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Functional analysis of genes during bovine preimplantation embryo development

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Dedicated to my mother, father, sister and all members of my family

Functional analysis of genes during bovine preimplantation embryo development

This study, the RNA interference (RNAi) approach was applied to suppress the expression of the maternal (C-mos) and embryonic (Oct-4) transcripts in bovine oocytes and embryos, respectively using microinjection of sequence specific double-stranded RNA (dsRNA). For this 435 bp C-mos and 341 bp Oct-4 dsRNA were synthesised and microinjected into the cytoplasm of immature oocytes and zygotes, respectively. In experiment 1, immature oocytes were categorized into three groups: those injected with C-mos dsRNA, water (RNase-free), and uninjected controls. In experiment 2, *in vitro* produced zygotes were categorized into three groups: those injected with Oct-4 dsRNA, water (RNase-free) and uninjected controls. The developmental phenotypes, the level of mRNA and protein expression were investigated after treatment in both experiments. Microinjection of C-mos dsRNA has resulted in reduction of C-mos transcript (70%) and protein after maturation compared to the water injected and uninjected controls ($P < 0.01$). From oocytes injected with C-mos dsRNA, 60% showed the extrusion of first polar body compared to 50% in water injected and 44% in uninjected controls. Moreover, only oocytes injected with C-mos dsRNA showed spontaneous activation. Microinjection of zygotes with Oct-4 dsRNA has also resulted in reduction in Oct-4 transcript abundance (72%) and protein at the blastocyst stage compared to the uninjected control zygotes ($P < 0.01$). The first cleavage, morula and blastocyst rate were not significantly different between three treatment groups. However, a significant reduction in the number of inner cell mass was observed in Oct-4 dsRNA injected embryos compared to the other groups. In conclusion, these results demonstrated that sequence specific dsRNA can be used to knockdown maternal or embryonic transcripts in bovine embryogenesis and therefor as a tool to study the function of genes.

Funktionelle Analyse von Genen in boviner preimplantativer Entwicklung

In dieser Arbeit wurde der RNA interference (RNAi) Ansatz angewendet, um die Expression maternaler C-mos und embryonaler Oct-4 Transkripte in bovinen Oozyten bzw. Embryos mittels Mikroinjektion sequenzspezifischer doppelsträngiger RNA (dsRNA) zu unterdrücken. Hierzu wurden 435 bp C-mos und 341 bp Oct-4 dsRNA synthetisiert und in das Zytoplasma immaturer Oozyten bzw. Zygoten mikroinjiziert. In Experiment 1 wurden die immaturen Oozyten in drei Gruppen eingeteilt: mit C-mos dsRNA injizierte, mit Wasser (RNase-freiem) injizierte und uninjizierte Kontrollen. In Experiment 2 wurden die in vitro produzierten Zygoten in drei Gruppen eingeteilt: mit Oct-4 injizierte, mit Wasser (RNase-freiem) injizierte und uninjizierte Kontrollen. Entwicklungsphänotypen, mRNA Level und Protein Expression wurden nach der Behandlung in beiden Experimenten untersucht. Die Mikroinjektion von C-mos dsRNA resultierte in einer Reduktion von C-mos Transkript (70%) und Protein nach Maturation verglichen mit mit Wasser injizierten und uninjizierte Kontrollen ($P < 0.01$). 60% der mit C-mos dsRNA injizierten Oozyten zeigten Extrusion des ersten Polrkörpers verglichen mit 50% der mit Wasser injizierten und 44% der uninjizierten Kontrollen. Überdies zeigten einzig mit C-mos dsRNA injizierte Oozyten spontane Aktivierung. Die Mikroinjektion von Zygoten mit Oct-4 dsRNA resultierte ebenfalls in einer Reduktion von Oct-4 Transkriptabundanz (72%) und Protein im Blastozystenstadium verglichen mit den uninjizierten Kontrollzygoten ($P < 0.01$). Teilungs-, Morula- und Blastozystenrate waren nicht signifikant unterschiedlich zwischen den 3 Behandlungsgruppen. Ferner wurde eine signifikante Reduktion der Anzahl der Zellen der inneren Zellmasse in Oct-4 dsRNA injizierten Embryos beobachtet im Vergleich zu den anderen Gruppen. Diese Resultate demonstrieren, dass sequenzspezifische dsRNA zum Knockdown maternaler oder embryonalen Transkripte in der bovinen Embryogenese genutzt werden um die Funktion von Genen zu untersuchen.

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List of abbreviations

°C	Degree celsius
2´,5´-A	2´-5´-oligoadenylate
2´,5´-OAS	2´-5´-oligoadenylate synthetase
AI	Artificial insemination
AP	Alkaline phosphatase
APS	Ammonium peroxydisulfate
A. thaliana	*Arabidopsis thaliana*
asRNA	Antisense RNA
ATP	Adenosine triphosphate
BMP-15	Bone morphogenetic protein 15
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
C. elegans	*Caenorhabditis elegans*
C-mos	Oocyte maturation factor Mos
CO_2	Carbondioxy
COC	Cumulus oocyte complex
cont.	Continue
CSF	Cytostatic factor
C_T	Threshold cycle
Ctcf	CCCTC-binding factor
DEPC	Diethyl pyrocarbonate
D. melanogaster	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
Doc 1r	Tumor suppressor deleted in oral cancer-related 1
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DTCS	Dye Terminator Cycle Sequencing
DTT	Dithiothreitol

eCG	Equine chronic gonadotropin
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetraacetic acid
EGA	Embryonic genome activation
eIF2α	α-subunit of eukaryotic protein synthesis initiation factor 2
ES	Embryonic stem
EST	Expressed sequence tag
Fgf-4	Fibroblast growth factor 4
FITC	Fluorescein isothyocyanate
FSH	Follicle stimulating hormone
G	Gauge
g	Gram
G2 phase	Second gap phase
Gdf-9	Growth differentiation factor 9
GFP	Green fluorescence protein
GVBD	Germinal vesicle breakdown
h	Hour
H2a	Histone 2a
hCG	Human chorionic gonadotropin
hpi	Hours post insemination
HRP	Horseradish peroxidase
ICM	Inner cell mass
IPTG	Isopropyl β-D-thiogalectosidase
Itpr 1	Inositol 1,4,5-triphosphate receptor 1
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
IU	International unit
kDa	Kilo dalton
L	Litter
LH	Latinizing hormone

M	Molar
MII	Second meiosis
mA	Milliampere
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minute
miRNA	Micro RNA
ml	Milliliter
mm	Millimeter
MPF	Maturation promotion factor
M phase	Meta phase
MPM	Modified parker medium
mRNA	Messenger RNA
MW	Molecular weight
MZT	Maternal-zygotic transution
n	Number
Nek2	Nelated expressed kinase 2
NF-κB	Nuclear factor-κB
nM	Nanomolar
nt	Nucleotide
OAS	Oligoadenylates synthetase
OCS	Oestrus cow serum
Oct-4	Octamer-binding transcription factor 4
OPN	Osteopontin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
PCR	Polymerase chain reaction
Ped	Promoted cell dead
PKR	Protein kinase R
Plat	Plasminogen activator tissue
PLCζ	Phosoholipase Cζ

PLK1	Polo-like kinase 1
PTGS	Post-transcriptional gene silencing
PVP	Polyvinyl pyrolidone
qr-PCR	Quantitative real-time PCR
RdRP	RNA dependent RNA polymerase
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNasin	Ribonuclease inhibitor
rpm	Rotations per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
s	Second
SAS	Satistical analysis system
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLS	Sample loading solution
S phase	Synthesis phase
ssRNA	Sense RNA
TE	Trophectoderm
TFNτ	Tau interferons
TGS	Transcriptional gene silencing
tRNA	Transfer RNA
U	Enzyme unit
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3indolyl-β-D-galactopyra-noside
ZGA	Zygotic gene activation
µm	Micrometer
µg	Microgram
µl	Microliter

