수의학박사학위논문

소 체외수정 및 체세포핵이식 수정란에서 interferon tau 를 조절하는 유전자들의 상관관계

Temporal interplay between the genes that control interferon tau expression in early *in vitro* fertilization- and nuclear transfer-derived bovine embryos

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수의학과 수의산과학 전공

모하메드이슬람모하메드삿드엘딘

Islam Mohamed Saad Eldin

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Temporal interplay between the genes that control interferon tau expression in early *in vitro* fertilization- and nuclear transfer-derived bovine embryos

By Islam Mohamed Saad Eldin Mohamed

Supervisor: Professor Byeong Chun Lee, DVM, PhD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF PHYLOSOPHY OF DOCTOR

In

Theriogenology and Biotechnology
Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard

Goo Jang _____

Byeong Chun Lee _____

Jong Ki Cho _____

Min Kyu Kim _____

Se Chang Park _____

Seoul National University
August 2012

Declaration

This thesis is submitted by the undersigned for the examination for the degree of Doctor of Philosophy to Seoul National University. This thesis has not been submitted for the purpose of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition, work and experiments of this thesis are entirely my own.

Islam M. Saad Eldin

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Islam M. Saad Eldin

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Islam Mohamed Saad Eldin Mohamed

(Supervisor: Professor Byeong Chun Lee, DVM, Ph.D.)

Department of Theriogenology and Biotechnology

Department of Veterinary Medicine

Graduate School of Veterinary Medicine, Seoul National University

ABSTRACT

In the current work, the embryonic development and the temporal behavioral interaction of the genes involved in IFNτ gene expression and how they behave in an orchestrated manner to increase the developmental competence of IVF and NT produced embryos were investigated. Behavior of genes included ETS2, CDX2, GATA2, GATA3, OCT4 and NANOG was analyzed in early bovine IVF produced embryos, (from compact morulae to the blastocyst hatching stages), by semi- and relative quantitative PCR and compared between two *in vitro* culture (IVC) systems, two-step chemically defined medium and modified synthetic oviductal fluid (mSOF) containing 8 mg/mL, BSA.

Early embryonic development was found to be better in two-step chemically

defined culture system than that of mSOF as indicated by the increment of blastocyst yield, 33.1% in two-step culture system vs. 18.8% in mSOF medium, and the blastocyst hatching, 52.3% in two-step culture system vs. 33.5% in mSOF medium. Relative quantitative gene expressions showed harmonic behavior in the two-step culture system rather than the culture in mSOF, IFNτ showed even increase throughout the embryonic development in the two-step culture medium while it decreased with blastocyst hatching in mSOF culture condition.

Temporal dominance of OCT4 over all the transcription factors was found in regulation of IFNτ expression (the major factor of expression regulation but in inverse manner). However, ETS2, CDX2, GATA2 and GATA3 are potent IFNτ stimulator in cumulative manner but in case of OCT-4 decrement. CDX2 directly related with IFNτ, but still under OCT4 dominance and also regulated by the subservient of OCT4 which is NANOG.

These findings confirmed the usefulness of using the two-step chemically defined culture medium for increasing the developmental competence of IVF produced embryos and elucidated the dominance of OCT4 over the other genes implicated in regulation of IFNτ expression.

Moreover, bovine trophoblast cells (BTs) from IVM/IVF oocytes and *in vitro* produced blastocysts, were cultured, isolated and used them, for the first time, as donor cells for nuclear transfer and compared them with adult fibroblasts (AFs) as donor cells. BTs were reprogrammed in enucleated oocytes to blastocysts with similar efficiency to AFs (14.5% and 15.6% respectively, $P \leq 0.05$). The levels of

IFNτ, CDX2 and OCT4 expression in IVF-, BT- and AF-derived blastocysts were analyzed using reverse transcription polymerase chain reaction and reverse transcription quantitative polymerase chain reaction (RT-PCR and RT-qPCR). IVF-produced embryos were used as reference to analyze the linear progressive expression of IFNτ through mid, expanded and hatching blastocysts.

RT-PCR and RT-qPCR studies showed that IFNτ expression was higher in BT-derived blastocysts than IVF- and AF-derived blastocysts. Both IVF- and BT-derived blastocysts showed a progressive increase in IFNτ expression as blastocyst development advanced when it compared with AF-derived blastocysts. OCT4 was inversely related with IFNτ expression, while CDX2 was found to be directly related with IFNτ temporal expression. Persistence of high expression of IFNτ and CDX2 was found to be higher in BT-derived embryos than in IVF- or AF-derived embryos. These results could be a useful tool for understanding the IFNτ genetics and epigenetics.

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Key Words: Cow, somatic cell nuclear transfer, trophoblast cell, gene expression, implantation, Interferon-τ.

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List of abbreviations

Symbol	Name
AC	Adenylate cyclase
AF	Adult fibroblast
bIFN	Bovine IFN
BSA	Bovine serum albumin
BT	Bovine trophoblast
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CDX2	Homeobox protein CDX-2
CL	Corpus luteum
COCs	Cumulus-oocyte complexes
COX	Cyclo-oxygenase
DAG	Diacyl glycerol
DEPC	Diethyl pyrocarbonate
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco’s modified Eagle's medium
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ET	Embryo transfer
Ets-2	E-twenty six-transcription factor-2
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenasec
GATA2	GATA binding protein 2 - a transcription factor
GATA3	GATA binding protein 3 - a transcription factor
GFP	Green fluorescence protein
GM-CSF	Granulocyte monocyte-colony stimulating factor
ICBSP	Interferon consensus sequence binding protein
ICM/TE	Inner cell mass/Trophectoderm
IETS	International Embryo Transfer Society
IFN	Interferon
IFNT	Interferon tau (gene)
IFNτ	Interferon tau (protein)
IL	Interleukin
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	IFN-stimulated responsive element

IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
JAK	Janus kinase
KRT8	Keratin-8
MEF	Mouse embryonic fibroblast
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
mSOF	Modified synthetic oviductal fluid
NANOG	Homeobox protein NANOG
NEAA	Non-essential amino acids
NT	Nuclear transfer
Oct-4	Octamer-binding transcription factor 4
oIFN	Ovine IFN
oTP-1	Ovine trophoblast protein-1
OTR	Oxytocin receptor
PCR	Polymerase chain reaction
PGCs	Porcine granulosa cells
PGE	Prostaglandin E
$PGF_{2\alpha}$	Prostaglandin F 2 alpha
PGFM	PGF metabolite (15-keto-13,14 dihydro-PGF)
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PVA	Poly venyl alcohol
qPCR	Quantitative PCR
RIA	radioimmuonassay
RT-PCR	Reverse transcription-PCR
STAT	Signal Transducer and Activator of Transcription
TCM	Tissue culture medium
TGC	Trophoblast giant cell
TYK	Tyrosine kinase

LIST OF PUBLICATION

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1. **Islam M. Saadeldin**, BongHan Kim, ByeongChun Lee and Goo Jang (**2011**): Effect of different culture media on the temporal gene expression in the bovine developing embryos. **Theriogenology** 75(6):995-1004 (doi:10.1016/j.theriogenology.2010.11.006) (PMID: 21220156).

2. **Islam M. Saadeldin**, Ali M. Fadel, Mohamed M. Z. Hamada, Adel A. El-Badry (**2011**): Effects of exposure to 50 Hz, 1 Gauss magnetic field on reproductive traits in male albino rats. **Acta Vet. Brno** 80(1):107-111 (doi: 10.2754/avb201180010107).

3. **Islam M Saadeldin**, OkJae Koo, JungTaek Kang, DaeKee Kwon, SolJi Park, SuJin Kim, JoonHo Moon, HyunJu Oh, Goo Jang, ByeongChun Lee (2012): Paradoxical effects of kisspeptin: it enhances oocytes *in vitro* maturation while it has an adverse impact on hatched blastocysts during *in vitro* culture. **Reprod. Fertil. Dev.** 24(5):656-68.

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2. BongHan Kim, **Islam M. Saadeldin**, ByeongChun Lee and Goo Jang: The synergic effect of nerve growth factor and vascular endothelial growth factor on *in vitro* maturation and developmental competence in bovine oocytes. The proceedings of 37th annual conference of IETS, **Florida, USA**, January 8-12, **2011** (Cited: ***Reprod Fertiy Dev, 2011, 23:169-170***).
3. **Islam M. Saadeldin**, BongHan Kim, Bego Roibas da Torre, OKJae Koo, Goo Jang, ByeongChun Lee: IVF- or NT-derived trophoblast culture: An easy model for revealing interferon tau epigenetics and transgenesis. **The 2nd International Joint Workshop** between the United Graduate School of Veterinary Sciences,

Gifu University (UGSVS-GU) and College of Veterinary Medicine, Seoul National University (SNU), February 19-21, **2011**. Shiba-Yayoi hotel, **Tokyo, Japan.**

4. **Islam M. Saadeldin**, Ok Jae Koo, Jung Taek Kang, Dae Kee Kwon, Sol Ji Park, Su Jin Kim, Joon Ho Moon, Hyun Ju Oh, Goo Jang, and Byeong Chun Lee: Kisspeptin enhances porcine oocyte *in vitro* maturation but may adversely affect early embryonic development. Poster presentation at the 44th SSR Annual Meeting, July 31 - August 4, **2011**, in Portland, **Oregon, USA (**Cited**; *Biology of Reproduction, 2011, 85:434*)**.

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10. SuJin Kim, **Islam M. Saadeldin**, SongJeon Lee, WonWu Lee, BongHan Kim, ByeongChun Lee and Goo Jang: Production of transgenic bovine cloned embryos using piggybac transposition. **The 3rd International Joint Workshop** between the United Graduate School of Veterinary Sciences, Gifu University (UGSVS-GU) and College of Veterinary Medicine, Seoul National University (SNU), February 8-9, **2012**, Lotte city hotel, **Gimpo, Korea.**

PART 1

GENERAL INTRODUCTION

&

REVIEW OF LITERATURE

Introduction

The trophoblast interferon (IFNτ) was discovered in 1987 through the purification of extracts of ovine blastocyst-secreted proteins with antiluteolytic properties. The active principles were monitored by biological assays involving administration into the uterus of cyclic sheep. After purification to homogeneity, the antiluteolytic proteins were identified by N-terminal sequencing (Stewart *et al.*, 1987) and cDNA cloning (Imakawa *et al.*, 1987). At the time of the identification of IFNτ, there was already a large body of information on the role of blastocyst secreted proteins in the maternal recognition of pregnancy, and on the physiological processes underlying luteolysis and the establishment of pregnancy. There was also an extensive literature on related interferons, and several type I interferons were immediately available in quantities sufficient for experiments *in vivo*. As a result, the identification of the antiluteolysin led to rapid progress, particularly in relation to the control of its synthesis, its mechanism of action and its immunomodulatory and possible therapeutic properties. These advances and the background to them have been reviewed elsewhere (Bazer *et al.*, 1996; Martal *et al.*, 1998; Roberts *et al.*, 1999).

The unique pattern of IFNτ expression is regulated by promoter/enhancer regions that are distinct from that of other type I IFN genes (Ealy and Yang, 2009). One key component of IFNτ expression is Ets-2 transcription factor. Ezashi *et al.* (1998) first identified it as a regulator of IFNτ transcription. Subsequent findings determined

that a consensus Ets-2 binding site is present in all transcriptionally active bovine IFNτ. Moreover, at least three trophoectodermal transcription regulators are also involved with IFNτ expression regulation. Caudal-type homebox 2 (CDX2) stimulates IFNτ promoter activity in the presence of Ets-2 (Ezashi *et al.*, 2008, Sakurai *et al.*, 2009). Two additional regulators, GATA transcription factors 2 and 3 (GATA2 and GATA3), were recently found to have role in regulation of IFNτ expression (Bai *et al.*, 2009). In addition, the POU homeodomain protein (Oct-4), that is best known as a marker of pluripotency (Pesce and Scholer, 2000), blunts the ETS2 induced IFNτ promoter activity (Ezashi *et al.*, 2001) beside the inhibition of other factors during early pregnancy like CDX2 (Niwa *et al.*, 2005).

According to Hernandez-Ledezma *et al.* (1992), they suggested that the best way to study IFNτ expression as an indicator of embryo quality is the temporal expression than the absolute expression at a particular stage because the latter is known to vary widely.

The aim of the work

This study is subdivided into 2 parts preceded by a general review of literature; in part 1 the work was undertaken using *in vitro* fertilization-derived embryos to: (1) compare the developmental competence of bovine embryos that were cultured *in vitro* cultured in a two-step chemically defined medium or modified synthetic oviductal fluid medium (mSOF) containing 8 mg/mL, BSA; (2) compare the gene

expression patterns in these embryos; and (3) elucidate the interplay and dominance among the major factors controlling the developmental competence markers of bovine embryos specifically IFNτ and its regulator genes (CDX2, ETS2, GATA2, GATA3, NANOG, and OCT4). In part 2, for detailed description of trophoblast function/IFNτ expression using nuclear transfer technique, the aim was to (1) elucidate if trophoblast, as an interferon tau secreting cell, can be reprogrammed in bovine enucleated oocyte; (2) determine the relative abundance of IFNτ expression in the resulting cloned preimplantation embryos; and (3) study the temporal gene interaction affecting IFNτ expression, especially the OCT4 and CDX2 genes, in comparison with other kinds of embryos.

LITERATURE REVIEW

1- Early embryonic development and implantation

Fertilization refers to the union of male and female gametes to form a zygote, a process that begins with the entry of a spermatozoon into a secondary oocyte. It is completed when the nuclear envelopes of the male and female pronuclei break down and the chromatin condenses into chromosomes which are oriented on a common mitotic spindle. As in other mammals, in cattle there is close apposition of pronuclei, followed by pronuclear membrane breakdown without fusion (Longo, 1973)

The period before attachment is correctly referred to as the pre-attachment

stage (Betteridge and Fléchon, 1988). The stage of the early embryo is very important from two perspectives. The first of these is ‘maternal recognition of pregnancy,' a crucial step in the successful establishment of pregnancy. The second issue is that this period is critical and important from the perspective of embryo manipulation.

The early embryonic period includes the following developmental processes: cleavage, compaction, blastulation, expansion, hatching, and elongation. Successful completion of these sequential events is important for establishment of pregnancy. Among the events that occur during the embryonic period, the most critical and culminating event leading to a successful pregnancy is ‘implantation’. The term implantation refers to the process by which the embryo implants or inserts itself to the endometrium. The implantation process requires a receptive endometrium, a functionally normal embryo at the blastocyst stage and a dialogue or cross-communication between the maternal and embryonic tissues especially through interferon tau, which is the major crucial cytokine for establishing the implantation (Peippo *et al.*, 2011).

2- Interferon tau (IFNτ)

2-1 IFNT mRNA and protein profiles during early pregnancy (Maternal recognition of pregnancy)

The antiluteolytic effects of the IFNτ are responsible for the maternal recognition of pregnancy, which is the term used to describe how a mother responds

(physiologically) to the presence of a conceptus in her reproductive tract. In domestic ruminants, the developing embryo does not implant until relatively late in development, although the conceptus is clearly capable of communicating with the mother well before implantation occurs, and before the conceptus has access to the maternal circulation. Failure of the conceptus to signal its presence at the appropriate time leads to pregnancy loss. The pioneering experiments of Moor and Rowson (1964, 1966a,b) revealed that the maternal recognition of pregnancy in ewes takes place at about day 12–13 of pregnancy. Transfer of blastocysts to the non-pregnant uterus before days 11–12 in sheep prolonged luteal function, whereas removal of blastocysts from the uterus of pregnant animals before this time did not extend the life of the corpus luteum. Furthermore, infusing homogenized day 14 or 15 conceptuses into the uterine lumen extended luteal function in non-pregnant sheep, although this antiluteolytic effect was absent when day 25 embryonic homogenates were infused. It was evident that a substance released by preimplantation conceptuses prevents luteolysis. Isolated trophoblastic vesicles transferred into the uterus extended the life of the corpus luteum in both sheep and cattle, so the antiluteolysin (termed ‘trophoblastine’ at this time) was known to be a product of the embryonic trophoblast (Martal *et al.*, 1979).

The expression of IFNT occurs during a defined period of pre- and peri-attachment conceptus development in cattle and sheep. As outlined in Fig. 1, IFNT mRNA and IFNτ protein is first detected as trophectoderm forms at the late morula to early blastocyst stage of development (Hernandez-Ledezma *et al.*,

1992, Kubisch *et al.*, 1998). Both mRNA and protein levels are relatively low at the blastocyst stage of development but increase with advancing age of the spherical conceptus (Kubisch *et al.*, 2001). At day 14–15 of pregnancy, IFNT mRNA levels increase dramatically in cattle (Ealy *et al.*, 2001). A similar surge in IFNT mRNA is observed in ovine conceptuses at day 12–13 of pregnancy (Anthony *et al.*, 1988, Winkelman *et al.*, 1999). In both species, increases in IFNT mRNA concentrations coincide with conceptus elongation, where trophectoderm proliferation causes substantial increases in overall conceptus size and mass of the trophectoderm. Bovine conceptuses progress from spherical masses of ~0.5 mm in diameter on day 10–12 of pregnancy to filamentous structures that are 10–30 cm in length on day 16 of pregnancy, the time when maternal recognition of pregnancy must occur for pregnancy to proceed in cattle, and 50–200 cm at day 21 of pregnancy when placental attachment begins (Betteridge and Flechon, 1988).

This extensive trophectoderm mass not only provides a substantial surface area for contact with the uterine lining prior to placental attachment but also amplifies the overall amount of IFNτ protein produced during early pregnancy (Fig. 1). When considering that the level of IFNT mRNA also increases at this time, profound amounts of IFNτ are available to interact with the maternal system and generate the maternal recognition of pregnancy signal. In one study, culture of individual filamentous ovine conceptuses produced 25–500 ng of IFNτ each hour over a 48-hr period (Ashworth and Bazer, 1989).

The constitutive expression of IFNT is not long-lasting; levels of IFNT mRNA

decline sharply after day 21 of pregnancy in cattle, coincident with trophectoderm attachment to the uterine lining (Bartol *et al.*, 1985, Helmer *et al.*, 1987, Ealy *et al.*, 2001). Mechanisms controlling this reduction in expression remain speculative but probably result from changes in gene regulatory events occurring because of trophectoderm differentiation and/or uterine attachment. Ruminant conceptuses do not invade into the uterine lining after blastoceol formation and zona hatching, as is the case for rodents and humans. Instead, the developing bovine conceptus remains free-floating in the uterine lumen before it begins to attach to the uterine lining until on or after day 19 of pregnancy (Betteridge and Flechon, 1988, Wooding, 1982, Wooding, 1992). Uterine invasion is limited to the erosion of the luminal epithelial lining. A specialized cell type derived from trophectoderm, termed the trophoblast giant cell (TGC) or binucleate cell is responsible for syncytium formation. This cell lineage is multinucleated and hyperploidic and possesses the ability to traverse the uterine endometrium and fuse with epithelial cells to create an endometrium that is nearly devoid of luminal epithelium by day 25 to 30 of pregnancy (Klisch *et al.*, 1999a,b).

