17 embryos. In addition, IFN-τ and PAG-9 mRNA were evaluated to determine trophoblast cell function among all groups. Expression of Ascl2 and

INF-τ mRNA was detected in all day 17 embryos regardless of group (Fig. 1A and Fig. 2A). Expression of Hand1 mRNA was detected in all AI and IVF embryos and in 7/8 NT embryos (Fig. 1B). PAG-9 mRNA was detected in 4/6, 3/6 and 0/8 day 17 embryos of AI, IVF and NT, respectively (Fig. 2B). Abundance of Ascl2 mRNA was greater in NT embryos than in AI embryos ($P < 0.05$, Fig. 1A). Abundance of Hand1 mRNA was greater in AI embryos than in NT embryos ($P < 0.05$, Fig 1B). Abundance of IFN-τ mRNA did not differ among groups (Fig. 2A) suggesting normal mononucleate cell function among all groups. Greatest abundance of PAG-9 mRNA was observed in AI embryos, with no detectable mRNA for NT embryos ($P < 0.05$, Fig. 2B), indicating an alteration in binucleate cell number or function in NT embryos. These results suggest abnormal trophoblast development in nuclear transfer produced embryos.

Semiquantification of Ascl2 and Hand1 mRNA at Day 40

To determine if altered expression of Ascl2 and Hand1 is normalized in NT embryos that survive to the stage of implantation, cotyledonary tissue was collected from Day 40 fetuses. Expression of Ascl2 and Hand1 mRNA was detected in all AI (n=3), IVF (n=2) and NT (n=3) cotyledons obtained from day 40 pregnancies (Fig. 3). Relative abundance of Ascl2 and Hand1 mRNA was greatest in day 40 NT cotyledons compared to IVF and AI cotyledons ($P < 0.05$, Fig. 3A and Fig. 3B). These results indicate that Ascl2 and Hand1 expression remains altered in NT embryos even though they successfully attach and develop placentomal structures.

Distribution of SBU-3-positive Cells in Day 40 Cotyledonary Tissue

To establish whether the altered gene expression of Ascl2 and Hand1 correlates with modified trophoblast cell populations in bovine NT embryos, binucleate cell frequency was evaluated. Day 17 embryos and day 40 cotyledonary tissues from AI, IVF and NT embryos were stained using the monoclonal antibody SBU-3, which specifically labels trophoblast binucleate cells in ruminants (Lee et al., 1986). In day 17 embryos, few trophoblast binucleate cells, as defined by observation of two nuclei were identified (Fig 4, Panel 1A and B). These cells were not positive for SBU-3 antibody (data not shown), presumably due to their early stage of development. At day 40, cotyledonary tissues from AI fetuses had more positive cells, than IVF and NT fetuses, for the binucleate cell specific antigen (Fig. 4, Panel 2B, 2D, 2F; and Table 2; $P < 0.01$). These results suggest that there are fewer binucleate cells or that there is altered binucleate cell function in NT placentomes.

Parental Allele Expression of Ascl2

A single polymorphism detected between *Bos taurus* and *Bos indicus* Ascl2 PCR products (Fig 5A) was used to determine the parental origin of Ascl2 mRNA. At day 17, expression of Ascl2 was found to be from both paternal and maternal origin regardless of group (Fig 5B). However, the maternal allele appears to produce more Ascl2 mRNA than the paternal allele (Fig 5B). At day 40, the paternal Ascl2 allele appears to be silenced,

with a few embryos still expressing from both alleles, regardless of experimental group (Fig 5C). These results indicate that the Ascl2 gene is parentally regulated in cattle, with expression primarily from the maternal allele.

Discussion

In the present study we provide the first demonstration that genes believed critical for trophoblast proliferation (Ascl2) and differentiation (Hand1) in rodents and humans are expressed during placental formation in a ruminant species. Moreover, there is altered expression of these genes in embryos derived from assisted reproductive techniques such as somatic cell nuclear transfer and this aberrant expression strongly associates with the abnormal placental development. In addition, we show that cotyledonary tissue from day 40 NT fetuses have fewer functional binucleate cells than do AI and IVF fetuses. As binucleate cells closely associate with placentome development, their reduced number in nuclear transfer pregnancies may be the cause of the reduced placentome, a common observation in cloned ruminants (Hashizume et al., 2002; Hill et al., 2000; Ono et al., 2001b; Stice et al., 1996; Wells et al., 1999).

Research in gene regulation of placental development has primarily focused on the mouse model. To establish the commonality of mechanisms, other species, including the cow, need to be investigated. Although gestation length, placental hormone secretory patterns and placental morphology of these two species differ, some characteristics are

shared. In mice, the spongiotrophoblast cells of the placenta undergo endoreduplication to develop into giant cells (MacAuley et al., 1998), similar to bovine trophoblast cells undergoing acytokinetic mitosis to become binucleate cells (Klisch et al., 1999). Another commonality is in the genes involved in trophoblast differentiation. The genes most investigated in mice are the basic helix-loop-helix (HLH) transcription factors Ascl2 and Hand1. In mice, Ascl2 is a critical transcriptional regulator of trophoblast differentiation, expressed in the ectoplacental cone (EPC), the chorion and the spongiotrophoblast cell layers during placental development (Guillemot et al., 1994). It is absent in primary and secondary giant cells (Guillemot et al., 1994). Homozygous mutant Ascl2 mice die at 10 days postcoitum (d.p.c.) because the spongiotrophoblast layer fails to develop, and an increase in giant cell number and size is observed (Guillemot et al., 1994). In the present study, we demonstrate that bovine trophoblast cells express Ascl2, and this expression is altered in day 17 embryos and day 40 cotyledonary tissue from NT embryos, compared to their in vivo derived counterparts. It is not unreasonable to infer that elevated Ascl2 in the NT context contributes to the placental failure common to cloned embryos.

Bi-allelic expression of Ascl2 was observed across all experimental groups of day 17 bovine embryos prior to implantation. In mice, Ascl2 is an imprinted gene in which the paternal Ascl2 allele is expressed in the early postimplantation conceptus (5.5 d.p.c.), then undergoes progressive decrease until silenced by 9.5 d.p.c. (Tanaka et al., 1999). These findings may explain the bi-allelic expression found in the current study since, at day 17, the bovine embryo has not formed any attachment to the endometrium. By day 40, the

paternal allele of Ascl2 appears to be silenced in the bovine embryo, even though a low level paternal expression was detected in a few samples. The imprinting of Ascl2 appears not to be controlled by DNA methylation, since expression from the paternal allele was not restored in mice deficient for the gene DNA-methyltransferase I (Dnmt1), which is involved in maintenance the methylated state (Tanaka et al., 1999). Imprinting of Ascl2 in the mouse and cow appears to occur around the time of implantation, suggesting that other Dnmt's that are involved with *de novo* methylation, such as Dnmt3a and Dnmt3b, play a role in regulation of Ascl2. Further studies are required to determine how the Ascl2 gene is imprinted in the bovine embryo at later stages of development.

Hand1, another gene critical for trophoblast differentiation, is predominately expressed in mouse trophoblast giant cells but has been detected in the extraplacental cone and areas of the spongiotrophoblast cell layer that also express Ascl2 (Scott et al., 2000). Development is arrested in mouse embryos carrying a mutated Hand1 gene around 7.5 d.p.c and there is a significantly reduced number of trophoblast giant cells (Riley et al., 1998). Cross et al. (1995) reported that the Rcho-1 trophoblast cell line over-expressing Hand1 differentiated into giant cells. Giant cell development was inhibited when $Hand1^{+/+}$ Rcho-1 cells co-expressed Ascl2 (Cross et al., 1995). In the present study, Hand1 mRNA expression was detected in day 17 bovine embryos. Hall et al. (2005) was not able to detect Hand1 expression from day 8 bovine blastocysts, suggesting that, at this stage, the trophoblast cells are undergoing extensive proliferation to elongate prior to implantation. In addition, expression of Hand1 in NT embryos is altered, first being under-expressed at

day 17 and then over-expressed at day 40. One possible explanation for the current results is that, in day 17 NT embryos, the over-expression of Ascl2 directly or indirectly inhibits factors involved in trophoblast differentiation (e. g. Hand1 expression). This action is expected to inhibit binucleate cell formation. The regulatory factors of Hand1 are poorly characterized. Nonetheless, by day 40, expression of Ascl2 and Hand1 are both higher in NT cotyledonary tissue. The higher Hand1 expression may be a direct response to fewer binucleate cells, given that Hand1 is responsible for the induction of differentiation of mononucleate cells. It must be kept in mind that 75% of NT embryos did not survive to day 40 in the present investigation. It is possible that those that survived did so because they were able to produce enough Hand1 to bring about binucleate cell formation allowing for implantation to occur.

IFN-τ, a secretory protein that inhibits the normal luteal regression, is a marker of trophoblast cell function early in gestation (Helmer et al., 1987; Roberts et al., 1992). IFN-τ mRNA expression is first seen around the time of blastocoel formation (Hernandez-Ledezma et al., 1992), and continues until just prior to attachment around day 25-28 of gestation (Roberts et al., 1992). Wrenzycki et al. (2001) detected elevated levels of IFN-τ mRNA in cloned blastocysts compared to in vitro produced blastocysts. In the present study, where later stage embryos (day 17) were used, no differences in INF-τ mRNA expression among AI, IVF and NT groups were detected. These results suggest that mononucleate trophoblast cells in the NT embryos are functioning normally with respect to synthesis of the pregnancy recognition factor IFN-τ. In contrast, Stojkovic et al. (1999)

reported that in vivo produced, in vitro produced and NT cloned bovine embryos produce IFN-τ in a linear manner from day 11 to day 15 in culture, however, after day 15, IFN-τ production from cloned embryos levels off, whereas in vivo and in vitro derived embryos continue to produce IFN-τ through day 23 (Stojkovic et al., 1999). In these experiments, embryos were cultured in vitro for the entire 23 days and did not elongate after day 9, as seen in vivo. In the present study, we have employed a more physiological model in which embryos were transferred into recipient cows and developed in vivo until day 17, resulting in appropriate elongation.

In the current study, mRNA expression of a pregnancy-associated glycoprotein isoform (PAG-9) mRNA was used as a marker of binucleate cell function in day 17 embryos. Ruminant binucleate cells are believed to migrate from the chorion into the maternal epithelium, from where they release several types of proteins including PAG's (Wooding, 1981). Several isoforms of PAGs have been identified and exhibit unique temporal and spatial expression patterns during pregnancy (Green et al., 2000). Of the 21 different isoforms, PAG-9 is expressed only by binucleate cells as early as day 25 of gestation (Green et al., 2000). In this study, expression of PAG-9 mRNA was detected in day 17 AI embryos, but was undetectable in NT embryos. This results have two possible conclusions, either the binucleate cell of day 17 NT embryos are not developed enough to produce PAG-9, or there are no binucleate cells at this stage.

The binucleate cells in the cotyledonary and intercotyledonary areas appear similar, but differences in antigen expression have been observed (Lee et al., 1986; Lee et al.,

1985). These researchers demonstrated that mono-clonal antibody SBU-3, that recognizes PAG's, was localized to all the binucleate cells in the cotyledonary area. Utilizing this antibody, we determined that placental cotyledon tissue from day 40 NT fetuses has fewer binucleate cells. These reduced numbers may lead to fewer cotyledons developing, which could be related to early pregnancy loss. The larger placentomes seen later in gestation may be a compensation mechanism to meet the nutrient requirements of the growing fetus.

In summary, we provide new information to demonstrate that genes critical for trophoblast proliferation (Ascl2) and differentiation (Hand1) in other species, are expressed during placental development in the cow. Using embryos derived from somatic cell nuclear transfer, which have recognizable placental abnormalities as a model, we determined that expression of these genes is altered. We believe that a plausible mechanism for altered trophoblast development in NT bovine embryos is the incomplete epigenetic reprogramming of the donor cells. It is more likely that this occurs with Ascl2, given its over-expression is maintained through day 40 in NT embryos, whereas Hand1 is first down-regulated at day 17 but over-expressed at day 40 in the NT context, indicating more of a response rather than a primary problem. Perhaps altered chromatin modification could account for the over-expression on Ascl2, given that imprinting is unlikely to be based on the bi-allelic expression observed for all experimental groups. Further, upstream factors may be causing the altered expression of Ascl2 and Hand1. In addition, placental tissue from NT fetuses have fewer binucleate cells, which could, in itself, lead to the abnormal placental development and pregnancy loss commonly associated with nuclear transfer. By

understanding the underlying molecular events involved in bovine trophoblast development, we can gain insight to regulatory mechanisms involved in successful placentation and how these events may be manipulated to improve assisted reproductive techniques such as somatic cell nuclear transfer.

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Table 1. Oligonucleotide primer sequences used for RT-PCR.

Gene	Primer (5'-3') sense and antisense	Length (bp)	Annealing Temperature (°C)
Gapdh	TGTTCCAGTATGATTCCACCC	791	58
	TCCACCACCCTGTTGCTGTA		
Hand1	GCTCTCCAAGATCAAGACTCTGC	224	58
	CGGTGCGTCCTTTAATCCTCTTC		
IFN-τ	GCTATCTCTGTGCTCCATGAGATG	353	58
	AGTGAGTTCAGATCTCCACCCATC		
Ascl2	CGCTGCGCTCGGCGGTTGAGTA	210	67.5
	GGGACCCGGGCTCCGAGCTGTG		
PAG-9	TCCTTTTGTACCATGCCAGC	330	58
	TGCCCTCCTGCTTGTTTTTG		

Figure 1. Graphs of representing the semiquantitative RT-PCR for Ascl2 (A) and Hand1 (B) in day 17 AI, IVF and NT embryos. The quantification represents means ± SEM of individual samples (AI and IVF, n=6; NT, n=8). Different superscripts represent significant differences in means ($P < 0.05$).