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1 Introduction

Presently, the genomes of various species including the bovine are largely sequenced. Moreover, several studies have been carried out during the last decade to investigate the expression patterns of genes in bovine embryogenesis in response to various culture and treatment conditions (Rizos et al. 2002, Lonergan et al. 2003, Rizos et al. 2003, Tesfaye et al. 2004, El-Halawany et al. 2005). Despite the fact that the bovine genome has been recently reported to be completely sequenced, the function of most of the genes is not yet known. Till recently, the function of a specific gene in bovine species has been predicted using knockout experiments conducted in mouse (Larue et al. 1994, Riethmacher et al. 1995). However, these knockout technologies are extremely laborious and need long time to see the effects. So what is needed is a technique that can be used to jump directly from sequence to function in the whole animal. For this, the post transcriptional gene silencing (PTGS) by double-stranded RNA (dsRNA) or RNA interference (RNAi), has emerged as a new tool for studying gene function in an increasing number of organisms (Fire et al. 1998, Fire 1999). To overcome this, the RNAi approach through introduction of sequence specific dsRNA into the cells has been reported for the first time in *Caenorhabditis elegans* as an effective tool to study gene function in this species (Fire et al. 1998). Due to its relative easy application and its effectiveness, this technique has been used to study gene function during early embryogenesis in mammalian species including mouse (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Svoboda et al. 2001, Grabarek et al. 2002, Kim et al. 2002, Stein et al. 2003a, 2003b, Svoboda et al. 2004, Alizadeh et al. 2005, Gui and Joyce 2005), swine (Cabot and Prather 2003, Anger et al. 2004) and bovine (Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b). This approach has been reported to be an effective tool to inhibit genes from both maternal and embryonic genome expressed in mouse (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Svoboda et al. 2001, 2004, Alizadeh et al. 2005, Gui and Joyce 2005, Shin et al. 2005). Most recently, our group (Nganvongpanit et al. 2006a, 2006b) and other (Paradis et al. 2005) have used the RNAi approach to suppress transcripts in bovine oocytes and embryos.

Oocyte maturation is a complex phenomenon involving both the nucleus and the cytoplasm. The meiotic resumption is characterized by geriminal vesicle breakdown (GVBD), chromosomal condensation, progression to metaphase of the first meiosis release of the first polar body and then arrest at the metaphase (M phase) of the second meiosis (MII) (Motlik and Kubelka 1990). The meiotic arrest (MII arrest) is maintained by the persistently high activity of cyclin B-$p34^{cdc2}$ kinase, also called maturation promotion factor (MPF) (Draetta and Beach 1988, Brizuela et al. 1989, Masui 1992, Fan and Sun 2004). MPF activity is necessary to maintain MII arrest in oocytes, and the function of a multi-component complex, known as cytostatic factor (CSF), is required to sustain MPF activity (Hirao and Eppig 1997). CFS activity is the coordinated function of at least two protein, mitogen-activated protein kinase (MAPK) and mos. The activation of MAPK mediates the activation of MPF, a key regulator of the M phase and results in the induction of GVBD in xenopus (Gotoh and Nishida 1995, Kosako et al. 1996), mouse (Araki et al. 1996), bovine (Fissore et al. 1996) and porcine (Ohashi et al. 2003). Mos, the C-mos protooncogene product, is one of the central regulators of meiosis in vertebrate oocytes (Sagata 1996). As it has been observed in C-$mos^{-/-}$ knock-out mice, inhibition of C-mos sythesis in mouse oocytes using RNAi results in parthenogenetic activation (Wianny and Zernicka-Goetz 2000). However, so far the effect of C-mos suppression in bovine oocytes is not yet studied.

Oct-4 belongs to the sub-group of octamer-binding protein that binds by the POU domain to promoter and enhancer regions of various genes with octamer sites (Nichols et al. 1998, Ovitt and Schöler 1998). The Oct-4 gene is presumed to be involved in the maintenance of an undifferentiated state, and also the determination or establishment of the germ line (Ovitt and Schöler 1998). Moreover, Oct-4 influences several genes expressed during early development, including Fgf-4, Rex-1, Sox-2, OPN, hCG, Utf-1 (Pesce and Schöler 2001) and IFNτ (Ezashi et al. 2001). However, the role and possible effect of bovine Oct-4 gene suppression is not yet investigated in bovine embryos.

Therefore, the main objectives of this study are:

1. To use the RNAi technology for study the function of targeted gene in bovine preimplantation embryos.
2. To investigate the effect of the suppression of C-mos gene on the mRNA and protein expression during oocyte maturation and observe the biological effects of the suppression of this gene on *in vitro* oocyte maturation.
3. To investigate the effect of the suppression of Oct-4 gene on the mRNA and protein expression during bovine embryogenesis, including biological effects of the suppression of this gene on embryo development.

2 Literature review

Life begins for animals when sperm fertilizes an oocyte to form a zygote. What do we know about this mechanism that activate gene expression in mammals and thereby turn on the developmental program? Historically, answers to this question have relied heavily on studies done with fertilized-oocytes from frogs and flies (Yasuda and Schubiger 1992). In mammals, the preimplantation embryo is defined by the development of the zygote through several cleavage divisions, the activation of embryonic transcription, and the morphogenetic events of compaction and cavitations resulting in the formation of a blastocyst. In the period from fertilization to implantation involves various morphological, cellular, and biochemical changes related to genomic activity (Stanton et al. 2003). These changes include the inside zona diameter of gamete from less than 30 μm in the primordial follicle to more than 120 μm in the bovine tertiary follicle (Humblot et al. 2005). Also, the elongation of embryonic tissues, cell-cell contact between the mother and the embryo, and placentation. The embryo begins to form the placenta around day 20 of gestation in the bovine (King et al. 1980, Yamada et al. 2002), while embryonic trophoblast and endometrial cells tightly until to form placentomes on day 30 (Wooding 1992, Wooding and Flint 1994). The bovine embryos at the blastocyst stage are approximately 170 μm in diameter, but become approximately 50 mm long by the time of implantation (Morris et al. 2001). Embryonic cells undergo both proliferation and differentiation to form the fetus and placenta throughout early embryogenesis. Reprogramming of the genome may be completed and reset during these steps, with embryonic development progressing to temporal and spatial gene expression (Lee et al. 2002, Tanaka et al. 2002).

The major reproductive wastage in farm animals is early embryo loss, i.e. the anomalous development of embryos or an aberration of placentation (Cross et al. 1994). Various technologies, such as artificial insemination, embryo transfer, and cloning, have been applied to bovine reproduction (Holm et al. 1999, Hashizume et al. 2002). Precise knowledge of the gene expression profile during preimplantation is necessary to reduce early losses and to improve the reproductive efficiency of these new technologies (Schultz et al. 1999, Lonergan et al. 2003, Vignesult et al. 2004). However, little is known about the complex molecular regulation of embryos and extra-embryonic

membrane development in cattle. Thus, the genes to be profiled include new, functional gene candidates. This suggests an assessment method for key gene to help clarify the complex mechanisms in early embryo development, including also trophoblast cell proliferation and differentiation.

2.1 Development of preimplantation embryo

The preimplantation embryo passes through distinct metabolic phases, undergoing changes in protein synthesis, energy requirements and amino acid uptake as it develops from a zygote to the blastocyst stage. Concurrently, it also undergoes morphological changes particularly at compaction when the first differentiation process is observed.

2.1.1 Developmental competence

Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization. The developmental competence of the oocyte is acquired within the ovary during the stages that precede ovulation or in case of *in vitro* maturation, precede the isolation of the oocyte from its follicle (Mayes 2002). It is a difficult parameter to assess since embryonic development may fail due to reasons independent of oocyte quality. Developmental competence is usually expressed as the percentage of oocytes that can develop to the blastocyst stage (Gandolfi 1997). However, development to the blastocyst stage does not guarantee that the embryo will develop to term. Other aspects used to evaluate developmental competence include morphological evaluations, such as number of blastomeres or the ratio between inner cells mass (ICM) and trophoectoderm (TE) cells number and metabolic rates (Crosier et al. 2001). The size and the quality of the follicle of origin (Hazeleger et al. 1995) influence the developmental capacity of bovine oocytes. It appears that oocyte requires an additional prematuration to express their competence (Hendriksen et al. 2000). If *in vivo*, this prematuration occurs during preovulatory growth before the lutenising hormone (LH) surge, the ovarian morphology, the number and size of the follicles present in the ovary at the time of aspiration, the composition of the follicular fluid (Madison et al. 1992, Hazeleger et al. 1995, Lonergan et al. 2003) may be critical for

the oocyte to acquire developmental competence. The developmental competence of the oocyte may also be lost during *in vitro* maturation (IVM) since the number and quality of cumulus cells surrounding the oocyte are important in this process (Blondin and Sirard 1995, Gandolfi et al. 1997).