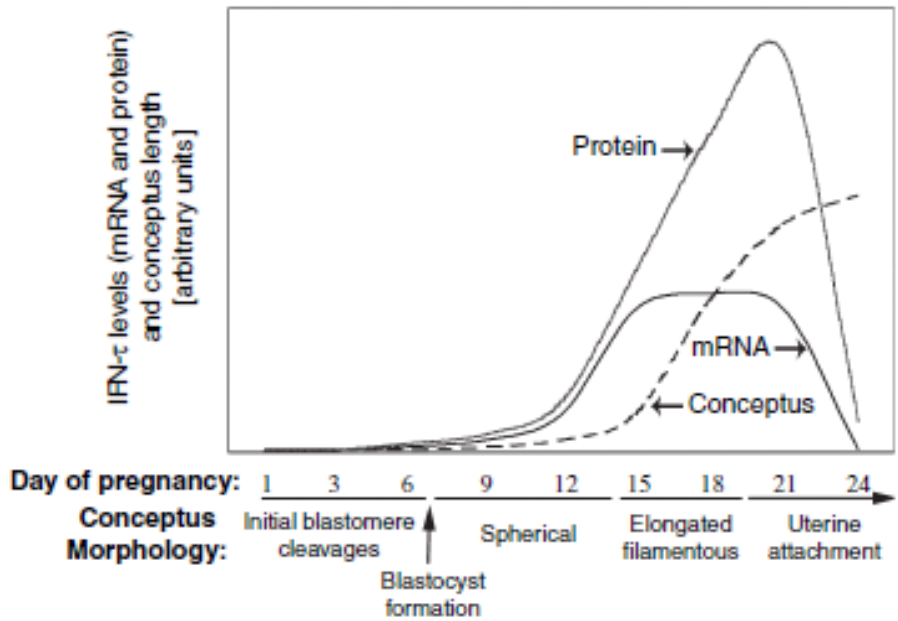

Figure 1. The ontogeny of IFNT expression during early pregnancy in cattle. Detectable quantities of IFNτ protein are found in conditioned medium at the late morula and early blastocyst stage of bovine development. IFNT mRNA is also first detected at these stages, and relative abundance of IFNT mRNA increases slowly until around day 14–16 of pregnancy when mRNA concentrations increase coincident with conceptus elongation. IFNT mRNA levels decrease sharply around days 19–21 of pregnancy coincident with trophectoderm attachment to the uterine lining. The bovine conceptus, and more specifically its trophectoderm layer, undergoes an exponential growth phase during this period of maximal IFNT expression. This event produces large quantities of IFNτ protein during pre- and peri-attachment development (Ealy and Yang, 2009).

2-2 Discovery of trophoblast interferon

Godkin *et al.* (1982) purified a protein secreted by the sheep conceptus that seemed to bear the hallmarks of the antiluteolysin. This protein consisted of several isoforms with a molecular weight of approximately 18000, and was the major secretory product of the conceptus trophoblast tissue between day 13 and day 15 of pregnancy, the time of maternal recognition of pregnancy. This protein, initially called ‘ovine trophoblast protein 1’ (oTP-1), is now known as ovine interferon–tau (oIFNτ). The trophoblast protein was produced maximally for only a few days of pregnancy, at a time when the trophoblast was attached only loosely to the uterine wall. Production of oIFNτ increased over three orders of magnitude from day 12 to day 16, when synthesis was maximal. When injected into the uterine lumen of cyclic ewes, purified oIFNτ mimicked the effect of conceptus homogenates by delaying luteal regression, and immunoneutralized extracts did not, indicating that IFNτ alone is sufficient for maternal recognition of pregnancy. The trophoblast signal was not detectable in the peripheral circulation of pregnant sheep, indicating that it does not leave the uterus to act on the corpus luteum. Instead, oIFNτ acts directly on the uterine endometrium, where it alters protein and PGF2α production. Endometrial cells express type I interferon receptor subunits IFNAR1 and IFNAR2 (Kaluz *et al.*, 1996) and, although the ruminant receptor subunits differ from those in man (with 67 and 58% sequence identity, respectively; Han *et al.*, 1997) they have the same structural features. A protein with similar properties, produced between day 16 and day 24 of gestation, was characterized subsequently as a

secretary product of bovine conceptuses. This protein, bIFNτ, was shown to be antiluteolytic and to alter endometrial PGF2α output. bIFNτ has molecular masses of 22000 and 24000 kDa, each with multiple isoforms, and is glycosylated with N-linked oligosaccharides. In contrast, oIFNτ is not glycosylated. A caprine IFNτ has been identified, which crossreacts with antiserum to oIFNτ and is secreted between day 16 and day 21 of pregnancy. The proteins of this complex consist of a mixture of both glycosylated and non-glycosylated polypeptides.

2-3 Isoforms and structures

The IFNτ genes expressed by ruminant conceptuses share approximately 70% homology with IFN-w (also known as IFNαII). A 595 bp open reading frame encodes a 195 amino acid pre-protein containing a 23 amino acid signal sequence which is cleaved to yield the mature protein. IFNτ shows remarkable homology of cDNA nucleotide sequence across ruminant species. Bovine, ovine and caprine IFNτ are more similar in sequence to each other than bIFNτ is to bovine IFN-w. In the coding region, the nucleotide sequences exhibit approximately 90% identity, and their inferred amino acid sequences about 80% identity. Eighteen IFNτ cDNA or genomic sequences have been described in sheep and 11 in cattle, although not all are transcribed. Parsimony analysis indicates that the ovine isoforms may be placed into five related groups. Within each species, IFNτ sequences are similar, as a result of the gene duplication events by which they arise having occurred relatively recently, and it is difficult to distinguish differences among individual genes from

allelic variation. In sheep, these genes appear to display distinct patterns of expression in the trophectoderm and are subject to different developmental regulation during pregnancy. Why there should be multiple isoforms of IFNτ is unclear since individual ovine IFNτ isoforms extend corpus luteum function when injected into the lumen of cyclic ewes. Ealy *et al.* (1998) have demonstrated that isoforms of ovine IFNτ vary in their biological potency, including their ability to extend corpus luteum lifespan in non-pregnant ewes. The additional six carboxy terminal amino acids in IFNτ (relative to IFNα or -β) do not affect the antiviral or antiluteolytic properties of the molecule, as shown by comparing the wild-type and mutated molecules (Ealy *et al.*, 1998). However, other mutations at the carboxyl terminus of the molecule do alter both antiviral and antiluteolytic properties. These experiments show that the antiluteolytic activity of IFNτ is related to its antiviral and antiproliferative properties and not necessarily to its receptor affinity (Niswender *et al.*, 1997).

2-4 Mechanisms of IFNτ action

2-4-1 Antiluteolytic properties

IFNτ suppresses the normal pattern of pulsatile release of uterine PGF2α leading to luteolysis at the end of the oestrous cycle (Fig. 2). In sheep, basal PGF2α production is not eliminated, and circulating concentrations of the PGF2α metabolite, 15-keto-13,14-dihydro-PGF (PGFM), are higher in pregnancy than

during the oestrous cycle. Nevertheless, in pregnant animals, pulses of PGF2α are diminished, and the corpus luteum remains functional. Generation of luteolytic episodes of PGF2α secretion requires luteal secretion of oxytocin and the interaction of circulating oxytocin with its receptor, which is located principally on endometrial epithelial cells. IFNτ modulates uterine PGF2α release by inhibiting endometrial oxytocin receptor expression (Flint *et al.*, 1992). Northern blotting shows that this effect is exerted at the level of gene transcription (Stewart *et al.*, 1993). In sheep, the endometrial oxytocin receptor is not expressed before day 10 after mating. After day 20, luteal concentrations of oxytocin mRNA are low and the corpus luteum is incapable of secreting oxytocin. Therefore, the time at which IFNτ is expressed coincides with the period over which luteal oxytocin is available to stimulate PGF2α secretion (Flint *et al.*, 1992). Since, in sheep and cattle, IFNτ blocks episodes of PGF2α secretion driven by luteal oxytocin, it might be expected that IFNτ would not be expressed by the blastocyst in those species not expressing oxytocin in the corpus luteum. This expectation raises the question of whether expression of oxytocin at high concentrations in the corpus luteum and trophoblast secretion of IFNτ have arisen in the same species, that is, in artiodactyls that have evolved during the last 36 million years? It appears they have: high concentrations of luteal oxytocin and trophoblast secretion of IFNτ are both present in the cervids (and are also involved in the maternal recognition of pregnancy in red deer Cervus elaphus; Demmers *et al.*, 1999, 2000) but neither is present (at least in

physiologically relevant concentrations) in equids, suids or camelids. The giraffids have IFNτ (Liu *et al.*, 1996), but it is not known whether they have luteal oxytocin. The only exception to this rule appears to be roe deer Capreolus capreolus in which luteal oxytocin is present, but the blastocyst enters a long period of preimplantation diapause before elongation, and does not express IFNτ (*Flint et al.*, 1994). IFNτ is not required in the roe deer because oxytocin does not cause PGF2α release, so luteolysis does not occur and there is no preimplantation maternal recognition of pregnancy. Because oxytocin receptor gene expression is induced by oestrogen (McCracken *et al.*, 1984), it has been suggested that IFNτ affects the oxytocin receptor through an inhibitory action on the oestrogen receptor (Fig. 3). There is a precedent for this mechanism, as type I interferons activate protein kinase C and, in many cells, phorbol esters, which also activate protein kinase C, increase the turnover of oestrogen receptor mRNA and reduce concentrations of oestrogen receptor protein (Martin *et al.*, 1995). However, IFNτ may also reduce the transcriptional activity of the oestrogen receptor without affecting receptor concentrations, as Robinson *et al.* (1999) have shown that the downregulation of oxytocin receptor expression precedes any change in oestrogen receptor concentration in bovine endometrial epithelium. Surprisingly, in the short term, IFNτ increases oestrogen receptor transcriptional activity (Flint *et al.*, 2000). Bazer *et al.* (1997) proposed that the mechanism by which IFNτ suppresses oxytocin receptor expression involves the type I interferon receptor signal transduction

system and several members of the interferon-induced transcription factor family (Fig. 3). This family includes interferon- stimulated gene factor-3 (ISGF3), interferon regulatory factor 1 (IRF-1), IRF-2, interferon consensus sequence binding protein (ICBSP) and lymphoid-specific IRF. Binding of a type I interferon to its receptor immediately activates the latent tyrosine kinases, JAK1 and tyk2, which phosphorylate tyrosine residues of signal transducers and activators of transcription 1 (STAT1), STAT1A and STAT2 (Stark *et al.*, 1998). These three phosphoproteins then bind a fourth DNA-binding protein and the resulting interferon-stimulated gene factor binding complex is transported to the nucleus, where it binds to an IFN-stimulated responsive element (ISRE) present in the promoter–enhancer region of interferon-responsive genes. This activates transcription of interferon-responsive genes such as IRF-1, which, in turn, activate expression of the negative acting transcription factor IRF-2. Bazer *et al.* (1997) suggest that an IFNτ-induced regulatory factor (possibly IRF-2) suppresses expression of the oestrogen receptor directly by binding to an IFNτ-responsive element in the oestrogen receptor gene. The same factor also blocks, either directly or indirectly, the expression of the oxytocin receptor gene, preventing the uterine luteolytic mechanism and ensuring the establishment of pregnancy.

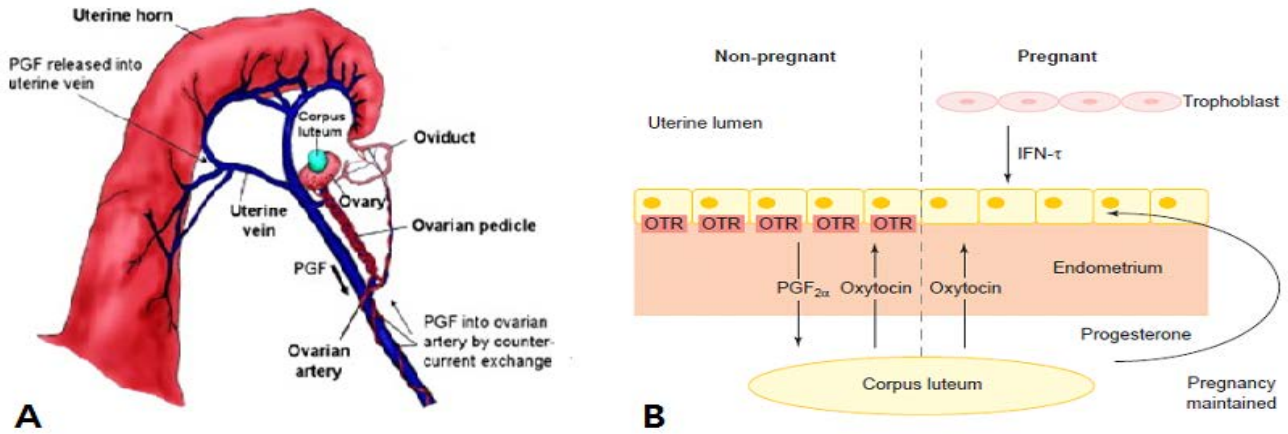

Figure 2: Tissues, hormones and hormone receptors involved in the maternal recognition of pregnancy. A) The countercurrent exchange system showing the flow of prostaglandin F2 (PGF2) from endometrium to ovary through uterine vein and ovarian artery respectively (Frode *et al.*, 2011). B) In non-pregnant animal, luteolysis occurs as a result of uterine secretion of PGF2 that driven by oxytocin to generate a positive feedback loop. In pregnancy, oxytocin receptor expression is blocked by trophoblast interferon. By blocking luteolysis, the conceptus ensures continued exposure of the endometrium to high circulating concentrations of progesterone, which, in turn, maintains the secretory activity of the endometrial glands, which provide nutrients required for blastocyst growth. Receptors for IFNτ and for PGF2 are expressed constitutively on endometrial and luteal target cells (modified after Demmers *et al.*, 2001).

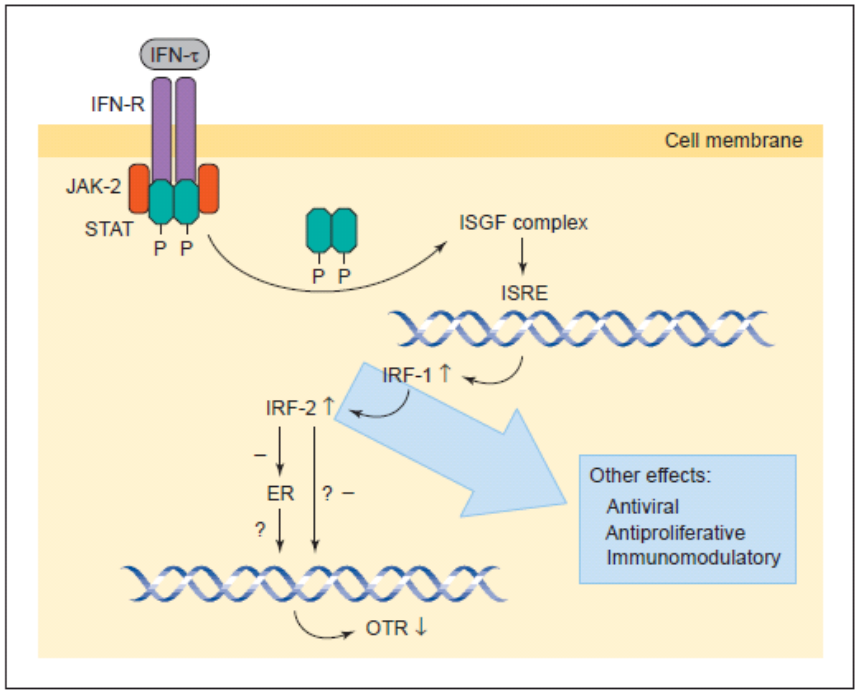

Figure 3: Proposed mechanism of action of interferon (IFNτ) on endometrial cells. IFNτ binds to a dimeric interferon receptor (IFN-R) spanning the cell membrane. The intracellular domain of the receptor binds tyrosine kinases (Janus kinases, JAKs), which are activated after interferon binding, and subsequently phosphorylate other proteins named signal transducers and activators of transcription (STATs). The STATs dimerize and bind two other proteins to form a trimeric interferon-stimulated gene factor (ISGF) complex, which is translocated to the nucleus, where it binds an interferon-stimulated regulatory element (ISRE), resulting in the expression of the IRF-1 gene. The product of this gene, in turn, activates expression of IRF-2, which interacts with other regulatory elements to control the expression of interferon-responsive genes, including the oxytocin (OTR) and oestrogen (ER) receptors. The effects of the IRFs include the induction of antiviral, antiproliferative and immunomodulatory responses to interferons (Demmers *et al.*, 2001).

2-4-2 Immune effects of IFNτ

In addition to controlling oxytocin receptor gene expression in the endometrium, IFNτ affects the synthesis of other cytokines that contribute to the immunomodulation required to prevent rejection of the conceptus and stimulate blastocyst growth. IFNτ increases expression of IFNγ and interleukin 4 (IL-4) by bovine lymphocytes *in vitro* (Tuo *et al.*, 1999) and reduces the proliferative responses of lymphocytes to IL-2 (Niwano *et al.*, 1989). IFNτ increases endometrial cyclo-oxygenase 2 (COX-2) concentrations and PGE2 production (Dannet-Desnoyers *et al.*, 1994), which may contribute to the reduction in IL-2 expression observed in bovine lymphocytes and endometrium (Emond *et al.*, 1998; Leung *et al.*, 2000). IFNτ may also affect immunomodulatory cell–cell interactions through increased expression of MHC class I molecules on endometrial cells (Todd *et al.*, 1998) and by decreasing the expression of transforming growth factor b (TGF-b) and retinol-binding protein (Godkin *et al.*, 1997). Pregnancy is thought to involve a shift from a Th1 towards a Th2 immune environment, with a reduction in inflammatory, cytotoxic T-cell responses in favour of B-cell activation and a decrease in proinflammatory interleukins such as IL-1a, IL-2 and IL-6 (Wegmann *et al.*, 1993). Leung *et al.* (2000) found no evidence for a change in lymphocyte populations at the time of the maternal recognition of pregnancy and so this shift presumably occurs principally after the cessation of IFNτ production. However, an increase in PGE2 production together with a decrease in IL-2 IFNτ may initiate these changes. PGE2 also enhances GM-CSF production by peripheral lymphocytes

and endometrium and, as described above, GM-CSF may induce further IFNτ synthesis and blastocyst growth. Other endometrial cytokines induced by IFNτ include bovine granulocyte chemotactic protein 2 (Hansen *et al.*, 1999). In addition, IFNτ induces the ubiquitin crossreactive protein or interferon-stimulated gene 17 product (ISG-17; Hansen *et al.*, 1999), which controls cytosolic protein processing through the proteosome; osteopontin (Johnson *et al.*, 1999), which promotes cell–cell attachment and may be involved in attachment of the blastocyst to the endometrial epithelial surface; and the antiviral Mx protein (Ott *et al.*, 1998).