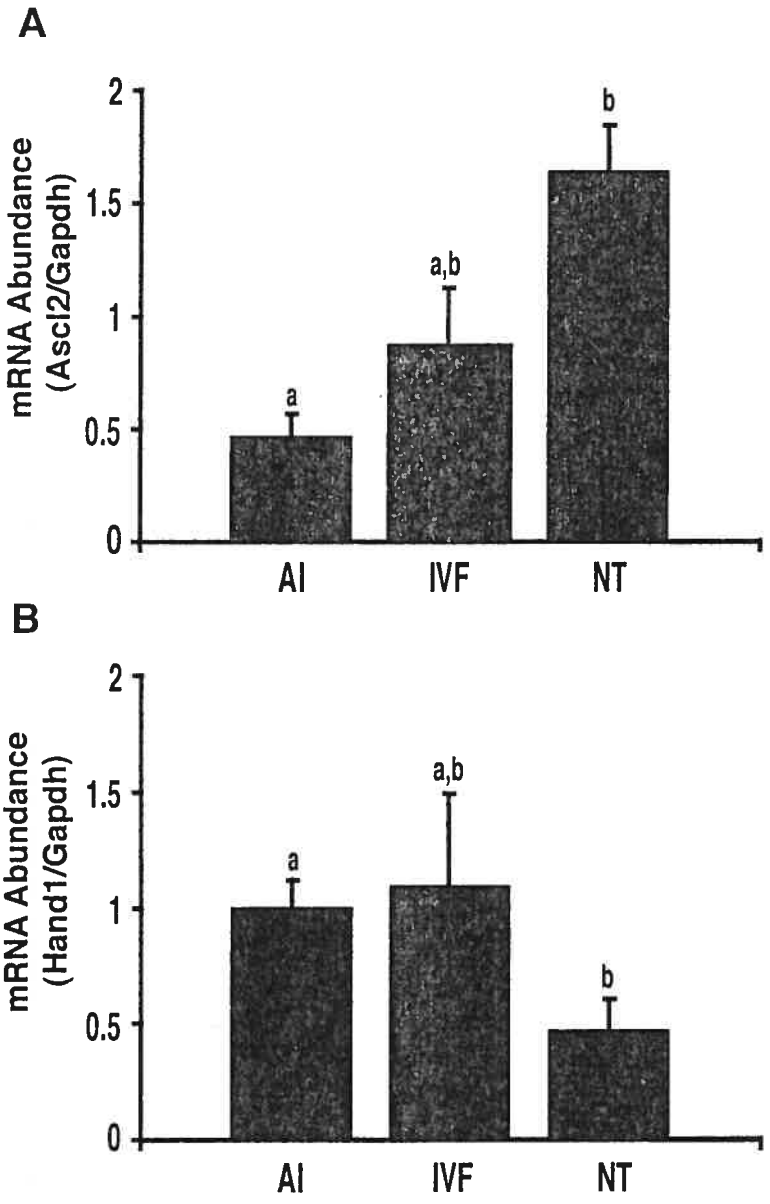

A
mRNA Abundance (Ascl2/Gapdh)
2
1.5
1
0.5
0
a
a,b
b
AI
IVF
NT
B
mRNA Abundance (Hand1/Gapdh)
2
1.5
1
0.5
0
a
a,b
b
AI
IVF
NT

Figure 2. Graphs of representing the semiquantitative RT-PCR for IFN-τ (A) and PAG-9 (B) in day 17 AI, IVF and NT embryos. The quantification represents means ± SEM of individual samples (AI and IVF, n=6; NT, n=8). Different superscripts represent significant differences in means ($P < 0.05$).

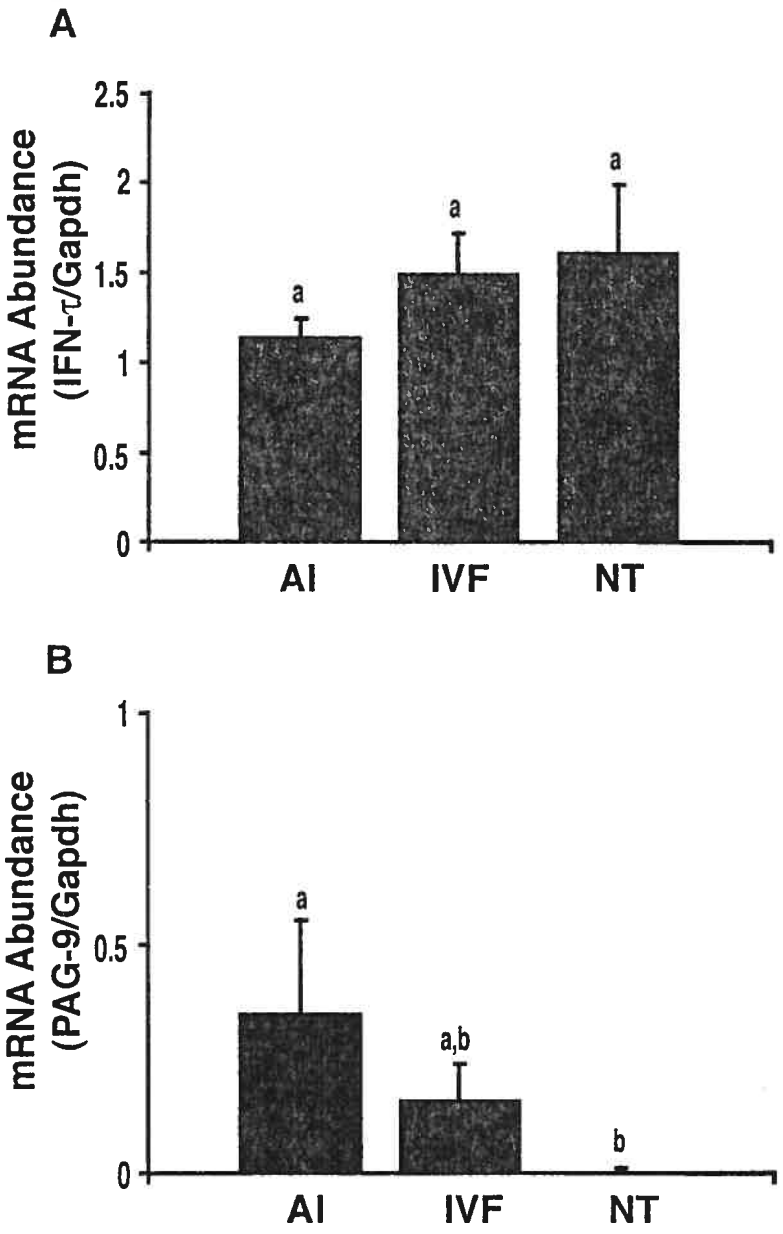

A
mRNA Abundance
(IFN-τ/Gapdh)
2.5
2
1.5
1
0.5
0
a
a
a
AI
IVF
NT
B
mRNA Abundance
(PAG-9/Gapdh)
1
0.5
0
a
a,b
b
AI
IVF
NT

Figure 3. Graphs of representing the semiquantitative RT-PCR for Ascl2 (A) and Hand1 (B) in day 40 AI, IVF and NT embryos. The quantification represents means ± SEM of individual samples (IVF, n=2; AI and NT, n=3). Different superscripts represent significant differences in means ($P < 0.05$).

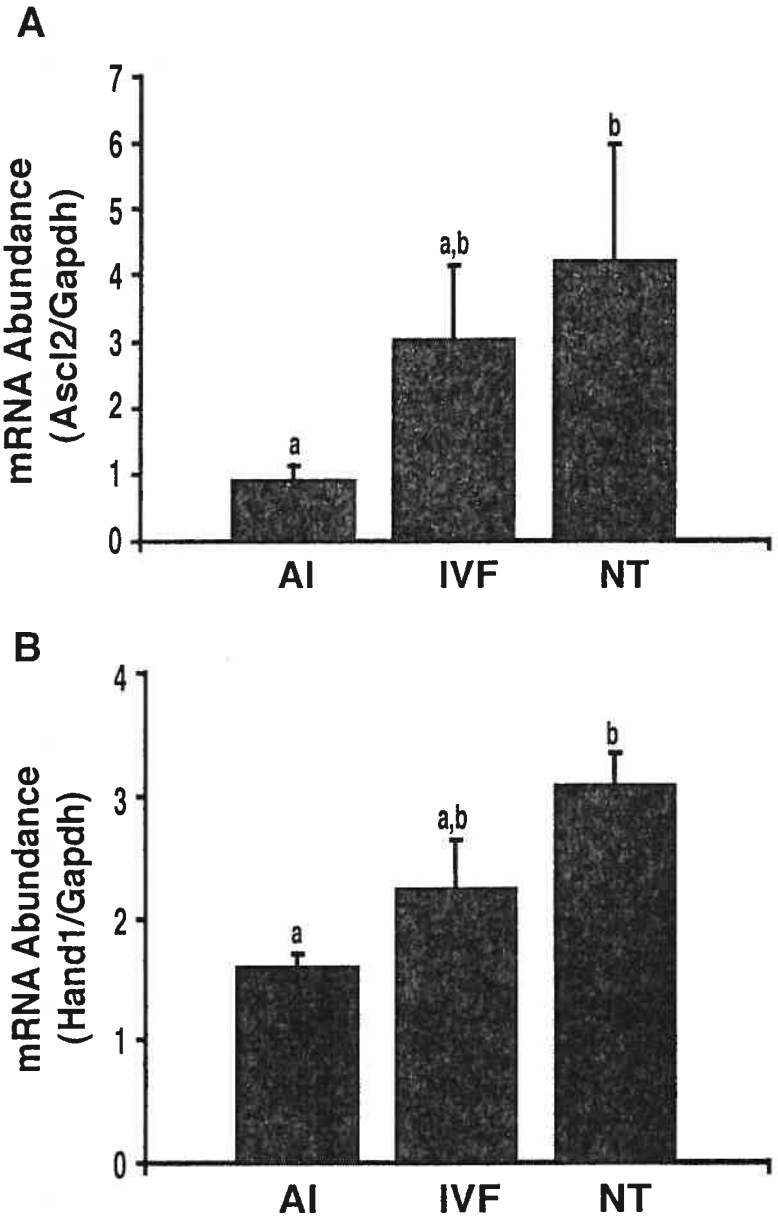
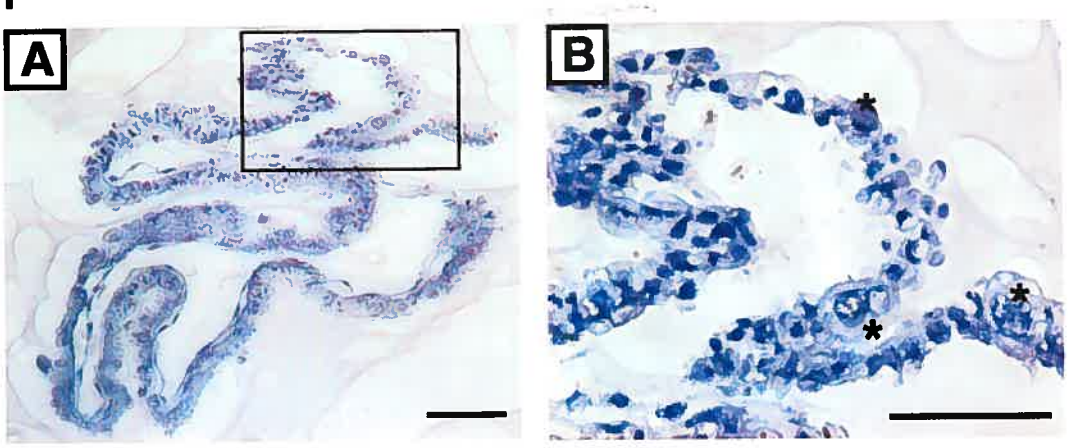

A
mRNA Abundance
(Ascl2/Gapdh)
7
6
5
4
3
2
1
0
a
a,b
b
AI
IVF
NT
B
mRNA Abundance
(Hand1/Gapdh)
4
3
2
1
0
a
a,b
b
AI
IVF
NT

Figure 4. Immunohistochemical characterization of binucleate cells in day 17 bovine embryos and cotyledonary tissue from day 40 AI, IVF and NT fetuses. Panel 1: cross section of day 17 embryo stained with H/PAS (A). At higher magnification (B) binucleate cells are detected (*). Panel 2: H/PAS staining of day 40 AI (A), IVF (C) and NT (E) cotyledonary tissues. Positive binucleate cells for SBU-3 (red) in day 40 AI (B), IVF (D) and NT (F) cotyledonary tissue. Nuclei counterstained with DAPI (blue). Small and large bars = 100 and 200μm, respectively.

