PLACENTATION IN THE BOVINE WITH SPECIFIC EMPHASIS ON THE ROLE OF THE TROPHOBLAST CELLS AND PLACENTAL ABNORMALITIES ASSOCIATED WITH THE NUCLEAR TRANSFER PROCESS

by

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(Under the direction of Dr. Steve Stice)

ABSTRACT

Placental abnormalities are proving to be a formidable obstacle to overcome in the nuclear transfer process. In the bovine, it is the trophoblast cells which make the major contribution to the parenchyma cells of the placenta. Previous studies have shown trophoblast cells to be feeder layer dependent, and, in the mouse, fibroblast growth factor-4 dependent, to remain undifferentiated and in a proliferating state. To date, there have been no reports of a bovine trophoblast cell line that is not feeder layer dependent, nor are there any studies determining the effects of fibroblast growth factor-4 on bovine trophoblast. It would be useful to have a stable line of bovine trophoblast cells, for in vitro studies, to determine if these cells are capable of contributing the trophectoderm lineage in vitro, and eventually in vivo, to aid in development of a normal, functioning placenta.

INDEX WORDS: Placenta, Bovine, Trophoblast, Fibroblast growth factor-4, Nuclear transfer, Feeder layers

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TABLE OF CONTENTS

Page

Acknowledgments ……iv

Chapter

1 Introduction.....1

2 Literature Review.....5

3 Effects of FGF-4 and Bovine Fetal Fibroblast Feeder Layers on Trophoblast Outgrowth of *In Vitro* Produced Bovine Embryos.....29

Conclusions.....50

CHAPTER 1

INTRODUCTION

The successes of nuclear transfer technology have been well touted since the creation of Dolly the sheep, the first mammal cloned from an adult somatic cell. This technology is truly astounding in its potential applications. For agriculture, it means a continuous supply of genetically superior animals, such as high producing dairy cows and leaner cows and pigs. For wildlife, it offers the potential to rescue species from the brink of extinction, or even to bring them back from extinction through surrogate mothers of a similar species. For humans it could mean a near limitless supply of organs, organs that would not be rejected because they would be grown from the recipient's own cells and thus no immune response would be generated. It means reduced costs of many medications, as they can be produced, en masse, in something as common and easy to acquire as cow's or goat's milk.

As with most new technology, there are advances and there are setbacks. Nuclear transfer has not been spared its fair allotment of obstacles to be overcome. Not only is the process time consuming, costly and nowhere near efficient, but it is also associated with a plethora of physiological abnormalities.

There have been many problems associated with the effect that nuclear transfer has on the normal development of the cloned animal. McCreath, et al. (2000) performed gene targeted nuclear transfer studies in the ovine and found a high occurrence of kidney defects, including renal pelvis dilation. They also found above normal, non-specified, liver and brain pathology (McCreath, Howcroft et al. 2000) and Hill, et al. (1999) reported cases of severe hepatic lipidosis and cardiac and circulatory abnormalities. The nuclear transfer process is also associated with

increased birth weights (Hill, Roussel et al. 1999) and a lack of spontaneous parturition (Hill, Roussel et al. 1999), both of which Hill suggests may contribute to the relatively high mortality rate.

By far, the most commonly reported theme of deformity is that of placental development. It has been well established that animals produced via the nuclear transfer method experience a wide range of placental abnormalities (Stice, Strelchenki et al. 1996; Hill, Roussel et al. 1999; Wells, Misica et al. 1999) that result in a substantial loss of live births, the birth of deformed offspring, and an inordinate amount of stress on the recipient animal. The high rate of fetal mortality, due to the placental problems associated with the nuclear transfer process, are so common it has led one researcher to suggest that "abnormal placental function in cloned fetuses may adversely affect an otherwise normal fetus"(Garry, Adams et al. 1996).

Placental deformities in the bovine include, but are not limited to, fewer placentomes (Stice, Strelchenki et al. 1996; Wells, Misica et al. 1999), lack of adventitial placentation (Hill, Roussel et al. 1999) enlarged umbilical vessels (Hill, Roussel et al. 1999; Wells, Misica et al. 1999), excessive accumulation of allantoic fluid (hydrallantois) (Wells, Misica et al. 1999), edematous membranes (Hill, Roussel et al. 1999; Wells, Misica et al. 1999), edematous amnion (Hill, Roussel et al. 1999), edematous chorioallantois (Hill, Roussel et al. 1999), intercotyledonary edema (Hill, Roussel et al. 1999), retarded allantoic growth (Wells, Misica et al. 1999), no development of cotyledonary tissue (Stice, Strelchenki et al. 1996), significant decrease in the number and size of cotyledons (Hill, Roussel et al. 1999; Wells, Misica et al. 1999) and enlarged placentomes (Hill, Roussel et al. 1999).

Many of the failed pregnancies abort during the middle of the first trimester, around day 40 (Stice, Strelchenki et al. 1996; Hill, Roussel et al. 1999), even though the fetus appears to be

developing in a normal manner (Wells, Misica et al. 1999). Stice, et al. (1996) have noted that the caruncles hemorrhage, implying a maternal response to the fetus; however, correct placentome formation does not necessarily follow (Stice, Strelchenki et al. 1996).

It is likely that this loss is due to the deformation in placentome development (Wells, Misica et al. 1999), as it is around this time (day 40) that the fetus must begin to receive at least some of its nutrients through functioning placentomes (Melton, Berry et al. 1951; Stice, Strelchenki et al. 1996). It has been suggested that the placentome deformities could be the result of an allantoic deficiency (Wells, Misica et al. 1999). Anatomically, anomalous formation of the placentomes is among the most commonly reported placental abnormalities. Everything from fewer and poorly developed placentomes (Stice, Strelchenki et al. 1996; Hill, Roussel et al. 1999; Wells, Misica et al. 1999), to enlarged placentomes (Hill, Roussel et al. 1999; Wells, Misica et al. 1999) have been described.

Later in gestation, during the third trimester, another peak in pregnancy loss occurs. The losses at this time appear to be largely the consequence of hydrallantois (Hill, Roussel et al. 1999; Wells, Misica et al. 1999), a condition resulting in "excessive accumulation of allantoic fluid" (Wells, Misica et al. 1999). Hill reported a diagnosis of hydrallantois at 20 liters of fluid in the allantoic cavity; this increased to 150 liters at the time of caesarean-section, and one case of possibly up to 200 liters. Even this condition, as late in pregnancy as it is, could likely be attributed to placentomal abnormalities, specifically, a reduced number of placentomes (Wells, Misica et al. 1999).

Considering the wide range of deformities which are being reported across mammalian species, it is not too presumptuous to postulate that there is a high possibility that placental, and other difficulties, may prove to be problematic in species which have yet to have

the nuclear transfer technology applied to them. This would pose severe restrictions on the practical and widespread use of this technology, and on the implications of integrating it into endangered species' reproduction programs. Before nuclear transfer can be utilized to its full potential, it is imperative that a solution to prevent, or correct, these problems be found.

CHAPTER 2

LITERATURE REVIEW

Placentation in the Bovine

From conception until parturition, fetal-maternal communication is of monumental importance in maintenance of pregnancy and the birth of live, healthy offspring. It is through the fetal-maternal exchange, via the placenta, that the fetus will receive all its nutrients and exchange waste and gasses, such as oxygen and carbon dioxide (Cross 1998; Bell, Hay Jr. et al. 1999). Proper formation of the mammalian placenta is paramount for this critical, bidirectional communication to occur.

There are many different types of placentas, from the relatively highly invasive hemochorial (Thomas 1997) type human placenta, to the relatively non-invasive synepithelial chorionic (Wooding and Morgan 1993; Martal, Chene et al. 1997; Klisch, Hecht et al. 1999) type ruminant placenta. No matter what the anatomical classification of the placenta, all are crucial for the production of healthy, viable offspring.