2-4-3 Antiviral and antiproliferative properties

The antiviral and antiproliferative properties of type I interferons have been reviewed by Stark *et al.* (1998). The antiproliferative effects of IFNτ, which in Daudi cells (a human Burkitt lymphoma cell line) are less marked than those of IFNα, result in cell cycle blockade at G1, probably through inhibition of the cyclin-dependent kinase, cdk2 (Subramaniam and Johnson, 1997). The differential cytotoxicity is due to different affinities of IFNα and -τ for the type I receptor. In Madin Darby bovine kidney cells, IFNα has a higher affinity than IFNτ for the receptor (Subramanian *et al.*, 1995). Antiviral activity depends on low receptor occupancy, but the antiproliferative effect is manifested only at higher occupancy, and so requires higher concentrations of IFNτ than of IFNα. Consistent with the lack of involvement of the carboxy terminal six amino acid extension in IFNτ, the differences in binding affinity reflect differences in the interactions of the N-

terminal ends of the molecules with the receptor. Because IFNτ has a reduced cytotoxicity compared with IFNα, -β or -γ (Soos and Johnson, 1999), it may be useful therapeutically. IFNτ causes fewer side effects than IFNβ at effective doses in experimental murine allergic encephalomyelitis, an autoimmune animal model for multiple sclerosis (Soos and Johnson, 1999) and has potent antiviral activity against the human and feline immunodeficiency retroviruses and against ovine lentivirus and human papilloma virus. However, it does have acute effects after systemic administration on T-cell populations, and can cause symptoms of acute cytokine poisoning in some species (for example, red deer; Demmers *et al.*, 2000).

2-5 Mechanisms controlling IFNτ synthesis

Stewart *et al.* (1989) showed by northern blotting of blastocyst mRNA, and Farin *et al.* (1990) demonstrated by in situ hybridization, that oIFNτ is expressed transiently during a limited period of development, and that its expression is localized to the extra-embryonic trophectoderm of bovid conceptuses. The mRNA is detectable in low concentrations in embryos from day 10 to day 12 of pregnancy, increases from day 13 to day 15, coincident with the time of maternal recognition of pregnancy, and decreases shortly afterwards. Peak production occurs at day 16 in sheep (Godkin *et al.*, 1982) and day 17 in cattle (Bartol *et al.*, 1985), and IFNτ mRNA is detectable in the trophectoderm until about day 20 in sheep or day 25 in cattle. At the time of peak production, IFNτ mRNA is present in blastocysts at a higher concentration than any other mRNA. Ashworth and Bazer (1989)

demonstrated by radioimmunoassay that IFNτ is detectable in the media of cultured sheep blastocysts shortly after hatching from the zona pellucida, which is at least 1 week before peak production at the time of conceptus elongation, but the concentrations involved are unlikely to elicit a physiological response at this stage. The marked increase in the expression of the IFNτ gene on day 13 in sheep and day 15 in cattle coincides with the morphological transition of the blastocyst from a spherical to a filamentous form, rather than strictly correlating with the day of pregnancy. The onset of IFNτ expression appears to be genetically programmed independently of the maternal uterine environment, since IFNτ is expressed *in vitro* after *in vitro* fertilization and maturation. However, conceptus IFNτ production is clearly affected by the uterine environment, as IFNτ production by ovine conceptus tissue *in vitro* is higher when cultured in the presence of endometrial tissue. Furthermore, the maternal plasma progesterone concentration, which controls uterine glandular secretion, is correlated with IFNτ production by the conceptus in cattle (Kerbler *et al.*, 1997; Mann *et al.*, 1999). Termination of IFNτ expression is dependent on implantation, since cessation of oIFNτ expression occurs in the regions of the trophoblast that have established cellular contacts with the uterine epithelium during the implantation process.

2-6 The transient nature of IFNτ secretion

The rapid onset and cessation of IFNτ gene expression in the trophectoderm of the elongating blastocyst makes this family of genes of particular interest in the

study of transcriptional regulation (Fig. 4). Furthermore, unlike other groups in the type I interferon family, the IFNτs are not induced by viruses (Guesdon *et al.*, 1996). The enhancer and promoter sequences upstream of the IFNα and -β genes are well characterized and contain sequences known to confer viral induction. In contrast, the upstream sequences 5' to the IFNτ genes, which are conserved among members of the group, are quite distinct, and do not contain viral induction sequences. In searching for the sequences responsible for controlling IFNτ expression, Ezashi *et al.* (1998) emphasized the role of the Ets family of transcription factors. They used yeast one-hybrid analysis of a day 13 conceptus cDNA library and electromobility shift assay, and suggested that Ets-2 activates gene transcription through a specific enhancer sequence, CAGGAAGTG, located between –78 and –70 bp upstream of the transcription start site. On co-transfection of an IFNτ promoter (–126 to +50 bp) luciferase reporter construct and an Ets-2 expression plasmid into human choriocarcinoma (JAR) cells, luciferase expression was increased up to 30-fold by concurrent Ets-2 expression. A mutated Ets-2 motif found in inactive IFNτ pseudogenes (with the central sequence TGAA in place of GGAA) was not activated by Ets-2 co-transfection. Ets-2 was shown to be present in day 15 trophectoderm nuclei by immunocytochemistry and, taken together, these data implicate Ets-2 in the onset of IFNτ gene expression. It is presumed that Ets-2 controls IFNτ expression since the gene duplication event leading to the evolution of the IFNτ group resulted in the new gene being placed immediately downstream of the Ets-2 enhancer sequence. Other factors that may activate IFNτ gene

expression include granulocyte–macrophage colony-stimulating factor (GM-CSF), acting via the proto-oncogene c-jun and an AP-1 site at –654 to –555 bp (Imakawa *et al.*, 1993; Yamaguchi *et al.*, 1999). It is not surprising that Ets-2 and AP-1 pathways are involved in an event (IFNτ gene expression) so closely associated with a rapid phase of blastocyst growth, as both these factors control genes responsible for cell proliferation. However, Ets-2 and AP-1 are expressed in a wide variety of tissues, while IFNτ is expressed only in the trophoblast, and it is unlikely that IFNτ expression is controlled by these factors alone. Guesdon *et al.* (1996) and Yamaguchi *et al.* (1999) have identified negative regulatory domains in the bovine IFNτ promoter that may be involved in the precisely timed cessation of gene expression.

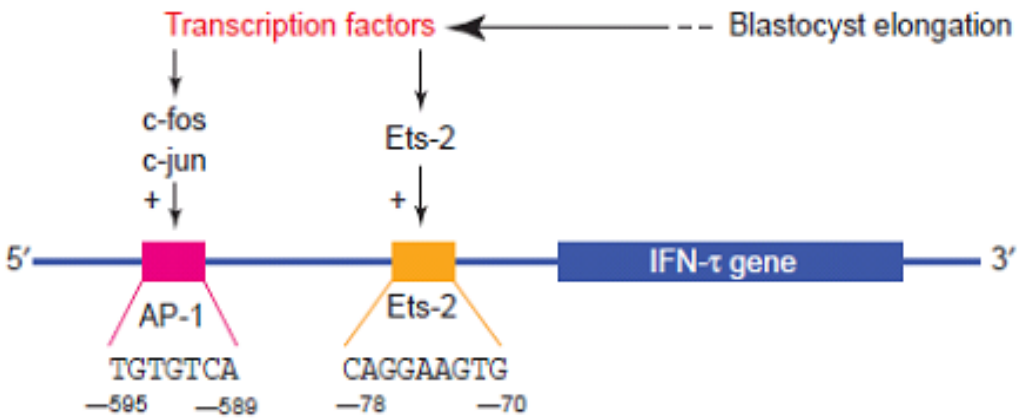

Figure 4: Control of interferon (IFNτ secretion by the developing conceptus). Expression of several IFNτ genes, which code for different isoforms, is induced through the actions of transcription factors. The activatory sequences respond to the transcription factors Ets-2 and c-fos–c-jun. (modified after Demmers *et al.*, 2001).

2-7 Transcriptional control of IFNT expression

The unique pattern of IFNT expression is regulated by promoter/enhancer regions that are distinct from that of other type I IFN genes. Much has been learned over the past 10–15 years about how IFNT transcription is controlled during early pregnancy (Fig. 5). One key component of IFNT expression is the Ets2 transcription factor; a fairly ubiquitous factor that regulates expression of other placental factors in various species, including placental lactogen-II in the mouse (Sun and Duckworth, 1999) and hCGβ in the human (Ghosh *et al.*, 2003, Johnson and Jameson, 2000). Ezashi *et al.* (1998) first identified Ets2 as a regulator of IFNT transcription by using the yeast one hybrid system to screen a day 13 ovine conceptus cDNA library for nuclear protein binding partners specific for a proximal IFNT promoter/enhancer region (−91 to −69 bp). This specific promoter/enhancer region associates with nuclear proteins in electromobility shift assays (Leaman *et al.*, 1994). Subsequent findings determined that a consensus Ets2 binding site is present in all transcriptionally active ovine and bovine IFNT. Also, promoters containing the cognate Ets2 site are transcriptionally active in human choriocarcinoma cells (Jar cells) when co-transfected with an Ets2 expression plasmid (Ezashi *et al.*, 1998).

At least two trophectodermal transcription regulators are also involved with regulating IFNT expression. Caudal-type homeobox 2 (Cdx2) is a transcription factor required for trophectoderm lineage emergence in the mouse (Ralston and Rossant, 2008, Strumpf *et al.*, 2005). It localizes to trophectoderm during ovine

conceptus development, and over-expression in JEG3 cells or a non-placental cell line (NIH3T3) stimulates IFNT promoter activity in the presence of Ets-2 and AP-1 (Imakawa *et al.*, 2006). A second factor, distal-less 3 (DLX3), is a homeodomain factor required for placental development in mice (Morasso *et al.*, 1999). It is expressed in trophectoderm of various mammals, including a bovine trophectoderm cell line and acts cooperatively with Ets2 in Jar choriocarcinoma cells to optimize IFNT transcription (Ezashi *et al.*, 2008).

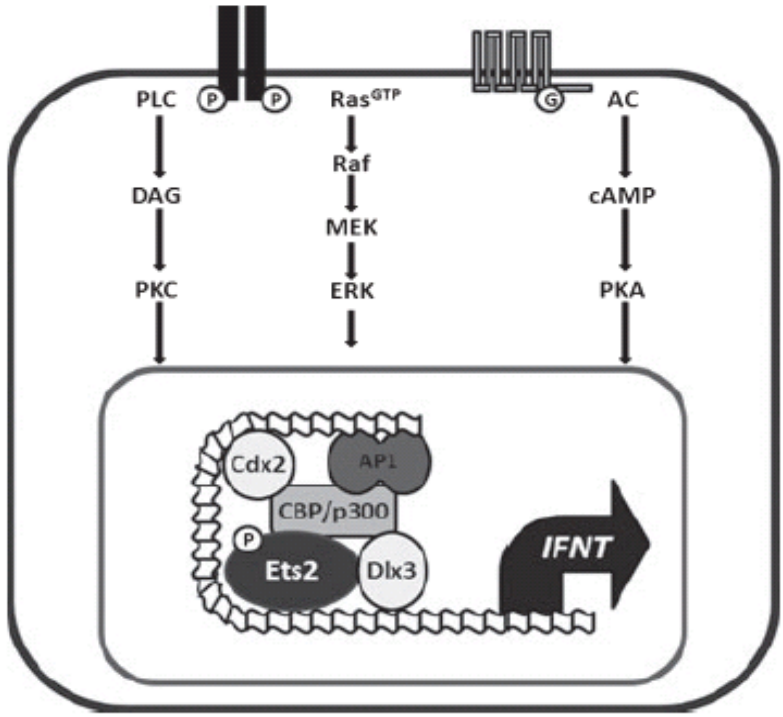

Figure 5. A model for describing how extracellular factors mediate IFNT expression. At least three signaling systems (PKC, MAPK, PKA) are implicated in stimulating IFNT expression in trophectoderm. Presumptive ligands may include but are not necessarily limited to GM-CSF and FGF2. These signaling cascades impact IFNT expression by affecting the activity of several transcriptional regulators, such Ets2 and AP1, and by promoting co-activator coupling, as depicted here by the assembly of a transactivation complex after recruitment of CBP (or its close relative p300). (Ealy and Yang, 2009).

2-8 How is the temporal expression pattern of IFNT controlled?

It is clear that Ets2 is a central component of the core transcriptional circuit that enables IFNT transcription in trophectoderm (Degrelle *et al.*, 2005). However, unlike IFNT, Ets2 production continues throughout gestation in trophectoderm. Also, Ets2 is expressed by other embryonic and adult tissues. The spatial and temporal control of IFNT expression in developing conceptuses must, therefore, be controlled by other factors. The trophectoderm-restricted expression of IFNT probably occurs in large part because of the regulatory activities of Cdx2, Dlx3 and potentially other trophectoderm-specific factors (Imakawa *et al.*, 2006). The temporal expression of IFNT is more difficult to quantify, but several ideas have been put forth (see Fig. 6).

There likely are at least two mechanisms involved with limiting IFNT expression early in embryo development (*i.e.* prior to the late morula/early blastocyst stages). The absence of Cdx2 and Dlx3 during the initial cleavage stages of embryo development likely compromises the ability of early embryos to produce IFNτ (Imakawa *et al.*, 2006, Ezashi *et al.*, 2008). Also, a functional block in Ets2 activity exists during these initial cleavage stages. The POU homeodomain protein, Oct4, blunts the Ets-2-induced IFNT promoter activity in Jar cells (Ezashi *et al.*, 2001) (see Fig 6a). Oct4 is best known as a marker of pluripotency in early embryonic cells, embryonic germ cells, and embryonic and adult stem cells (Pesce *et al.*, 1998, Pesce and Scholer, 2000). Oct4 also inhibits the expression of other factors during early pregnancy. For example, Oct4 inhibits Cdx2 expression in

mouse embryos and embryonic stem cells (Niwa *et al.*, 2005). Oct4 expression is restricted to the inner cell mass (ICM) after blastocyst formation in the mouse. However, Oct4 expression is not as restrictive in other species. Oct4 expression can be detected in both the ICM and trophectoderm in bovine and porcine blastocysts (Kirchhof *et al.*, 2000) and immunoreactive Oct4 can be detected in bovine trophectoderm up to day 10 of pregnancy (Van Eijk *et al.*, 1999), or approximately 3 days after blastocyst formation but several days before the massive increase in IFNT mRNA (see Fig. 1). To summarize, the lack of IFNT expression before blastocyst formation likely is caused by a combination of Oct4 repression of Ets2 activity and the lack of Cdx2 and Dlx3 activity, and the slow increase in IFNT mRNA levels between days 7 and 14 of pregnancy may be because of the reduction and eventual loss of Oct4 in trophectoderm (Fig. 6).

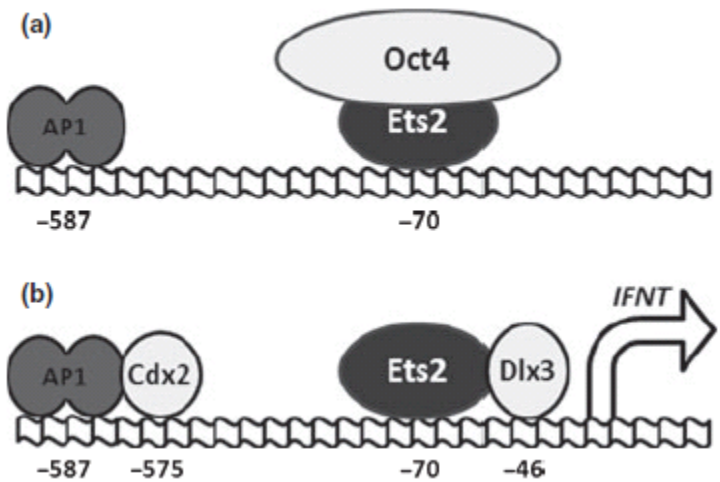

Figure 6. Transcriptional regulation of IFNT expression. Ets2 and AP1 are constitutively produced throughout early conceptus development. Hence, the unique temporal expression pattern for IFNT is controlled by additional factors. Panel a: IFNT is not transcribed during the first few days of pregnancy coincident with the production of Oct4, an embryonic lineage specifying factor that interacts with Ets2 to limits its ability to stimulate IFNT expression. Also, trophectoderm-lineage factors, such as Cdx2 and Dlx3, are not present during initial cleavage stages. Panel b: Oct4 expression subsides as the trophectoderm lineage emerges at the blastocyst stage, allowing Ets2 to stimulate IFNT expression. Also, at least two trophectoderm factors, Cdx2 and Dlx3, facilitate IFNT expression (Ealy and Yang, 2009).

3 *In vitro* production (IVP) of cattle embryos

3-1 *In vitro* fertilization

In vitro fertilization represents an interesting step in the *in vitro* cattle embryo production procedure. The overall success of the *in vitro* fertilization process requires good control of different steps: sperm selection, sperm capacitation and fertilization itself. Semen is known to contain factors that can prevent capacitation and/or fertilization. Abnormal spermatozoa, dead cells, enzymes and bacteria are also present. In addition, the concentration of sperm suspensions has to be controlled to ensure the continuity of the IVF method. Numerous techniques have been proposed for removing undesirable semen components and concentrating the motile sperm fraction in a suspension of known concentration. The most conventional is the swim-up method (Parrish *et al.*, 1986) but other methods have been described involving either centrifugation on BSA (Wall *et al.*, 1984) or Percoll (Bolton and Braude, 1984) density gradients or Sephadex column separation (Drobnis *et al.*, 1991). Centrifugation on Percoll density gradients has been reported as the most suitable method for human IVF, the pregnancy rate increased from 18% after swim-up to 31.5% after Percoll sperm separation (Guerin *et al.*, 1989). The next critical point encountered in IVF is sperm capacitation. Ejaculated spermatozoa are more suitable than epididyme collected sperm for bovine IVF and give rise to more normal eggs (Pavlok *et al.*, 1988) but they have to undergo a capacitation treatment prior to fertilization (First and Parrish, 1987). The mechanisms of mammalian sperm capacitation, which normally takes place in the

female genital tract, are not yet well understood but several *in vitro* capacitation methods have been proposed: washing with high ionic strength solution (Brackett *et al.*, 1982); treatment with bovine follicular fluid (Fukui *et al.*, 1983) or oviduct fluid (Parrish *et al.*, 1989). However, the most common method used in bovine IVF involves heparin (Parrish *et al.*, 1986). Heparin, as well as other glycosaminoglycans present in the female genital tract (Lenz *et al.*, 1982), is able to capacitate bovine fresh sperm within 4 h (Parrish *et al.*, 1988) and frozen-thawed sperm within 15 min (Parrish *et al.*, 1986). In general, the medium used for sperm/oocyte co-culture is TALP (Parrish *et al.*, 1986) supplemented with the appropriate heparin concentration, in droplets under mineral oil and containing 10 oocytes and lxl0^6 sperm/ml (Parrish *et al.*, 1986).