Panel 1



Panel 2

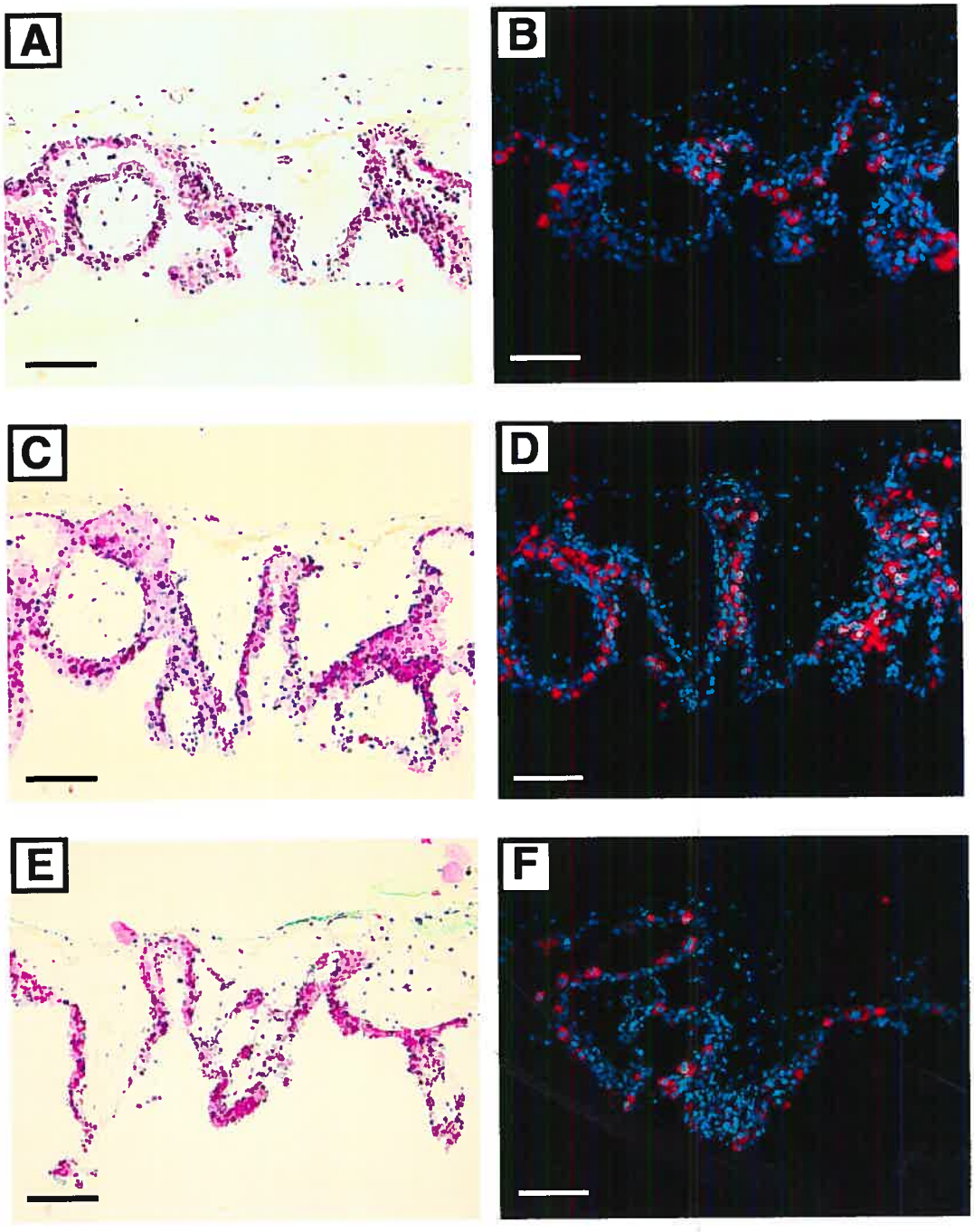
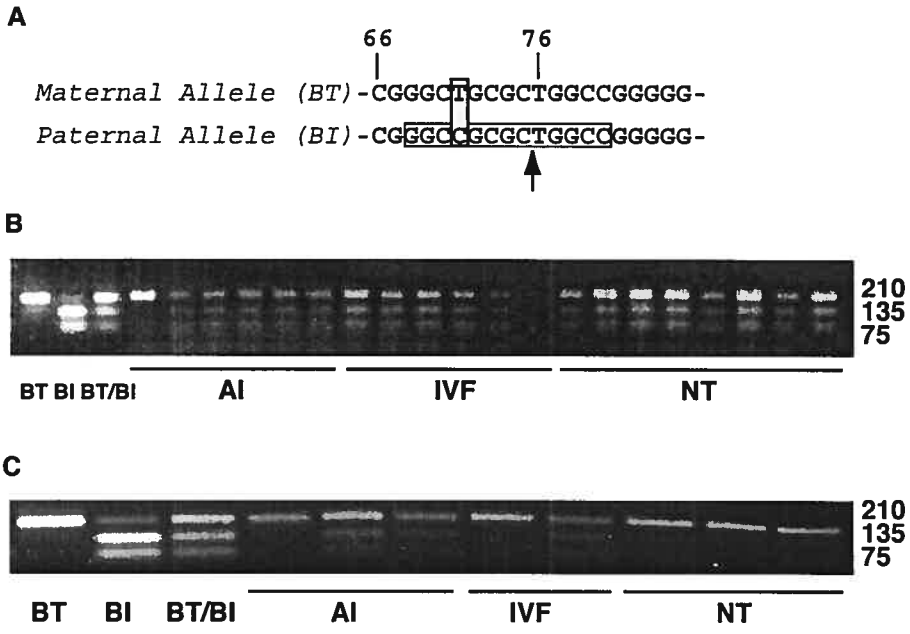


Table 2. Percentage of SBU-3 positive binucleate cells from AI, IVF and NT day 40 cotyledonary tissue.

Group	n	Nuclei	SBU-3 Positive Cells	%
AI	3	7603	1123	14.8 ± 0.27^{a}
IVF	2	7293	729	9.8 ± 0.84^{b}
NT	3	11020	740	6.6 ± 0.60^{c}

$^{a,b,c}P < 0.01$

Figure 5. Analysis of parental expression of bovine Ascl2 in day 17 embryos and day 40 cotyledonary tissue. (A) A diagram of the single polymorphism difference (grey box) between the maternal (*Bos taurus*) and paternal (*Bos indicus*) Ascl2 PCR products as well as the recognition sequence (white box) and cleavage psotion (arrow) for the restriction enzyme *Sfi* I. Digestion of Ascl2 PCR products from day 60 *Bos taurus* (BT), *Bos indicus* (BI), mixed (BT/BI) cotyledonary tissues, and AI, IVF and NT day 17 embryos (B) and day 40 cotyledonary tissue (C).


A
66
76
Maternal Allele (BT)-CGGGCTGCGCTGGCCGGGGG-
Paternal Allele (BI)-CGGGCCGCGCTGGCCGGGGG-
B
210
135
75
BT BI BT/BI
AI
IVF
NT
C
210
135
75
BT
BI
BT/BI
AI
IVF
NT

CHAPTER IV

ABUNDANCE OF EPIGENEITC MODIFYING TRANSCRIPTS IN BOVINE TROPHOBLAST CELLS AND EMBRYOS PRODUCED BY SOMATIC CELL NUCLEAR TRANSFER

Short Title: Abundance of epigenetic regulators in SCNT embryos and trophoblast cells.

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Abstract

In somatic cell nuclear transfer (SCNT), donor cells must undergo epigenetic reprogramming to reach a totipotent state similar to fertilized zygotes. However, complete reprogramming appears to be elusive and is considered a major barrier to normal development. The placenta of SCNT embryos may be one of the main organs affected by incomplete reprogramming due to the role played by a vast number of imprinted genes. Our objectives were to evaluate the expression of genes involved in epigenetic modifications (Dnmt1, Dnmt3b, HAT1 and HDAC1) in embryos and placental tissues of embryos produced by in vitro fertilization (IVF) and SCNT, as well as develop trophoblast (TS) cell lines from both groups as a model for trophoblast development. Abundance of Dnmt1 mRNA was less in SCNT blastocyst stage embryos than their IVF counterparts, whereas Dnmt3b, HAT1 and HDAC1 mRNA were not different. In day 40 placental tissues no difference in mRNA was detected for all genes. Abundance of Dnmt3b mRNA was less and HAT1 mRNA was more in the SCNT TS cell lines. In addition, TS cell line produced from SCNT embryos grew at a faster rate and expressed more Ascl2 mRNA, an imprinted trophoblast profileration gene, than IVF TS cells. These results suggest that the altered expression of epigenetic modifying genes may not be the cause of incomplete reprogramming associated with SCNT. In addition, the ability of TS cells developed from SCNT embryos to maintain elevated levels of Ascl2 mRNA, similar to those found in vivo

suggests these cell lines are a model for investigating bovine trophoblast development in culture.

Introduction

Since the development of assisted reproductive techniques, such as somatic cell nuclear transfer (SCNT), there have been several reports of abnormal placental development. One of the most common observations seen in bovine embryos produced by SCNT is the reduced and underdeveloped placentomes early in gestation. Several researchers have reported poor vascular development and absence or reduced number of cotyledons in day 40 to day 60 NT placental tissue (Hashizume et al., 2002; Hill et al., 2000; Stice et al., 1996). The abnormal placental development can be seen as early as the formation of the trophoblast cells (epithelial cells of the placenta) in the SCNT blastocyst. Cell allocation in SCNT blastocysts seems to favor the trophoblastic cells rather than the inner cell mass, compared to in vivo produced embryos (Du et al., 1996). Given that the placenta is derived from the trophoectoderm, the alteration in cell number may influence placental size and/or function.

To have a better understanding of trophoblast development, several researchers have utilized trophoblast stem (TS) cell lines. These TS cell lines have been isolated from blastocyst stage embryos and cultured in vitro for extended periods of time (Desmarais et al., 2004; Shimada et al., 2001; Talbot et al., 2000; Tanaka et al., 1998). To maintain these

cells lines, researchers must co-culture with a feeder cell layer, usually comprised of fibroblast cells. However, several reports have shown maintenance of the TS cells in the absence of co-cultures when cultures were performed with fibroblast conditioned media (Desmarais et al., 2004; Shimada et al., 2001; Tanaka et al., 1998). In mouse, TS cells have been reported to require fibroblast growth factor 4 (Fgf4) to be maintained in culture (Tanaka et al., 1998). Removal of Fgf4 caused these cells to stop proliferating and differentiate into giant cells.

One gene that appears to play an important role in trophoblast development is achaete scute-like homologue 2 (Ascl2; also known as Mash2). The Ascl2 gene, a mammalian homologue of the Drosophilia achaete-scute gene, belongs to the basic helix-loop-helix transcription factor family (Johnson et al., 1990). In mice, Ascl2 stimulates cell proliferation and inhibits progression of trophoblast to their terminally differentiated giant cell form (Cross et al., 1995; Rossant et al., 1998; Tanaka et al., 1997). Using homozygous mutant mice, Guillemot et al. (1994) demonstrated that inactivation of Ascl2 was embryonic lethal, and also a reduced number of spongiotrophoblast cells along with an excessive number of trophoblast giant cells. In the bovine, altered abundance of Ascl2 mRNA has been detected in SCNT day 8 blastocyst and Day 17 filamentous embryos, compared to their in vitro produced (IVF) counterparts (Arnold et al., 2003; Wrenzycki et al., 2001). Interestingly, Ascl2 is an imprinted gene in mice with the paternal allele only being expressed until day 7.5 postcoitum (p.c) then silenced (Guillemot et al., 1995).

The poor efficiency of SCNT and abnormal development of the placenta and fetus has been attributed to the incomplete reprogramming of the donor cells (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). Somatic donor cells that fail to reprogram completely and form totipotent nuclei ultimately contain epigenetic alterations that persist through development. The epigenetic process of DNA methylation appears to be altered in SCNT animals. Genome wide methylation of SCNT embryos, regardless of species, was greater than IVF controls (Dean et al., 2001). The methylation of CpG islands is through the DNA methyltransferase (Dnmt) enzymes (Bestor, 2000). Three Dnmt's have been identified to play critical roles in methylation patterns. Dnmt1 is involved in maintenance of DNA methylase activity (Yoder et al., 1997). Dnmt3a and Dnmt3b have poor maintenance activity, but have a high affinity for causing de novo methylation (Okano et al., 1998). Wrenzycki and co-authors (2001) detected altered expression of Dnmt1 in bovine SCNT day 8 blastocysts compared to in vitro controls.

Another epigenetic process is histone modification. In contrast to DNA methylation, which is generally associated with gene repression, histone modifications can either result in gene expression (as commonly seen with histone acetylation) or gene repression (as commonly seen with histone methylation; for review see Fischle et al., 2003). Acetylation and deacetylation are controlled by the enzymatic proteins histone acetyltransferase (HATs) and histone deacetlyase (HDACs), respectively. Expression of HAT1 and HDAC isoforms 1, 2, 3 and 7 have been detected in bovine oocytes to blastocyst

stage embryos (McGraw et al., 2003). However, expression in SCNT embryos has not been reported.

Even though the Ascl2 gene is located within an imprinting cluster on chromosome 7 in mice, its imprinting appears to be regulated by something other than methylation. Expression from the paternal allele was not restored in mice deficient for the gene DNA-methyltransferase I (Dnmt1), which is involved in maintenance of the methylated state (Tanaka et al., 1999). Imprinting of several genes involved in mouse placental development have been linked to histone modification independent of DNA methylation. Lewis and co-authors (2004) demonstrated that the imprinting center 2 on mouse chromosome 7 recruits histone methylation to repress the paternal alleles of the placenta specific genes in the region (including Ascl2). These findings suggest that imprinting in this region involves histone modification as well as DNA methylation.