The ruminant placenta is novel in that it has a fetomaternal hybrid tissue, the placentome (Wooding 1992; Wooding and Morgan 1993), which is composed of both maternal epithelium and fetal cellular chorion (Wooding and Morgan 1993). The placentome is bidirectional, it has the ability to convey signals from the fetus to the maternal environment and from the maternal environment to the fetus (Wooding and Morgan 1993). Correct development of the placentomes is a fundamental requirement to the overall development of the ruminant placenta, and consequently, to the proper development of the fetus (Wooding 1992).

Growth of the placentome is initiated by trophoblast multinucleated giant cells (Wooding 1992) and consists of a tuft of fetal villi (cotyledons) (Wooding 1992; Schlafer, Fisher et al. 2000) with intimate connections to maternal crypts (caruncles) (Wooding 1992; Schlafer, Fisher et al. 2000). The placentome is highly vascularized and is the major site of exchange between the fetus and the maternal environment (Wooding and Morgan 1993). The "extensive interdigitation" (Schlafer, Fisher et al. 2000) of the fetal and maternal tissues prodigiously increases the surface area for exchange (Schlafer, Fisher et al. 2000), which is estimated to be 130m^2, "the entire surface area of a large room" (Schlafer, Fisher et al. 2000).

Placentomes are domed, oval structures (King, Atkinson et al. 1980; Schlafer, Fisher et al. 2000) and are 10-12 cm long and 2-3 cm thick (Schlafer, Fisher et al. 2000). During gestation, they are arranged in 4 rows which run lengthwise along both uterine horns (Schlafer, Fisher et al. 2000). They will be larger in the horn containing the fetus and decrease in size as they approach the point at which both horns connect (Schlafer, Fisher et al. 2000). Numbers of placentomes vary per individual animal, from as few as 70 to as many as 120 (Schlafer, Fisher et al. 2000).

The placenta is a highly specialized organ (Bell, Hay Jr. et al. 1999) which serves many functions necessary for the survival of the eutherian fetus. The placenta plays an immunologic role in that it prevents the fetus from being rejected by the maternal immune response (Soares, Faria et al. 1993; Cross 1998; Bell, Hay Jr. et al. 1999). It plays an endocrine role in that it regulates and/or produces a "growing list" of pregnancy specific hormones and growth factors (Soares, Faria et al. 1993; Cross 1998; Bell, Hay Jr. et al. 1999; Schlafer, Fisher et al. 2000). The placenta is responsible for the de novo synthesis of proteins and the formation of a physiological barrier against infection (Soares, Faria et al. 1993; Cross 1998; Bell, Hay Jr. et al. 1999;

Schlafer, Fisher et al. 2000). It is the trophoblast cells which allow for these specialized functions of the placenta (Soares, Faria et al. 1993).

If the correct fetal-maternal connections are not made during the attachment phase of gestation, days 21-30 in the bovine (King, Atkinson et al. 1981), thus providing the stage for placentation, development of the fetus will stop (Melton, Berry et al. 1951). Or, as is being witnessed in nuclear transfer pregnancies, the pregnancy will continue, but various abnormalities will ensue.

The consequences of abnormal placentation can not only lead to the loss of a viable fetus, but also to many other fetal abnormalities and an inordinate amount of stress of the recipient animal. One of the more obvious of these consequences is decreased placental transport between the fetus and the maternal environment (Bell, Hay Jr. et al. 1999). This decreased transport can result in decreased oxygen uptake leading to fetal hypoxaemia (Bell, Hay Jr. et al. 1999). A decrease in glucose transport can lead to fetal hypoglycemia (Bell, Hay Jr. et al. 1999). Another consequence is an overall decreased surface area for exchange (Bell, Hay Jr. et al. 1999).

Development of the Bovine Blastocyst: An Overview

It is during early development that cells are allocated to form either the trophectoderm or the embryo proper. The differentiation into trophectoderm or inner cell mass is the first differentiation of the blastocyst (Soares, Faria et al. 1993; Keefer, Stice et al. 1994; Flechon, Laurie et al. 1995). In the mouse, cellular allotment to the trophectoderm has been reported to occur as early as the 8 cell stage (Balakier and Pedersen 1982), and as late as the morula to blastocyst transition (Cross 1998). The first cellular division of a fertilized bovine embryo occurs at 24-28 hours after ovulation (Betteridge and Flechon 1988) and second cleavage begins

on day 2 (Betteridge and Flechon 1988). On day 5, repeated cell divisions have led to an approximately 32 cell embryo, the morula (Betteridge and Flechon 1988). It is at this stage that compaction begins (Betteridge and Flechon 1988) and cells are allocated, by polarized division, to either the inner cell mass cell lineage (from which the fetus will develop) or the trophectoderm cell lineage (from which the extraembryonic tissues will develop) (Balakier and Pedersen 1982).

On day 7 the blastocoele appears and the embryo is now referred to as a blastocyst (Betteridge and Flechon 1988). The early blastocyst is 160-180um in diameter and consists of around 100 cells (Betteridge and Flechon 1988). The embryo has not yet hatched and the zona pellucida is at its maximum thickness of 12um (Betteridge and Flechon 1988). It is at this time that the inner cell mass (the inner cells of the blastocyst)(Balakier and Pedersen 1982; Cross 1998) and the trophectoderm (the outer cells of the blastocyst) (Balakier and Pedersen 1982; Cross 1998) become easily distinguishable from one another (Hernandez-Ledezma, Sikes et al. 1992). Later in development, the inner cell mass will become the embryo and some extraembryonic structures, including part of the placenta (Soares, Faria et al. 1993). The trophectoderm will form the bulk of the placenta and the blastocoele will form the yolk sac cavity (Schlafer, Fisher et al. 2000), which contains an early source of nutrients for the young embryo (Thomas 1997).

Hatching occurs on days 9-10 and at this time the blastocyst will consist of approximately 200 cells (Betteridge and Flechon 1988). By days 11-12 the blastocyst has enlarged to 375um in diameter and contains approximately 1,000 cells (Betteridge and Flechon 1988). Cellular allocation to the inner cell mass is very low (Betteridge and Flechon 1988; Thompson 1997), comprising less than 25% of the 1,000 cell blastocyst (Betteridge and Flechon

1988). Elongation will begin between days 12 and 14 (Betteridge and Flechon 1988), except for in *in vitro* derived blastocysts, in which no elongation is witnessed (Betteridge and Flechon 1988).

The maternal recognition of pregnancy, interferon tau, must be received by days 16-17 to avoid corpus luteum regression (Wathes and Wooding 1980; Betteridge and Flechon 1988; Mann, Lamming et al. 1999) and subsequent loss of pregnancy. It is possible to retrieve elongated blastocysts up until day 18 (Betteridge and Flechon 1988). On day 20 the allantois begins to form (Betteridge and Flechon 1988) and there is clear attachment between the embryo and the uterine epithelium (Guillomot and Guay 1982).

Blastocyst Attachment and Placentome Development

Early in gestation, before day 40, in the bovine, a fetus can receive all of its nutrients through "uterine milk", (Melton, Berry et al. 1951; Stice, Strelchenki et al. 1996; Thompson 1997) absorbed by the chorionic sac (Melton, Berry et al. 1951). After day 40, the fetus must begin to receive at least some of it nutrients via fetal-maternal exchange, through the placentomes, to support further development of the fetus and maintain a viable pregnancy (Stice, Strelchenki et al. 1996). Because of this, the attachment of the embryo to the uterine endometrium is critical for survival. Attachment involves the migration of trophoblast binucleate/giant cells to come into intimate contact with epithelial cells of the uterus (Wooding and Morgan 1993; Thie, Rospel et al. 1998).