3-2 Nuclear transfer (NT)

The NT procedure was first devised by the German Nobel Laureate Hans Spemann in 1938 when he proposed an experiment involving the insertion of a nucleus into an enucleated oocyte. The idea was not pursued, however, because he did not have the equipment required to perform such an experiment (Spemann, 1938). Briggs and King were the first to successfully utilize NT in the production of live offspring from metazoan cells. They reported the successful production of Northern Leopard Frog, *Rana pipiens*, tadpoles via NT in May of 1952 (Briggs and King, 1952). Success with NT in mammals was not reported until the 1980's. Initially experiments involving the transfer of pronuclei from one mouse embryo to

another proved successful in producing live births; however they were unable to produce viable embryos beyond the blastocyst stage using blastomeres from cleavage-staged embryos as nuclear donors (McGrath and Solter, 1984). Finally in 1986 Willadsen reported the production of completely viable sheep embryos derived from the transfer of 8- and 16-cell blastomeres to enucleated oocytes that result in the birth of live lambs (Willadsen, 1986). In 1987, Prather *et al.* used essentially the same procedure to produce live cattle (Prather *et al.*, 1987). Over the next few years, a number of other species successfully cloned from cells of preimplantation embryos followed including mice, rats, rabbits, pigs, goats, and monkeys (Di Berardino, 2001). With continuing advances in nuclear transfer technology, the question remained: could offspring be produced by NT from differentiated cells? A number of embryonic stem cell-like lines were produced from mice (Piedrahita *et al.*, 1990), cattle (Stice *et al.*, 1996), sheep (Notarianni *et al.*, 1991), and pigs (Notarianni *et al.*, 1990), but attempts at producing cloned animals from stem cell-like lines proved ineffective (Stice *et al.*, 1996; Tsunoda and Kato, 1993). Based on early work with somatic cells and cultured stem cell-like cells it was believed that it was not possible to produce viable offspring from adult cells; however, in 1994 Sims and First came a step closer reporting the successful production of cloned calves using inner cell mass (ICM) cells cultured *in vitro* for up to 28 days (Sims and First, 1994). In 1996 Campbell *et al.* announced the production of five cloned sheep derived from *in vitro* cultured, putative differentiated ICM cells (Campbell *et al.*, 1996). The success with cultured cells

was closely followed by the announcement of the birth of Dolly, the first cloned animal derived from an adult cell (Wilmut *et al.*, 1997). The announcement of Dolly was significant in that it demonstrated a differentiated mammary cell derived from an adult animal was able to be reprogrammed to an embryonic state and give rise to a complete and healthy animal. Since the first successful SCNT experiments in sheep, the technology has been applied in the production of a number of other species including mice (Wakayama *et al.*, 1998), cattle (Wells *et al.*, 1999), goats (Baguisi *et al.*, 1999), pigs (Polejaeva *et al.*, 2000), mouflon sheep (Loi *et al.*, 2001), rabbits (Chesne *et al.*, 2002), mules (Woods *et al.*, 2003), cats (Shin *et al.*, 2002), rats (Zhou *et al.*, 2003), horses (Galli *et al.*, 2003), dogs (Lee *et al.*, 2005), ferrets (Li *et al.*, 2006), buffaloe (Shi *et al.*, 2007), wolves (Kim *et al.*, 2007) and camels (Wani *et al.*, 2010) and the list continues to grow. The production of animals by NT involves multiple steps including recipient cytoplast preparation, culture of donor cell, embryo reconstruction, activation, culture and/or embryo transfer.

PART 2

Effect of different culture media on the temporal gene expression in the bovine developing embryos

ABSTRACT

The embryonic development and the temporal behavioral interaction of the genes involved in IFNτ gene expression were explored and how they behave in an orchestrated manner to increase the developmental competence of IVF produced embryos by culturing in the chemically defined medium. Behavior of genes included ETS2, CDX2, GATA2, GATA3, OCT4 and NANOG were analyzed in early bovine IVF produced embryos, (from compact morulae to the blastocyst hatching stages), by semi- and relative quantitative PCR and compared between two *in vitro* culture (IVC) systems, two-step chemically defined medium and modified synthetic oviductal fluid (mSOF) containing 8 mg/mL, BSA. Early embryonic development was found to be better in two-step chemically defined culture system than that of mSOF as indicated by the increment of blastocyst yield, 33.1% in two-step culture system vs. 18.8% in mSOF medium, and the blastocyst hatching, 52.3% in two-step culture system vs. 33.5% in mSOF medium. Relative quantitative gene expressions showed harmonic behavior in the two-step culture system rather than the culture in mSOF, IFNτ showed even increase throughout the embryonic development in the two-step culture medium while it decreased with blastocyst

hatching in mSOF culture condition. Temporal dominance of OCT4 over all the transcription factors was found in regulation of IFNτ expression (the major factor of expression regulation but in inverse manner). However, ETS2, CDX2, GATA2 and GATA3 are potent IFNτ stimulator in a cumulative manner but in case of OCT-4 decrement. CDX2 directly related with IFNτ, but still under OCT4 dominance and also regulated by the subservient of OCT4 which is NANOG. In conclusion, this study confirmed the usefulness of using the two-step chemically defined culture medium for increasing the developmental competence of IVF produced embryos and elucidated the dominance of OCT4 over the other genes implicated in regulation of IFNτ expression.

Keywords: Cattle, Embryonic development, *In vitro* fertilization, Gene expression, Interferon tau.

1. Introduction

In vitro production (IVP) of bovine embryos has become a reliable alternative to conventional superovulation techniques and has been used as a tool to study pre-implantation embryo development (Bavister, 1995). In particular, the 5-6 days post-fertilization period of IVP embryos is the time when various developmentally important events occur, including the first cleavage division that is critical in determining the subsequent development of the embryo (Lonergan *et al.*, 1999), activation of the embryonic genome, compaction of the morula and blastulation (Memili and First, 2000). This phase in development is also characterized by distinct morphological changes that must include a well-orchestrated expression of genes derived from both maternal and embryonic genomes to allow for compaction, cavitation and blastocoel expansion (Kidder, 1992). Significant losses in IVP occur during the *in vitro* culture (IVC) period suggesting the post-fertilization environment is critical for development (Lonergan *et al.*, 2006, Wrenzycki *et al.*, 1996). The relative abundance of numerous transcripts varies through the early pre-implantation period and is strongly influenced by the culture environment (Wrenzycki *et al.*, 1999, 2001, 2004).

Over the past 25 years, much attention has been given to interferon tau (IFNτ) which is produced by the trophoectoderm during a defined period of peri-attachment of ruminant embryos (Bartol *et al.*, 1985, Godkin *et al.*, 1984, Helmer *et al.*, 1987, Imakawa *et al.*, 1987, Martal *et al.*, 1979). IFNτ are first detected at the late morula to early blastocyst stages of development. Both mRNA and protein level

are relatively low at the blastocyst stage but increase with advancing age of the spherical conceptus (Kubisch *et al.*, 1998, 2001). IFNτ acts on the uterus to interrupt the luteolytic pathway caused by oxytocin-dependant pulses of prostaglandin F2α (PGF2α) so that corpus luteum (CL) function may continue. IFNτ also promotes luteal function by regulating uterine metabolism of various prostaglandins in a concentration dependant manner (Binelli *et al.*, 2000, Guzeloglu *et al.*, 2004, Parent *et al.*, 2003). A majority of failed pregnancies occur within the first 6 weeks after mating in cattle and it is estimated that 10-40% of all failed pregnancies occur during the critical period when IFNτ must interact with the uterus for continuation of pregnancy (Thatcher *et al.*, 2001).

The unique pattern of IFNτ expression is regulated by promoter/enhancer regions that are distinct from that of other type I IFN genes (Ealy and Yang, 2009). One key component of IFNτ expression is Ets-2 transcription factor. Ezashi *et al.* (1998) first identified it as a regulator of IFNτ transcription. Subsequent findings determined that a consensus Ets-2 binding site is present in all transcriptionally active bovine IFNτ. Moreover, at least three trophoectodermal transcription regulators are also involved with IFNτ expression regulation. Caudal-type homebox 2 (CDX2) stimulates IFNτ promoter activity in the presence of Ets-2 (Ezashi *et al.*, 2008, Sakurai *et al.*, 2009). Two additional regulators, GATA transcription factors 2 and 3 (GATA2 and GATA3), were recently found to have role in regulation of IFNτ expression (Bai *et al.*, 2009). In addition, the POU homeodomain protein (Oct-4), that is best known as a marker of pluripotency (Pesce and Scholer, 2000), blunts the

ETS2 induced IFNτ promoter activity (Ezashi *et al.*, 2001) beside the inhibition of other factors during early pregnancy like CDX2 (Niwa *et al.*, 2005). This study, as a continuation to our previous work, was undertaken to: (1) compare the developmental competence of bovine embryos that were cultured *in vitro* cultured in a two-step chemically defined medium or modified synthetic oviductal fluid medium (mSOF) containing 8 mg/mL, BSA. (2) compare the gene expression patterns in these embryos and (3) elucidate the interplay and dominance among the major factors controlling the developmental competence markers of bovine embryos specifically IFNτ and its regulator genes (CDX2, ETS2, GATA2, GATA3, NANOG, and OCT4).

2. **Materials and Methods**

2.1. Oocyte collection and *in vitro* maturation (IVM)

Ovaries were collected from a local abattoir into saline at 35 °C and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18 gauge needle attached to a 10 ml disposal syringe. The COCs with evenly-granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 2 mM NaHCO3 (Sigma–Aldrich Corp., St. Louis, MO, USA), and 1% penicillin–streptomycin (v/v). For IVM, COCs were

cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton-Dickinson Ltd., Plymouth, UK) for 22 h in 450 µl TCM-199 supplemented with 10% FBS, 0.005 AU/mL FSH (Antrin, Teikoku, Japan), 100 µM Cysteamine (Sigma-Aldrich) and 1 µg/mL 17β-estradiol (Sigma–Aldrich) at 39 °C in a humidified atmosphere of 5% CO2.

2. Sperm preparation, *in vitro* fertilization (IVF) and *in vitro* culture of embryo s (IVC)

Motile spermatozoa were purified and selected using the Percoll gradient method (Machado *et al.*, 2009). Briefly, spermatozoa were selected from the thawed semen straws by centrifugation on a Percoll discontinuous gradient (45–90%) for 15 min at 1500 rpm. The 45% Percoll solution was prepared with 1 ml of 90% Percoll (Nutricell, Campinas, SP, Brazil) and 1 mL of capacitation-TALP (Nutricell). The sperm pellet was washed two times with capacitation-TALP by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet used for insemination of matured oocyte (At 24 h of IVM). Oocytes were inseminated (day 0) with 1-2 × 10^6 spermatozoa/mL for 18 h in 30 µL microdrops of IVF-TALP medium (Nutricell) overlaid with mineral oil at 39 °C in a humidified atmospher e of 5% CO_2. Presumptive zygotes were denuded and cultured in two different media, the two-step defined culture medium (first 5 days with D1 then transferred to the later stage medium D2) and mSOF and overlaid with mineral oil (Sigma–Aldrich). All incubations were done at 39 °C in an atmosphere of 5% O_2, 5% CO_2

and 90% N_2. Cleavage rates were recorded on days 2 and embryonic development was monitored according to the stages of the International Embryo Transfer Society (IETS) (Robertson and Nelson, 2010); C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts (Figure 7.A). Hatching of blastocysts was recorded on day 9.

2.3. Counting total cells of blastocysts

The blastocysts of stages C7, C7H and C8 were washed in PBS then stained by Hoechst 33342 (Sigma–Aldrich) for 20 min, mounted on glass slides and the number of nuclei in the embryos were counted using a fluorescence microscope (Nikon TE2000, Tokyo, Japan).

2.4. Determination of the relative abundance of genes transcripts in single embryo by semi-quantitative RT-PCR

Single embryo of each stage (C4-C8) were washed in PBS three times then transferred into 5 µL of diethylpyrocarbonate (DEPC) treated water (Invitrogen) and stored at -80 °C or used freshly for total RNA extraction using the RNeasy total extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Reverse transcription was carried out at 50 °C for 50 min. Individual RT reaction was performed using random hexamer and superscript TM III reverse transcriptase (Invitrogen) in a 20 µL reaction. 1-2 µL cDNA subjected to reverse

transcription-polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-i-starTaq (Intron Biotech., Seoul, Republic of Korea). Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in table 1. The PCR amplification carried out for one cycle of denaturation at 95 °C for 5 min and subsequent cycles with denaturation at 95 °C, annealing for 30 sec, extension at 72 °C for 45 sec and final extension at 72 °C for 5 min. and then ten µl of PCR products were fractioned on 1% agarose gel (Intron Biotech.) and stained with RedSafe TM (Intron Biotech.). Expression level for each gene was determined densiometrically by Image J software (1.40g, NIH, USA). Relative expression levels of each gene at specific stages of embryo development were represented as a ratio to that of GAPDH gene expression.

2.5. Relative quantification (RQ) of transcripts by real-time RT-PCR (qPCR)

Real-Time RT-PCR was done according to the Takara Bio Inc. guidelines. Total 22 µL PCR reaction was made by adding 2 µL cDNA, 1 µL forward primer, 1 µL reverse primer, 8 µL SYBR Premix Ex Taq, 0.4 µL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 µL of Nuclease-free water (Ambion Inc., Austin, TX). The reaction was done by using 7300 Real Time PCR System (Applied Biosystems, Forest City, CA) according to the company instructions. The thermal profile for real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 40 sec. All primers are described in Table 1.

2.6. Experimental design

In this study, we compared embryonic developmental competence between a two-steps chemically defined media (D1-D2) and mSOF, by incubating presumptive zygotes produced via *in vitro* fertilization in the two media. Cleavage rates, morulae compaction, blastocyst formation and blastocyst hatching were observed in both groups with regard to the stage codes by IETS (Robertson and Nelson, 2010). Total cell numbers of three developing blastocysts of codes C7, C7H and C8 from each group were counted after staining the nuclei with Hoechst 33342 (Sigma-Aldrich). The relative abundance of gene expression (IFNτ, ETS2, CDX2, GATA2, GATA3, OCT4 and NANOG) was evaluated and represented as a ratio to that of GAPDH gene expression. Three embryos from each stage were subjected to RT-PCR and qPCR with repeating each sample three times. In real-time RT-PCR, the relative quantification of each gene expression (IFNτ, OCT4 and CDX2) was compared to the calibrator which is the relative quantity of the target gene of C4 stage with its GAPDH expression in both experimental groups.

2.7. Statistical analysis

In each experimental group, presumptive zygotes were randomly distributed. All data were subjected to one-way ANOVA followed by Tukey test to deter mine differences among the two experimental groups using GraphPad Version 4.0 (Motulsky, 2003). Statistical significance was determined when a *P* value was less than 0.05.

3. Results

Embryo development and fate of blastocysts after IVC in the two-step chemically defined media and mSOF

A total of 184 and 170 presumptive zygotes were incubated in the two-step culture system and mSOF, respectively (Table 2). There was no difference in cleavage rate (78.3% vs. 74.7%) and morulae compaction (50.5% vs. 45.3%) between the two groups (Table 2). However, the blastocyst yield was significantly increased in D1-D2 medium than that of mSOF (33.7% vs. 18.8%) and significantly more blastocysts hatched in D1-D2 medium (52.3% vs. 33.5%).

Total cell numbers of blastocysts after IVC in the two-steps chemically defined media and mSOF

After blastocyst formation, expansion (C7) and while hatching (C7H); there was no significant difference between the two groups (Table 2 and Figure 7.B and C), while the total cell number of blastocysts was higher in D1-D2 medium after hatching (C8) than that of mSOF (140 ± 8.25 vs. 122 ± 6.42; $P<0.05$).

Semi-quantitative analysis and relative quantification of transcripts among the different stages of embryonic development

In order to analyze the semi-quantitative differential expression of the eight designated genes, the level of GAPDH expression from single embryo compared to the expression of the other genes by pixel comparison using imaging analysis

program. The gene expression in embryos cultured in two-step culture medium as shown in figure 2A were; IFNτ and CDX2 showed an even increase in expression then CDX2 showed slight decrease in expression at the stage of blastocyst hatching while OCT4 and NANOG showed a decreased expression with the advancement of embryo growth. GATA2 and GATA3 expression were low in embryos until the mid-blastocyst stage but became high after blastocyst expansion. However, the gene expression of mSOF embryos (Figure. 8B) were; IFNτ and CDX2 showed increased expression until the blastocyst expansion and mid-blastocyst respectively then they showed decrease in expression after these stages while OCT4 behave in inverse manner to IFNτ. NANOG showed an even increase with the advancement of blastocyst growth. Both GATA2 and GATA3 showed intermittent expression with tendency to increase after blastocyst hatching. ETS2 expression level showed no difference among the stages of embryonic development between the two groups. Relative quantification (RQ) of the IFNτ, OCT4 and CDX2 was confirmed by qPCR analysis and the results were found to be similar to that of RT-PCR (table 2).

Temporal dominance of the genes at each stage of embryo development in relation to IFNτ

We summarize the relative gene expression after comparing the pixel of each gene with its expression along the stage of embryo development (Fig. 9 and Table 3). In the two-steps culture system, the prominent gene in both stages C4 and C5 was OCT4 then GATA3 was added versus OCT4 in C6. At C7 CDX2 added to the

prominent genes. At C7H CDX2 became a stable then at C8 GATA2 and added to the prominent genes. IFNτ showed even increase in expression with the blastocyst advancement. However, in case of mSOF medium; the prominent gene in both stages C4 and C5 was OCT4 like the two-step culture medium, GATA3 and CDX2 added versus OCT4 in C6 and then they became the prominent gens at C7. At blastocyst hatching OCT4 resumed its expression with NANOG then at C8 GATA2 and GATA3 began to express again. IFNτ showed increased expression until C7 when it showed a decrease in expression with blastocyst hatching (C7H).

4. Discussion

IVP embryos derived from chemically defined medium has showed comparable development *in vitro* and higher efficiency of calving rate compared to undefined culture medium, mSOF (Lim *et al.*, 2007). This chemically defined medium can allow us to produce safer embryos without unknown pathogens in FBS or BSA. Here, it is confirmed again that the importance of using the chemically defined two-step culture system for IVC of bovine IVP embryos because of the elevated blastocyst yield, cell number and hatching rate. Furthermore, this system of IVC demonstrated the harmonic and orchestrated interplay among the genes expressed at each embryo developmental stage responsible for the embryonic-maternal interaction, especially the IFNτ signal. According to Hernandez-Ledezma *et al.* (1992), they suggested that the best way to study IFNτ expression as an

indicator of embryo quality is the temporal expression than the absolute expression at a particular stage because the latter is known to vary widely.

Gene expression between the two groups showed marked variation in their behaviors (Fig. 2 and 3) and this variation is surely due to the culture environment (Corcoran *et al.*, 2006, Purpera *et al.*, 2009, Rizos *et al.*, 2003). IFNτ expression was even and progressively increased with the advancement of embryonic development in two-step chemically defined system while it showed a decrease in expression by the blastocyst hatching in mSOF medium. This finding is consistent with our result in blastocyst quality and reflects the developmental competence and its ability to establish pregnancy after embryo transfer (ET) (Lonergan *et al.*, 2003). From the studied genes, ETS2, this was firstly recorded as an essential gene for IFNτ promoter activation (Ezashi *et al.*, 1998). We found that ETS2 expression accompanied IFNτ expression but without a difference in its relative expression either among the stages of embryonic development or between the two experimental group confirming that ETS is a basic component of IFNτ expression regardless its relative expression. GATA2 and GATA3 transcriptional factors were described as regulators involved in IFNτ expression. We found that GATA2 and GATA3 varied even with the embryonic stages of the same culture but they seem to be increased toward the blastocyst hatching in both experimental groups. This suggests the supportive effect of GATA2 and GATA3 for increase IFNτ transcription in cumulative and overlapping pattern (Dorfman *et al.*, 1992, George *et al.*, 1994, Ng *et al.*, 1994). CDX2 expression was found to be directly related to

IFNτ expression along the embryo development until the hatching stage in two-step chemically defined culture system where it tends to be slightly decreased suggesting that CDX2 is a potent regulator of IFNτ expression (Kim *et al.*, 2007, Kurosaka *et al.*, 2004, Chen *et al.*, 2009). OCT4 and NANOG were found to be inversely affected the IFNτ expression in both experimental groups. In the two-step chemically defined culture system, OCT4 expression becomes minimal with the hatched blastocyst stage that is a good sign of blastocyst quality (Yao *et al.*, 2009) but in case of mSOF, OCT4 resumed its high expression with blastocyst hatching. It appeared from these results that many genes are involved with IFNτ expression, some of them are directly related (CDX2 and GATA2 combined with GATA3) and other genes are inversely related (OCT4 and NANOG) to IFNτ expression. From these results, we can say that the interplay of gene expression in the two-step chemically defined culture system showed consistent and orchestrated interaction resulting in an increase of IFNτ expression and subsequent IFNτ secretion which is essential indicator of blastocyst developmental competence. To clarify which gene is the dominant over others, blastocysts hatching stage (C7H) answering this question because we found that CDX2 is directly related to IFNτ but begins to be slightly decrease with this stage, but IFNτ is still dramatically increased. So, the decrease in OCT4 leads the way for the other genes to increase IFNτ like CDX2, GATA2 and GATA3. Niwa *et al.* (2005) showed that OCT4 is a potent negative regulator of CDX2 and Chen *et al.* (2009) showed that OCT4 directs its subservient, NANOG, to suppress CDX2 expression. This result is also confirmed in mSOF

culture in where the maximal level of OCT4 and NANOG the level of CDX2 was the minimal and even GATA2 or GATA3 were unable to increase the IFNτ after blastocyst hatching. Our study suggests the presence of glucose and other additives (PVA, myoinositol and phosphate) in two-step culture medium as our previous published work cause harmonic gene expression especially for the IFNt, OCT4 and CDX2. Addition of some nutrients in the 2nd stage of culture stables the gene expression of these genes with blastocysts development. Especially CDX2, which showed marked decrease in mSOF medium while it still stable in two-step culture sysyetm. The stable expression of CDX2, as a marker of trophoblast, might be due to the presence of glucose in the 2nd stage of two-step culture medium as glucose is essential for trophoblast (Augustin *et al.*, 2001, Brandão *et al.*, 2004), while in mSOF, these genes disturbed due to lack of such nutrients.