The absence of reliable markers for the identification of viable embryos for transfer at the early cleavage stage is likely to contribute to the generally low implantation rates in *in vitro* fertilization (IVF) treatment (Fenwick et al. 2002). Early cleavage is indicative of increased developmental potential in embryos and may be useful as a criterion in the selection of embryos for transfer. To improve the selection of the embryo with the highest implantation potential, Van Montfoort et al. (2004) suggested that selection for transfer should not be based on cell number and morphology on the day of transfer alone but also on early cleavage status.

2.1.2 Oocyte maturation

Oocyte maturation is a complex phenomenon during which the oocyte progresses from the diplotene to the MII stage (nuclear maturation). The oocyte resumes meiosis in response to the ovulatory LH surge or removal from the follicle. In cattle, GVBD occurs within hours after removal from the follicle or the ovulatory LH signal. The oocyte remains arrested at the MII stage until fertilization takes place and the oocyte completes meiosis and forms the pronucleus. However the completion of nuclear maturation alone does not guarantee subsequent embryo development (Yang et al. 1998). Oocyte maturation also involves transformations at the cytoplasmic level that prepare the cell to support fertilization and early embryonic development (cytoplasmic maturation).

Oocytes matured *in vitro* or *in vivo* have similar rates of nuclear maturation, fertilization and cleavage, but clearly differ in their developmental potential (Blondin and Sirard 1995). Differences in development between *in vivo* and *in vitro* cultured bovine oocytes are expressed at the morula and blastocyst stage (Farin and Farin 1995). Important factors either in the form of proteins or stable mRNAs are stored during oocyte growth and final follicular maturation after the growth has been completed (Blondin and Sirard 1995). The ability of the oocyte to complete meiosis is known as meiotic competence, which is acquired gradually during follicular growth. It is closely correlated with oocyte

size, which in turn is correlated with follicle size and the size of the antral follicle at which the oocyte acquires meiotic competence is species-specific (Mayes 2002). Cleavage and blastocyst rates increased in parallel with meiotic competence and significantly higher developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 µm (Hazeleger et al. 1995). Once the oocyte becomes meiotically competent, inhibitory factors are necessary to maintain the oocyte in meiotic arrest. The nature of meiotic arrest in bovine follicles is poorly understood.

Oocyte maturation is a complex phenomenon involving both the nucleus and cytoplasm as mentioned above. The meiotic resumption is characterized by GVBD, chromosomal condensation, progression to M phase of the first meiosis release of the first polar body and then arrest at the MII (Motlik and Kubelka 1990). Most mammalian oocytes are ovulated at MII and remain arrested at this stage until activated by a fertilizing spermatozoon or by artificial stimuli (Ozil 1990). The meiotic arrest is maintained by the persistently high activity of cyclin B-p34^{cdc2} kinase, also called MPF (Draetta and Beach 1988, Brizuela et al. 1989, Masui 1992, Fan and Sun 2004). MPF activity is necessary to maintain MII arrest in oocytes, and the function of a multi-component complex, known as CSF, is required to sustain MPF activity (Hirao and Eppig 1997). CFS activity is the co-ordinated function of at least two protein, MAPK and mos. The MAPK has a role in promoting MPF activation and in assisting meiotic resumption (Fissore et al. 1996, Ohashi et al. 2003). The activation of MAPK mediates the activation of MPF, a key regulator of the M phase and results in the induction of GVBD in xenopus (Gotoh and Nishida 1995, Kosako et al. 1996), mouse (Araki et al. 1996), bovine (Fissore et al. 1996) and porcine (Ohashi et al. 2003). From these findings, it is clear that MAPK activity is necessary for the maintenance of MPF activity and for prevention of MII arrest release of matured oocytes. Mos, the C-mos protooncogene product is one of the central regulators of meiosis in vertebrate oocytes (Sagata 1996). In 1988, Sagata et al. had been shown, the C-mos was required for activation of MPF in G2 arrested xenopus oocytes. In agreement with the study done by Roy et al. (1990) on xenopus species showed that injecting C-mos mRNA into the oocyte induces oocytes maturation, while inhibiting C-mos prevents oocytes maturation. In mouse, C-mos clearly shows important role to control the oocytes arrest at MII (O'Keefe et al. 1989, Araki et al. 1996, Choi et al. 1996, Sovoboda et al. 2000, Wianny and Zernicka-Goetz

2000). $Mos^{-/-}$ oocytes undergo GVBD and progress through the first meiosis but fail to arrest at MII. Instead, they undergo spontaneous parthenogenetic activation (Colledge et al. 1994, Hashimoto et al. 1994). Similarly, inhibition of mos sythesis in mouse oocytes using RNAi also resulted in parthenogenic activation (Wianny and Zernicka-Goetz 2000). Thus, C-mos probably functions to maintain MII arrest by promoting MAPK activity, which in turn may either inactivate the cyclin B degradation system, prevent an increased rate of degradation, or both (Hirao and Eppig 1997). In mammalian oocytes other than mouse, the requirement of MAPK activity for meiotic resumption is still controversial (Ohashi et al. 2003). Although C-mos has been shown to play as an important roles in porcine oocytes maturation (Ohashi et al. 2003). But the function of C-mos in bovine oocyctes is unclear, which need to be investigating in this study.

2.1.3 Embryo morphological change

During the ealry development from day 1 to day 8 the bovine embryo remained within the zona pellucida, approximately 170μm in diameter (Morris et al. 2001). The first cleavage occurred at 2 days after fertilization. Between day 3 and 4 after fertilization the embryo contained 8-16 cells. Following 16-32 cells at day 5 and 6, which began to form junctions and resulting in a compact of cells termed the morula. Compaction is known to be the first essential step in differentiation and is fundamental and essential for viable blastocyst formation. The embryos formed blastocoelic cavities at day 8. This stage, the cells differentiate into ICM, destined to become the fetus, surrounded by TE cells, distined to become the placental tissue. At this stage the embryo had a tatal cells complement about 120-140 cells which the ICM comprising about 25% and the TE cells about 75% of the total cell number. The blastocyst continued to expand by 1.5 times to reach a diameter about 200μm with a complement of 160 cells. Around day 9 and 10, explanded blastocysts hatched from the zona pellucida and the hatched blastocysts underwent further expansion before they started to elongate at day 13. The embryos were spherical, ovoid or elongated in shape, and diameter increased from about 5.2 mm at day 13 to about 52 mm at day 16 (Morris et al. 2001).

2.1.3.1 Embryo cleavage

The embryos enter into several divisions after fertilization. The zygote is large cell, having a low nuclear to cytoplasmic ratio. To attain a ratio similar to somatic cells, cell divisions occur without an increase in cell mass. This process is referred as cleavage. The resulting daughter cells are called blastomeres. Blastomeres from the 2- to 8-cell stage in the rabbit and sheep are totipotent, that is fully capable to give rise to an intact embryo. In 4-cell stage, no more than three of four blastomers are totipotent cells, and in 8-cell stage not more than one of eight blastomers is totipotent cells (Hafez and Hafez 2000). Mouse embryos take about three and half days to develop from the 1-cell stage to the blastocyst stage containing 32 or more cells. The first (1- to 2-cell) and second (2- to 4-cell) cell cycles of the mouse embryo take between 16-20 h and 18-22 h respectively, depending on the strain of mice (Harlow and Quinn 1982). The duration of certain phases of the cell cycle differ considerably between 1- and 2-cell mouse embryos. The duration of the synthesis phase (S phase) increases from 4 h to 7 h from the pronuclear stage to the 2-cell stage, whereas the duration of second gap phase (G2 phase) and M phase increases from 8 h to nearly 12 h (Streffer et al. 1980). The duration of the G2 and M phase of the second cell cycle is strain-specific leading to differences in the length of the 2- to 4- cell cycle in different mouse strains (Molls et al. 1983). Morover, the rate of cleavage has also been linked to genetic influences. Warner et al. (1987a,b) have described a H-2 linked gene, called the preimplantation embryo development (Ped) gene, that influences the rate of cleavage divisions of preimplantation mouse embryos. The Ped gene has two functional alleles, fast and slow, as defined by the rate of development of preimplantation embryos, with the fast allele being dominant. In a more recent study, Brownell and Warner (1988) demonstrated that the Ped gene phenotype of embryos cultured in vitro is maintained thus, the control of embryo cleavage is largely dependent on the genes of the embryo itself and is not a function of the uterine environment.