Attachment is a gradual process which occurs over several weeks (Melton, Berry et al. 1951) with the primary attachment phase being days 21-30 of gestation (King, Atkinson et al. 1981), and the first points of attachment being around the embryo itself (Melton, Berry et al.

1951). The preattachment period is 8-10 days after hatching (MacLaren and Wildeman 1995), with attachment beginning at days 18-19 (Wathes and Wooding 1980; MacLaren and Wildeman 1995). This early attachment is extremely fragile with only slight trophectoderm invasion into the uterine glands (Wathes and Wooding 1980; MacLaren and Wildeman 1995).

By days 20-21 there is a definite union between the trophectoderm of the embryo and the epithelium of the uterus (Wathes and Wooding 1980; Guillomot and Guay 1982; Perry, Norman et al. 1999). At this time, the first firm contacts between fetal binucleate cells and uterine epithelial microvilli can be found (Guillomot and Guay 1982). At day 27 intimate attachment can be seen (King, Atkinson et al. 1980) and at day 28 there is "complete interdigitation" between the embryo and the uterus (Perry, Norman et al. 1999).

It is only after the embryo has attached that the process of placentation and placentome formation occurs. In the ruminant, placentation is initiated by the trophoblast cells of the young embryo and is believed to be under the control of circulating steroid hormones and locally produced growth factors (Gerstenberg, Allen et al. 1999).

At 30 days, the placentomes (cotyledons) begin to develop on the surface of the chorioallantoic membrane (King, Atkinson et al. 1980; Schlafer, Fisher et al. 2000). The number of cotyledons will vary considerably between individual animals, with some having as few as 70 and others having as many as 120 (Schlafer, Fisher et al. 2000). By day 33, fetal villi and maternal crypts have formed, and placentomes are easily seen as slightly raised oval structures (King, Atkinson et al. 1979). At this time, the fetal villi and maternal crypts begin to lengthen and develop secondary branching (King, Atkinson et al. 1979). Extensive branching of the placentomal villi occurs by day 42 and continues until day 67 (King, Atkinson et al. 1979). The increase in branching functions to increase the surface area for exchange (Perry, Norman et

al. 1999), thus producing a more functionally efficient placenta. Individual placentomes will increase in size and complexity, but there is little variation in cellular composition throughout gestation (King, Atkinson et al. 1979).

In both the sheep (Perry, Norman et al. 1999) and the cow (Bell, Hay Jr. et al. 1999), placental growth is at a maximum during the first half of gestation. Perry (1999) found that during early placentation, the time of extensive villi growth, environmental conditions can have a profound effect on the functionality and size of the placenta in later gestation. Restricting protein in the first trimester in heifers resulted in a larger placenta at partuition as a result of more extensive microvilli formation (Perry, Norman et al. 1999). If the protein restricted diet is followed with a well balanced diet, containing the recommended amount of protein, the increased number of microvilli will allow more exchange between the fetus and the maternal environment (Perry, Norman et al. 1999).

Perry (1999) suggests that the substantial growth in the first half of gestation could have profound effects on placental size and function later in gestation. Attachment and subsequent placentation/placentome development sets the stage for the production of a live, healthy animal.

Interferon Tau

Interferon tau is the maternal recognition of pregnancy signal produced by the trophoblast cells of the conceptus (Klemann, Kazuhiko Imakawat et al. 1990; Bazer 1992) of the sheep, cow and goat (Bazer, Ott et al. 1994; Stojkovic, Wolf et al. 1995). Interferon tau was initially called trophoblastin (Bazer 1992; Martal, Chene et al. 1997) and then trophoblast protein-1 (Hernandez-Ledezma, Sikes et al. 1992), because it is the first major protein secreted by the trophoblast in ruminants (Bazer, Ott et al. 1994). It was later determined, using cDNA

cloning and amino acid sequencing that "trophoblastin" was actually a Type I Interferon and given the designation tau (Bazer, Spencer et al. 1997). It is a subclass of the Type I interferon, the alpha II omega interferons (Bazer, Ott et al. 1994; Bazer, Spencer et al. 1997). At maturity, a tau protein has 172 amino acids with cysteine residues conserved at 1, 29, 99 and 139 (Bazer, Ott et al. 1994) and a molecular weight of 20kDa (Martal, Chene et al. 1997).

Interferon tau is a trophoblastic, secretory, antiluteolytic protein (Klemann, Kazuhiko Imakawat et al. 1990; Bazer, Ott et al. 1994; Stojkovic, Wolf et al. 1995; Martal, Chene et al. 1997). Interferon tau functions in a paracrine manner to inhibit the pulsatile release of luteolytic prostaglandin (Hernandez-Ledezma, Sikes et al. 1992; Bazer, Spencer et al. 1997) from the uterine epithelium (Bazer, Ott et al. 1994) thus extending the life span of the corpus luteum (CL), (Hernandez-Ledezma, Sikes et al. 1992; Bazer, Ott et al. 1994; Bazer, Spencer et al. 1997) and preventing a return to the estrous cycle (Hernandez-Ledezma, Sikes et al. 1992). Martal et al. (1997) comment that if interferon tau had been identified 20 years ago, it would have been designated a hormone of reproduction.

Interferon tau mRNA is easily detected in hatched blastocysts by day 12 (Bazer 1992) and production may persist until at least day 38 (Bazer 1992). Using mRNA amplification, Hernandez-Ledezma et. al. (1992) were able to detect tau mRNA as early as day 8 in non-hatched blastocysts. They also reported that embryos at the blastocyst stage, actively making interferon tau, had a cell count of 115 +/- 22 (Hernandez-Ledezma, Sikes et al. 1992). Kubisch et al. (1998), reported that interferon tau production did not highly correlate with the number of cells in a blastocyst or on the quality of the blastocyst itself (Kubisch, Larson et al. 1998).

It is only the mononuclear cells of the trophectoderm which have the ability to secrete interferon tau (Bazer, Spencer et al. 1997; Martal, Chene et al. 1997; Mann, Lamming et al.

1999), and they have this ability for only a very short time (Martal, Chene et al. 1997), with secretion being maximum during the periimplantation period of days 15-24 days (Martal, Chene et al. 1997; Kubisch, Larson et al. 1998). In the bovine, tau secretion is critical for maternal recognition of pregnancy on day 14-17 (Bazer, Ott et al. 1994) and in the ovine it is critical on days 12-13 (Bazer, Ott et al. 1994).

Interferon tau is crucial in preventing the pulsatile release of prostaglandin (PGF) which results in the regression of the CL (Bazer 1992; Mann, Lamming et al. 1999) and a return to the estrous cycle. The CL is responsible for producing progesterone, the hormone of pregnancy (Bazer 1992), which is required for successful implantation, placentation and fetal development (Bazer 1992).

In 1992, Bazer reviewed several mechanisms by which interferon tau was postulated to work. He suggested an extension of the "progesterone block" period by up-regulation of progesterone receptors, direct inhibition of estrogen receptors, direct inhibition of endometrial oxytocin receptors or inhibition of non-specified post-receptor mechanisms (Bazer 1992). In 1994, theories on the mechanism of action appeared to lean toward the inhibition of endometrial estrogen receptors and oxytocin receptors (Bazer, Ott et al. 1994). And by 1997, it was determined that tau suppresses the gene transcription of estrogen receptors and oxytocin receptors by acting on the uterine epithelium (Bazer, Spencer et al. 1997). In 1999, Mann, et al. stated that "it is the development of oxytocin receptors on the luminal epithelium that is the key event in the development of the luteolytic mechanism" (Mann, Lamming et al. 1999). That interferon tau acts on oxytocin receptors to prevent luteolysis, appears to be the current method by which it is believed to act in the bovine.