In conclusion, the efficiency of using two-step chemically defined media was confirmed as a good tool for bovine embryos *in vitro* culture system because of; 1) Increased blastocyst yield, 2) Increased blastocyst cell number, 3) Increased blastocyst hatching rate and 4) harmonic and orchestrated gene interplay reflected by progressive increase in IFNτ expression which is a good sign for blastocyst developmental competence. Finally, the dominance of OCT4 over all the other transcripts in regulation of IFNτ expression was recorded.

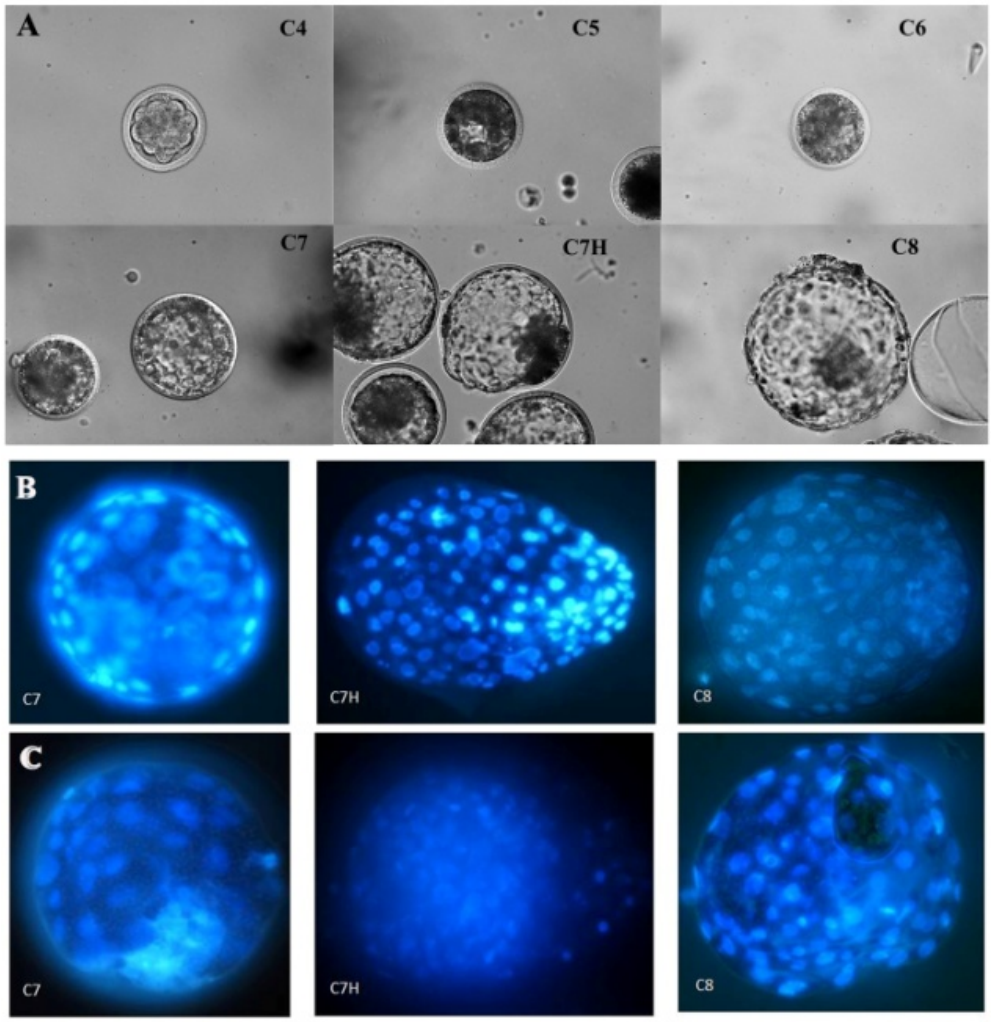

Figure 7. A) Representative developmental embryos (from compact morulae (C4) to hatched blastocyst (C8), B) blastocysts in stages C7, C7H and C8 stained by Hoech st 33342 cultured in two-steps culture system and C) in mSOF medium. C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts.

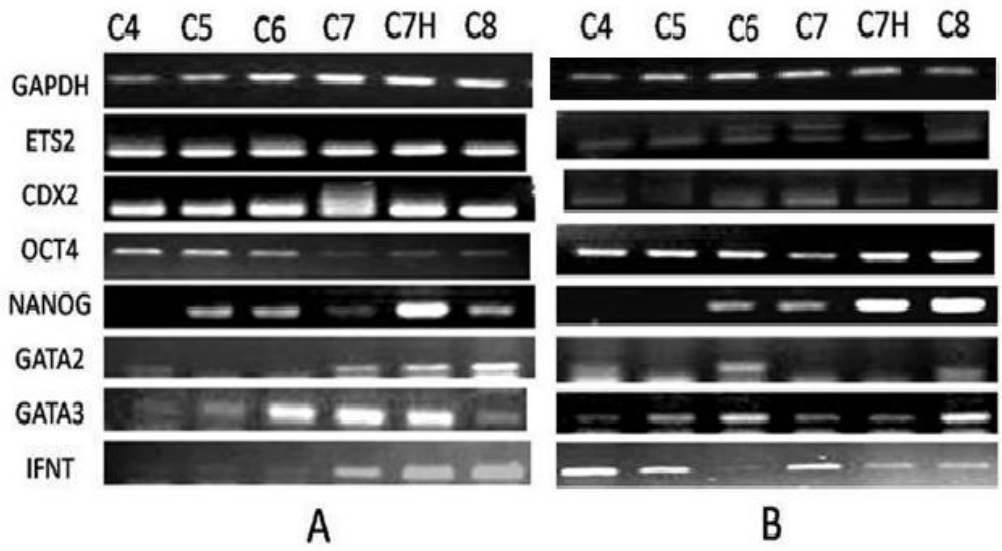

Figure 8. Representative gel photographs of the mRNA expression of GAPDH, ET S2, CDX2, OCT4, NANOG, GATA2, GATA3, and IFNτ in embryos cultured in (A) two-steps culture system and (B) in mSOF medium. C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts.

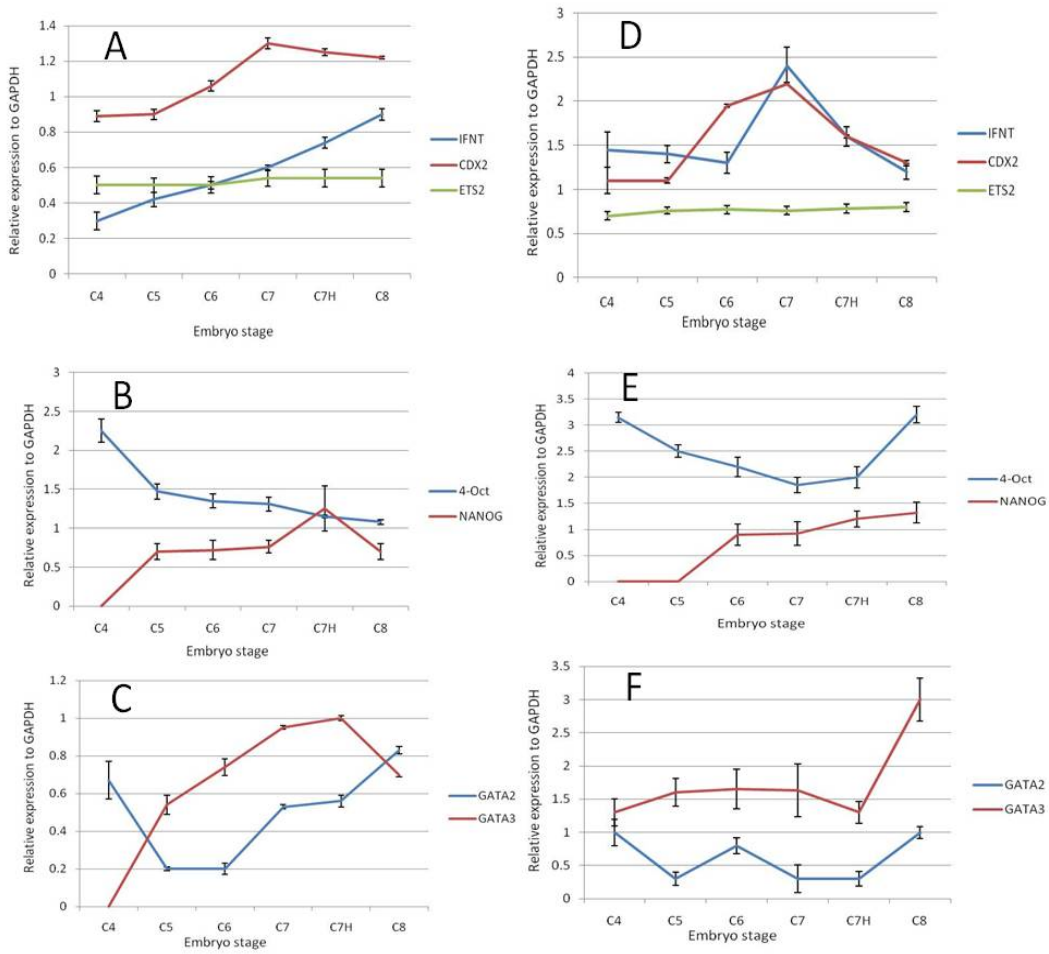

Figure 9. Densiometric pixel comparison of relative gene expression between two-step culture medium (A, B, C) and mSOF medium (D, E, F). C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts.

Tables 1. Primers used for RT-PCR and real-time RT-PCR

Gene	Primer sequences (5'----3')	Annealing temperature (°C)	Cycle numbers	Fragment size (bp)	GenBank accession number	References
GAPDH*	F: TGCACCACCAACTGCTTGGC R: CACGTTGGGAGTGGGGACGC	60	38	267	NM_001034034.1	(Perez *et al.*, 2008)
	F:GGCGTGAACCACGAGAAGTA R:CCCTCCACGATGCCAAAGT	60	40	120		
IFNτ*	F:GACGATCTCTGGGTTGTTAC R:GTG ATGTGGCATCTTAGTCA	55	38	565	AF238611.1	(Yao *et al.*, 2009)
	F:TCCATGAGATGCTCCAGCAGT R:T GTTGGAGCCCAGTGCAGA	60	40	103		
OCT4*	F:GGTTCTCTTTGGAAAGGTGTTC R:ACACTCGGACCACGTCTTTC	60	38	314	AF022987	(Kurosaka *et al.*, 2004)
	F: CCACCAGCAGGCAAACAC R: GAGAAGGCGAAGTCAGAAGC	60	40	223		
NANOG	F:TTCCCTCCTCCATGGATCTG R:ATTTGCTGGAGACTGAGGTA	58	38	219	NM_001025344	(Pant *et al.*, 2009)

CDX2	F:GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	59	38	140	DQ126146	(Degrelle *et al.*, 2005)
ETS2	F:GTGGGCCTATCCAGCTGTG R: TTCCCTGACGTCTTGTGGAT	58	38	227	DQ126148	(Degrelle *et al.*, 2005)
GATA2	F: GAGGACTGTAAGCGTAAAGG R: AAGAACCAAGTCTCCCCAT	60	38	140	XM_583307	(Bai *et al.*, 2009)
GATA3	F: ATGAAACCGAAACCCGATGG R: TTCACAGCACTAGAGAGACC	60	38	185	NM_001076804	(Bai *et al.*, 2009)

*The upper and lower line primers of GAPDH, IFNτ and OCT4 were for RT-PCR and real-time PCR, respectively.

Table 2. Embryonic development, total blastocyst number and fate of blastocyst after IVC of bovine IVF produced embryos in chemically defined two-steps medium (D1-D2) and mSOF*

Item		Two-steps culture system			*mSOF*		
Oocytes		184			170		
****Cleaved (%)**		144 (78.3)			127 (74.7)		
****Morulae (%)**		93 (50.5)			77 (45.3)		
****BL*** (%)**		62 (33.7) [a]			32 (18.8) [b]		
****BL hatching (% of BL)**		52.3 [a]			33.5 [b]		
BL total cell number	**C7**	97 ± 9.21			93 ± 8.88		
	C7H	119 ± 11.34			113 ± 6.69		
	C8	140 ± 8.25[a]			122 ± 6.42 [b]		
RQ* of gene expression**	Embryo stage	IFNτ	OCT4	CDX2	IFNτ	OCT4	CDX2
	C4	1	1	1	1	1	1
	C5	1.16	2.05	0.95	1	0.94	0.99
	C6	2.14	1.86	1.15	2.43	1.45	1.0 1
	C7	2.89	1.57	1.3	2.45	0.95	1.03
	C7H	3.18	0.65	1.33	2.5	1.37	1.08
	C8	3.59	0.38	0.97	1.28	1.83	1.07

[a, b] Values for different superscripts in the same row are significantly different ($P < 0.05$).

*mSOF: modified synthetic oviductal fluid

**Cleaved, Morulae, BL and BL hatching were recorded on Day 2, 5, 7 and 9, respectively.

***BL: Blastocyst.

***RQ: realtive quantitification by real-time RT-PCR.C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts.

Table 3. Relative pixel folds comparison of the analyzed RT-PCR gel photographs of embryos *in vitro* cultured in the two culture systems. C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts.

Gene	**Two-steps culture system**						**mSOF medium**					
	C4	**C5**	**C6**	**C7**	**C7H**	**C8**	**C4**	**C5**	**C6**	**C7**	**C7H**	**C8**
ETS2	+	+	+	+	+	+	+	+	+	+	+	+
CDX2	+	+	+	+++	++	++	+	+	++	+++	+	+
OCT4	+++	++	++	++	+	+	+++	++	++	+	++	+++
NANOG	-	+	+	+	++	+	-	-	+	+	++	+++
GATA2	+	-	-	+	+	++	++	-	++	-	-	++
GATA3	++	+	++	++	+++	++	+	++	++	++	+	+++
Prominent gene/s	OCT4	OCT4	OCT4 GATA3	OCT4 CDX2 GATA3	CDX2 GATA3 NANOG	CDX2 GATA2 GATA3	OCT4	OCT4	OCT4 CDX2 GATA3	CDX2 GATA3	OCT4 NANOG	OCT4 NANOG GATA3
IFNτ	+	+	++	++	+++	++++	+	+	+	+++	++	+

PART 3

Embryonic development and implantation related gene expression of oocyte reconstructed with bovine trophoblast cells

Chapter 1

Isolation of bovine trophoblast using different feeder cells

Abstract

The trophectoderm cells, arise from the outer side of blastomere on blastocyst stage, are the first differentiated embryonic cells with specific potential as a stem cell. Physiology of trophectoderm cells has been studied however their functions are still remain unclear, because the lack of definitive information of cell lineages. This study aimed to establish in culture different feeder-dependent trophectoderm cell lines from a 9 day, pre-implantation, in vitro produced bovine embryos. Two different feeders were used; porcine granulosa cells (PGCs) and mouse embryonic fibroblasts (MEF). Both cells were mitotically inactivated by mitomycin-C and then cultured with a density $5x10^4$/ml on 0.1% (w/v) gelatin coated 4-well dishes. Trophectoderm cells were observed by light microscopy and characterized by reverse transcription-PCR using specific primers. In results, trophectoderm cells display epithelial characteristics, cuboidal morphology and express mRNA of homebox protein CDX2, cytokeratin 8 (KRT8) and interferon tau (IFNτ). PGCs

were highly proliferative with doubling time of approximately 24 h when compared to MEF one ($P \leq 0.5$), easy to recover, and provide a reasonable source of steroids, 17β-estradiol (E2) (31.21±3.1 ng/ml) and progesterone (P4) (6.36±0.4 ng/ml). Moreover, primary trophectoderm cell colonies cultured on PGCs grew faster, with a doubling time of approximately 48 h when compared to MEF one ($P \leq 0.5$). We speculate that the continuous supplement of steroids and other cytokines during the co-culture of trophoblasts with granulosa cells might help the trophectoderm cells growth than that of MEF. Further investigations are required in this regard. In conclusion, porcine granulosa cells can be good alternative feeders to culture porcine and bovine trophectoderm.

Keywords: Trophectoderm, granulosa cells, feeder cells, cow.

Introduction

The mammalian blastocyst consists of two distinct cell populations, the inner cell mass and the trophoblast. Trophoblastic cells are thought to be the first to differentiate during embryogenesis (Handyside and Johnson, 1978). The inner cell mass forms the embryo and its associated membranes, while the trophoblastic cells form a large portion of the placenta (Carlson, 1996). While the differentiation of the trophoblast is essential for implantation and placental formation, this process remains poorly understood due to the complex interaction of the numerous participating factors. During bovine peri-implantation, trophoblastic cells produce a number of specific molecules such as IFNτ (Flint *et al.*, 1979; Morgan *et al.*, 1989). To investigate trophoblast development and function, an *in vitro* model is required. Toward this end, several trophoblastic cell lines were recently developed in various species including swine (Flechon *et al.*, 1995), mouse (Tanaka *et al.*, 1998) and cattle (Talbot *et al.*, 2000). Maintenance of these trophoblastic cell lines required feeder cells. Thus, the development of a culture system in the presence of new feeder cell would be highly desirable, and would facilitate the investigation of the

characteristics and function of trophoblastic cells *in vitro*. In the present study, a different feeder cell, porcine granulosa cell, has been used for culturing trophoblastic cells which were isolated from the *in vitro* produced blastocyst.