2.1.3.2 Embryo compaction

Compaction is the first event of morphogenic and cellular differentiation. The most significant event occurring at compaction is the emergence of 2 distinct cell populations: the blastomeres remaining in contact with the outside are destined to form the TE cells lineage while the blastomeres inside the embryo are destined to form the ICM. In mammalian embryos, during the 8-, 16- and 32-cell stages, specific cells are induced to change their morphological and functional phenotype to a polarized form. This commences with the division of the 8-cell stage embryos generating an average of 9 outside and 7 inside cells in the embryo (Johnson and Ziomek 1981), with the outer cells being polarised and larger than the inner cells that remain apolar. The trigger to the development of a polarized phenotype in the outer cells may be related to the pattern of intercellular contacts. Polarization is suppressed when a cell is completely surrounded by other cells, while when contact with other cells is incomplete polarity develops. The close cell contacts that develop are due to the presence of the cell adhesion molecule uvomorulin, which progressively becomes distributed to areas of cell-cell contact and remains absent from the apical areas of the outer polarised cells (Johnson et al. 1986). Polarization of the outer cells is evident by the basal migration of the nucleus and the apical accumulation of actin, clathrin, endosomes and microvilli. Once a cell acquires polarity, the progeny of the cell will be influenced by the orientation of the subsequent cleavage plane, hence either two polar or one polar and one non-polar cell will arise. It is only in fully expanded blastocysts that ICM and TE cells can not cross lineages. This initial differentiation is also the first decrease in cell totipotency in the embryo whereby the internalized apolar cell subpopulation in the morula will preferentially form the ICM of the blastocyst and the outer polar cells develop trophectodermal characteristics. Trophectoderm cells therefore are polar, enveloping and fluid transporting (Ducibella and Anderson 1975, Gardner and Johnson 1972) whereas ICM cells are highly adhesive, compact readily on each other and, when aggregated to a morula, move to its center (Johnson and Ziomek 1981).

2.1.3.3 Blastocyst formation

Blastocyst formation (cavitation) is essential for implantation and subsequent development and implantation failure is a principal cause of early pregnancy loss

(Edmonds et al. 1982, Wiley et al. 1990, Edwards 1997). The TE is the first ion transporting epithelium formed during development and provides an important model allowing the investigation of cell polarity during development (Watson 1992, Watson et al. 1999). The trophectoderm cells initiates implantation via direct contact with the uterus and eventually contributes to the trophoblast giant cells and extra-embryonic membranes such as the chorion. The differentiation of TE cells has direct ties to compaction, since this cell layer is derived from the polar outer cells of the compacted morula (Watson 1992, Watson et al. 1999). Blastocyst formation is dependent upon TE cell differentiation. The TE cells acquire the characteristics of epithelial cells in being flattened and joined together by tight junctional complexes (Ducibella and Anderson 1975). When the mouse embryo has about 32 cells, TE cells begin to pump fluid into intracellular spaces and later into extracellular spaces, forming the blastocoelic cavity (Borland et al. 1977). The trophectoderm ion transport systems play an important role in establishing ion concentration gradients across the epithelium, and thereby in providing the force that drives water into the blastocoelic fluid. Electron probe microanalyses of Na^+, Cl^-, K^+, Ca^{2+} and Mg^{2+} have shown that all these ions are concentrated within the blastocoelic fluid (Borland et al. 1977). The active transport mechanisms required to move these ions against their concentration gradients are thought to involve the transport of Na^+ and Cl^-. The main contributor is the Na,K-ATPase that has been localised to the basolateral domain of the TE (Watson 1992, Watson et al. 1999). The presence of the tight junctional complex is also necessary and plays a multifunctional role. It provides an impermeable seal allowing fluid accumulation, regulates paracellular transport (Manjewala et al. 1989) and contributes to a polarization of the distribution of the Na,K-ATPase (Watson et al. 1990). The development of the epithelial junctional complex, such as democollins, desmogleins family, E-cadherin and catenins are important in the initiation and maintenance of the cell polarized state (Fleming et al. 1989, 1991, Citi 1993, Larue et al. 1994, Riethmacher et al. 1995). Since the blastocoelic fluid is largely composed of water, the TE-ion transport systems establish ion concentration gradients across the epithelium which facilitates the osmotic accumulation of water via water channels to form the blastocoelic fluid. The establishment of apical junctional complexes provides a TE seal that regulates the leakage of water from the blastocyst cavity. As metioned above, the blastocyst contains

two distinct cell types: the ICM cells which go on to form the embryo proper, and the TE cells which are involved in the initial contact with and the infiltration of the uterine wall and eventually contribute to the placenta and the extra-embryonic membranes. The first week of mammalian development is a perilous time for the early embryo. There is a great need to understand the mechanisms controlling this interval to develop rational interventions to either promote or inhibit fertility in a variety of mammalian species. This research addresses this need and is aimed at providing an eventual understanding of the causative events underlying early embryo loss and to ensuring that assisted reproductive technologies are applied in a safe and efficient way.

In mammalian embryogenesis the first morphological indication of differentiation is the formation of the TE cells at the early blastocyst stage. While the ICM cells remain totipotent, TE cells are restricted to extra-embryonic cell lineages. In mice, a key factor for the first differentiation step in embryogenesis is the POU domain transcription factor Oct-4 (Okamoto et al. 1990, Rosner et al. 1990). Oct-4 belongs to the sub group of octamer-binding protein that binding with the POU domain to promoter and enhancer regions of various with octamer sites (Ovitt and Schöler 1998). In mouse, Oct-4 is expressed in pluripotent cells such as cleavage stage blastomeres, ICM of the blastocyst, epiblast of the early postimplantation embryo and embryonic stem (ES) cells (Yeom et al. 1991, Palmieri et al. 1994, Pesce and Schöler 2001), while TE differentiation correlates with downregulation of Oct-4 (Palmieri et al. 1994). Bovine Oct-4 shares high sequence homology with its mouse orthologue, but its protein product is found at similar levels in pluripotent and differentiating cells of the bovine preimplantation embryo (Kurosaka et al. 2004). The function of Oct-4 gene is presumed to involve the maintenance of an undifferentiated state, and also the determination or establishment of the germ line (Ovitt and Schöler 1998). In murine embryogenesis, Oct-4 has been shown to be essential for the development of totipotent ICM cells (Nichols et al. 1998), Oct-$4^{-/-}$ embryos die at the time of implantation due to a failure to form the ICM (Pesce and Schöler 2001). Moreover, Oct-4 influences several genes expressed during early development, including Fgf-4, Rex-1, Sox-2, OPN, hCG, Utf-1 (Pesce and Schöler 2001) and IFNτ (Ezashi et al. 2001). The transcription factor Oct-4 is thought to be very important to early embryonic development and differentiation because Oct-4 is the earliest known as transcription factor to be developmentally expressed and such studies

would further elucidate the mechanisms of embryonic development as mentioned above. Most studies have been performed on mouse embryos (Ovitt and Schöler 1998, Boiani et al. 2002, Bortvin et al. 2003, Kehler et al. 2004). Although, the early preimplantation development of mouse, porcine and bovine is similar in that the embryos of these mammalian species all progress through three major morphologenetic transition, compaction, cavitation and expansion, finally leading to hatching and implantation (Crosier et al. 2000, Kirchhof et al. 2000, Crosier et al. 2001). However, some differences are evident. Therefore, each species must be studied for a better understanding of the development process.