Cells of the Bovine Placenta

The first differentiation of the bovine blastocyst is that which forms the ICM and TE (Soares, Faria et al. 1993; Keefer, Stice et al. 1994; Flechon, Laurie et al. 1995). The cells' position within the undifferentiated morula controls their developmental potential (Cross 1998). Those destined to become inner cell mass are on the inside and those destined to become trophectoderm are on the outside.

The inner cell mass is made up of undifferentiated embryonic stem cells, the embryonic ectoderm (Cross 1998). The first differentiation of the inner cell mass results in the epiblast and the hypoblast (Thomas 1997). The epiblast will give rise to the three embryonic germ layers (Thomas 1997) of the developing fetus and the amniotic ectoderm (an extraembryonic tissue) (Thomas 1997). The hypoblast will differentiate into the extraembryonic endoderm, the yolk sac and the extraembryonic mesoderm, which is the contribution of the inner cell mass to the placenta (Balakier and Pedersen 1982; Wooding and Morgan 1993; Thomas 1997; Cross 1998; Tanaka, Kunath et al. 1998). The extraembryonic mesoderm will form the umbilical cord (Cross 1998)

It is the trophectoderm, the first differentiated tissue of the pre-implantation blastocyst stage embryo (Betteridge and Flechon 1988; Wooding and Morgan 1993; Flechon, Laurie et al. 1995), which makes the principal contribution to the parenchymal cells of the fetal portion of the placenta (Wooding and Morgan 1993; Tanaka, Kunath et al. 1998). It is these trophectoderm cells that account for the specialized functions of the placenta (Wooding and Morgan 1993), allowing attachment and implantation to occur and thus establishing "the necessary conduit for

pregnancy to proceed" (Wooding and Morgan 1993). Trophoblast cells are found only in eutherian mammals and make contributions only to the placenta (Cross 1998).

The trophectoderm further differentiates to form the cytotrophoblast and the syncytiotrophoblast (Thomas 1997). The cytotrophoblast is a thin inner layer of trophoblast and is composed of cuboidal cells (Thomas 1997). The syncytiotrophoblast is the outer syncytial layer of trophoblast that will develop an intimate relationship with the uterine endometrium (Sunderland, Bulmer et al. 1985; Thomas 1997) In the bovine, trophoblast cells show little or no penetration beyond the maternal epithelium (Lindenberg, Hyttel et al. 1989). The cells grow out upon the surface of the uterine epithelium in a non-invasive manner, as is characteristic of the epitheliochorial type of placentation (Lindenberg, Hyttel et al. 1989). There is little, if any, displacement of maternal endometrial cells, instead of dislocation, the trophoblast cells of the bovine placenta locate on top of them (Lindenberg, Hyttel et al. 1989).

The trophoblast epithelium can be cellular, syncytium or both (Wooding and Morgan 1993). These cells will grow to form a continuous epithelial layer of the chorion that will cover the entire chorioallantoic surface (Schlafer, Fisher et al. 2000). This is both a physiological and structural barrier which surrounds the developing fetus (Wooding and Morgan 1993).

Beginning on day 8, endoderm cells of the trophectoderm begin to spread out from under the inner cell mass (Betteridge and Flechon 1988). By day 10, these cells completely line the "cuboidal and polygonal" trophectoderm cells that surround the blastocoel, hence, the formation of the trophoblast (Betteridge and Flechon 1988). Basement lamina separates the trophectoderm from the maternal endoderm (Betteridge and Flechon 1988). Mesodermal cells, which have differentiated from the inner cell mass by days 14-16, begin to migrate outwards between the trophectoderm and endoderm forming an inner and an outer layer as it travels (Betteridge and

Flechon 1988). The outer layer will line the trophectoderm and become the chorion (Betteridge and Flechon 1988). The inner layer will cover the endoderm and form the wall of the yolk sac (Betteridge and Flechon 1988).

Trophoblast cell growth and differentiation is controlled by many transcription factors (Cross 1998), unknown factors in fetal calf serum (Wooding and Morgan 1993), and on cell-cell interactions (Cross 1998). In the mouse, only cells above the inner cell mass continue proliferation (Cross 1998). There are at least 4 different endpoints for trophoblast stem cells in the rat, trophoblast giant cell, spongiotrophoblast cell, glycogen cell and syncytial trophoblast cell (Wooding and Morgan 1993). The majority of trophoblast cells are columnar but irregular in shape and vary from 14-22um in height (King, Atkinson et al. 1979).

A spongiotrophoblast cell is terminally differentiated (Faria and Soares 1991) and is found in the junctional zone of the chorioallantoic placenta after mid-gestation (Faria and Soares 1991). Also terminally differentiated is the trophoblast giant cell that appears just after the initiation of attachment (Faria and Soares 1991). Mouse trophoblast cells are “end cells” and depend upon contact with the inner cell mass to remain proliferative (Betteridge and Flechon 1988). It is only the polar trophectoderm, that is in direct contact with the inner cell mass, which continues to proliferate (Tanaka, Kunath et al. 1998). It will proceed to form the extraembryonic ectoderm, the ectoplacental cone and the secondary giant cells (Tanaka, Kunath et al. 1998). The mural trophectoderm, that not in direct contact with the inner cell mass, discontinues proliferation and becomes the primary giant cells. However, this does not appear to be the case in the bovine, where mitosis is witnessed throughout the entire trophoblastic tissue, and the trophectoderm immediately above the inner cell mass degenerates (Betteridge and Flechon 1988).

The outer surface of the inner cell mass will remain covered with trophectoderm cells until approximately day 12 (Betteridge and Flechon 1988). At this time, the trophoblast cell plasma membrane is highly differentiated (Betteridge and Flechon 1988). By scanning electron microscopy Guillomot (1982) found slender microvilli uniformly over the entire apical surface (Guillomot and Guay 1982; Betteridge and Flechon 1988), and laterally, junctional complexes composed of interdigitations, tight junctions of the adherons type and desmosomes were found by Betteridge and Flechon (1988). When attachment begins (days 18-19), the microvilli disappear and the trophoblast cell surface becomes smooth (Guillomot and Guay 1982). At day 16, the cells are again covered with long microvilli that disappear by day 20 leaving a "wrinkled" cell surface (Guillomot and Guay 1982). At this time (day 20-21) the cells are spindle shaped with an "irregularly ridged" cellular surface (Guillomot and Guay 1982). Microvilli remain on non-adherent trophoblast cells (Guillomot and Guay 1982).

Using transmission electron microscopy, Guillomot (1982) found "extracellular material and numerous vesicles wrapped in the network of trophoblast microvilli". Also seen were endocytotic invaginations into the plasma membrane and dense bodies in the cytoplasm (Guillomot and Guay 1982). Because of these findings Guillomot (1982) postulates that the microvilli covering the trophoblast cell surface in early gestation could be involved in nutrient absorption by the free floating embryo.

The cytoskeletal constituents of the trophectoderm are those that are characteristic of epithelial cells (Betteridge and Flechon 1988). Bundles of intermediate filaments spread from desmosome to desmosome (Betteridge and Flechon 1988) and micro filaments are copious in the microvilli and cortical cytoplasm (Betteridge and Flechon 1988). The most obvious indication

of mitotic activity in the trophoblast is the distribution of microtubules throughout the cytoplasm and grouped in bundles as midbodies (Betteridge and Flechon 1988).