Materials and methods

Oocyte collection and *in vitro* maturation (IVM)

Cow ovaries were collected from a local abattoir, place in saline at 35 °C and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposal syringe. The COCs with evenly-granulated cytoplasm that were enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium199 (TCM-199, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% FBS, 2 mM NaHCO3 (Sigma–Aldrich Corp., St. Louis, MO, USA) and 1% penicillin–streptomycin (v/v). For IVM, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton, Dickinson U.K. Limited, Plymouth,UK) for 22 h in 450 μl TCM-199 supplemented with 10% FBS, 0.005 AU/ml FSH (Antrin, Teikoku, Tokyo, Japan)

and 1 μg/ml 17β-estradiol (Sigma–Aldrich Corp.) at 39 °C in a humidified atmosphere of 5% CO2.

Sperm preparation, *in vitro* fertilization (IVF) and *in vitro* culture of embryos (IVC)

Motile spermatozoa were purified and selected using a Percoll gradient (Machado *et al.*, 2009). Briefly, spermatozoa were selected from thawed semen straws by centrifugation on a Percoll discontinuous gradient (45–90%) for 15 minutes at 1500 rpm. A 45% Percoll solution was prepared with 1 ml of 90% Percoll and 1 ml of TALP medium. The sperm pellet was washed two times with TALP medium by centrifugation at 1500 rpm for 5 minutes. The active motile spermatozoa from the pellet were used for insemination of matured oocyte (at 24 h of IVM). Oocytes were inseminated (day 0) with 1-2 × 106 spermatozoa/ml for 18 h in 30 μl microdrops of IVF-TALP medium overlaid with mineral oil at 39 °C in a humidified atmosphere of 5% CO2. Presumptive zygotes were denuded and

cultured in a two-step defined culture medium as described previously (Lim *et al.*, 2007) and overlaid with mineral oil (Sigma–Aldrich Corp.).

Preparation of feeder cells

Two different feeders were used; porcine granulosa cells (PGCs) and the conventional method using mouse embryonic fibroblasts (MEF). PGCs were obtained through aspiration of follicular fluid of 4-6 mm porcine ovarian follicles with 18-gauge needle (Fig. 10). The follicular fluid was centrifuged 1500 rpm for 2 min then washed three times with PBS then with culture medium (DMEM and FBS 10%). Both cells were mitotically inactivated by mitomycin-C and then cultured with a density $5x10^4$/ml on 0.1% (w/v) gelatin coated 4-well dishes in DMEM-199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), non essential amino acids (NEAA), β-meracptoethanol and nucleosides (Talbot *et al.*, 2000)

Measurement of steroid hormones in the culture medium

Steroids (E2 and P4) were measured in the culture medium through commercial

kits. Progesterone concentrations were measured by radioimmunoassay (RIA) using a Coat-a-Count Progesterone Kit (Siemens Medical Solutions Diagnostics, U.S.A.). The kit contains rabbit anti-progesterone antibody and has a minimum detection limit of 0.02 ng/ml. The intra- and interassay precision (coefficient of variation, CV %) of the samples are ranged from 2.7 to 8.8 and from 3.9 to 9.7, respectively. Estradiol concentrations were measured by electrochemiluminescence immunoassay using Estradiol II kit (Roche Diagnostics Corp., Indianapolis, IN, U.S.A.). The kit contains rabbit anti-estradiol antibody and has a minimum detection limit of 5.0 pg/ml. The intra- and interassay precision (CV %) of the samples ranged from 2.3 to 6.2 and from 6.2 to 13.0, respectively.

Isolation and culture of trophoblasts

Hatched blastocysts produced by IVF were plated into 4-well tissue culture dishes on day 10 to 11 of their development (Nunc, Thermo Scientific, Roskilde, Denmark). The dishes were coated with 0.1% gelatin and containing either feeder layers of mouse embryonic fibroblasts (MEF) or porcine granulosa cells (PGCs)

treated with mitomycin C (Sigma-Aldrich Corp.). Blastocysts were cultured in 1 ml of DMEM-F12 (mixture of DMEM-F12 supplemented with 10% FBS, 0.1 mM, β-mercaptoethanol, 1% nonessential amino acids [Invitrogen], 2 mMGlutaMax, and 1% Penicillin/Steptomycin [Invitrogen]). Fresh medium was added to the primary cultures every 3–4 days. Secondary passage of the trophoblast cell cultures was performed by physical dissociation. Secondary and subsequent cultures were done by removing the monolayer of cells from the tissue culture plate surface followed by mechanical dissociation and chopping of the primary colonies and subculture of the small chops on new feeder plates. The cells were pelleted by centrifugation (1500 rpm for 2 minutes) in a 1.5 mL round-bottom centrifuge tube. The resulting relatively small clumps of cells were resuspended in 10% DMEM-F12 and plated onto a feeder layer, typically at a 1:4-6 split ratio. Incubation performed at 39 °C in a humidified atmosphere of 5% CO_2 air.

RT-PCR

Total RNA was extracted using an RNeasy total extraction kit (Qiagen, Valencia,

CA, USA) according to the manufacturer instructions. Reverse transcription was carried out at 50 °C for 50 min. Individual RT reactions were performed using a random hexamer and SuperScript™ III Reverse Transcriptase (Invitrogen) in a 20 µl reaction. One to two microliters cDNA were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using aMaxime PCR PreMix kit (i-starTaq) (Intron, Seoul, Republic of Korea). Primer sequences, annealing temperatures and approximate sizes of the amplified fragments are listed in table 4. PCR amplification was carried out with one cycle of denaturation at 95 °C for 5 min and subsequent cycles of denaturation at 95 °C, annealing for 30 seconds, extension at 72 °C for 45 seconds and final extension at 72 °C for 5 min. Ten µl of PCR products were fractionated on 1% agarose gel (Intron) and stained with RedSafe™ (Intron).

Statistical analysis

Data were subjected to one-way ANOVA followed by Tukey's test to determine differences among the experimental groups using GraphPad (Version 4.0). Statistical significance was determined when a P value was less than 0.05.

Results and discussion

Primary culture of trophoblasts was done by placing of hatching or hatched blastocysts on a feeder layer. Outgrowths from the attached blastocysts were seen and left to expand for 3 weeks until reaching about 1 cm in diameter. Trophoblasts were morphologically large cuboidal cells (Fig. 11). Secondary and subsequent cultures were done by mechanical dissociation and chopping of the primary colonies and subculturing of the small chops on new feeder plates every 7 days after reaching a diameter of about 0.5 cm. After several passages, cells maintained the same morphology and strongly expressed mRNA of trophoblast markers, IFNτ, keratin (KRT8) and CDX2 (Fig. 11 and 12). The cells showed fast growth pattern in primary culture when they grew on PGCs; the colony diameter measures 7mm in one week while those which cultured on MEF grew by 5.7 mm in one week ($P \leq 0.05$). While there were no differences between the patterns of secondary cultures when trophoblast cultured on different feeders (Fig. 13). PGCs were highly proliferative with doubling time of approximately 24 h when compared to MEF one ($P \leq 0.5$), easy to recover, and provide a reasonable source of steroids, 17β-estradiol

(E2) (31.21±3.1 ng/ml) and progesterone (P4) (6.36±0.4 ng/ml). We speculate that the continuous supplement of steroids and other cytokines during the co-culture of trophoblasts with granulosa cells might help the trophectoderm cells growth than that of MEF.

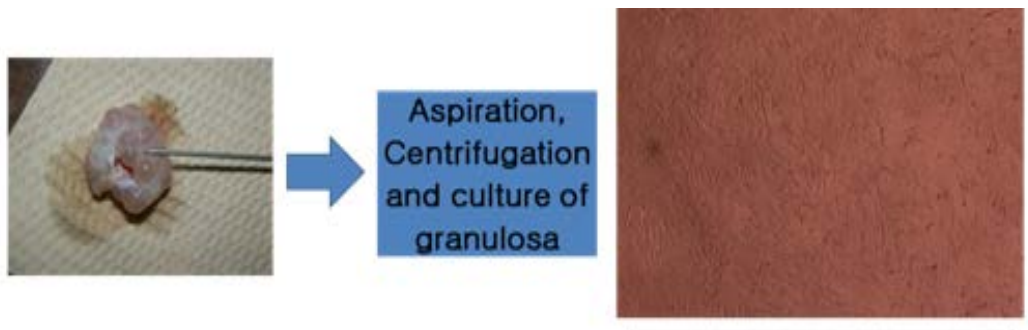

Fig. 10: Isolation of porcine granulosa cells (PGCs). Follicular fluid undergone centrifugation then the cells were washed and cultured in DMEM with 10% FBS.

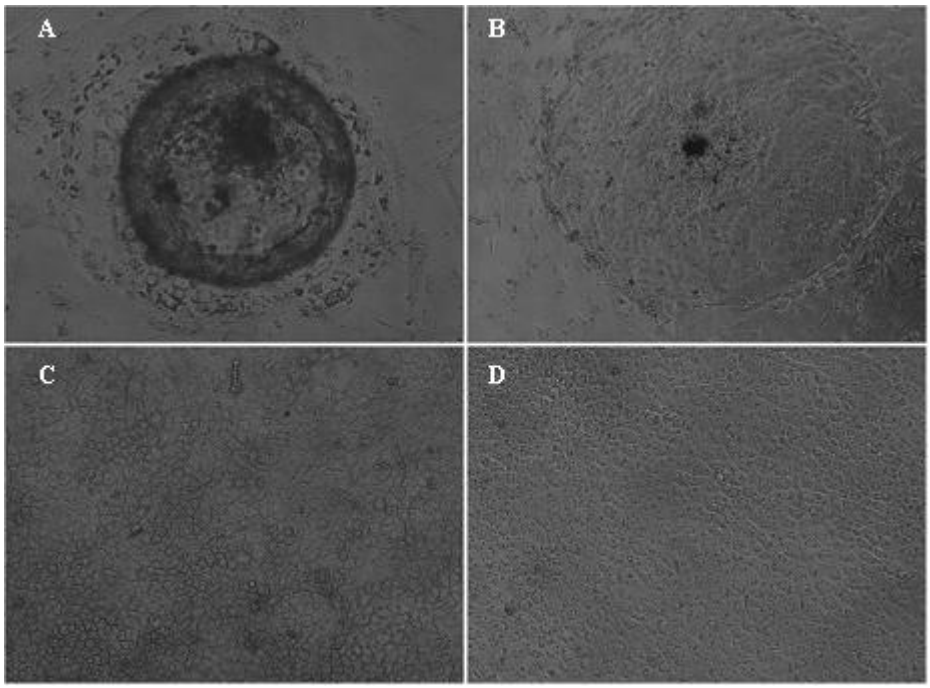

Figure 11. Isolation of trophoblasts from *in vitro* fertilized embryos. The embryo, a hatched blastocyst, was put on a feeder layer (Day 0) (X40). After 4 (A) (X40) and 7 days (B), the embryo was attached and exhibited trophoblast outgrowth. Primary trophoblast cells, which are large cuboidal cells on the feeder cells (C) (X100), were stable after serial subculture (D) (X100).

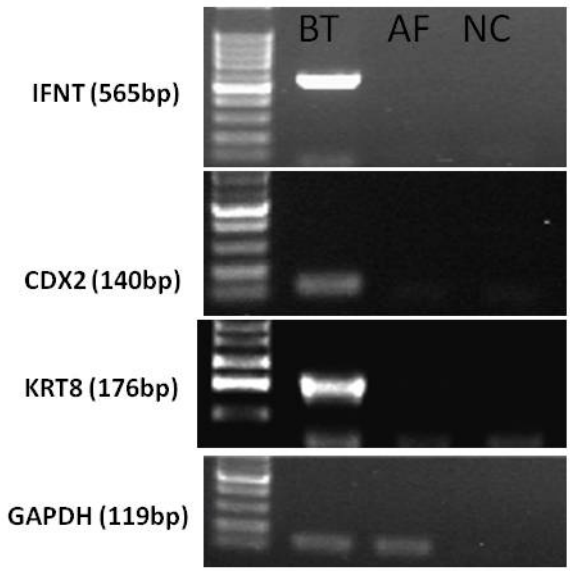

Figure 12. Gene expression screening in bovine trophoblasts (BT) and adult fibroblast cell lines (AF). Isolated trophoblasts cells strongly expressed IFNτ, KRT8 and CDX2, but adult fibroblasts (AF) did not. NC: negative control cDNA template.

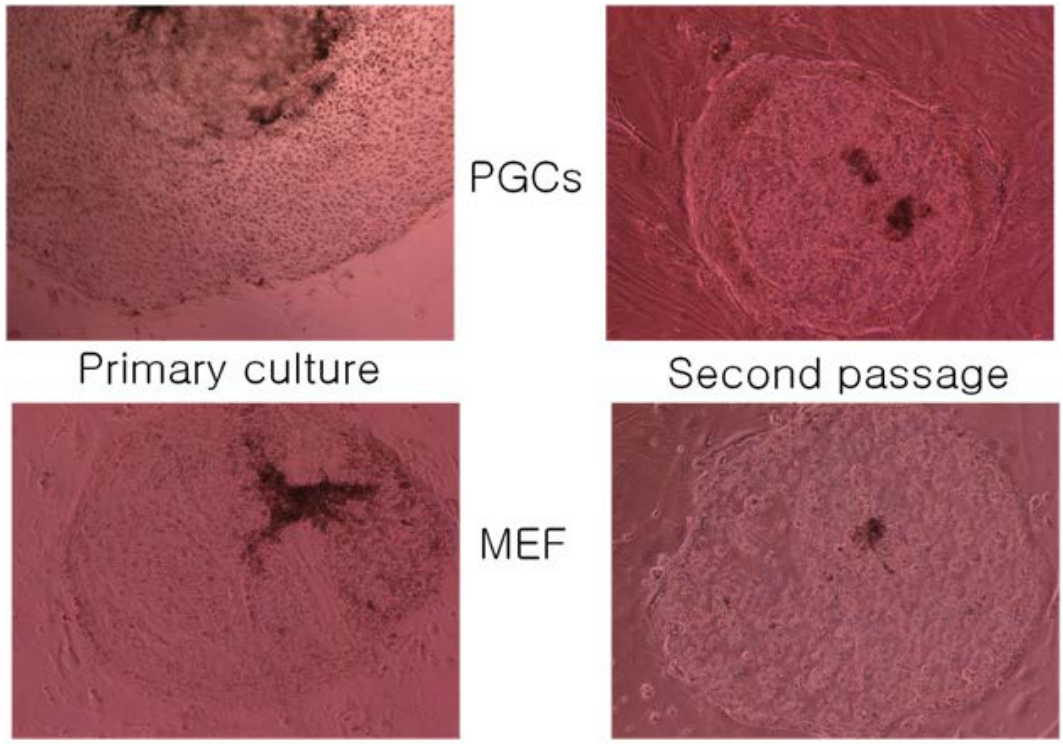

Fig. 13: Culture of bovine trophoblast on porcine granulosa cells (PGCs) and mouse embryonic fibroblast (MEF). The cells were subcultured for the 2nd passage.

Table 4. Primers used for RT-PCR.

Gene	Primer sequences (5'----3')	Annealing temperature (°C)	Fragment size (bp)	GenBank accession number
IFNT	F*: GACGATCTCTGGGTTGTTAC R*: GTG ATGTGGCATCTTAGTCA	55	565	X65539
CDX2	F:GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	60	140	DQ126146
KRT8	F: CACCAGTTCCAAGCCTGTGG R: TCAGGTCTCCTGTGCAGATGC	55	176	NM_001033610.1
GAPDH	F:GGCGTGAACCACGAGAAGTA R:CCCTCCACGATGCCAAAGT	60	119	NM_001034034.1

Chapter 2

Using of trophoblasts as donor cells for nuclear transfer

Abstract

Nuclear transfer (NT) has been used to produce many cloned offspring using several types of cells, including embryonic cells. Even though inner cell mass cells have been used as donor karyoplast for producing cloned animals, there are few studies using trophoblast. In mice, clones were born by nuclear transfer of trophoblasts from the expanded blastocyst into enucleated oocytes as a trial to show the totipotency of both inner cell mass and trophectoderm cells isolated from blastocysts. However, bovine trophoblast cell (BT) lines have not been used in NT to date. The purpose of this study was to elucidate whether BT as donor cell can be reprogrammed in bovine enucleated oocyte and be able to develop blastocyst. Hatched blastocysts produced by IVF were used to isolate BTs on mouse embryonic fibroblasts (MEF) treated with mitomycin C as feeder cells. BTs and adult fibroblasts (AF, control group for NT) were microinjected to perivitelline space of *in vitro* mature enucleated oocytes and electrically fused. Reconstructed embryos

were chemically activated and cultured in a 2-step chemically defined medium. As a result, BTs expressing IFNτ were successfully isolated and cultured on feeder layers were reprogrammed in the enucleated oocytes to blastocyst with similar efficiency to AF (14.5% and 15.6%, respectively; $P \leq 0.05$). Reprogramming of BTs was either negatively confirmed through injection of MEF into enucleated oocytes (no cleavage was observed) or positively confirmed through injection of transgenic trophoblasts.

Introduction

Since the first cloned lamb was born, nuclear transfer (NT) has been challenging in several species and has produced many cloned offspring (Keefer, 2008, Mastromonaco and King, 2007, Vajta, 2007). To produce cloned offspring, fetal fibroblasts have been chosen as a preferential donor cell line for NT so far because they have high proliferative potentials (Boquest *et al.*, 2002, Cibelli *et al.*, 1998, Forsberg *et al.*, 2002, Hyun *et al.*, 2003). In cattle, fetal and adult fibroblasts have been dominantly used for NT to produce cloned calves. Additionally, several types

of cells, like granulosa, cumulus, oviduct epithelial cells, skin, tongue and other cells, have been used for NT (Gong *et al.*, 2004, Kato *et al.*, 2000).

After fertilization of an egg with a sperm, the one-cell stage embryo grow up through several mitosis and reaches the preimplantation stage, becoming a blastocyst, which consists of an inner cell mass (ICM) that is capable of differentiation into all embryo organs and trophoblasts, which are the first differentiated cells from the embryo, and contributes formation of the placenta and fetal membranes but does not participate the formation of the fetus proper (Hyun *et al.*, 2003). In this study, for the first time, the trophoblasts isolated from bovine blastocysts were used as donor cells in nuclear transfer.

Materials and methods

Preparation of donor cells

Two donor cell types were used for NT. For donor cells, BT, the cell sheets were carefully removed by pipetting, then transferred to washing medium (DMEM-F12 with 10% FBS) and washed 2 times. Trypsin-EDTA was added for 6-8 minutes with

interval pipetting every 2 minutes. Cells were centrifuged (1500 rpm for 2 minutes) and washed with PBS 2 times and then suspended in PBS containing 0.5% FBS (v/v). For adult fibroblasts, confluent cell cultures were washed 2 times with PBS. Trypsin-EDTA was added for 2-3 minutes to detach the cells.

Differential cell staining of blastocysts

The cell numbers of blastomeres, inner cell mass (ICM) and trophectoderm (TE) cells in blastocysts were counted after chemically defined staining as described by Thouas *et al.* (2001). Blastocysts were incubated in 500 μl of BSA-free, Hepes-buffered TCM-199 supplemented with 1% (v/v) Triton X-100 and 100 μg/ml propidium iodide for 30 sec. When the TE color visibly changed to red and shrank slightly during treatment, blastocysts were incubated at 4 °C for overnight in 500 μl fixative solution consisting of 25 μg/ml bisbenzamide in absolute ethanol. The blastocysts were then treated in 99% (v/v) glycerol and mounted onto a glass microscope slide in a droplet of glycerol solution, and cell numbers of each parameter were counted using epifluorescence microscopy. The ICM cell nuclei

labeled with bisbenzimide appeared blue, and TE labeled with both bisbenzimide and propidium iodide appeared pink.