Our objectives were to measure mRNA levels of genes involved in DNA methylation (Dnmt1 and Dnmt3b) and histone modification (HAT1 and HDAC1) in blastocyst stage embryos and placental tissue of embryos produced by IVF or SCNT. In addition, primary trophoblast cell lines from IVF and SCNT embryos were developed and analyzed as a possible in vitro model for studying the altered trophoblast development associated with SCNT offspring. Knowing the expression profiles of genes involved in epigenetic modifications would provide a better understanding of the reprogramming of donor cells required for successful placental development and production of SCNT animals.

Materials and Methods

Animals, Embryos and Fetuses

All treatment protocols involving the use of animals were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. All investigations were performed using F1 interspecies crossings of the *Bos taurus* (Holstein) and *Bos indicus* (Nelore) sub-species for the maternal and paternal genomes, respectively.

Preparation of Donor Cells

Donor cells for nuclear transfer were a primary fibroblast cell line from a male F1 hybrid fetus collected at 60 days of gestation. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and 50 units/ml Penicillin-Streptomycin (GibcoBRL) and were used between 2 to 5 passages. Donor cultures were allowed to progress to confluency and maintained for two days prior to use so cells would be in the G1/G0 phase of the cell cycle.

Oocyte Collection and In Vitro Maturation (IVM)

Oocytes of *Bos taurus* origin were obtained from slaughterhouse ovaries and matured in vitro as previously described (Bordignon et al., 2003). After 20-22 h of IVM, matured COCs were randomly assigned to either IVF or NT groups.

Production of Somatic Cell Nuclear Transfer Embryos (SCNT)

Nuclear transfer was performed as described previously (Bordignon et al., 2003), with minor modification. Briefly, cumulus cells were removed by gentle vortexing in a 0.2% hyaluronidase (Sigma) solution. Micro-manipulation of oocytes was conducted in PBS containing 7.5 μg/ml of cytochalasin B (Sigma). To remove completely the chromatin, approximately 30% of the host cytoplasm adjacent to the first polar body was removed. To confirm removal of host oocyte DNA, oocytes were placed into medium containing 5 μg/ml of Hoechst 33342 (Sigma) for 15 minutes and then subjected briefly to ultraviolet irradiation. A single fetal fibroblast cell was introduced into the perivitelline space and couplets were placed in a 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and subjected to a 1.5-kV electric pulse lasting 70 μsec. Couplets were then washed and placed into 50 μl drops of modified synthetic oviductal fluid (mSOF;108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM NaHCO3, 1.7 mM CaCl2-H2O, 0.5 mM glucose 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 μg/ml gentamicin, 0.01% phenol red, 1.4 mM glycine, 0.4 mM alanine, 1 mM glutamine, 2% essential amino

acids, 1% non-essential amino acids; Gardner et al., 1994). After 1-2 h in culture, couplets were examined to determine fusion and then exposed to 5 mM ionomycin (Sigma) for 4 min to induce parthenogenetic activation. Reconstructed oocytes were cultured in mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days.

Production of In Vitro Fertilized Embryos

In vitro fertilized (IVF) embryos were produced as previously described (Parrish et al., 1986). Briefly, frozen *Bos indicus* semen was thawed and processed by a Percoll gradient. Sperm were cultured in 100 ml drops of Tyrode's medium (Parrish et al., 1986) supplemented with 6 mg/ml fatty-acid free bovine serum albumin (BSA; Sigma), 2 μg/ml heparin (Sigma), 10 mM pyruvic acid (Sigma) and 50 μg/ml gentamycin (Sigma). Groups of 25 *Bos taurus* COCs were cultured with sperm for 18-20 hrs. After fertilization, cumulus cells were removed by gentle vortexing and presumptive zygotes were cultured in 50 μl drops of mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 7 days.

On day 8 of culture, blastocyst stage SCNT and IVF embryos were either snap-frozen in liquid N_2 and stored at -70°C, placed in culture for primary trophoblast cell lines or non-surgically transferred to synchronized non-lactating Holstein cows. One to two embryos were transferred per recipient. On day 40, placental tissues were collected from pregnant uteri, snap-frozen in liquid N_2 and stored at -70°C until further processing.

Establishment of Primary Trophoblast Stem Cell (TS) Lines

Primary TS cell lines for SCNT and IVF were produced as previously described (Shimada et al., 2001) with minor modifications. Briefly, pools of 3-10 blastocyst stage embryos per groups were placed into 35-mm or 100-mm culture dishes coated with acid soluble collagen type I (Sigma). Culture media consisted of a 1:1 mixture of Dulbecco's modified Eagle's/F-12 medium (DME/F12, Invitrogen; supplemented with 50 units/ml Penicillin-Streptomycin, 0.5 μg/ml amphotericin B) and bovine uterine fibroblast conditioned media supplemented with 10% FBS, 25 ng/ml fibroblast growth factor 4 (Sigma) and 25 ng/ml heparin (Sigma). Uterine fibroblast conditioned media was prepared by incubating DME/F12 supplemented with 10% FBS on confluent bovine uterine fibroblast cells cultures for 2 to 3 days. Conditioned media was filtered and stored at -20°C until needed.

Blastocysts attached to the culture dishes and trophoblast outgrowths were observed within 1 week. Upon confluency, primary TS cells line from SCNT and IVF blastocysts were collected for RNA extraction.

RNA Extraction, Purification and Reverse Transcriptase (RT) Reaction

Individual SNCT and IVF blastocyst stage embryos were homogenized in buffer RLT (Qiagen) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Micro kit (Qiagen), as recommended by the manufacturer. Total RNA was eluted

in 10 μl and used for RT using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation, Madison, WI) according to the instructions from the manufacturer.

Day 40 placental tissues and primary TS cells were homogenized in buffer RLT (Qiagen) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Protect Mini kit (Qiagen), as recommended by the manufacturer. Total RNA was measured by spectrophotometry at 260 nm and 1.0 μg/sample of total RNA was used for the RT reaction with M-MLV RT.

Quantitative RT-PCR of Day 8 Blastocysts and Day 40 Placental Tissues

To analyze mRNA steady state amounts of Dnmt1, Dnmt3b, HAT1, HDAC1, peptidylprolyl isomerase A (PpiA) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) in day 8 IVF and SCNT blastocyst stage embryos and day 40 placental tissues, specific quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were performed by utilizing a modified technique previously described (Parrott and Skinner, 1998). Briefly, PCR products using gene specific primers (Table 1) were gel extracted and pooled using Qiaquick Gel Extraction and QIAquick Purification kits, respectively (Qiagen) to generate standard curve ranging from 0.001 fg/μl to 100 fg/μl. Five known standard concentrations and samples were subjected to PCR amplification in 50 μl of 1/10 embryo equivalent cDNA or 1ul day 40 cDNA and PCR master mix (0.2 μM gene specific primers; 0.2 μM of dNTP's; 5 μl 10X PCR buffer containing; 500mM KCl,

15 mM MgCl2, and 100mM Tris-HCl, pH 9.0). After initial denaturing for 5 min at 94°C, samples were subjected to temperature cycles of 30 sec at 94°C; 30 sec at gene dependent annealing temperature (Table 1); and 30 sec at 72°C with a final elongation at 72°C for 4 min. Optimal cycle number for amplification during the exponential phase was determined for each gene. Cycle numbers for blastocyst samples were: Dnmt1, 40; Dnmt3b, 33; HAT1, 43; HDAC1, 33; Gapdh, 30 and PpiA, 30. For day 40 samples and TS cells cycle numbers were: Dnmt1, 29; Dnmt3b, 29; HAT1, 35; HDAC1, 25; Gapdh, 24 and PpiA, 24.

PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Densities of the amplified fragments were calculated with a computer imaging system and analyzed with Macintosh NIH software (National Institute of Health, Bethesda, MD). Values for each gene were calculated by comparing them to a gene specific standard curve (Fig. 1). Results were expressed as fg of gene of interest: fg of Gapdh.

Characterization of IVF and SCNT Trophoblast Cell Lines

To analyze primary trophoblast cell lines from IVF and SCNT embryos, semi-quantitative PCR was conducted. Trophoblast stem cell markers caudal type homeo box 2 (Cdx2) and eomesodermin (Eomes), as well as bovine specific marker for trophoblast function, interferon tau (IFN-τ), were analyzed. Inner cell mass specific gene fibroblast growth factor 4 (Fgf4) was analyzed to determine contamination of trophoblast cells. Gene specific primers (Table 1) were used as stated above. For genes without known bovine sequences, primers were designed based on homologous sequences between human and

mouse sequences. PCR products of the expected size were excised and purified using a Gel Extraction kit (Qiagen). Purified cDNA was then ligated into a pGEM-T Easy Vector System I (Promega Corp., Nepean, Canada) according to the instructions of the manufacturer, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the use of a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA), and at least 3 independent samples were sequenced for verification of authenticity. Quantitative RT-PCR was performed on IVF and SCNT TS cell cDNA for Dnmt1, Dnmt3b, HAT1, HDAC1, PpiA and Gapdh as stated above using the same cycle number as those for day 40 samples. In addition, qRT-PCR was conduction at 35 cycle to analyze Ascl2 mRNA concentrations in TS cells.

Determination of Parental Allele Expression of Ascl2 in IVF and SCNT Trophoblast Cell Lines

To analyze the parental origin of Ascl2 mRNA in IVF and SCNT TS cells, digestions of PCR products were conducted as previously described (Arnold et al., 2004). Briefly, a single polymorphism was detected (T/C) at the 86 bp position of a 210 bp Ascl2 PCR product between the *Bos taurus* (maternal) and *Bos indicus* (paternal) that is within the recognition site of the restriction enzyme *Sfi* I, which allowed the paternal allele to be digested whereas the maternal allele remained intact. PCR products from IVF and SCNT

TS cell samples were digested with *Sfi* I at 50° C for 15 hrs. Digestions were separated in a 1.5% agarose gel and stained with ethidium bromide.

Statistical Analysis

Relative expression for each gene was normalized to PpiA (fg/fg) and data was analyzed using the least square analysis of variance by the General Linear Model procedures of SAS. Data are presented as means ± SEM. For day 8 samples, data is presented as 1 embryo equivalent. A probability level of $P<0.05$ was defined as significant.

Results

Relative mRNA Abundance in Day 8 IVF and SCNT Blastocyst Stage Embryos

To analyze the abundance for genes involved with DNA methylation (Dnmt1 and Dnmt3b) and histone acetylation (HAT1 and HDAC1), IVF (n=12) and SCNT (n=12) blastocyst stage embryos were utilized. No difference was detected in abundance of the housekeeping genes PpiA (IVF, 0.14 ± 0.024 fg; SCNT, 0.11 ± 0.016 fg) and Gapdh (IVF, 4.44 ± 1.15 fg; SCNT, 3.88 ± 1.10 fg) mRNA between IVF and SCNT blastocysts ($P>0.10$). Relative abundance of Dnmt1 mRNA was greater in IVF blastocyst than for SCNT blastocysts ($P < 0.05$, Fig. 2A). Similar expression levels for Dnmt3b were detected in the two groups (Fig. 2A). These results suggest that the abnormal methylation status of genes in SCNT embryos may be caused by the altered maintenance rather than altered de

novo methylation. No detectable difference was observed for HAT1 and HDAC1 (Fig. 2B) between groups. These results suggest that altered histone acetylation associated with SCNT may not be due to the expression of histone modifying enzymes HAT1 and HDAC1.

Relative mRNA Abundance in Day 40 Placental Tissue from IVF and SCNT produced Embryos

To determine if altered expression of epigenetic genes persist during development, day 40 placental samples were collected from embryos produced by IVF and SCNT (n=3 per group). Abundance of mRNA for Dnmt1 and Dnmt3b (Fig. 3A) as well as HAT1 and HDAC1 (Fig. 3B) were similar between the two groups.

Characterization of Primary Trophoblast Stem Cells

Trophoblast stem (TS) cell lines were developed from IVF and SCNT embryos as a potential model for analyzing the altered trophoblast development associated with SCNT animals. Day 8 blastocysts attached and started to form outgrowth within one week (Fig. 4A and B). As cell growth continued dome shaped groups of cells would form (Fig. 4C). These domes would continue to grow and eventually form fluid filled trophoblastic vesicles that would detach and float in culture (Fig. 4D). These vesicles could be harvested and placed into the new collagen-coated plates, where they would attach and form outgrowths. In addition, gene markers for trophoblast stem cells, Cdx2 and Eomes were detected in both IVF and SCNT TS cell lines (Fig. 5A). No detection of Cdx2 or Eomes mRNA was

detected in the negative control fetal kidney (Fig. 5A). In addition, a marker for trophoblast cell function in bovine, IFN-τ, was detected in both TS cell lines and not in the negative control (Fig. 5A). Fibroblast growth factor-4 which is specifically expressed by the ICM, was not detected in either TS cell lines, indicating no cell contamination (Fig. 5B). These results suggest the cells in culture are of trophectoderm lineage and may be useful for comparing trophoblast development in vitro between IVF and SCNT embryos. Interestingly, the SCNT TS cells proliferate at a greater rate than IVF TS cells, reaching confluency in less days with fewer embryos ($P < 0.05$, Table 2).