The organelles of the trophectoderm cytoplasm are those familiar to most mammalian cell types, mitochondria with transverse cristae, ribosomes and polyribosomes, rough and smooth endoplasmic reticulum, Golgi apparatus, many lysosomes and/or residual bodies and many lipid droplets (Betteridge and Flechon 1988). The nuclei of the trophoblast have dense chromatin that is found mostly in the periphery (Betteridge and Flechon 1988). Also found in the nuclei is parachromatin granules and typically active nucleoli (Betteridge and Flechon 1988).

Trophoblast cells are responsible for the maternal recognition of pregnancy signal that ensures continued progesterone production via a functional corpus luteum (Wooding and Morgan 1993). The trophoblast initiates migration of the fetal trophoblast binucleate cells and these are the cells that fuse with individual cells in the uterine epithelium (Wooding and Morgan 1993). The trophoblast produces a variety of pregnancy related hormones, placental lactogen, retinol-binding protein, pregnancy associated glycoproteins I, II and III, interferon tau, progesterone and transforming growth factor beta (Stojkovic, Zakhartchenko et al. 1997; Schlafer, Fisher et al. 2000).

Trophoblast binucleate cells and trophoblast giant cells, are two types of differentiated trophoblast cells which play a very important role in placentation. A binucleate cell contains two distinct nuclei, a giant cell contains three or more distinct nuclei. These cells migrate across the microvillar junction and fuse with individual maternal epithelial cells of the uterus, forming the placentome (MacLaren and Wildeman 1995). It is through the placentome, the fetal maternal hybrid tissue of the bovine placenta, which communication between the mother and fetus actually occurs (Wooding 1992; Wooding and Morgan 1993).

There are 2 major functions attributed to the binucleate/giant cells (Wooding 1992). The first is formation of the maternofetal hybrid tissue that is required for implantation and placentomal growth (Wooding 1992). The second major function of these cells is the production and delivery of protein and steroid hormones (Wooding 1992).

It is believed that giant/binucleate cells develop from the mononucleate trophoblast cells (Wooding 1983; Klisch, Hecht et al. 1999) by nuclear division without subsequent cellular division (Wooding 1983; Faria and Soares 1991; Cross 1998). This polyploidism is normal and common in many endothelio and haemochorial type placentas (Klisch, Hecht et al. 1999). In the bovine, the majority of trophoblast cells are binucleate, with only a small percentage containing 3 or more nuclei (Klisch, Hecht et al. 1999), though giant cells have been found to contain up to 8 nuclei (Wathes and Wooding 1980). The average size of these cells is 20x40um (Wathes and Wooding 1980) but they can be as large as 20x50um (Wooding 1983).

The first granulated giant cells appear in the chorionic epithelium at days 16-17 (Wooding and Wathes 1980) and by the 18th day, and throughout the rest of gestation (Wooding and Wathes 1980), they will comprise approximately 20% of the trophectoderm epithelial cells (Wooding 1983; Wooding 1992; Klisch, Hecht et al. 1999). In the pregnant horn they can comprise up to 70% of the cell area of the uterine epithelium on days 26 and 27 (Wathes and Wooding 1980).

At early gestation, giant cells have no microvilli and are totally surrounded by mononucleate trophoblast cells, prohibiting any direct contact between them and the maternal cells of the placenta (King, Atkinson et al. 1979). Giant cells contain very little cytoplasm (King, Atkinson et al. 1979) and the concentration of organelles within them is higher than seen in any of the other cell types that comprise the placentome (King, Atkinson et al. 1979).

At any period during gestation, 15-20% of the giant cells will be migrating through the apical tight junctions of the trophectoderm (Wooding 1983; Wooding 1992). During the first 28 days of pregnancy the percentage of giant cells actively migrating is significantly higher than at later stages of gestation (Wooding 1983). This intense migration during early pregnancy is due to implantation and the extensive syncytium formation that is required at this time (Wooding 1983). The continued migration and fusion of giant cells, right up until parturition, (Wooding 1983) increases the surface area of exchange between mother and fetus by 10,000 times (Wooding and Morgan 1993).

Giant cells are also responsible for delivery of fetal products which are carried in their granules (Wooding 1983). Once migration to the uterine epithelium is complete and the products delivered, the cell will condense and become a "cell remnant" which is then phagocytosed by the chorionic epithelium (Wooding and Wathes 1980).

Fibroblast Growth Factors (Please note that the following discussion on fibroblast growth factors is in relation to the mouse unless otherwise indicated.)

Fibroblast Growth Factors (FGF's) are a family of polypeptide hormones (Rappolee, Basilico et al. 1994) consisting of at least 9 (Rappolee, Basilico et al. 1994; Yamaguchi and Rossant 1995; Fuminori, Harada et al. 1998) and as many as 14 (Wilder, Kelly et al. 1997) members which interact with 4 receptors (Yamaguchi and Rossant 1995) to produce a wide variety of results depending on the "target cell and its developmental history" (Yamaguchi and Rossant 1995). In early embryonic development, at least 3 members of this family are known to play important roles: acidic FGF (aFGF, FGF-1) (Rappolee, Basilico et al. 1994), basic FGF

(bFGF, FGF-2) (Rappolee, Basilico et al. 1994) and Kaposis's sarcoma type (FGF-4) (Rappolee, Basilico et al. 1994).

The role of FGF-4 in the development and embryogenesis of the mouse and the frog (*Xenopus)* have been extensively studied (Liu and Nicoll 1988; Paterno and Gillespie 1989; Hebert, Basilico et al. 1990; Haimovici and Anderson 1993; Rappolee, Basilico et al. 1994; Slack 1994; Wilder, Kelly et al. 1997; Fuminori, Harada et al. 1998; Tanaka, Kunath et al. 1998). Its role in the bovine has received much less attention.

FGF-4 appears to be the first FGF to be expressed during embryonic development (Wilder, Kelly et al. 1997). The mRNA of FGF-4 is expressed in the preimplantation mouse blastocyst stage embryo (Rappolee, Basilico et al. 1994) and the FGF-4 polypeptide is found in the cells of the inner cell mass (ICM) (Rappolee, Basilico et al. 1994), and is believed to be required for the proliferation, differentiation and tissue patterning of those cells (Yamaguchi and Rossant 1995). The mRNA for FGF-4 is first found at the 1 cell stage and the RNA is first seen at the 4 cell stage (Wilder, Kelly et al. 1997), at which time the expression becomes limited to the inner cell mass (Wilder, Kelly et al. 1997). mRNA expression continues to increase in accumulation until the blastocyst stage (Rappolee, Basilico et al. 1994). FGF-4 expression is also found in the embryonic ectoderm before gastrulation occurs (Yamaguchi and Rossant 1995). FGF-4 is angiogenic (Liu and Nicoll 1988) and could possibly be involved in the development of the extensive vascularization which must occur during placentation (Liu and Nicoll 1988).

Inactivation of FGF-4 alleles or the FGF-4 receptor alleles, in the mouse, will stop development shortly after implantation (Wilder, Kelly et al. 1997; Arman, Haffner-Krausz et al. 1998). *In vitro*, FGF-4 negative mouse embryos will degenerate unless FGF-4 is supplemented in the culture medium (Wilder, Kelly et al. 1997). It has been established that adding FGF-4 to the

culture medium of *in vitro* produced mouse embryos will significantly enhance the growth and proliferation of the trophoblast *in vitro* (Fuminori, Harada et al. 1998; Tanaka, Kunath et al. 1998). A literature search resulted in finding no information available on the effects FGF-4 has on trophoblast proliferation in the bovine.