Derivation of GFP-transgenic trophoblast cell lines

Fibroblasts were isolated from bovine fetuses on Day 45 of gestation. Fetal tissues were minced with a surgical blade and dissociated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 0.25% (w/v) trypsin and 1 mM EDTA (Invitrogen) for 1 h at 37 ^{o}C. Trypsinized cells were washed once in Ca^{2+}- and Mg^{2+}-free DPBS by centrifugation at 1,500 rpm for 2 min, and subsequently seeded into 100-mm plastic culture dishes. Seeded cells were subsequently cultured for 6 to 8 days in DMEM supplemented with 10% (v/v) FBS (Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ and 1% (v/v) minimal essential medium (MEM) nonessential amino acid solution (Invitrogen) at 39 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until confluent, subcultured at intervals of 4 to 6 days by trypsinization for 5 min using 0.1% trypsin and 0.02% EDTA, allocated to three new dishes for

further passaging and then stored in freezing medium in liquid nitrogen at –196 ^{o}C. The freezing medium consisted of 80% (v/v) DMEM, 10% (v/v) DMSO and 10% (v/v) FBS (Invitrogen). Prior to SCNT, cells were thawed, cultured for 3 to 4 days until confluency for contact inhibition, and retrieved from the monolayer by trypsinization for 30 sec.

Gene Construction and transfection

GFP (Clontech) was amplified with PCR. Gateway PCR primers were GFP-Forward: ggggacaagtttgtacaaaaaagcaggcttcACCATGGCCAGCAA-AGGAGAAGAACTT, GFP-Reverse: ggggaccactttgtacaagaaagctgggtc-TTATTTGTAGAGCTCATCCATGCC. The amplified PCR fragments were used in a gateway cloning system (Invitrogen). The GFP -PCR fragments were recombined with BP and LR clonase (Invitrogen). As an entry vector, pDonor (Invitrogen) was used. Destination vectors, PB-CA with p-CCAGG promoter (from Addgene, http://www.addgene.org) were used to produce the final expression vector. Transposase expression vector (named pCy43, which is provided by Sanger Institute, Hinxton, UK) was used to transpose PB-CA-GFP. Approximately 18-24 hr

before transfection using Fugene HD (Roche, Mannheim, Germany), donor fibroblasts were plated in a 6-well plate. Once growing cells were 50-60 % confluent, transfection were carried out as described in the manufacturer's instructions. Serum-free DMEM (Invitrogen) containing a ratio of one to three (DNA: transfection reagent) was added as a culture medium. PB-CA-GFP and pCy43 were employed for GFP expression. To make GFP expressing cell line, GFP positive cells were mechanically collected and expanded in culture medium. These transgenic cells were used as donor cells for nuclear transfer (see below) and the resulted blastocysts were cultured to isolate trophoblast as in Fig. 14.

The nuclear transfer (NT)

The cells were centrifuged and washed with PBS 2 times and then suspended in PBS containing 0.5% FBS (v/v). A single trophoblast or adult fibroblast was deposited into the perivitelline space of enucleated oocytes. The couplets were subsequently placed in a fusion medium comprising 0.26 M mannitol, 0.1 mM $MgSO_4$, 0.5 mMHEPES and 0.05% BSA (Sigma) and transferred into a cell fusion chamber with a stainless steel wire electrodes (BTX 453, 3.2 mm gap; BTX, San

Diego, CA, USA) after equilibration for 3 min. Fusion was induced by two DC pulses of 1.75 to 1.85 kV/cm for 15 sec using a BTX Electro Cell Manipulator 200. Fusion of the donor cell and ooplast was observed 1 h after electric stimulation under a stereomicroscope. Only fused embryos were selected and cultured for 4 h in TCM 199 supplemented with 10% FBS. Reconstructed embryos were activated for 4 minutes with 5mM ionomycin (Sigma-Aldrich Corp.) followed by 4 h of culture in 1.9 mM 6-DMAP (Sigma-Aldrich Corp.) microdrops. Cloned embryos were cultured in 25 μl microdrops of a two-step defined culture medium overlaid with mineral oil (Sigma-Aldrich Corp.) for 7 to 8 days at 39 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

Statistical analysis

In each experimental group, presumptive zygotes were randomly distributed. All data were subjected to one-way ANOVA followed by Tukey's test to determine differences among the experimental groups using GraphPad (Version 4.0). Statistical significance was determined when a P value was less than 0.05.

Results and discussion

For this study, we isolated trophoblast cells from *in vitro* fertilized embryos. Hatched blastocysts were attached to a mouse feeder layer, and BT cells with a cuboidal morphology (Talbot *et al.*, 2000) were maintained for further culture (as in Fig. 11). The BT cell line had high proliferative capability and outgrowth into visible colonies occurred within 5 to 7 days after subculture (Fig. 11). IFNτ was well expressed in these cells but not in adult fibroblasts were not expressed (as in Fig. 12).

A trophoblast cell was injected into enucleated oocytes, and the oocytes and trophoblast cells were then fused, activated and cultured in a two-step defined culture medium. Fusion of BTs was lower than in AFs (71.3% vs. 93.1%, respectively), while there were no difference in cleavage, morula, blastocysts formation rates and ICM/TE ratio (71.3%, 30.6%, 14.5% and 31.27±4.2%, respectively) between the two types of donor cell (Table 5 and Fig. 15). For more confirmation, we injected mouse embryonic fibroblasts as a negative control donor cell (the feeder cells of BT-1 in culture), and no reprogramming occurred (morula

and blastocyst compaction were both 0%, n=60 oocytes), which provide that the reprogramming was solely derived from the BT donor cells. Moreover, we used transgenic BTs derived from green fluorescence protein (GFP) embryos as donor cells for nuclear transfer, and the resultant embryos expressed GFP (Fig. 16). In the current study, the blastocysts derived from AFs or BTs showed no difference in developmental competence parameters, including cleavage, blastocyst formation and total and differential blastocyst cell counts, while they did show a significant difference in membrane fusion that might have been due to the large cell size of the BTs compared with the AFs (Fig. 17) (Prather *et al.*, 1987, Zhang *et al.*, 2009).

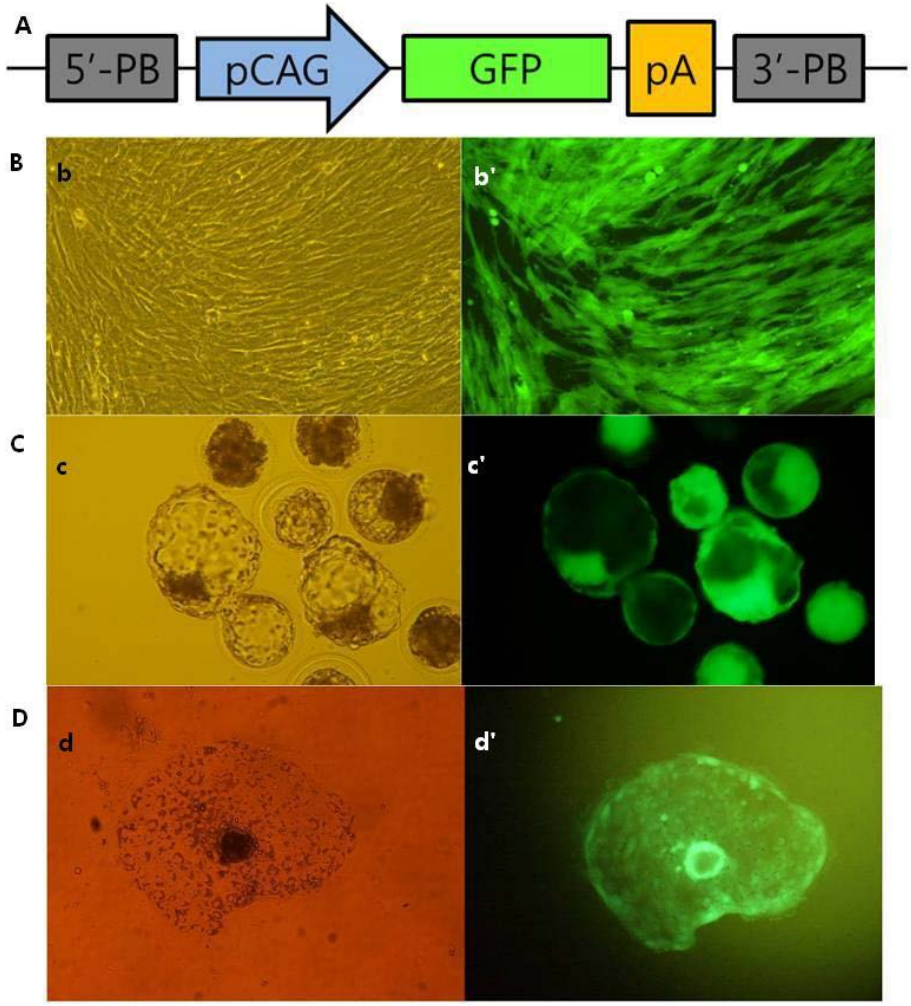

Figure 14: Production of Green fluorescent protein (GFP) expressing cloned embryos and trophoblast cell line. A) Illustration of GFP expressing vector map, B) GFP expression in bovine donor fibroblasts (b-b`); cloned bovine pre-implantation embryos (c-c`) and transgenic trophoblast cell line (d-d'). Images under visible light (b-c-d) and fluorescent light (b'-c`-d`).

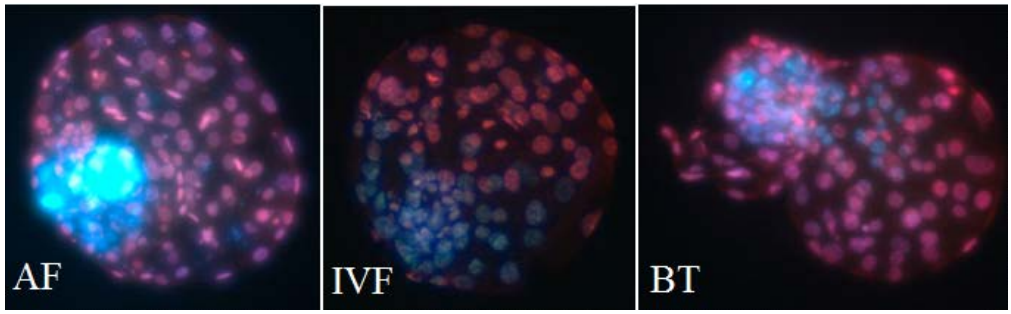

Figure 15. Differential staining of different blastocysts, trophectoderm cells stained red and ICM cells stained blue.

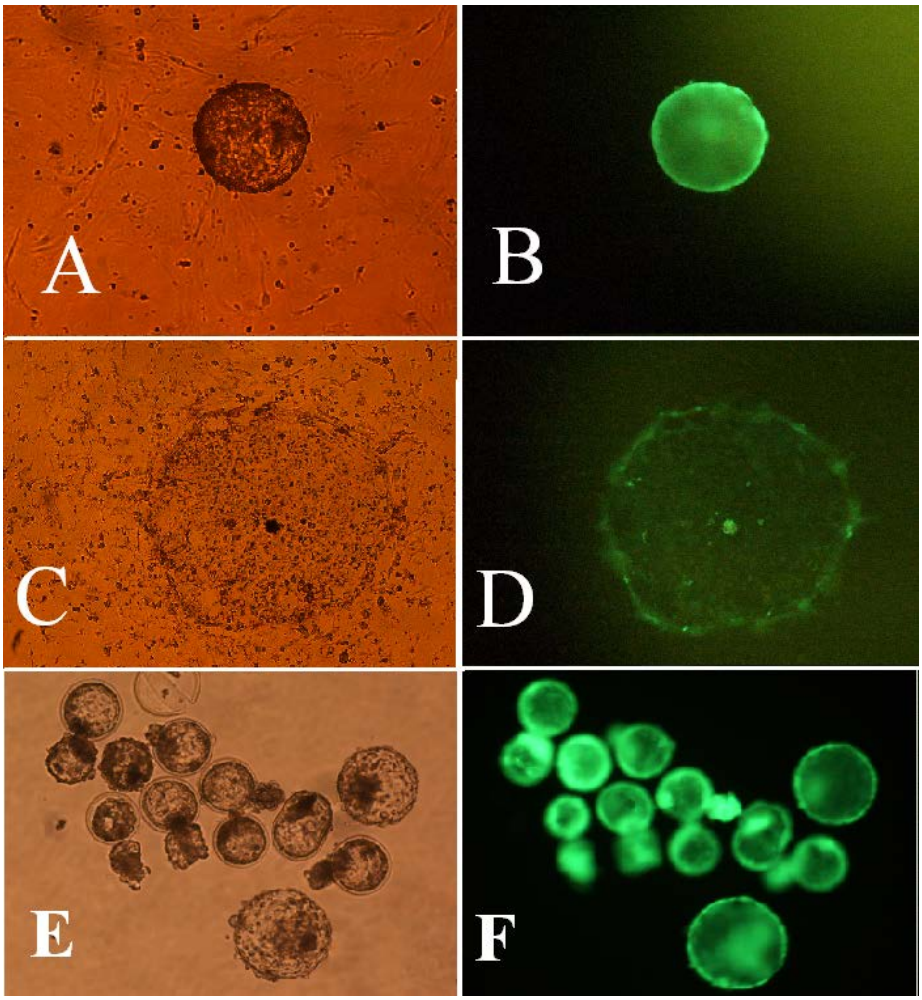

Figure 16: A& B, Day 1 transgenic blastocyst cultured on feeder cells (MEF) expressing GFP; C& D, Day 7 cultured blastocyst on feeder cells with apparent GFP expression in trophoblast outgrowths; E& F, blastocysts derived from using transgenic BT as donor cell expressing GFP. A, C, E were seen under visible light microscope; B, D and F were see under UV lamp. Contact us for more information about GFP vector map and transfection-related protocols.

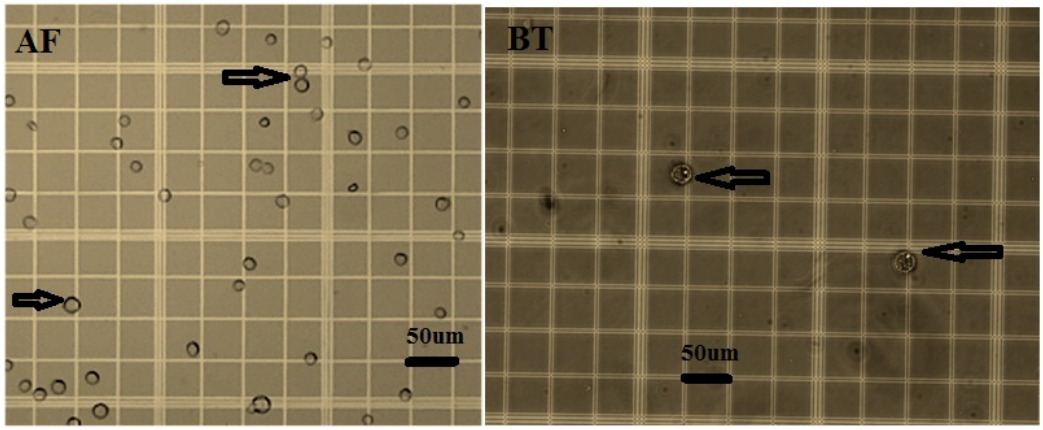

Figure 17. Different bovine cell sizes on hemocytometer slide. Trophoblast (BT) ranges from 20-25 µm however the adult fibroblast (AF) cell size ranges from 12-15 µm.

Table 5. Development of embryos reconstructed by using an adult fibroblast or trophoblast as a donor cell

Type of donor cell	Total oocytes	Fused oocytes (%)	Cleaved embryos (%)	Morula (%)	Blastocyst (%)	Total blastocyst cell count on 7^{th}day (ICM/TE)
Adult Fibroblast	90	83 (93.1)*	63 (75.9)	24 (28.9)	13 (15.6)	74.02±5.1 (31.27±4.2%)
Trophoblast	87	62 (71.3)	49 (79.0)	19 (30.6)	9 (14.5)	76.71±4.6 (28.92±5.1%)

*The value is significant (P≤0.05).

Chapter 3

Analysis of temporal gene expression in embryos resulted from using trophoblast as donor cells for nuclear transfer

Abstract

The temporal progressive increase of interferon tau (IFNτ) secretion from the bovine trophoblast is a major embryonic signal of establishing pregnancy. Here, we compared the temporal expression of implantation related genes between blastocysts derived from reprogrammed BTs and AF in enucleated oocytes. The levels of IFNτ, CDX2 and OCT4 expression in IVF-, BT- and AF-derived blastocysts were analyzed using reverse transcription polymerase chain reaction and reverse transcription quantitative polymerase chain reaction (RT-PCR and RT-qPCR). IVF-produced embryos were used as reference to analyze the linear progressive expression of IFNτ through mid, expanded and hatching blastocysts. RT-PCR and RT-qPCR studies showed that IFNτ expression was higher in BT-derived blastocysts than IVF- and AF-derived blastocysts. Both IVF- and BT-derived blastocysts showed a progressive increase in IFNτ expression as blastocyst

development advanced when it compared with AF-derived blastocysts. OCT4 was inversely related with IFNτ expression, while CDX2 was found to be directly related with IFNτ temporal expression. Persistence of high expression of IFNτ and CDX2 was found to be higher in BT-derived embryos than in IVF- or AF-derived embryos. In conclusion, using BTs expressing IFNτ as donor cells for bovine NT could be a useful tool for understanding the IFNτ genetics and epigenetics.

Keywords: Interferon tau, bovine trophoblast, reprogramming, nuclear transfer, real-time PCR

Introduction

Some reports have demonstrated trophoblast isolation and its function *in vitro* in cattle (Talbot *et al.*, 2000, 2004, 2008). In mice, living pups were born by nuclear transfer of trophectoderm cells from the expanded blastocysts into enucleated oocytes as a trial to show the similarity into tipotencyof both ICM and trophoblast cells from a single blastocyst (Tsunoda and Kato, 1998). However, bovine trophoblast cell lines have not been employed in NT so far.

Over the past two decades, there has been much interest in interferon tau (IFNτ), which is produced by the trophectoderm during a defined period of peri-attachment in ruminant embryos. IFNτ is a type I IFN under a unique transcriptional control that limits its expression to ruminant trophoblasts prior to implantation (Ezashi *et al.*, 2001, Hernandez-Ledezma *et al.*, 1992, Spencer and Bazer, 2004). A major role of this cytokine is to mute the pulsatile release of prostaglandin $F_{2\alpha}$ from the maternal uterine endometrium, thereby, blocking luteolysis (Hansen *et al.*, 1999, Roberts *et al.*, 1992).

The unique pattern of IFNτ expression is regulated by promoter/enhancer regions that are distinct from those of other type I IFN genes (Ealy and Yang, 2009,

Thatcher *et al.*, 2001). One key component of IFNτ expression is caudal-type homeobox 2 (CDX2), which stimulates IFNτ promoter activity in the presence of Ets-2 (Ezashi *et al.*, 2008, Sakurai *et al.*, 2009). In addition, the POU homeodomain protein (Oct-4), which is best known as a marker of pluripotency (Pesce and Scholer, 2000), blunts the ETS2-induced IFNτ promoter activity (Ezashi *et al.*, 2001) in addition to inhibiting other factors during early pregnancy like CDX2 (Niwa *et al.*, 2005). So this study, as a continuation of our previous work, was undertaken to (1) elucidate if BT, as an interferon tau secreting cell, can be reprogrammed in bovine enucleated oocyte; (2) determine the relative abundance of IFNτ expression in the resulting cloned preimplantation embryos; and (3) study the temporal gene interaction affecting IFNτ expression, especially the OCT4 and CDX2 genes.