Relative mRNA Abundance in Primary Trophoblast Stem Cell Lines from IVF and SCNT produced Embryos

The TS cell lines were analyzed to determine if the expression profiles detected in day 8 blastocysts remain stable after several days in culture. Abundance of Dnmt1 was similar for IVF and NT groups (Fig 6A). Abundance of Dnmt3b was greater in IVF TS cells compared to SCNT TS cells ($P<0.05$, Fig. 6A). These results suggest that prolonged in vitro culture may effect expression of DNA methylation enzymes. Abundance of HAT1 was greater for the SCNT TS cells ($P < 0.05$, Fig. 6B). Relative levels of HDAC1 mRNA tended to be higher in SCNT TS cells than their IVF counterparts ($P = 0.08$, Fig. 6B). These results indicate that in vitro culture has different effects on IVF and SCNT trophoblast cells in regards to expression of histone acetylating genes. Abundance of the Ascl2 mRNA, a gene involved in trophoblast proliferation, was greater in SCNT TS cells ($P < 0.05$, Fig.

6C). To analyze the imprinting status of Ascl2 mRNA, digestion of PCR products from IVF and SCNT TS cells was evaluated. Expression of Ascl2 was found to be from both paternal and maternal origin (Fig 7) with a greater amount of Ascl2 mRNA produced by the maternal than the paternal allele. These results indicate that altered expression of Ascl2 mRNA reported for SCNT embryos is maintained in culture (Wrenzycki et al., 2001; Arnold et al., 2003).

Discussion

In the present study, we provide new information on the expression of genes involved in epigenetic modifications in embryos produced by somatic cell nuclear transfer. To date, this is the first study to analyze the expression of the DNA methylation enzyme Dnmt3b, histone acetylation enzyme HAT1 and the histone deacetylase enzyme HDAC1 between IVF and SCNT produced embryos. In addition, we characterized trophoblast (TS) cell lines produced from IVF and SCNT embryos as a model for investigating trophoblast development. By utilizing TS cell lines, we were able to investigate further the abnormal trophoblast development commonly associated with SCNT embryos.

Incomplete epigenetic reprogramming of the donor cell to a zygotic-like state appears to be one of the leading causes of SCNT failure. In early development, SCNT embryos have been shown to have altered DNA methylation compared to IVF embryos. After fertilization, bovine zygotes undergo genome-wide demethylation followed by

remethylation at later stages (Dean et al., 2001). However, in cloned embryos at the one cell stage, demethylation started to occur but then stopped as development continued (Dean et al., 2001). In addition, these cloned embryos started to undergo remethylation so at the morula stage, methylation in the blastomeres resembled that of the donor cells (Dean et al., 2001). Two genes that are involved in DNA methylation are Dnmt1 and Dnmt3b. Dnmt1 is involved in maintenance of DNA methylase activity (Yoder et al., 1997), whereas Dnmt3b is involved in de novo methylation (Okano et al., 1998). In the present study Dnmt1 mRNA was lower in day 8 blastocyst stage SCNT embryos compared to their IVF counterparts. This may indicate an alteration in genome-wide methylation due to less Dnmt1. These results support those found by Wrenzycki and co-authors (2001), where embryos produced by SCNT had lower Dnmt1 levels compared to IVF embryos. However, they also demonstrated that IVF embryos had higher levels of Dnmt1 than in vivo produced embryos (Wrenzycki et al., 2001). These authors conclude that in vitro culture may be playing a greater role than the SCNT procedure. The present results may also reflect the Dnmt1 status of the donor cells utilized. Robertson et al. (1999) reported a significant down-regulation in Dnmt's 1, 3a and 3b mRNA levels in cultured fibroblast cells that were in the G0/G1 stage of the cell cycle. The donor cells used in the current study were utilized at the same cell cycle stage. The expression of Dnmt3b in the current study was not different between IVF and SCNT blastocysts, suggesting transcription of this enzyme is not regulated in the same manner as Dnmt1 and may not be involved in the altered genome-wide methylation associated with SCNT embryos.

To determine if Dnmt1 is altered throughout gestation or Dnmt3b transcripts are affected at later stages, we investigated expression in day 40 placental tissue. At day 40, no difference was detected for either Dnmt1 or Dnmt3b, regardless of group. These results may suggest that only embryos with normal expression levels survive to this stage of development or other DNA methylation enzymes may be altered. It must be mentioned that in the present study protein levels or enzymatic activity were not analyzed. Further studies are required to determine if these enzymes are functioning properly in SCNT animals.

Enzymes involved in another epigenetic process, histone modification, were also investigated between IVF and SCNT blastocyst as well as day 40 placental tissues. Depending on the type of modification that occurs, the nucleosome will either open up to allow for transcriptional factors to bind or remain tightly bound. Histone acetylation is commonly associated with activated transcription, whereas deacetylation is associated with transcriptional repression (Fischle et al., 2003; Struhl, 1998; Tazi and Bird, 1990). Two proteins involved with controlling the acetyl groups are histone acetyltransferase (HAT; adds acetyl groups) and histone deacetylase (HDAC; removes acetyls groups). In the present study, HAT1 and HDAC1 mRNA were not different between groups in either day 8 blastocysts or day 40 placental tissue. These results suggest that mRNA abundance for HAT1 and HDAC1 may not be playing a critical role in the altered histone modification that has been associated with SCNT embryos. Santos and co-authors (Santos et al., 2003) demonstrated that SCNT bovine embryos had hypermethylated histone H3-K9 associated

with genome-wide hypermethylation. Methylation of histones is usually associated with gene silencing.

To further investigate trophoblast development in SCNT embryos, primary TS cell lines were developed. Bovine trophoblast cell lines have been developed utilizing several culture conditions. By utilizing a mouse fibroblast feeder layer, Talbot et al., (2000) reported the development of bovine trophectoderm cells lines from day 10-11 in vitro produced blastocysts that were continuously cultured for 2 years (76 passages) without noticeable morphological changes occurring. Overall morphology of the trophectoderm cells was similar to in vivo expanded preimplantation blastocysts (Talbot et al., 2000). To control for any effects caused by co-culturing trophoblast cells with feeder cells, Shimada et al. (2001) developed a trophoblast cell line (BT-1) utilizing uterine fibroblast-conditioned media. These researchers were able to continuously culture these BT-1 cells for 18 months (75 passages) without senescent or morphological changes. Fibroblast-conditioned media stimulated cellular growth better than serum or serum-free media (Shimada et al., 2001). Cellular markers for bovine trophoblast cell function, such as interferon tau (IFN-τ) and placental lactogen (PL) have been detected from bovine TS cell lines (Shimada et al., 2001). In the present study, we utilized a modified TS cell culture procedure described by Shimada and co-authors (2001) to develop TS cell lines from IVF and SCNT embryos. Morphology of these TS cells were similar between groups and to those reported by Shimada et al. (2001). An interesting observation seen in the present study was the formation of domes of cells within the culture that eventually formed free

floating vesicles (Fig. 4). These free floating trophoblastic vesicles have been reported previously (Shimada et al., 2001; Talbot et al., 2000), and display the tight cellular junction that allow the trophoblast cells to elongate in the uterine lumen prior to implantation. Expression of trophoblast stem cell genes (Cdx2 and Eomes) and trophoblast function (IFN-τ) and the absence of the ICM expression marker FGF4 in both groups provide evidence that the TS cell line were not contaminated with other cell lineages. However, the TS cells produced by SCNT embryos appear to reach confluency at a faster rate than their IVF counterparts. These results suggest that the altered trophoblast development associated with SCNT embryos is maintained in culture.

Expression of the epigenetic modifying genes were analyzed in TS cell cultures and provide further insight into their regulation of trophoblast cell development. The expression of Dnmt1 and Dnmt3b mRNA in TS cells followed similar patterns of expression as seen in the day 8 blastocysts. However, unlike the day 8 blastocysts, Dnmt1 was no longer significantly different. In addition, the expression of Dnmt3b was greater in IVF TS cells than SCNT TS cells, whereas in day 8 blastocysts this difference was not present. The expression of histone modifying genes HAT1 and HDAC1 appear to be altered in culture. HAT1 mRNA abundance was greater in SCNT TS cells than their IVF counterpart. These results may indicate the different expression patterns between the ICM and trophectoderm or effects caused by culturing these cell line. Interestingly, the abundance of HAT1 and HDAC1 in IVF day 8 blastocysts and IVF TS cells appears to be

similar. Further studies are required to determine if expression patterns for these genes is different between the ICM and trophectoderm.

As stated previously, the SCNT TS cells appear to grow at a faster rate than IVF TS cells. Associated with this finding is the expression of the trophoblast profilerating gene Ascl2 in these TS cell lines. Expression of Ascl2 was greater in SCNT TS cells than IVF TS cells suggesting a potential mechanism for the greater proliferating rate observed. Interesting, Ascl2 has been reported to be expressed at a higher rate in SCNT day 8 blastocyst embryos (Wrenzycki et al., 2001), as well as day 17 SCNT embryos compared to IVF embryos (Arnold et al., 2003). The observation that expression of Ascl2 mRNA remains elevated in SCNT TS cells suggests a direct alteration in the Ascl2 gene rather than an upstream effects from the ICM. The Ascl2 gene has been demonstrated to be a maternally expressed imprinted gene in mouse after implantation (Tanaka et al., 1999). In the current study Ascl2 mRNA from TS cells was expressed by both maternal and paternal alleles. Previous studies have reported similar bi-allelic Ascl2 expression in day 17 filamentous embryos (Arnold et al., 2004). These results suggest that Ascl2 expression from TS cells mimic expression from preimplantation embryos. In mice, the imprinting appears to be controlled by factors other than methylation. The paternal allele was not restored in mice deficient for Dnmt1 (Tanaka et al., 1999). Lewis et al. (2004) demonstrated that the paternal allele of Ascl2 was repressed by histone methylation and not DNA methylation. The control of bovine Ascl2 requires further investigations.

In summary, epigenetic alterations associated with SCNT bovine embryos do not appear not to be caused by altered expression of the enzymes involved with DNA methylation (Dnmt1 and Dnmt3b) or histone acetylation status (HAT1 and HDAC1). Other epigenetic modifying genes, or the activity of these genes, may play a greater role in the reprogramming of donor cells for successful SCNT development. Trophoblast cell lines from embryos produces by SCNT appear to maintain their altered gene expression and cell development similar to those seen in SCNT embryos and placental tissues. By utilizing these cell lines, we will be able to gain further insight into the altered trophoblast development of embryos produced by assisted reproductive techniques.

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Table 1. Primer sequences used for RT-PCR.

Gene	Sense and Antisense Primer (5'-3')	Anneal Temperature (°C)	Sequence Reference (GenBank No.)
Dnmt1	GAGGAGGGCTACCTGGCTAAA	61	NM_182651
	CCCGTGGGAAATGAGATGTGAT		
Dnmt3b	CTCCGAAGTGTGTGAAGAG	58	NM_181813
	TCAGCAGGTGGTAGAACTC		
Hat1	CAGATATATAAGGCTGACATGAC	56	BT021536
	GCTGTAATATCAAGAACTGTAGG		McGraw et al., 2003
HDAC1	ACTACTACGACGGGGATGTTG	58	McGraw et al., 2003
	GCCAAGACGATATCATTGACG		
Ascl2	GAGCTGCTCGACTTCTCCAG	60	Arnold et al., 2003
	TGGAAGGTCTCTGCGGACAG		
Cdx2	CAGCCAAGTGAAAACCAGGACGA	57	NM-001265
	GAACCGCAGAGCAAAGGAGAGG		NM_007673
Eomes	CCACCGCCACCAAATGAGATG	56	NM_005442
	TTCACCCAGAGTCTCCTAATACTG		XM_135209
IFN-τ	GCTATCTCTGTGCTCCATGAGATG	58	AF270471
	AGTGAGTTCAGATCTCCACCCATC		
FGF4	TTCTTCGTGGCCATGAGCAG	59	Daniels et al., 2000
	AGGAAGTGGGTGACCTTCAT		
Gapdh	TGTTCCAGTATGATTCCACCC	59	AF077815
	TCCACCACCCTGTTGCTGTA		
PpiA	ACCGTCTTCTTCGACATCGC	62	AY247029
	CTTGCTGGTCTTGCCATTCC		

Figure 1. Standard curves for (A) housekeeping genes Gapdh (square) and PpiA (circle); (B) DNA methyltransferase enzymes Dnmt1 (square) and Dnmt3b (circle); and (C) histone acetylation/deacetylation enzymes HAT1 (square) and HDAC1 (circle). Sample values were calculated using the formula: Y= A*Ln(X) + B, where Y= fg of gene, A= slope Ln(X)= natural log of the Density units measeured, and B= the y intercept. The coefficient of determination (R^2) values were: Gapdh=0.98, PpiA=0.95, Dnmt1=0.96, Dnmt3b=0.98, HAT1=0.96, and HDAC1=0.98.