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CHAPTER 3

THE EFFECTS OF FGF-4 AND BOVINE FETAL FIBROBLAST FEEDER LAYERS ON TROPHOBLAST OUTGROWTH OF IN VITRO PRODUCED BOVINE EMBRYOS

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Abstract

The effects of fibroblast growth factor-4 (FGF-4) on trophoblast outgrowth and proliferation in vitro has been well documented in the mouse, but little is known about the effects of this growth factor in the growth and proliferation of bovine trophoblast tissue. Also, the use of bovine mitotically inactivated fetal fibroblast feeder layers in the propagation of bovine cells is largely unknown. Classically, it is mitotically inactivated mouse fetal fibroblasts that have been used for feeder layers, even in the bovine. The purpose of this study was to determine whether media that was either supplemented, or not, with FGF-4, with feeder layers, or with both, affected the isolation and outgrowth of bovine trophoblast cells. The medium used was that published by Talbot, et al. (v:v DMEM and TCM-199). The bovine feeder layers significantly increased ($p<.05$) the initial embryo attachment rate. The culture conditions using a feeder layer and medium supplemented with FGF-4 resulted in cultures which reached a point at which they were able to be passaged at a significantly higher rate ($p<.05$). It is possible that feeder layers aid in initial embryo attachment and FGF-4 enhances the viability of bovine trophoblast cells in culture.

Introduction

Each advance made by science is accompanied by its share of setbacks and pitfalls. Even the highly celebrated process of nuclear transfer has not escaped this fate. For all the promising advances, both medically and agriculturally, that this technique puts within the grasp of the research scientist, there are still many kinks which need to be unraveled before nuclear transfer can live up to its full potential. One such kink that is posing tremendous losses, both financially and in the viability of potential offspring, is that of abnormal placentation by the recipient of

nuclear transfer embryos. One researcher has even suggested that "abnormal placental function in cloned fetuses may adversely affect an otherwise normal fetus" (Garry, Adams et al. 1996).

Many and various placental deformities are being reported across species in which this new technology has been applied. In the bovine alone, reported placental abnormalities include, but are not limited to, fewer placentomes (Stice, Strelchenki et al. 1996; Wells, Misica et al. 1999), lack of adventitial placentation (Hill, Roussel et al. 1999), enlarged umbilical vessels (Hill, Roussel et al. 1999; Wells, Misica et al. 1999), excessive accumulation of allantoic fluid (hydrallantois) (Wells, Misica et al. 1999), edematous membranes (Hill, Roussel et al. 1999; Wells, Misica et al. 1999), edematous amnion (Hill, Roussel et al. 1999), edematous chorioallantois (Hill, Roussel et al. 1999), intercotyledonary edema (Hill, Roussel et al. 1999), retarded allantoic growth (Wells, Misica et al. 1999), no development of cotyledonary tissue (Stice, Strelchenki et al. 1996), significant decrease in the number and size of cotyledons (Hill, Burghardt et al.; Hill, Roussel et al. 1999; Wells, Misica et al. 1999) and enlarged placentomes (Hill, Roussel et al. 1999).

It is the trophoblast cell line, the first differentiated tissue of the preimplantation blastocyst (Betteridge and Flechon 1988; Wooding and Morgan 1993; Flechon, Laurie et al. 1995) that is the major contributor to both formation and function of the placenta. It is these cells which migrate to form the placentomes (MacLaren and Wildeman 1995) which are vital, in the bovine, for the bidirectional fetal-maternal communication to occur and which aid in maintaining a viable pregnancy. Because of the crucial nature of trophoblast cells in early mammalian development, and the associated problems of nuclear transfer placentas, it is of paramount importance to study these cells to determine if they may hold the key, or at least a clue, to

unraveling the problems of the placental deformities that have come to be associated with the nuclear transfer process.

Previous studies by Tanaka, et al., (1998) in the mouse, have shown that by supplementing trophoblast medium with fibroblast growth factor-4 (FGF-4), stable lines of murine trophoblast stem cells could be cultured for extended periods of time. Upon removal of the FGF-4, the cells differentiated to become "other trophoblast subtypes" (Tanaka, Kunath et al. 1998). Talbot, et at.(2000), recently reported a stable line of bovine trophoblast cells, in which FGF-4 was not required to maintain the differentiated state (Talbot, Caperna et al. 2000). In both studies, the cells were feeder layer dependent for the first several passages.

Feeder layers are generally used in the culture of embryonic stem cells because they aid in maintaining the cells in an undifferentiated state (Tucker and Burke 1996; Xu, Inokuma et al. 2001). It is suggested that feeder layers may act by secreting specific growth factors that may stimulate protein synthesis and cell proliferation (Stojkovic, Wolf et al. 1995) or they may simply be providing a substrate for the cells that grow on them (Stojkovic, Wolf et al. 1995). Classically, it is mouse embryonic fibroblast cells that have been used to produce feeder layers (Flechon, Laurie et al. 1995). This study involves the use of bovine fetal fibroblast cells in production of the mitotically inactivated feeder layers.

The purpose of this study was first to determine if medium supplementation with FGF-4 has an affect on the proliferation of bovine trophoblast cells produced and maintained in vitro and second to determine if bovine trophoblast cell cultures, from in vitro produced embryos, could be established in the absence of feeder layers.

Materials and Methods

Establishment of Bovine Fetal Fibroblast Cell Line

The reproductive tract of a pregnant female was obtained from a local slaughterhouse and a 3.5 inch male fetus removed and placed in phosphate buffered saline. Small pieces of skin tissue were removed from the mid torso area and placed in trypsin/EDTA. The tissue was minced using a razor blade, transferred into a 15ml conical tube, vigorously aspirated 6-7 times and then placed in the incubator (37°C and 5% CO_2) for 20 minutes. Five additional ml of trypsin/EDTA was added to the mixture, vigorously pipetted and then placed in the incubator for 15 minutes.

The tube was removed from the incubator and the contents allowed to settle. The supernatant was removed and the pellet placed in a fresh 15ml conical tube in which the volume was increased to 15ml using fresh bovine fetal fibroblast (BFF) medium (DMEM w/L-Glutamine, 10-15% Fetal Calf Serum (FCS), 1% non-essential amino acids, 1000X penicillin-streptomycin and sodium bicarbonate). This was centrifuged at 1,000 x g for 5 minutes at 10°C. The pellet was then resuspended in 1ml of BFF medium, divided evenly into two T-150 tissue culture grade flasks with medium sufficient to cover the bottom of the flask, and incubated for 24h at 37°C and 5% CO_2. After 24h, the medium was changed and cells were again left undisturbed for a period of 24h.

Upon approximately 70% confluency, cells were passaged by being exposed to 2ml of trypsin with EDTA for 10 minutes. After 10 minutes the cellular solution was aspirated from the T-150 flask, placed in a 15ml centrifuge tube and centrifuged at 1,000 x g for 5 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of BFF medium. Cells were placed into 4 T-150 tissue culture grade flasks at 100,000 cells per flask.

The culture was further expanded and upon third passage a stock was created by freezing 20 vials of BFF cells in liquid nitrogen. The freezing protocol consisted of exposing the cells to trypsin, as if for passage, and centrifuging at 1,000 x g for 5 minutes. The supernatant was removed and the pellet resuspended in 10 ml of BFF medium. One half ml of cells and .5 ml of freezing medium was allocated to each of 20 cryotubes that were frozen at -70°C for 1 week and then transferred to liquid nitrogen. Freezing medium consisted of 30 ml BFF medium, 10 ml FCS and 10 ml DMSO.

These cells were maintained in culture and used as feeder cells for subsequent bovine trophoblast cell culture experiments. BFF cells for feeder layers were inactivated by placing them in 1 ug/ml of Mitomycin C for approximately 4 hours. Following mitomycin C treatment, BFF cells were thoroughly rinsed 4 times, using Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium, to remove all traces of the Mitomycin C.