2.1.4 Genes expression in preimplantation embryo

Gene expression (also protein expression or often simply expression) is the process by which a gene's information is converted into the structures and functions of a cell. Gene expression is a multi-step process that begins with transcription and translation and is followed by folding, post-translational modification and targeting. The amount of protein that a cell expresses depends on the tissue, the developmental stage of the organism and the metabolic or physiologic state of the cell. Gene expression is one of the most important principles underlying the development and control of cells, systems and organisms. Essentially gene expression is the process by which genetic information is converted into entities (mainly proteins) that contribute to the structure and operation of a cell. The study of gene expression encompasses the transcription of DNA to RNA (transcription) by predominantly messenger RNA (mRNA), but also transfer RNA (tRNA) and ribosomal RNA (rRNA), following synthesis the protein (translation). Initiation of transcription is the most important step in gene expression. Without the initiation of transcription, and the subsequent transcription of the gene into mRNA by RNA polymerase, the phenotype controlled by the gene will not be seen. Therefore in depth studies have revealed much about what is needed for transcription to begin. In conjunction with the activation of the embryonic genome, conventional one dimensional of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has shown that major changes occur in protein synthesis between day 1 (2-cell stage) and 2 (4- to 8-cell stage) of preimplantation mouse embryo development (Epstein and Smith, 1973,

Van Blerkom and Brockway 1975). The first proteins synthesized in the late 2-cell embryos coinciding with embryonic genome activation appear to be heat shock proteins (67,000-70,000 daltons) (Bensaude et al. 1983). During the late 4- and 8-cell stage new transcription is necessary to prepare the embryo for compaction, while during the morula to blastocyst transition there is also a change in transcriptional activity in line with the increase in the rate of protein synthesis (Braude 1979). These changes ultimately lead to the appearance of tissue or stage specific polypeptides in the ICM and TE cells at the blastocyst stage (Handyside and Johnson 1978, Howe et al. 1980).

2.1.4.1 Maternal-zygotic transition

Fertilization initiates a cascade of events leading to the temporal and spatial expression of genes prerequisite for embryo and fetal development. However, the earliest stages of development are largely dependent on maternally derived messages stored in the oocyte prior to fertilization. As development progresses maternal RNAs and proteins are depleted and embryo-derived messages become key controlling factors. The shift from dependence on oocyte-derived messages to embryo-produced messages is referred to as the maternal-zygotic transition (MZT). This crucial transition occurs during the first few post-fertilization cell cycles in a species dependent manner. Maternal-zygotic transition takes place at different periods, depending on the species (reviewed in Telford et al. 1990, Kanka 2003). For example, MZT occurs at roughly the 2-cell stage in mice (Schulz 1993), 4- to 8-cell stage in human (Telford et al. 1990) and the 8- to 16-cell stage in bovine (Memili and First 1999, 2000). The transition from maternal to embryonic control of development is characterized by a degradation of maternal RNA and protein, sensitivity to transcriptional inhibitors such as α-amanitin, and a burst of transcriptional activity from the embryonic genome. The content of RNAs (mRNA and rRNA) decline from the oocyte to the morula stage after which there is a marked increase (Bilodeau-Goeseels and Schultz 1997). Inhibition of polymerase dependent transcription by α-amanitin during the earliest stages of development has shown that embryos can survive in the absence of transcription from embryonic genome until a certain species dependent stage of development. This generally is the point at which MZT is considered to occur. Sensitivity of α-amanitin has been identified at the 2-cell

stage in mouse (Telford et al. 1990, Memili and First 2000) and the 4- to 8-cell stage in cattle (Plante et al. 1994, Natale et al. 2000), although partial sensitivity was detected at the 2- to 4-cell stage (Natale et al. 2000). In mouse, transcription from the embryonic genome has been identified within hours of fertilization in the male pronucleus of the zygote (Telford et al. 1990, De Sousa et al. 1998, Memili and First 2000). In bovine, a similar pattern of transcription before the MZT has emerged. The production of embryo-derived RNA has been detected at the zygote stage by incorporation of (^{35}S)UTP (Memili et al. 1998), at the 2-cell stage by incorporation (^{3}H) uridine (Plante et al. 1994, Viuff et al. 1996), and differential display RT-PCR (Natale et al. 2000) at the 4-cell stage by two dimention (2-D) electrophoresis (Barns and First 1991) northern blot analysis (Bilodeau-Goeseels and Schultz 1997) and sequence specific PCR (McDougall et al. 1998). Activation of the embryonic genome thus appears to occur in two phases with a minor activation prior to the MZT at the zygote to the 8-cell stage followed by a major activation at the 8- to 16-cell stage that coincides with the MZT. That transcription from the embryonic genome and MZT are required for a normal progression of development is clear, what remains to be determined is the chronology and cellular location of expression of development regulating genes from the embryo's genome. During the transition from maternal to embryonic control of development, maternal transcripts are depleted and embryo specific transcripts involved in early embryogenesis are generated (Adjaye et al. 1999). The transcription of the 18S, 5.8S and 28S rRNA, polymerase I and their subsequent processing lead to the formation of a distinct nuclear structure, the nucleus (Viuff et al. 1998). Further more, the transition is accompanied by modifications in chromatin structure and post-translational modifications of transcriptional abilities in early embryos (Pacheco-Trigon et al. 2002). In addition, a dramatic reprogramming of gene expression occurs during this transition, and the molecular foundation for transforming the highly differentiated oocyte in the totipotent blastomeres of the early cleavage stage preimplantation embryo (Ma et al. 2001). Vigneault et al. (2004) have demonstrated that the 15 genes (YY1, HMGA1, RY-1, P300, CREB, YAP65, HMGN1, HMGB1, NFAR, OCT-4, TEAD2, ATF-1, HMGN2, MSY2 and TBP) examined in their studies are all present in bovine oocytes throughout pre-MZT embryonic development in the form of mRNA. These results support the hypothesis that these factors could be implicated in the activation of

embryonic transcription. It also supports the idea that transcription factors may be found as maternally stored mRNA in the oocyte until their recruitment for translation just in time for MZT.

2.1.4.2 Embryonic genome activation

The variations in the duration of the cell cycle during the early stages of embryo development can perhaps be linked to specific developmental events that occur at this time. The lengthened cycle from the 2- to 4-cell stage in mouse embryos, in particular, may be related to one of the major events of preimplantation development, i.e. embryonic genome activation (EGA) or zygotic gene activation (ZGA). The earliest developmental changes are under post-transcriptional maternal control. They rely on changes in the translation of mRNAs synthesized during oocyte growth or post-translational protein modifications. The triggers for the initiation of embryonic transcription remain unclear (Memili et al. 1998, Ma et al. 2001). However, the activation of the mouse embryonic genome occurs at the late 2-cell stage (Goddard and Pratt 1983), corresponding with the long second cell cycle. In mouse, the embryonic genome is activated in two phases, a limited activation occurring between 18 and 21 h post-insemination and a major activation occurring between 26 and 29 h post-insemination (Flach et al. 1982). Although the first sign of major transcription by the embryonic genome appears during the 2-cell stage mouse embryo, recently a more sensitive assessment of the 1-cell stage mouse embryo has led to the suggestion that EGA may begin in the 1-cell stage mouse embryo and that differences between the transcriptional activity of the male and female pronuclei exist (Ram and Schultz 1993). The timing of EGA, or competence to sustain appreciable transcriptional activity in bovine embryos may be controlled temporally by a time dependent mechanism referred to as zygotic clock rather than by developmental stage (Nothias et al. 1995, Watson et al. 1999). These was confirmed by transfering a reporter gene in to 1-cell stage bovine embryos and examine the expression at a particular stage (Watson et al. 1999) and the similar test has been done with mouse zygote (Nothias et al. 1995). In bovine embryos, EGA has definitely occurred by the 8- to 16-cell stage as evidenced by incorporation of (^{3}H)-uridine into nucli and nucleolei at the 8-cell stage (De Sousa et al. 1998). This

activation is responsible for controlling subsequent development and, different transcripts are expressed in a stage specific manner. However, first transcript initiation at 2- to 4-cell stage was observed in bovine embryo development and this initiation is α-amanitin insensitive and is not required for progression of embryonic development to advanced preimplantation stage (Memili and First 1999). The data obtained in many references (Viuff et al. 1996, De Sousa et al. 1998, Memili et al. 1998, Memili and First 1999) suggest that there is a low level of transcriptional activity (mRNA sythesis, i.e. RNA polymerase II dependent transcription) that can be called minor gene activation between the 1- and late 4-cell stage, and the high level of transcriptional activity that can be called major gene activation at the 8-cells stage in bovine embryos (Figure 2.1).