Materials and methods

The blastocysts resulted from experiments in chapter 3 were used for further analysis in the current experiment.

Semi-quantitative and relative quantitative PCR

Three embryos from each stage, mid, expanded and hatching blastocysts (C6, C7 and C7H respectively according to the IETS embryo codes, Robertson and Nelson, 2010), were subjected to RT-PCR and qPCR with three repetitions per sample. A single embryo from each stage was washed with PBS three times, transferred into 5 µl of diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C or used freshly for total RNA extraction using an RNeasy total extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer instructions. Reverse transcription was carried out at 50 °C for 50 min. Individual RT reactions were performed using a random hexamer and SuperScript™ III Reverse Transcriptase (Invitrogen) in a 20 µl reaction. One to two microliters cDNA were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using aMaxime PCR PreMix kit (i-starTaq) (Intron, Seoul, Republic of Korea). Primer sequences, annealing temperatures and approximate sizes of the amplified fragments are listed in table 1. PCR amplification was carried out with one cycle of denaturation at 95 °C for 5 min and subsequent cycles of denaturation at 95 °C, annealing for 30

seconds, extension at 72 °C for 45 seconds and final extension at 72 °C for 5 min. Ten µl of PCR products were fractionated on 1% agarose gel (Intron) and stained with RedSafe™ (Intron). The expression level for each gene was determined densiometrically with the Image J software (Version 1.40g, NIH, Besthesda, MD, USA). Relative expression levels of each gene at specific stages of embryo development were represented as a ratio to GAPDH gene expression. Relative quantitative PCR (RT-qPCR) was done according to the Takara Bio Inc. guidelines. A 22 µl PCR reaction mix was made by adding 2 µlcDNA, 1 µl forward primer, 1 µl reverse primer, 8 µl SYBR Premix Ex Taq, 0.4 µl ROX Reference (Takara Bio Inc., Shiga, Japan) and 9.6 µl of nuclease-free water (Ambion Inc., Austin, TX, USA). The reaction was done by using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the company instructions. The thermal profile for real-time PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec and 72 °C for 40 sec.

Gene expression in blastocysts derived from different donor cells

Interferon tau (IFNT), CDX2 and OCT4 expression were studied by reverse transcription PCR of blastocysts derived from NT using bovine trophoblasts (BT) and adult fibroblasts (AF) or by *in vitro* fertilization (IVF). Relative quantitative PCR for these genes of different embryonic stages, mid (C6), expanded (C7) and hatching (C7H) blastocysts, were evaluated using the pixel intensity of the agarose gel bands and the using imaging analysis program ImageJ (1.40 g, NIH, Bethesda, MD, USA). The relative expression of each specific gene was calculated and presented as a ratio to the same gene in the same stage of IVF-produced embryos as arbitatory units.

Relative progression of gene expression in embryos derived from different donor cells

The stages of blastocyst expansion till blastocyst hatching showed variable behaviors in the temporal gene expression patterns, especially for the IFNτ, CDX2 and OCT4 genes. Here, we studied the relative temporal expression of these genes in mid, expanded and hatching blastocysts in relation to their expression in the

morula stage within the same group as an internal reference in order to determine the relative progressive expression of each gene separately.

Statistical analysis

In each experimental group, presumptive zygotes were randomly distributed. All data were subjected to one-way ANOVA followed by Tukey's test to determine differences among the experimental groups using GraphPad (Version 4.0). Statistical significance was determined when a P value was less than 0.05.

Results

Relative gene expression in blastocysts derived from different donor cells

IFNτ, CDX2 and OCT4 expressions in individual developing blastocyst (mid, expanded and hatching or C6, C7 and C7H, respectively, according to the IETS embryo codes) were compared among the different donor cell types (Fig. 18). RT-PCR showed that the IFNτ expression was increased by more in BTs than in AFs when the relative expressions of the same stages of IVF-produced blastocysts, stage

C7 and C7H but not C6, were compared (Fig. 18). Similarly, in BT-derived blastocyts, CDX2 showed an increase in expression when compared with AF produced hatching blastocysts. On the other hand, OCT4 showed a decrement in expression in BT-derived blastocysts compared with AF-derived blastocysts in both hatching and hatched stages (Fig. 18).

Temporal progression of gene expression

Fig. 19 illustrates the RT-qPCR comparison of IFNτ, CDX2 and OCT4 relative to the progression of the developmental stages of BT-, AF- and IVF-produced blastocysts in relation to their expression in the morula stage as an internal reference of the same group. IFNτ progressively increased along with blastocyst development, i.e., from the mid-blastocyst stage until hatching in both BT- and IVF-produced blastocysts; however, it showed a decrease in expression by advancement of blastocyst growth in AF-produced blastocysts. On the other hand, OCT4 expression progressively decreased along with blastocyst development in both BT- and IVF-produced blastocysts; however, it tended to be stable or slightly increased

(but not significant) in conjunction with blastocyst hatching in AF-derived blastocyts. CDX2showed a similar expression pattern of IFNτ in BT- and AF-derived blastocysts (i.e., progressive increase), while it did not show any significant change with the advancement of growth in IVF-produced blastocysts.

Discussion

Many cloned offspring have been produced using several types of donor cells including fibroblasts to date (Kato *et al.*, 2000, Keefer, 2008, Jang *et al.*, 2010). Most embryonic or somatic cells are reprogrammed in enucleated oocytes and develop into preimplantation embryos with different efficiencies. Although embryonic cells, particularly, ICM cells, which are pluripotent, have been well applied to NT for producing cloned offspring in mice, trophoblast cells have not been given much attention. In ruminants, trophoblasts cells at the implantation stage strongly expressed IFNτ, a type I IFN that is considered to be an important signal for anti-luteolysis in early pregnancy (Binelli *et al.*, 2000). Here, we proved the hypothesis that trophoblasts cells expressing IFNτ would be reprogrammed in

enucleated oocytes and develop into cloned blastocysts. In addition, IFNτ expression increased along with development from early to hatching blastocysts.

Hernandez-Ledezma *et al.* (1992) suggested that the best way to study IFNτ expression as an indicator of embryo quality is to examine the temporal expression rather than the absolute expression at a particular stage because the latter is known to vary widely. Therefore, the progressive increase in IFNτ expression from IVF- or BT-derived blastocysts indicates the similarity in behavior of IFNτ transcripts between the two groups, while its level was found to be decreased as growth of blastocysts produced with AFs progressed, reflects the blastocyst quality.

OCT4 was found to be inversely affected by the IFNτ expression in all experimental groups, confirming our previous results that OCT4 is the major dominant gene affecting IFNτ expression. CDX2 expression was found to be directly related to IFNτ expression along with the embryo development until the hatching stage in both BT- and AF-derived blastocysts suggesting that CDX2 is a potent regulator of IFNτ expression (Chen *et al.*, 2009, Kim *et al.*, 2007, Sakurai *et al.*, 2009) while it showed no change in IVF-produced blastocysts.

The significant increase in IFNτ expression in BT-derived blastocysts compared with IVF-produced blastocysts might be because of the increased expression of CDX2 and the decrease in OCT4. We speculate that the increase in IFNτ might be the result of a retained epigenetic status of the donor trophoblast (high expression of IFNt and CDX2) cell because it was found that the epigenetic state of the donor nucleus was retained in cloned embryos and that it affected the reprogramming and development of the embryos (Polo *et al.*, 2010, Yamazaki *et al.*, 2003). In addition, reprogramming by NT keeps Oct-4 and Nanog stably silenced by methylation (Polo *et al.*, 2010, Yamazaki *et al.*, 2003), which is another factor increasing IFNτ expression. Further investigations are required in this regard.

This is the first report to show successful reprogramming of bovine trophoblast cells expressing IFNτ by NT into preimplantation embryos and that the embryos expressed a progressive increase in IFNτ along with development from early to hatching blastocysts. Also, it showed the persistence of high expression of IFNτ and CDX2 in embryos derived from a cell in which these genes are highly expressed. Finally, use of BT cells in NT will be one resource for further understanding of the

interaction between OCT4 and CDX2 genes in the regulation of IFNτ expression and epigenetic regulation of this gene.

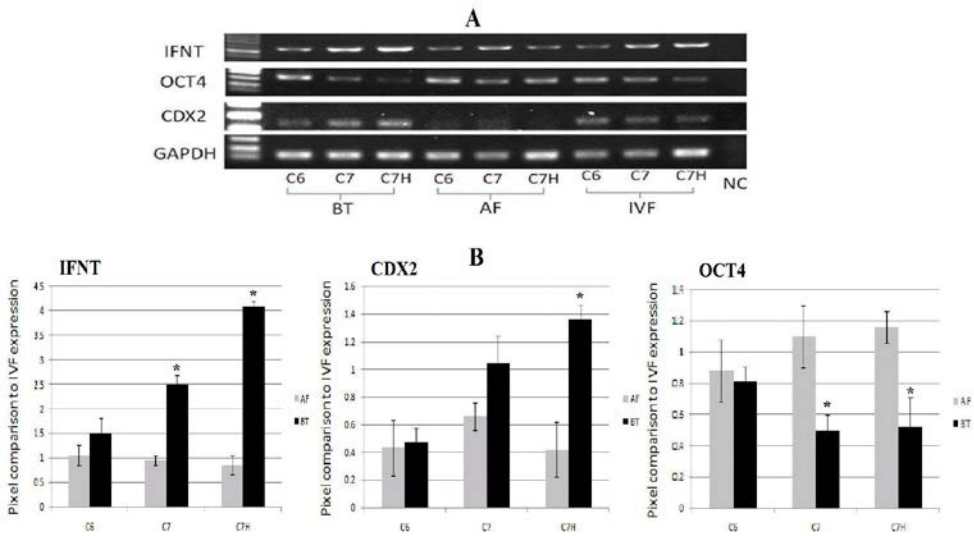

Figure 18. Interferon tau (IFNτ), CDX2 and OCT4 expression by (A) reverse transcriptase PCR of blastocysts derived from NT by bovine trophoblasts (BT) and adult fibroblasts (AF) or by *in vitro* fertilization (IVF); (B) comparison of relative quantitative PCR of IFNτ, OCT4 and CDX2 in different stages of blastocysts, mid (C6), expanded (C7) and hatching (C7H), in BT- and AF-produced blastocyst. The values (mean±SE) are presented as the relative expression to the same stage of IVF-produced blastocysts in arbitatory units. *The value is significant (P≤0.05).

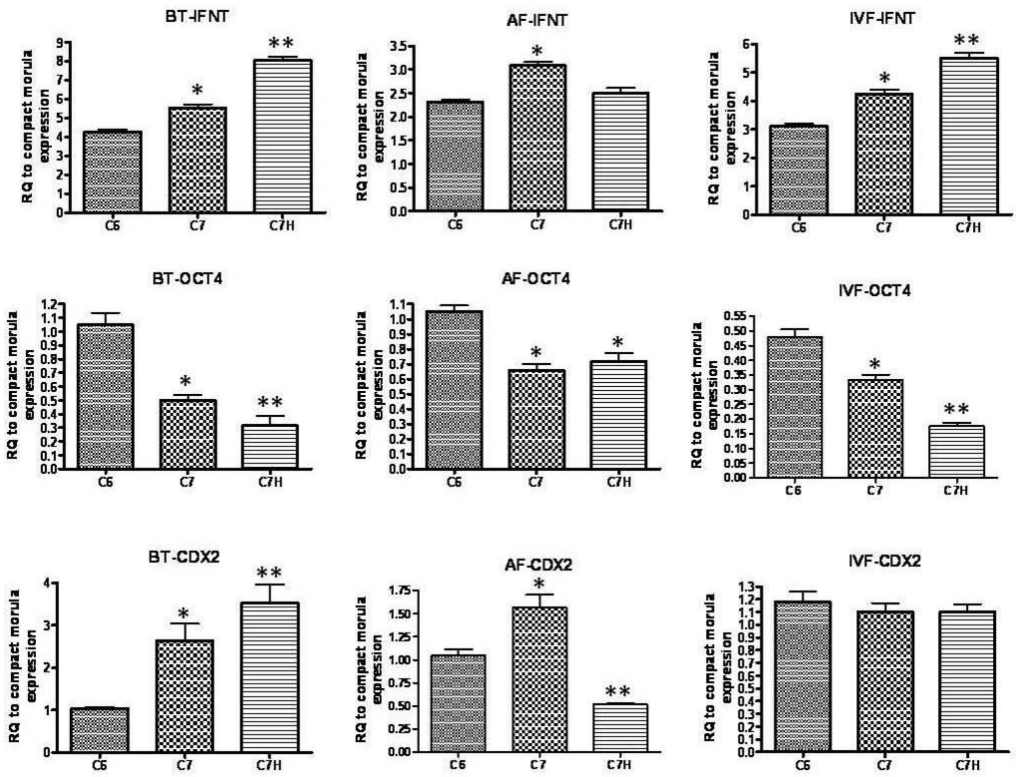

Figure 19. Real-time PCR values show the temporal relative progress of IFNT, OCT4 and CDX2 expression along with embryo development in IVF-, BT- and AF-derived blastocysts. The values (mean±SE) are presented as the relative quantitation (RQ) of the specific gene to its expression in the morula stage of the same group. *The value is significant (P≤0.05). C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching

Table 6. Primers used for RT-PCR* and real-time qRT-PCR

Gene	Primer sequences (5'----3')	Annealing temperature (°C)	Fragment size (bp)	GenBank accession number
IFNT	F:TCCATGAGATGCTCCAGCAGT R:TGTTGGAGCCCAGTGCAGA	60	103	X65539
	F*: GACGATCTCTGGGTTGTTAC R*: GTG ATGTGGCATCTTAGTCA	55	565	
OCT4	F:GGTTCTCTTTGGAAAGGTGTTC R:ACACTCGGACCACGTCTTTC	60	314	AF022987
CDX2	F:GCCACCATGTACGTGAGCTAC R: ACATGGTATCCGCCGTAGTC	60	140	DQ126146
KRT8	F: CACCAGTTCCAAGCCTGTGG R: TCAGGTCTCCTGTGCAGATGC	55	176	NM_001033610.1
GAPDH	F:GGCGTGAACCACGAGAAGTA R:CCCTCCACGATGCCAAAGT	60	119	NM_001034034.1

PART 4

GENERAL CONCLUSION

In the current thesis the temporal interaction among the genes that control IFNT was studied using different *in vitro* culture media and different donor cells for nuclear transfer (NT), including the IFNτ-secreting cell, the trophoblast.

The importance and the efficiency of using two-step chemically defined media was confirmed as a good tool for bovine embryos *in vitro* culture system because of; 1) Increased blastocyst yield, 2) Increased blastocyst cell number, 3) Increased blastocyst hatching rate, 4) harmonic and orchestrated gene interplay reflected by progressive increase in IFNτ expression which is a good sign for blastocyst developmental competence. 5) the dominance of OCT4 over all the other transcripts in regulation of IFNτ expression and subsequent IFNτ secretion.

The current study included the first attempt to isolate bovine trophoblast on a cheap and easily recovered feeder cells which are porcine granulosa cells instead of the conventional method which used mouse embryonic fibroblast. Morever, it showed the first attempt also to use bovine trophoblast as donor cells for nuclear transfer and produced *in vitro* competent embryos.

The temporal expression of IFNT showed a cumulative pattern in the embryos

derived from triophoblast as donor cells, that can be a useful method for further studying IFNT epigenetics.

Using of trophoblast cells in NT could be one resource for further understanding of the interaction between OCT4 and CDX2 genes in the regulation of IFNT expression and trophoblast physiology.

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초 록

소 체외수정 및 체세포핵이식 수정란에서

interferon tau 를 조절하는 유전자들의 상관관계

초기 수정란의 착상 과정에서는 관련 유전자들이 상호작용을 통해 착상을 돕는 것으로 보고되었다. 체외 배양 조건에 따른 착상 관련 유전자 발현 양상을 알아보기 위해 소 체외수정란을 2단계 한정 배지군과 BSA가 첨가된 mSOF 배지군에서 발육율 및 ETS2, CDX2, GATA2, GATA3, OCT4 및 NANOG 등의 유전자 발현을 semi-qPCR 및 relative PCR 방법으로 분석하였다. 체외발육을 비교한 결과 한정 배지군에서 유의적으로 높은 배반포 형성율(33.1% vs. 18.9%; p<0.05)과 배반포 부화율(52.3% vs. 33.5%)을 보였다. 착상관련 유전자인 IFNτ를 조절하는 유전자들의 상대적 발현량을 분석한 결과 한정 배지군에서 수정란 발육단계에 따라 발현량이 증가하였고 mSOF군에서는 배반포 부화 단계에서 감소함을 보였다. IFNτ 발현 조절에서 ETS2, CDX2, GATA2 및 GATA3는 발현을 증가하는 반면 OCT4는 감소시켰으나 OCT4가 다른 전사 인자에 비해 상대적으로 강하게 작용하였다. OCT4 및 그 수동적 인자인 NANOG의 영향을 받는 CDX2도 IFNτ 발현에 직접 영향을 미치는 것으로 나타났다. 결론적으로 체외수정란의 발육은 mSOF군에 비해 한정배지를 이용한 2단계 배양법에서 증가하였으며 OCT4가 다른 유전자보다 상대적으로 강하게 IFNτ 의 발현을 조절함을 알 수 있었다.

공여세포에 따른 체세포핵이식란의 발육율을 비교하기 위해 소 체외수정란 유래 배반포에서 수립된 영양아세포주와 성체 섬유아세포를 체세포핵이식에 공여하였다.

체외수정란을 대조군으로 하여 공여핵원에 따른 체세포핵이식란의 배반포에서 IFNτ, CDX2 및 OCT4 발현 정도를 RT-PCR 과 RT-qPCR로 비교 분석하였다. 공여세포에 따른 배반포 발육율은 유의적인 차이가 없었으나 (14.5% vs. 15.6%, P≤0.05) IFNτ 발현을 분석한 결과 영양아세포 유래 배반포에서 체외수정란과 섬유아세포 유래 배반포보다 높았다. 또한 체외수정란과 영양아세포 유래 배반포에서는 발육 단계에 따라 IFNτ와 OCT4는 유사한 발현 패턴을 보였다.

결론적으로 한정배지군에서 체외수정란의 높은 발육율 및 착상관련 유전자의 발육단계에 따라 발현 증가를 확인하였고 OCT4는 IFNτ 의 발현을 억제하는 반면 CDX2는 IFNτ 의 일시적 발현을 촉진시키는 것으로 나타났다. 착상관련 유전자의 발현에 있어서 본 연구에서 수립된 영영아세포 유래 복제배반포가 성체섬유아세포 유래 복제배반포보다 체외수정란과 유사한 양상을 보여 새로운 공여세포원으로 제시되었다. 이러한 결과는 IFNτ의 유전학적 및 후생학적 역할을 이해하는데 유용할 것으로 사료된다.

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핵심어: 소, 체세포복제, 영양아세포, 유전자발현, 착상, Interferon-τ.
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