In Vitro Fertilization

Eight to eleven day *in vitro* matured and fertilized bovine embryos were received from the laboratory of Dr. Ben Brackett after being prepared by Saksiri Sirisathien

. In brief, oocytes were harvested from slaughterhouse ovaries and washed twice in maturation medium (IVM) containing TCM-199 (Sigma, M-3769), 50 ug/ml sodium pyruvate, 25 mM $NaHCO_3$, 1 mg/ml polyvinyl alcohol, .25 mM glutamine, .1 mM cystine, .1 mM cysteamine, 10 mM HEPES, 50 ug/ml gentamicin sulfate, .1 IU/ml rh FSH (1.7 IU/ug) and 5 ng/ml rh IGF-I. They were matured overnight in 100uL drops of maturation medium in groups of 20-22 oocytes per drop.

Two straws of Holstein semen (10^8 sperm/straw) were selected via the swim up method in mDM (Dinkins and Brackett 2000) without hypotaurine or epinephrine. Straws were thawed at 37°C for 30 seconds at which time the semen was layered under 1.5ml of mDM in each of several 12x75 mm vials which were then placed at a 45° angle for 45 minutes at 39°C under moist 5% CO_2 in air. After 45 minutes, the uppermost 850 uL aliquots from each tube were pooled into a 15 ml tube and centrifuged at 320 x g for 10 minutes. The pellet was resuspended to a volume of 400 uL with mDM and 200 ug/ml heparin, incubated for 10 minutes and then 12-14 uL of the sperm suspension was introduced to the matured oocytes for coincubation for 18h.

At 18h post insemination, the presumptive zygotes were cultured in groups of 20 in 50 uL of glucose free synthetic oviductal fluid (SOF) which was supplemented with .1 mM non essential amino acids (Sigma, M-7145), .5 mM glutamine, .4 mM threonine and 3 mg/ml polyvinyl alcohol. At 72h post insemination, embryos with at least 4 cells were selected for further culture in SOF containing citrate (c-SOF+NEA) (Keskintepe and Brackett 1996) without glutamine. At 144h post insemination, embryos were transferred into IVM medium without FSH or IGF-I for further culture. At 192h post insemination, embryos were transferred into the same medium, freshly prepared for culture until hatching occurred.

Trophoblast Cell Culture

Hatched blastocysts were transferred to 4-well tissue culture grade plates and cultured in Talbot's Media (Talbot, Caperna et al. 2000) consisting of 41.5% of 10% Dulbecco's Modified Eagles Medium (DMEM), 41.5% TCM-199, 2% glutamine, 1% 2-mercaptoethanol, 1% each of 3uM guanosine, adenosine, cytidine, uridine and thymidine, penn/strep and 1% non-essential

amino acids. In the FGF-4 experimental group, FGF-4 was supplemented at a concentration of 25ng/ml with 1 ug/ml of heparin as per Tanaka et. al. (1998).

Hatched blastocysts were randomly allotted, as media and feeder layer availability permitted, to 1 of the following 4 treatment groups: Treatment 1: feeder layer and medium supplementation with FGF-4; Treatment 2: medium supplemented with FGF-4 in the absence of a feeder layer; Treatment 3: feeder layer but no supplementation with FGF-4; and Treatment 4: culture on tissue culture plastic only, no supplementation or feeder layer.

Embryos were placed, one per well per treatment, in 4 well tissue grade culture plates in 200 ul of medium, a volume just sufficient to cover the bottom of the well. Cultures were placed in the incubator at 37°C and 5% CO_2 and left undisturbed for 24h, after which time they were observed for embryo attachment. If attachment had occurred, the culture was fed with 100ul of appropriate medium. If attachment had not occurred, the embryo was punctured and pushed onto the culture dish, using a sterile 26g needle. This process was repeated 2 times at approximately 24h intervals. Cultures were often exposed to the environment and consequently, contamination of cultures was not an infrequent occurrence. Embryos that had not attached after 4 attempts, or approximately 96 hours, were recorded as no attachment and discarded. Cultures were fed with small volumes of medium until attachment was firmly established, at which time, medium was changed at approximately 48h intervals.

Since it has been found that treating trophoblast cells with trypsin is harmful (Talbot, Caperna et al. 2000), passage was accomplished by physical dissociation without the aid of any chemicals. Physical dissociation consisted of carefully lifting the individual trophoblast colonies from the culture dish and pipetting them until they were broken up into several pieces. The cell fragments were then centrifuged at 1,000 x g for 5 minutes after which time the supernatant was

removed and the cell pellet resuspended in enough medium to allocate 10ul of suspension into each new culture. Cells were initially placed in 4-well tissue culture grade dishes in 200 ul of medium, with 100 ul of medium being added at 24h intervals until the volume reached 500 ul, after which time the medium was changed at 48 hour intervals.

All cell cultures were assessed by visual examination. Colonies were considered to be actively dividing if growth outward, from the point of attachment, was observed. Cells were passaged when outgrowth covered approximately 60-70% of the culture dish.

Statistical Analysis

Statistical computations were made through the use of chi-square analysis for all data (Cochran and Cox 1957).

Results

Of 58 embryos cultured under treatment condition 1 (FGF-4 and feeder layer)(Table 1), 39 attached. Of the 39 that attached, 30 remained viable and were passaged (Table 2). There were 59 embryos grown under treatment condition 2 (FGF-4 and no feeder layer). Of this 59, 32 attached but only 12 remained viable for passage. Seventy-five embryos were cultured under treatment condition 3 (feeder layer only) and of this, 54 attached and 28 remained viable and were passaged. This number (initial attachment using a feeder only) is significantly different ($p<.05$) from all other treatment groups. There were no other significant differences among treatment groups for initial attachment. There were 75 embryos cultured under treatment condition 4 (plastic only), of this, 40 attached and 14 remained viable for passage.

When the number of cultures which remained viable for first passage was examined (Table 2), treatment 1 (FGF-4 and feeder layer) was significantly better ($p<.01$) than all other treatments.

Overall, the use of feeder layers (Table 3) significantly improved the rate of initial attachment ($p<.01$) and the maintenance of viability until passage ($p<.01$). FGF-4 did not have a significant effect on the initial attachment rate (Table 4), however, it did have a significant effect in the maintenance of viability until passage ($p<.1$).

The growth patterns and morphology of all treatments were similar, with embryos attaching and proceeding to grow out in thin sheets of cuboidal shaped cells. Dome-like structures often formed within the colonies, and appeared to be more prevalent in those colonies that were grown on feeder layers. Colonies had well defined edges with larger cells toward the outside. Colonies that were grown on feeder layers were easily lifted off indicating no intimate attachment between the colony and the feeder cells. Many floating and attached vesicles were common among all treatments (Figure 1). When a feeder layer was not used, it was common to see the sheet of cells gradually losing adhesion to the tissue culture plastic (Figure 2).

Discussion

Results indicate that feeder layers produced from BFF cells are able to establish and maintain trophoblast cell cultures in vitro. The use of BFF cells for feeder layers was shown to significantly improve the initial attachment rate ($p<.01$) and the maintenance of viability ($p<.01$) in these cells. However, since no murine STO cells were used as feeder layers, it remains unknown whether a species specific effect is involved in the improved attachment and longevity

of the trophoblast cultures. Comparison of efficiency of BFF cells and murine STO cells as feeders is indicated.

Unlike observations of Talbot, et al. (2000) and Tanaka, et al. (1998), these cells could be established without the use of a feeder layer. However, using a feeder layer significantly enhanced the rate of attachment of embryos and subsequent growth and proliferation. It should also be noted that even in the embryos which did attach without a feeder layer, obtaining that initial attachment proved to be much more difficult than when a feeder layer was present. If was often necessary to manipulate the blastocysts on the culture dish several times before a culture could become established.