2.2 Functional analysis of differentially expressed genes in animals

Today, with numerous genome projects adding tens of thousands of nucleotide sequences to the public databases each day, the exploration of gene function often begins with a DNA sequence. Here the challenge is to translate sequence into function. Traditional systems for studying developmental biology have access to a variety of methods for functional analysis of developmentally regulated genes: forward genetics (mutational analysis) for example is particularly suited to species with a short generation time such as the worm *C. elegans*, the fruit fly *Drosophila melanogaster* and more recently the zebra fish, while reverse genetics (transgenesis, knockout technology) is more typically associated with the mouse. This technology e.g. transgenesis, has so far only been of limited applicability to the study of development in farm animal species. However, the increasing efficiency of cloning via somatic nuclear transfer in these species, in combination with prior expression of ectopic genes or success in attempts at endogenous gene mutation in somatic cells by homologous recombination may soon be expected to extend the range of tools available for functional analysis substantially. Nevertheless, we might also expect that, before then, new technologies to map differential gene expression in the small amounts of material available from preimplantation embryos of domestic species might identify many of cDNA sequences, ESTs or complete genes of potential significance for the progression of normal

development either *in vivo* or following IVM, IVF and *in vitro* culture. What avenues are currently available to analyse function and how best can we utilize resources?

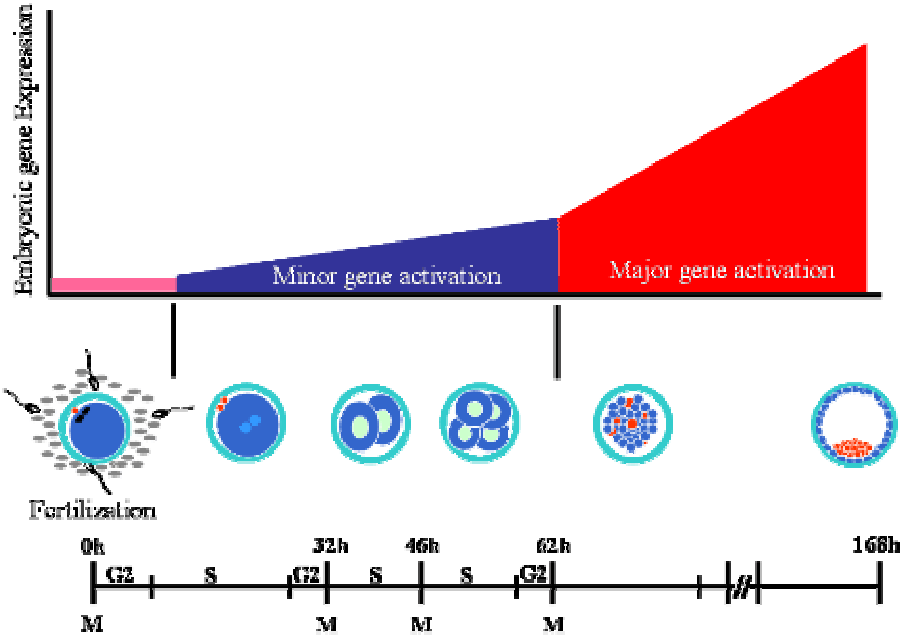

Figure 2.1: Bovine embryonic cell cycles and embryonic gene expression (mRNA synthesis). There is minor gene activation between the 1- and 4-cell stages. Changes in the transcriptional machinery and chromatin structure play an important role in the control of early gene expression. While the major gene activation starting at the 8-cell stage (Memili and First 2000).

2.2.1 Antisense RNA

In order to gain insight into the function of protein product of a gene of interest, antisense (as) RNA has been widely applied in eukaryotes in which the asRNA transcript expressed successfully inhibit the expression of specifically targeted mRNA (Robert et al. 1990, Iwaki et al. 1994, Wu and Welsh 1996). The asRNA is the technique designed to specifically and selectively inhibit production of proteins in cells (Crooke, 1998). It has been applied extensively in the amphibian, to a lesser extent in other species. Website of P.Vize provides a wealth of data (accessible under Fons Verbeek, at www.niob.knaw.nl, the website of the Hubrecht Laboratory, Netherlands Institute of Developmental Biology). This is a recently developed variant of asRNA

technology where morpholinos are used to modify anti-sense oligonucleotides in such a way that they are highly specific inhibitors of translation. Successful in zebra fish and being tried out in other species (Nasevicius and Ekker 2000).

2.2.2 Gene silencing

The effect of dsRNA or small interfering RNA (siRNA) to silence genes in animals was first shown in year 1998 by Fire and colleagues (Fire et al. 1998). It has been shown to have a potent effect on gene expression, particularly on mRNA stability. After it has been particularly successfully applied in *C.elegans* to analyse the function of a number of genes and has recently been used for the first time in mammals (mouse) in year 2000 by two research groups (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000). Although the possibility of non-specific effects has been discussed, it might be considered as an option worth exploring in farm animal species (Plasterk and Ketting 2000).

2.2.3 Protein knock down

The selective degradation of cellular proteins is mediated primarily by the ubiquitin-proteasome pathway. Manipulation of the ubiquitin-dependent proteolytic machinery to eliminate specific gene products at the protein level has been previously attempted with some success *in vitro* and *in vivo* (Zhou et al. 2000). The protein knock down strategy can be utilized not only as a novel method to dissect the role of oncoproteins in tumorigenesis, but also as a unique tool to delineate the function of a subpopulation of proteins localized to a specific subcellular compartment (Cong et al. 2003).

2.3 RNA interference

RNAi was originally discovered as endogenous properties of plants, but the real breakthroughs came when this phenomenon was discovered in nematode (Fire et al. 1998). Since then, RNAi related phenomena have been reported for a wide range of species in fungi, plants, invertebrates and vertebrates (Table 2.1), and the underlying

mechanism has become better understood. The successful application of RNAi to mammalian system in year 2000 (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000) has become a powerful tool for functional genomics. RNAi as commonly defined, is a phenomenon leading to post-transcriptional gene silencing (PTGS) after endogenous production or artificial introduction into a cell of siRNA with sequences complementary to the targeted gene (Bosher and Labouesse 2000, Elbashir et al. 2002). Whereas the transcription of the gene is normal, the translation of the protein is prevented by selective degradation of its encoded mRNA. However, PTGS is not restricted to RNAi and has emerged as a more complex mechanism that involves several different proteins and small RNAs. It is presumed that cells employ RNAi to tightly regulate protein levels in response to various environmental stimuli, although the extent to which this mechanism is employed by specific cell types remains to be discovered. However, the fact that RNAi is operative in cells of organisms ranging from plants, to nematodes and flies, and to mammals attests to its fundamental importance in the selective suppression of protein translation by targeted degradation of the encoding mRNA. Beyond its biological relevance, PTGS is emerging as a powerful tool to study the function of individual proteins or sets of proteins. User-friendly technologies for introducing siRNA into cells, in culture or *in vivo*, to achieve a selective reduction of targeted mRNA. The present article reviews this emerging technology, findings obtained to date using such RNAi methods, and the potential of RNAi based therapeutics for treating human disease (Brown and Catteruccia 2006, Gaur 2006, Rondinone 2006, Rossi 2006).