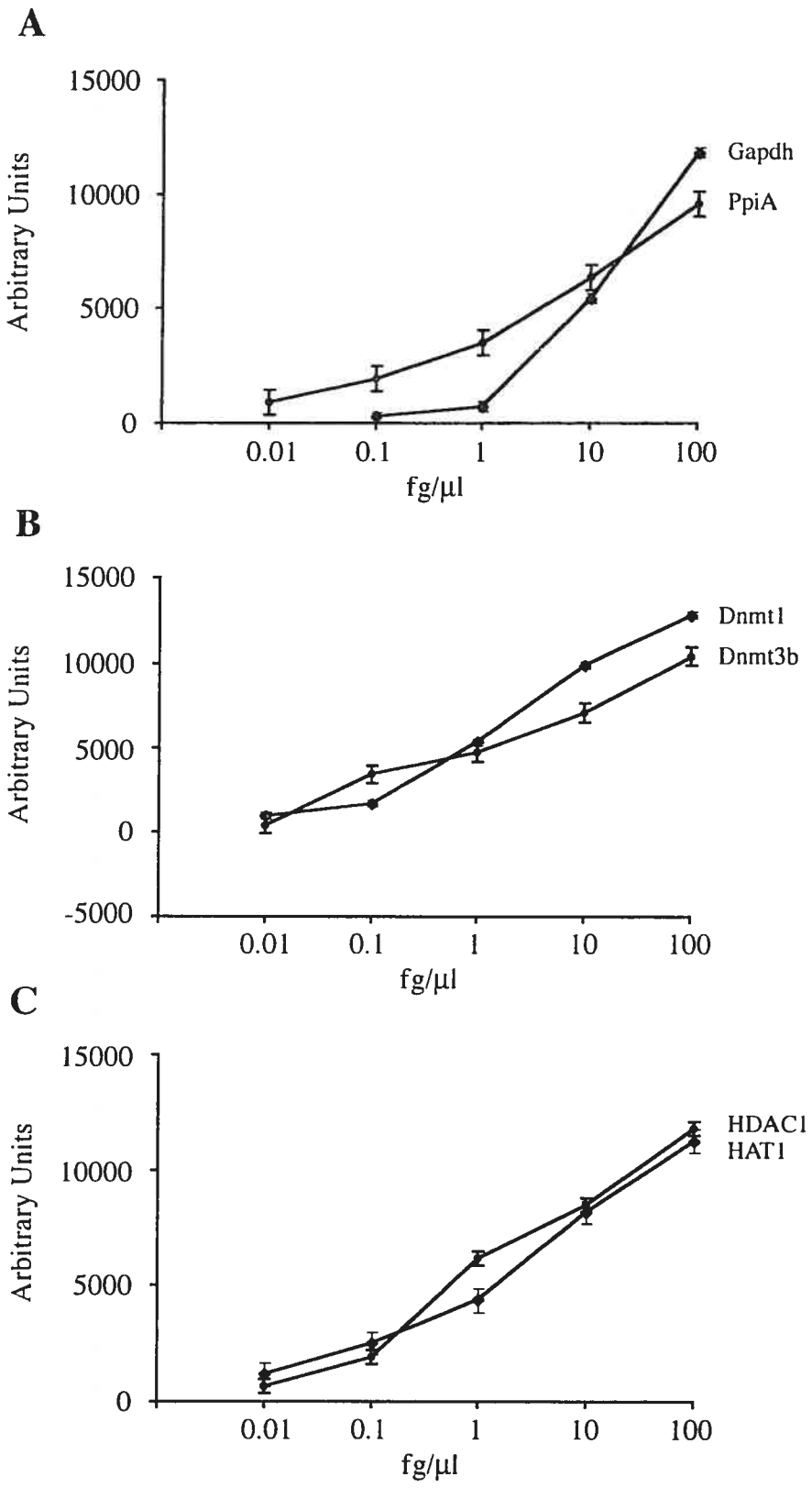

A
15000
10000
5000
0
Arbitrary Units
0.01
0.1
1
10
100
fg/µl
Gapdh
PpiA
B
15000
10000
5000
0
-5000
Arbitrary Units
0.01
0.1
1
10
100
fg/µl
Dnmt1
Dnmt3b
C
15000
10000
5000
0
Arbitrary Units
0.01
0.1
1
10
100
fg/µl
HDAC1
HAT1

Figure 2. Relative abundance of DNA methyltransferase enzymes (A) Dnmt1 and Dnmt3b and (B) histone acetylation/deacetylation enzymes HAT1 and HDAC1 mRNA in blastocyst stage embryos produced by IVF (black bars) or SCNT (white bars). Histograms represent ratio fg:fg PpiA. The quantification represents means ± SEM of individual samples (n=12 per group). Different superscripts represent significant differences in means ($P < 0.05$).

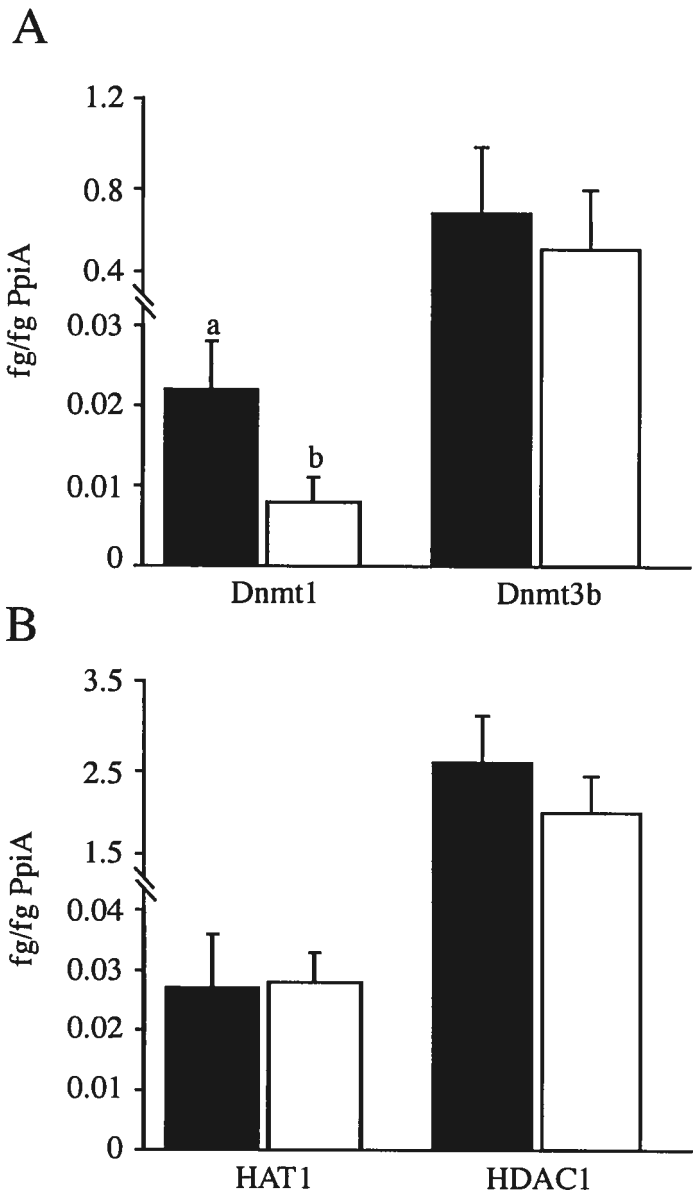

A
1.2
0.8
0.4
0.03
0.02
0.01
0
fg/fg PpiA
a
b
Dnmt1
Dnmt3b
B
3.5
2.5
1.5
0.04
0.03
0.02
0.01
0
fg/fg PpiA
HAT1
HDAC1

Figure 3. Quantification of DNA methyltransferase enzymes (A) Dnmt1 and Dnmt3b and (B) histone acetylation/deacetylation enzymes HAT1 and HDAC1 mRNA in day 40 placental tissues from IVF (black bars) or SCNT (white bars) produced embryos. Histograms represent ratio fg:fg PpiA. The quantification represents means ± SEM of individual samples (n=3 per group).

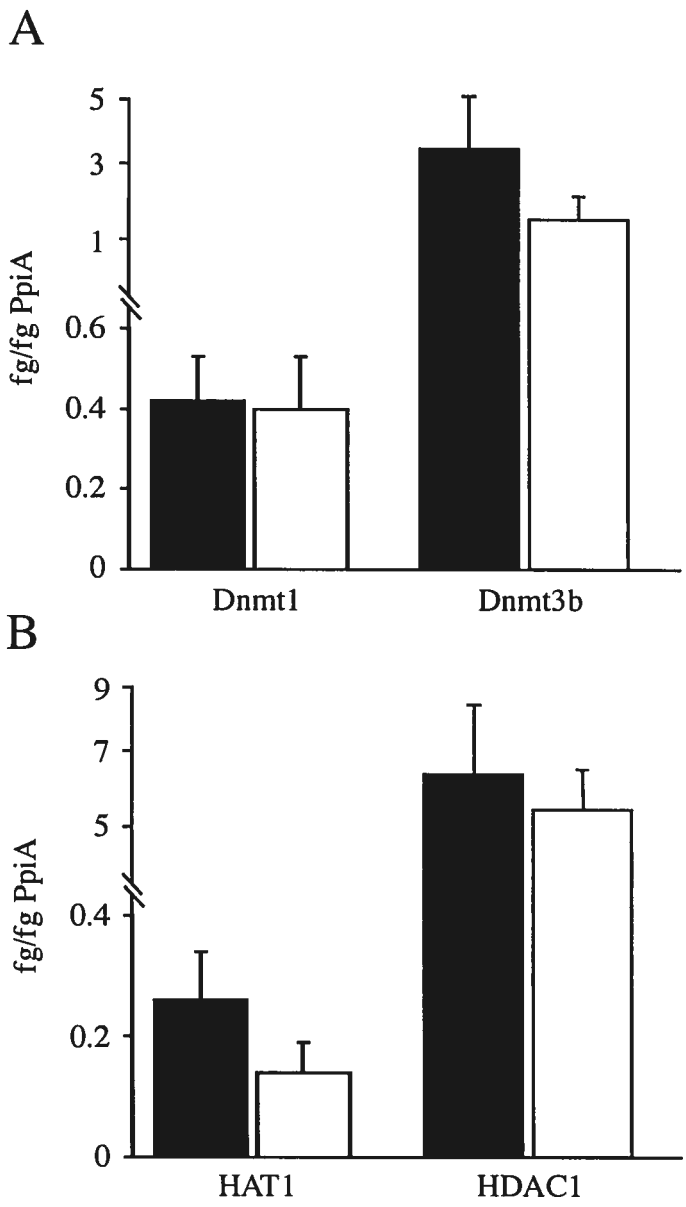

A
5
3
1
0.6
0.4
0.2
0
fg/fg PpiA
Dnmt1
Dnmt3b
B
9
7
5
0.4
0.2
0
fg/fg PpiA
HAT1
HDAC1

Figure 4. Development of bovine trophoblast cell lines. (A) Attachment of embryos and outgrowth of trophoblast cells; (B) complete attachment of trophoblast cells; (C) formation of domes of cells (*); and (D) floating trophoblastic vesicles. (bars = 200 μm)

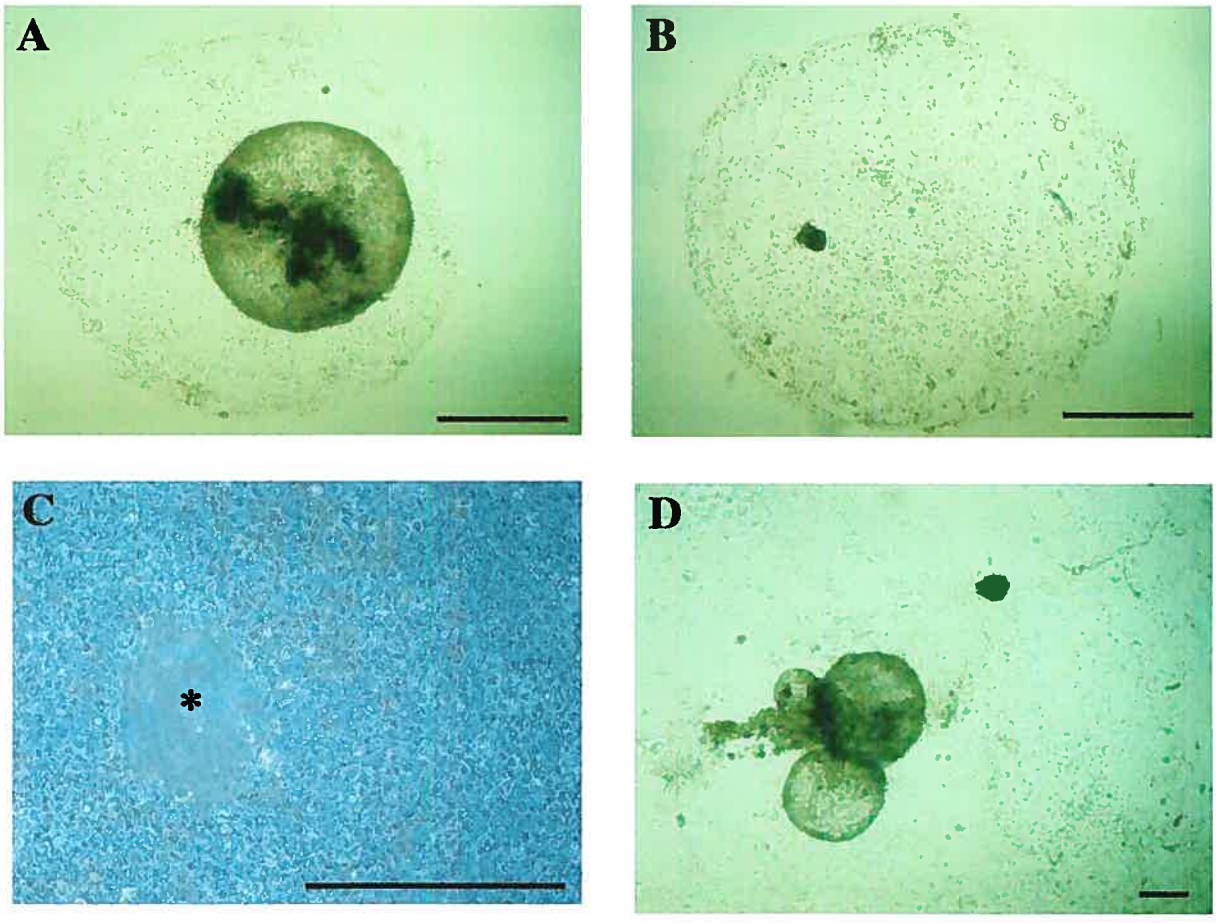

A
B
C
*
D

Table 2. Growth of IVF and SCNT Trophoblast Cells

Group	Replicates	Number of Embryos/Replicate	Number of Attached Embryos/Replicate	Days to Confluency
IVF	4	10 ± 0.5	8.5 ± 0.5	84 ± 5.3[a]
SCNT	4	5 ± 0.5	3.5 ± 0.5	37 ± 7.5[b]

$P < 0.05$

Figure 5. Expression of trophoblast specific genes (A) Cdx2, Eomes and IFN-τ and inner cell mass specific gene (B) Fgf4 in IVF and SCNT primary trophoblast cell lines. Gapdh served as internal control. Day 60 fetal bovine kidney served as a negative control (A) for trophoblast specific genes and blastocyst stage embryos served as positive controls for Fgf4 (B).