Though FGF-4 did not have an affect on initial attachment of the blastocysts, it did have an effect on the continued growth and proliferation once the cultures were established. Using both a feeder layer and supplementation of medium with FGF-4 significantly enhanced the number of cultures which remained viable and were able to be passaged. Tanaka et al. (1998) reported that FGF-4 was necessary for the trophoblast cells of the mouse to remain in an undifferentiated and proliferative state. This does not, however, seem to be the case in the cow, where cultures of trophoblast cells can be established, remain undifferentiated and proliferate without the use of FGF-4. It would appear, that in the bovine, FGF-4 may act simply to elongate the viability and health of trophoblast cells.

The cell cultures were morphologically similar, at a gross level, as those described by Talbot et al. (2000). However, since the cells were not stained intra-cellular morphology was not assessed in detail. Additional studies are indicated to confirm that these are in fact trophoblast cells and not an endothelial cell type. Because many of these cultures were established from hatched blastocysts 11 days old, an age at which the ICM should be degenerated (Talbot, Caperna

et al. 2000), it is likely that further studies would confirm the trophoblastic nature of these cells. Only after positive PCR, with the primer for interferon tau, which is known to be specific to trophoblast cells (Talbot, Caperna et al. 2000), should these cells be used further.

A trophoblast cell line could prove invaluable in improving the effectiveness of the nuclear transfer technique. Further studies should include the production of early stage chimeric embryos, utilizing the trophoblast cells, to determine if they are able to make a contribution to the placenta after being cultured in vitro.

Table 1 Attachment Results of Original Cultures in Talbot's Media[A] by Treatment

	#embryos[B]	#attached[C]	%attached
Feeder+FGF	58	39	67.2
FGF Only	59	32	54.2
Feeder Only	75	54	72.0[d]
Plastic Only	75	40	53.3

A: Talbot, et al. (2000)
B: Total number of embryos used.
C: Number of embryos used that attached.
d. Significantly different as compared to other treatment groups ($P<.05$).

Table 2 Passage Results of Original Cultures in Talbot's Media[A] by Treatment

	#attached[B]	#passaged[C]	%passaged
Feeder+FGF	39	30	76.9[d]
FGF Only	32	12	37.5
Feeder Only	54	28	51.8
Plastic Only	40	14	35.0

A: Talbot, et al. (2000)
B: Number of embryos with initial attachment.
C: Number of attached embryos which remained viable for passage.
d: Significantly different as compared to other treatment groups ($p<.05$).

Table 3 Effects of Feeder Layers on Attachment and Passage

	#Embryos[A]	#Attached[B](%)	#Passaged[C](%)
Feeders	133	93[d](69.9)	58[d](62.3)
No Feeders	134	72 (53.7)	26(36.1)

A: Total number of embryos.
B: Number of embryos that attached.
C: Number of attached embryos that remained viable for passage.
d: Significantly different as compared to no feeders ($p<.01$).

Table 4 Effects of FGF-4 on Attachment and Passage

	#Embryos[A]	#Attached[B](%)	#Passaged[C](%)
FGF-4	117	71(60.7)	42[d](59.1)
No FGF-4	150	94(62.7)	42(44.6)

A: Total number of embryos.
B: Number of embryos that attached.
C: Number of attached embryos which remained viable for passage.
d: Significantly different as compared to No FGF-4 ($p<.1$).

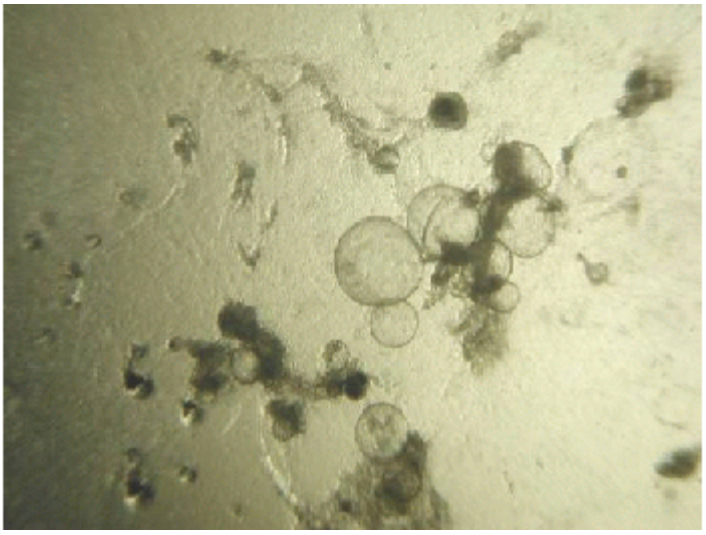

Figure 1: This culture was established from a 9 day hatched blastocyst with the use of FGF-4. Many vesicles of various sizes can be seen in this photo.

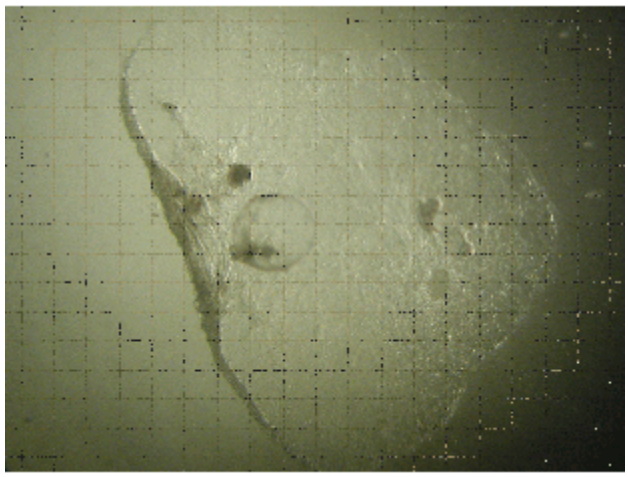

Figure 2: This photo shows the "sheet" nature of the culture. It also shows the beginning of the cells loss of adhesion from the culture dish (left side). This culture was established from a 10 day hatched blastocyst and grown without a feeder layer and without FGF-4.

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CONCLUSIONS

Placental deformities are a major hindrance in the production of nuclear transfer animals, and as such, the importance of establishing a line of trophoblast cells, the principle contributor to the placenta, cannot be over estimated. This study was undertaken to determine ideal culture conditions for bovine trophoblast cells. Studies in the mouse have shown that FGF-4 is required for trophoblast cells to remain undifferentiated. This is not the case in the bovine, as cultures could be established without the use of FGF-4. However, the use of FGF-4 did appear to increase the longevity of the cultures.

Results demonstrated that, while feeder layers do enhance the attachment rate of the blastocyst, they are not required to establish a culture of bovine trophoblast cells. All other known reports indicate the initial dependence of these cells on feeder layers, only becoming feeder layer independent after several passages.

Further research, into the making of chimeric embryos, was conducted, though the results were not conclusive and time did not permit the completion of that project. In brief, trophoblast cells from established cultures were labeled with a cell membrane marker and micro-injected into the periviteline space of bovine early blastocyst stage embryos. This was done to determine if the cells would contribute to the inner cell mass, the trophectoderm, or simply be extruded by the embryo as foreign material.

Only a minimal number of embryos were manipulated in this fashion, and it would be presumptuous to even assume any conclusive data resulted from these trials. However, by casual observation, under a fluorescent microscope, it is speculated, that with mastery of the technique, positive results should be forthcoming. Pursuit of experimentation initiated should facilitate

further understanding of placental abnormalities inherent with nuclear transfer embryos, and is an important step toward finding a remedy for those abnormalities.