RNA interference most likely evolved as a mechanism for cells to eliminate unwanted foreign gene products. Foreign genes are often present in cells at high copy numbers, being present as viral genes, transposable elements, or as plasmids introduced experimentally in cell transfection protocols (Marathe et al. 2000). It has been known for several decades that the level of expression of transgene usually decreases as the number of copies present in the cell increases and that endogenous homologous genes can also be suppressed by the presence of the transgene (Napoli et al. 1990). Although such gene silencing can occur at the transcriptional level, it is now recognized that a major mechanism of gene suppression occurs post-transcriptionally, and that a major mechanism for this PTGS is RNAi, the selective degradation of mRNAs targeted by

siRNAs (Van Blokland et al. 1994). Such PTGS via RNAi can occur very rapidly with proteins for many genes, being decreased within hours, and completely absent within 24 h (Pruss et al. 1997). Based upon these and other findings initially made in studies of plants (Ratcliff et al. 1997), it seems very likely that RNAi evolved as a mechanism to defend plant cells against viral infections.

Table 2.1: The applications of gene silencing in different species

Phylum	Species	Mechanism	Effector	References
Fungi	Neurospora	Quelling	Transgenes	Cogoni and Maciano 1999
Plants	Arabidopsis	PTGS	Transgenes	Elmayan et al. 1998
	Nicotiana	Transcriptional gene silencing	Transgenes, Virus	Furner et al. 1998
	Pitunia	PTGS	Transgenes	Dehio and Schell 1994
Invertebrates	*C. elegans*	RNAi	dsRNA	Fire et al. 1998, Ketting et al. 1999
		Transcriptional gene silencing	Transgenes	Kelly and Fire 1998
	D. melanogaster	RNAi	dsRNA	Misquitta and Peterson 1999
			shRNA	Paddison et al. 2002a
	Paramecium	Homology-dependent silencing	Transgene	Ruiz et al. 1998
	Trypanosome	RNAi	dsRNA	Wang et al. 2000
Vertebrates	*Danio rerio*	RNAi	dsRNA	Wargelius et al. 1999
	Mus musculus	RNAi	dsRNA	Wianny and Zernicka-Goetz 2000, Knott et al. 2005, Plusa et al. 2005
		RNAi	siRNA	Haraguchi et al. 2004
	Sus scrofa	RNAi	dsRNA	Cabot and Prather 2003 Anger et al. 2004
	Bos turus	RNAi	dsRNA	Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b
	Homo sapien	RNAi	dsRNA	Brown and Catteruccia 2006
			shRNA	Rossi 2006
			siRNA	Gaur 2006, Rossi 2006

2.3.1 Post transcriptional gene silencing and the discovery of RNA interference

Post transcriptional gene silencing and RNAi were discovered in genetic transformation studies of eukaryotic cells, principally plants and worms, wherein it was shown that mRNAs for the encoded transgene alone, or together with mRNAs for homologous endogenous genes are very low or absent despite high levels of transcription (Fire 1999, Marathe et al. 2000). The ability to manipulate and monitor gene expression in the plant, *Arabidopsis thaliana* and the roundworm, *C. elegans* (the genomes of both species are now complete) revealed the process of RNAi and allowed the relatively rapid identification of several genes that regulate the RNAi process. Transgenes insert into the genomes of plants by recombination in an apparently random manner so that the number of inserted copies, their chromosomal location, and their local arrangement within the chromosome vary among transformants. The observation of an inverse correlation between copy number and the level of gene expression suggested that an increased copy number of a particular gene results in silencing of that gene (Assaad et al. 1993). It was initially thought that such gene silencing was due to reduced gene transcription resulting from interactions between closely linked copies that result in the formation of secondary structures that promote methylation and inhibition of transcription (Ye and Signer 1996). Further studies showed that transcriptional gene silencing (TGS) could also occur in *trans*, such that one transgene can be silenced by another transgene introduced either by crossing or transformation. It was then proposed that a silencing RNA is produced by one locus that somehow affects the silencing of the other gene by a mechanism involving RNA-mediated inhibition of transcription (Mette et al. 2000). Although some data were consistent with such mechanisms of transcriptional silencing, additional data suggested the involvement of PTGS. The presence of dsRNA and their cleavage into siRNAs of approximately 23 nucleotides (nt) were demonstrated, and it was then shown that expression of dsRNA with sequences corresponding to open reading frames in plants results in PTGS (Hamilton and Baulcombe 1999). Similarly, expression of dsRNA with sequences complementary to those of endogenous genes results in the selective silencing of those genes in *C. elegans* (Zamore et al. 2000). Collectively, the studies of *A. thaliana* and *C. elegans* showed that both TGS and PTGS can be initiated by the same RNA degradation

pathway. Transcriptional gene silencing occurs when the dsRNA includes promoter sequences, whereas PTGS occurs when the dsRNA includes coding sequences. Although degradation of dsRNA is common to both mechanisms of gene silencing, the results also indicated that dsRNA-mediated TGS and PTGS involve different specific steps. Although RNAi as a mechanism of PTGS was first discovered in plants and may have evolved as a cellular defense mechanism against foreign DNA and RNA, it is very clear that RNAi is widely employed in most if not all eukaryotic cells as a mechanism to regulate the expression of endogenous genes. In 1998, it was discovered that injection of dsRNA was much more effective for silencing of gene expression in *C. elegans* than was asRNA (Fire et al. 1998). This experimentally induced PTGS, the first report of the use of RNAi as a tool in biology was very potent, and remarkably, the PTGS occurred not only in the worms to which the dsRNA was administered, but also in their progeny (Fire et al. 1998). It was then demonstrated that the endogenous mRNA was the target of the injected dsRNA by a post-transcriptional mechanism and involving degradation of the targeted mRNA (Montgomery et al. 1998). Surprisingly, it was further shown that the dsRNA is effective at very low concentrations, such that the copy numbers of the targeted mRNA are far greater than the number of dsRNAs present in the cell (Fire et al. 1998, Kennerdell and Carthew 1998). In addition, the suppression of the protein encoded by the targeted mRNA was found to persist through many rounds of cell division. Two observations (Fire et al. 1998, Kennerdell and Carthew 1998) strongly suggested that cells possess a mechanism for amplifying the RNAi mechanism. Not only can the RNAi process be maintained within cells of a common lineage, but it can also be transferred between cells, as shown in *C. elegans* where injection of dsRNA into the intestine results in silencing of the targeted gene in all cells of the F1 progeny of that worm (Fire et al. 1998). Indeed, dsRNA can enter cells and induce PTGS when worms are soaked in a solution containing the dsRNA or when the worms are fed bacteria expressing dsRNA (Tabara et al. 1998, Timmons and Fire 1998). Recently, a transmembrane protein called SID-1 was identified as a possible mediator of intercellular transfer of RNAi (Winston et al. 2002). Subsequently, other organisms were assayed for their capacity to induce RNAi. Evidence for RNAi in *D. melanogaster* was first demonstrated by Kennerdell and co-workers (Kennerdell and Carthew 1998) who showed the involvement of the Frizzled and Frizzled2 genes in the wingless

pathway after introduction of dsRNA into embryos. Again, several techniques were developed in order to use dsRNA in this organism leading to the establishment of cell-free (Tuschl et al. 1999) and cell culture models (Caplen et al. 2000). A system that employed dsRNA as an extended hairpin-loop RNA was developed to induce heritable gene silencing (Kennerdell and Carthew 2000). The *D. melanogaster* system has allowed the identification of several endogenous genes that play key roles in the RNAi process. An RNA nuclease activity called RNA-induced silencing complex (RISC) was discovered that is responsible for the degradation of endogenous mRNAs, as well as small nucleotide fragments (~25 nt in length), which could be used as guides by RISC (Hammond et al. 2000). They later characterized RISC as a ribonucleoproteic complex (Hammond et al. 2001). These results were soon extended by showing that RNAi is an ATP-dependent and translation-independent event where the introduced dsRNA is processed into 21–23 nt fragments that guide the cleavage of endogenous transcripts (Zamore et al. 2000). The enzyme responsible for the processing of the dsRNA was later discovered as an RNase III family nuclease named dicer, a protein with high homology to the *C. elegans* Rde-1 gene (Bernstein et al. 2001). To study the functions of RNAi in yeast, Volpe et al. (2002) deleted argonaute, dicer, and RNA-dependent RNA polymerase homologs; deletion resulted in the accumulation of complementary transcripts from centromeric heterochromatic repeats and de-repression of transgenes integrated at the centromere and impairment of centromere function. So the authors proposed that dsRNA arising from centromeric repeats targets the formation and maintenance of heterochromatin through RNAi. In mammalian cells, RNAi was first employed as a tool to induce the silencing of the targeted gene (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000). This approach was partially successful in mouse embryos (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Svoboda et al. 2001, Stein et al. 2003b, Knott et al. 2005, Plusa et al. 2005) and embryonic cell lines (Billy et al. 2001, Yang et al. 2001, Paddison et al. 2002b) where specific gene silencing was achieved. On the other hand, the introduction of dsRNA into mammalian somatic cells presents a major problem because it can induce (in a manner similar to the silencing observed during viral infection) to the activation of the protein kinase R (PKR) and RNAseL pathway, resulting in the inhibition of protein synthesis and induction of apoptosis (Baglioni and Nilsen 1983, Clarke and Mathews 1995, Gil and Esteban 2000).