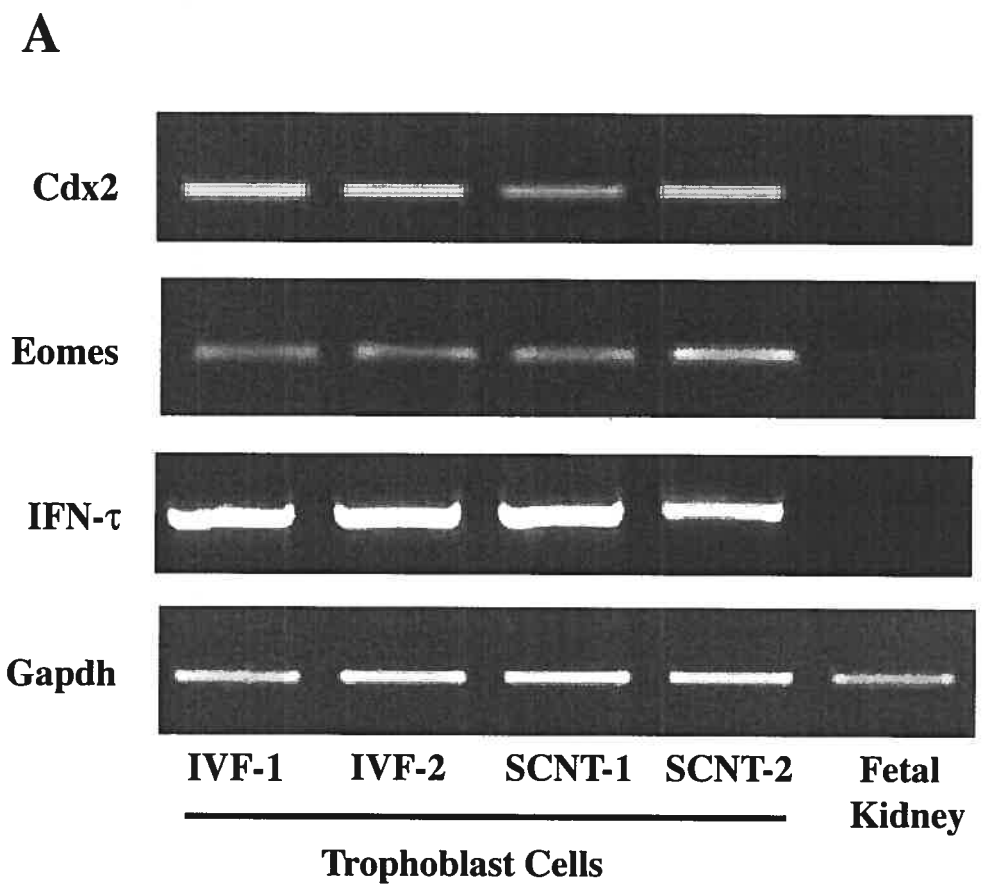

A
Cdx2
Eomes
IFN-τ
Gapdh
IVF-1
IVF-2
SCNT-1
SCNT-2
Fetal Kidney
Trophoblast Cells
B
FGF4
Gapdh
IVF-1
IVF-2
SCNT-1
SCNT-2
Blastocyst
Trophoblast Cells

Figure 6. Quantification of DNA methyltransferase enzymes (A) Dnmt1 and Dnmt3b, (B) histone acetylation/deacetylation enzymes HAT1 and HDAC1 and (C) Ascl2 mRNA in IVF (black bars) and SCNT (white bars) primary trophoblast cell lines. Histograms represent ratio fg:fg PpiA. The quantification represents means ± SEM of individual samples (n=3 per group). Different superscripts represent significant differences in means ($P < 0.05$).

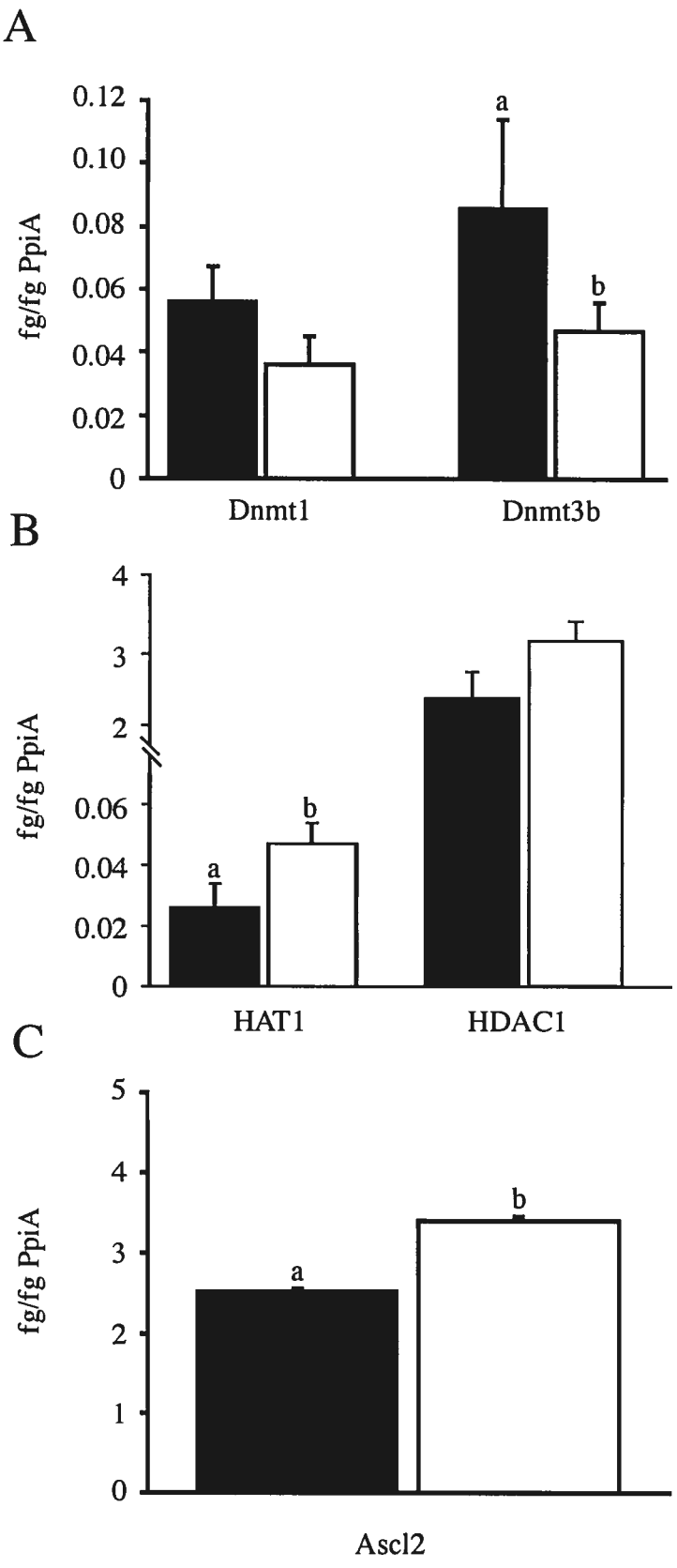
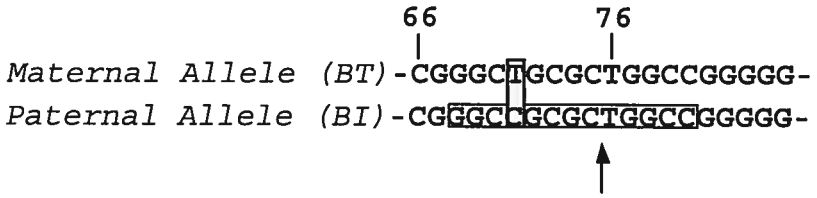
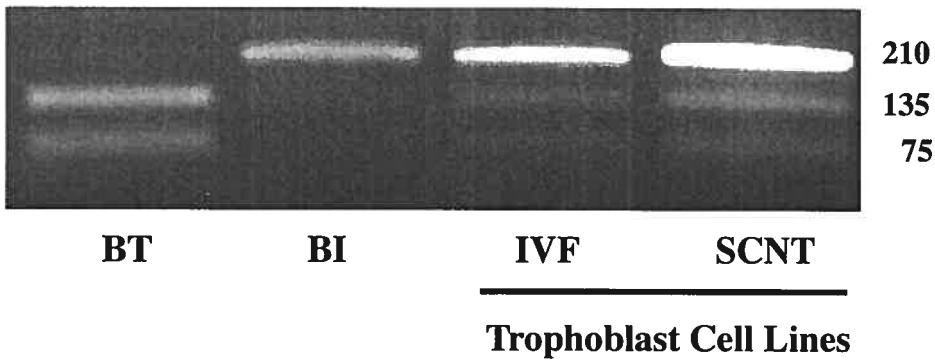

A
0.12
0.10
0.08
0.06
0.04
0.02
0
fg/fg PpiA
a
b
Dnmt1
Dnmt3b
B
4
3
2
0.06
0.04
0.02
0
fg/fg PpiA
a
b
HAT1
HDAC1
C
5
4
3
2
1
0
fg/fg PpiA
a
b
Ascl2

Figure 7. Analysis of parental expression of bovine Ascl2 in IVF and SCNT primary trophoblast cell lines. A diagram of the single polymorphism difference (grey box) between the maternal (*Bos taurus*) and paternal (*Bos indicus*) Ascl2 PCR products as well as the recognition sequence (white box) and cleavage psotion (arrow) for the restriction enzyme *Sfi* I (A). Digestion of Ascl2 PCR products from day 60 *Bos taurus* (BT), *Bos indicus* (BI) cotyledonary tissue and IVF and SCNT TS cells with *Sfi* I restriction enzyme (B).

A


66
76
Maternal Allele (BT)-CGGGCTGCGCTGGCCGGGGG-
Paternal Allele (BI)-CGGGCCGCGCTGGCCGGGGG-

B


210
135
75
BT
BI
IVF
SCNT
Trophoblast Cell Lines

General Discussion

The present series of studies provide novel information into the molecular regulation of bovine trophoblast development. In addition, altered regulation of these genes (Ascl2, Hand1) is associated with assisted reproductive techniques such as somatic cell nuclear transfer (SCNT), and may cause the abnormal placenta development observed in these animals. In addition, the number of functional binucleate trophoblast cells is reduced in SCNT bovine placental tissue, which could play a vital role in implantation. Expression of epigenetic modifying genes appear not to be the cause for the altered reprogramming commonly seen in SCNT embryos. Taken together, this series of studies provide further evidence into the regulation involved in successful bovine trophoblast development.

Placental development is a vital component of successful pregnancy in mammals. Until birth, this temporary organ will function as the sole source of nutrient/waste exchange and protection for the embryo/fetus. The size, shape and type of placentation of this unique organ is highly variable between species. In ruminants, placentation is of the epithiliealchorial-syndesmochorial or placentomal type. If the formation and/or function of the placenta is compromised, pregnancy is lost. The most critical time of development is early in gestation, prior to implantation. During this time the placental tissue must undergo rapid yet controlled growth. Most pregnancy losses occur during this time in the bovine; survival rates of in vitro produced conceptuses diminish from 93% on day 8 to 55% by day 40 (Diskin and Sreenan, 1980). Many of these losses are due to abnormal embryonic

development not allowing for the fetal/maternal interface to form properly (Thatcher et al., 1995).

Morphological development of the bovine placenta is well understood. Several researchers have documented the cellular population as gestation progresses. As in all mammals, the outermost cells of the placenta are of trophectoderm lineage and are the most studied in regards to placental development. The bovine trophoblast contains three cell types: stem cell-like, mononucleate and binucleate/giant cells (Greenstein et al., 1958). The stem cells are believed to give rise to the other two cell populations. The mononucleate cells make up the largest trophoblast cell population and play a key role in placental nutrient exchange. The binucleate cells are the unique population of trophoblast cells that play a key role in implantation and production of pregnancy related hormones.

Unlike the extensive number of morphological studies, studies on the molecular regulation of bovine trophoblast development are limited. Research in gene regulation of placental development has focused primarily on the mouse and human models, where gene mutation studies and gene screening of diseases associated with placental abnormalities have been utilized. By utilizing these techniques researchers have been able to identify key genes of trophoblast development.

A gene highly investigated in mice is the basic helix-loop-helix (HLH) transcription factor achaete scute-like homolog 2 (Ascl2 or Mash2). Expression of mouse Ascl2 mRNA has been localized to the chorion, the ectoplacental cone (EPC) and the spongiotrophoblast cell layers during placental development (Guillemot et al., 1994). Ascl2 mRNA is absent in

primary and secondary giant cells (Guillemot et al., 1994). Mice that carry a homozygous mutation for Ascl2 are embryonic lethal at 10 days postcoitum (d.p.c.) due to the lack of development of the spongiotrophoblast layer (Guillemot et al., 1994). These mice also have an increase in giant cell number and size. In humans, the homologue gene Hash2, is expressed in the corresponding extravillous cytotrophoblast cells (Alders et al., 1997). In the present set of studies, expression of the bovine Ascl2 mRNA was only in the cotyledonary tissue of placenta and the trophoblast cells of the bovine embryos. The temporal expression of Ascl2 corresponds to the developmental pattern of bovine trophoblast cells with the greatest expression during the time the trophoblast cells are undergoing rapid cell proliferation (day 17) to elongate into the uterine lumen prior to attachment. Ascl2 expression starts to decline after implantation, which occurs around day 28-30 of gestation (gestation = to 285 days), and by day 60 when the placentomes (area of fetal/maternal nutrient exchange in ruminants) are clearly visible, expression of bovine Ascl2 is minimal. The decrease in Ascl2 mRNA at this time would correspond to trophoblast maturation and differentiation.

The mouse Ascl2 gene appears to be a maternally expressed, imprinted gene with the paternal Ascl2 allele being expressed in the early postimplantation conceptus (5.5 d.p.c.), then decreasing until silenced by 9.5 d.p.c. (Tanaka et al., 1999). In study one, the parental expression of bovine Ascl2 appears to mimic that of the mouse with paternal silencing occurring after implantation. However, by analyzing the abundance of Ascl2 mRNA in parthenogenetic embryos (containing only two copies of the maternal genome),

the first evidence that the paternal genome is required for normal expression of bovine Ascl2 mRNA from the maternal allele is demonstrated.

Since their development, assisted reproductive techniques in cattle have been associated with abnormal placental development. Altered placental development and high pregnancy losses appear to be the norm for somatic cell nuclear transfer (SCNT) rather than the exception. The most common observation is reduced and underdeveloped placentomes early in gestation. Several researchers have reported poor vascular development and absence or reduced number of cotyledons in SCNT placental tissue during the early part of gestation (Hill et al., 2000a; Stice et al., 1996). The low number of SCNT fetuses that are able to survive through pregnancy are usually associated with fewer number of placentomes that are 2-fold larger that those observed in corresponding in vivo produced animals (Cibelli et al., 1998; Hill et al., 1999).

To date, the studies devoted to placental development in SCNT bovine fetuses have focused primarily on the morphology. In regards to gene expression, day 8 blastocysts stage embryos have been the most examined due to their availability. However, to fully investigate the developmental regulation of the SCNT placenta later stages of gestation are required. In study two, day 17 filamentous embryos, as well as day 40 placental tissues from embryos produced by SCNT were utilized. These studies provide valuable insight to the regulation of placental development at critical times of gestation when fetal loss of SCNT pregnancies range from 35- 78% (Cibelli et al., 1998; Hill et al., 2000a; Yamada et al., 2001).

Over-expression of Ascl2 was detected in SCNT day17 embryos and day 40 placental tissue compared to in vivo controls. In addition, expression of a gene critical in trophoblast differentiation in the mouse heart and neural crest cell derivative 1 (Hand1) appears altered. Hand1, another bHLH transcription factor, is predominately expressed in mouse trophoblast giant cells, but has been detected in the extraplacental cone and areas of the spongiotrophoblast cell layer that also express Ascl2 (Scott et al., 2000). Mouse embryos carrying a mutated Hand1 gene are embryonic lethal with development arresting around 7.5 d.p.c (Riley et al., 1998). Associated with these mutant mice are a significantly reduced number of trophoblast giant cells. Over-expression of Hand1 in Rcho-1 trophoblast cell line differentiated into giant cells (Cross et al., 1995), and this differentiation was inhibited when Hand1$^{+/+}$ Rcho-1 cells co-expressed Ascl2 (Scott et al., 2000). In the present study, expression of Hand1 in SCNT embryos is under-expressed at day 17 and then over-expressed at day 40. One possible explanation is that in day 17 SCNT embryos the over-expression of Ascl2 directly or indirectly inhibits factors involved in trophoblast differentiation (e. g. Hand1 expression). This action is expected to inhibit binucleate cell formation. The regulatory factors of Hand1 are poorly characterized. Nonetheless, by day 40, expression of Ascl2 and Hand1 are both higher in SCNT cotyledonary tissue. The higher Hand1 expression may be a direct response to fewer binucleate cells, given that Hand1 is responsible for the induction of differentiation of mononucleate cells. In study two, 75% SCNT embryos did not survive to day 40, suggesting that the placental tissue examined was from embryos that were able to produce

enough Hand1 to bring about binucleate cell formation allowing for implantation to occur and pregnancy to progress.

In study two, a mono-clonal antibody SBU-3 that recognises pregnancy-associated glycoproteins produced by binucleate cells (Lee et al., 1986) was utilized to determine that placental cotyledon tissue from day 40 SCNT fetuses have fewer functional binucleate cells. These reduced numbers may lead to fewer cotyledons developing, which could be related to early pregnancy loss. The larger placentomes seen later in gestation may be a compensation mechanism to meet the nutrient requirements of the growing fetus.

As seen in study one, the expression of Ascl2 in day 17 filamentous embryos was bi-allelic regardless of embryo origin (in vivo, in vitro or SCNT produced). Expression at day 40 is still elevated in SCNT placental tissues, even though expression is primarily from the maternal genome for both in vivo and SCNT groups. These findings provide evidence that the altered expression of Ascl2 in SCNT embryos is not related to effect associated with parental expression. The over-expression may be due to alterations in upstream factors that stimulate Ascl2 mRNA transcription or epigenetic effects that allow the Ascl2 gene to be more available for transcription.

Incomplete epigenetic reprogramming of the donor cells is thought to be one of the leading causes of SCNT developmental failure (Jouneau and Renard, 2003; Piedrahita et al., 2004; Smith and Murphy, 2004; Tamada and Kikyo, 2004). Two epigenetic processes most studies and have been shown to be altered in SCNT embryos are DNA methylation and histone modification. DNA methylation plays a critical role in imprinting. Genome-

wide demethylation that normally occurs in zygotes, appears to fail in cloned embryos with the methylation patterns of these embryos similar to the donor cells utilized (Dean et al., 2001). The other process involves modification to the histone proteins bound to DNA. Santos and co-authors (2003) demonstrated that SCNT bovine embryos had altered histone acetylation causing inhibition of transcription. These results provide further evidence towards the incomplete epigenetic reprogramming of SCNT embryos.

DNA methylation is mediated through DNA methyltransferase (Dnmt) enzymes (Bestor, 2000). The Dnmt1 enzyme is involved in maintenance of DNA methylase activity (Yoder et al., 1997), whereas Dnmt3a and Dnmt3b are involved in de novo methylation (Okano et al., 1998a). In contrast to DNA methylation, which is generally associated with gene repression, histone modifications can result in either gene expression (as commonly seen with histone acetylation) or gene repression (as commonly seen with histone methylation; for review see Fischle et al., 2003). Acetylation and deacetylation are controlled by the enzymatic proteins histone acetyltransferase (HATs) and histone deacetlyase (HDACs), respectively. Expression of HAT1 and HDAC isoforms 1,2,3 and 7 have been detected in bovine oocytes to blastocyst stage embryos (McGraw et al., 2003). However, expression in SCNT tissues has not been reported.

In the third study, expression of enzymes involved in DNA methylation (Dnmt1 and Dnmt3b) and histone modifications (HAT1 and HDAC1) were analyzed in day 8 blastocysts and placental tissues from SCNT produced embryos. Dnmt1 mRNA abundance was higher in IVF blastocyst than SCNT blastocysts in the present study similar to that

previously reported (Wrenzycki et al., 2001). However, the epigenetic alterations associated with SCNT do not appear to be controlled through regulating the expression of these enzymes since no difference in mRNA abundance of either DNA methylating or histone modifying enzymes were detected in day 40 placental tissues. Other epigenetic modifying genes may play a greater role than those investigated in the current study. Santos and co-authors (2003) demonstrated that SCNT bovine embryos had hypermethylation histone H3-K9 associated with genome-wide hypermethylation. Methylation of histones is usually associated with gene silencing.

In addition to analyzing blastocyst and placental tissues, primary bovine trophoblast (TS) cell lines were developed from IVF and SCNT embryos. The development of bovine TS cell lines have been reported utilizing co-cultures with mouse fibroblast feeder layer (Talbot et al., 2000) or with uterine fibroblast-conditioned media (Shimada et al., 2001). In the present study, we utilized a modified TS cell culture procedure described by Shimada and co-authors (2001) to develop TS cell lines from IVF and SCNT embryos. Morphology of these TS cells was similar between groups and to those reported by Shimada et al. (2001). Pure TS cell lines for both groups was confirmed by presence of trophoblast stem cell genes (Cdx2 and Eomes) and trophoblast function genes (IFN-τ) and the absence of the ICM expression marker FGF4. The TS cells produced by SCNT embryos appear to reach confluency at a faster rate than their IVF counterparts. These results suggest that the altered trophoblast development associated with SCNT embryos are maintained in culture.

Abundance of the epigenetic modifying genes was analyzed in the TS cell cultures and provides further insight into their regulation and roles in trophoblast cell development. No difference in Dnmt1 mRNA was observed in IVF and SCNT TS cells, whereas day 8 IVF blastocysts had more Dnmt1 mRNA than SCNT blastocysts. Abundance of Dnmt3b mRNA was greater in IVF TS cells than SCNT TS cells, whereas in day 8 blastocysts this difference was not detected. These results may indicate differing expression from trophoblast cells and the ICM. The expression of histone modifying genes HAT1 and HDAC1 appear to be altered in culture. HAT1 mRNA abundance was greater in SCNT TS cells than their IVF counterpart. These results could suggest an effect of culture or trophoblast cell expression. Interestingly, the abundance of HAT1 and HDAC1 in IVF day 8 blastocysts and IVF TS cells does not appear to differ.

Expression of Ascl2 was greater in SCNT TS cells than IVF TS cells suggesting a potential mechanism for the greater proliferating rate observed. Interesting, Ascl2 has been reported to be expressed at a higher rate in SCNT day 8 blastocyst embryos compared to IVF embryos (Wrenzycki et al., 2001), as well as later stage as shown in study 2. The observation that expression of Ascl2 mRNA remains elevated in SCNT TS cells suggests a direct alteration in the Ascl2 gene (epigenetic effect) rather than upstream regulators produced by the ICM. The expression of Ascl2 mRNA from TS cells was from both maternal and paternal alleles, similar to those found for pre-implantation embryos in the early studies.

Even though the Ascl2 gene is located within an imprinting cluster on chromosome 7 in mice, its imprinting appears to be regulated by something other than methylation. Expression from the paternal allele was not restored in mice deficient for the gene Dnmt1 (Tanaka et al., 1999). Histone modification independent of DNA methylation has been linked to imprinting of several genes involved in mouse placental development. Lewis and co-authors (2004) demonstrated that the imprinting center 2 on mouse chromosome 7 recruits histone methylation to repress the paternal alleles of the placenta specific gene in the region (including Ascl2). These findings suggest that imprinting involves histone modification as well as DNA methylation. The control of bovine Ascl2 requires further investigations.

By understanding the underlying molecular events involved in bovine trophoblast development, we can gain insight to regulatory mechanism involved in successful placentation and how these events may be manipulated to improve assisted reproductive techniques such as somatic cell nuclear transfer.

General Conclusion

1. In the bovine, as in other species, Ascl2 is vital for trophoblast development and is not expressed in other tissue of the developing fetus. The expression of bovine Ascl2 mimics the proliferative/differentiative status of the trophoblast cells. Utilizing parthenogenetic embryos suggests that the paternal genome is required for normal Ascl2 expression. In addition, the parental expression of bovine Ascl2 is similar to the mouse with paternal silencing occurring after implantation. By understanding the underlying molecular events involved in bovine trophoblast development, and how these events are conserved across species, we can gain insight to regulatory mechanisms involved in successful placentation.

2. Over-expression of Ascl2 in SCNT embryos causes altered trophoblast development leading to placental abnormalities. In addition, placental tissue from SCNT fetuses have fewer functional binucleate cells, which could, in itself, lead to the abnormal placental development and pregnancy loss commonly associated with nuclear transfer. Insight to regulatory mechanisms involved in successful placentation and how these events are altered in assisted reproductive techniques such as somatic cell nuclear transfer provides valuable information that could lead to the novel techniques for increasing its efficiency.

3. Epigenetic alterations associated with SCNT bovine embryos appears not to be caused by altered expression of the enzymes involved with DNA methylation (Dnmt1 and Dnmt3b) or histone acetylation status (HAT1 and HDAC1). Other epigenetic modifying genes or the activity of these genes may play a greater role in regards to reprogramming donor cells for successful SCNT development. The development of trophoblast cell line from embryos produces by SCNT appear to maintain altered cell development, providing a novel tools for understanding the mechanisms required for successful placental development of animals produced by assisted reproductive techniques.

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