Interestingly, this shows that, in mammalian cells, the mechanisms for RNAi are not identical to those in lower organisms although RNAi does operate in at least a subset of mammalian cell types, in a dicer-dependent manner via post-transcriptional mechanisms (Billy et al. 2001, Paddison et al. 2002b). Elbashir et al. (2001a) had the idea of directly introducing 21–23 nt dsRNA (siRNAs) into mouse and human cells to try to avoid the problems associated with the expression of longer dsRNAs. They showed that the siRNA could efficiently trigger silencing in the mammalian cells.

2.3.2 Mechanism of RNA interference

A clearer picture of PTGS emerged from several different basic observations, including the necessity of transcriptionally active genes and the ability of RNA viruses to silence a homologous endogenous gene (English et al. 1997). Within the last 3 years, a flurry of studies has identified several of the molecules that mediate RNAi, and the mechanism whereby these molecules affect the selective degradation of targeted mRNAs (Hamilton and Baulcombe 1999, Kenner and Carthew 2000, Tavernarakis et al. 2000, Wang et al. 2000). It is now clear that the production of dsRNA with sequence complementary to the mRNA being targeted is fundamental to the process of PTGS, while single-stranded RNA is not sufficient to induce PTGS. The importance of dsRNA is supported by a wealth of data. Transgenes engineered to synthesize dsRNA require only a few copies of the dsRNA to achieve PTGS and can induce cosuppression. There are several ways such transgenes produce dsRNA including the synthesis of long hairpin mRNAs by transcription of an inverted repeat (Kennerdell and Carthew 2000, Tavernarakis et al. 2000), and transcription of complementary sense and antisense strands by opposing promoters (Wang et al. 2000). Other studies have shown that although cells may initially produce very long dsRNAs, they are cleaved into smaller dsRNAs, 21-25 nt in length, that actually mediate RNAi (Hamilton and Baulcombe 1999). However, in mammals those exists two different pathways that respond to dsRNA. The RNAi pathway is the more ancient and, called a sequence-specific mechanism. Another mechanism that recognizes dsRNA and mounts an orchestrated response to it, the PKR and interferon pathway is known as sequence independent, which evolved relatively recently and it is specific to mammals.

2.3.2.1 The protein kinase R or interferon pathway

The protein kinase R (PKR) or interferon pathway response to dsRNA in mammalian cell was discovered 30 years ago (Figure 2.2). First described by Hunter et al. (1975), that exposed mammalian or cells to dsRNA, regardless of their sequence, triggers a global repression of protein synthesis, and eventually leads to apoptosis. In most mammalian somatic cells, exposure to dsRNA activates PKR, which catalyzes phosphorylation of translation initiation factor namely: α-subunit of eukaryotic protein synthesis initiation factor 2 (eIFα), which in turn inhibits translation. Protein kinase R is also involved in regulating Nuclear factor-κB (NF-κB), which produces 2′-5′-oligoadenylate synthetase (2′,5′-OAS), which in their produces 2′,5′ oligoadenylates with 5′-terminal triphosphate residues that subsequently induce activation of RNAse L which is responsible for general RNA degradation (Barber 2001). Protein kinase R and 2′,5′-OAS mutant mice demonstrate that these two components are essential for the apoptotic response to dsRNA (Der et al. 1997).
However, long dsRNA induced RNAi but not the PKR or interferon response in initial experiment in mammalian oocytes and embryos (Svoboda et al. 2000, Wianny and Zermicka-Goetz 2000, Yang et al. 2001, Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b). A better understanding of the RNAi mechanism also allowed the elimination of a non-specific response to dsRNA in somatic cells because although siRNAs can induce RNAi, they are believed to be too short to trigger the PKR and 2′,5′-OAS pathways (Zamore et al 2000, Elbashir et al. 2001a).

2.3.2.2 The RNA interference pathway

A working model for RNAi is shown in figure 2.3. The first step is the production of dsRNA directed against an mRNA. The second step involves the recognition of dsRNA and its processing to produce 21-23 nt siRNAs. The effector step is the recognition of the target mRNA by the siRNAs and the selective degradation of that mRNA. In this section, three mechanistic features of RNAi relevant to the mammalian pathway will be shown: 1) processing of dsRNA into siRNA; and 2) recognition and cleavage of the cognate mRNA;

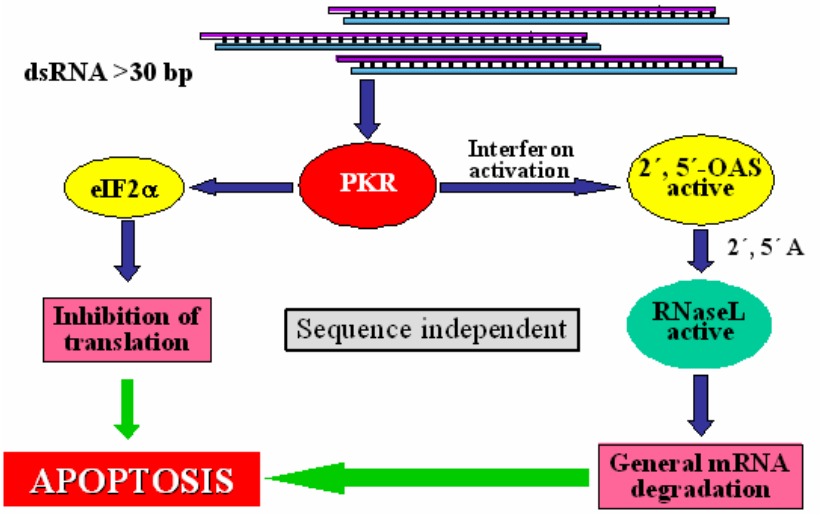

Figure 2.2: The sequence independent pathways responding to dsRNA in mammalian cells. The dsRNA activates protein kinase R (PKR), which catalyzes phosphorylation of translation initiation factor eIF2α and lead to inhibit the translation. PKR is also involved in interferon induction (through NF-κB). Interferon and dsRNA also activate 2´,5´-oligoadenylate synthetase (2´,5´-OAS) which produces 2´,5´oligoadenylates (2´,5´A) with 5´-terminal triphosphate residues. Oligoadenylates subsequently induce activation of RNAse L, which is responsible for general RNA degradation. Both PKR and 2´,5´-OAS are essential for the apoptotic response to dsRNA (Svoboda 2004).

2.3.2.2.1 Processing of dsRNA into siRNA

The introduction of dsRNA into cells, whether produced endogenously from exogenous plasmids or viral vectors, results in its recognition by an enzyme that cleaves the dsRNA into 21 to 23 nt double-stranded fragments in an ATP-dependent, processive manner with a 2 nt at 3´-overhang and 5´-phosphorylated end (Zamore et al. 2000, Elbashir et al. 2001b). This nuclease was identified as an enzyme called dicer that is highly conserved among plants, fungi, worms, flies and mammals (Zamore et al. 2000, Ketting et al. 2001, Elbashir et al. 2001b). It is a member of the RNase III family of dsRNA-specific ribonucleases (Bernstein et al. 2001). Dicer enzymes recognize and

process dsRNA which are essential for RNAi (Bernstein et al. 2001, Grishok et al. 2001, Ketting et al. 2001). Dicer is thought to function as a dimer based upon knowledge of bacterial RNase III and structural evidence, crystallographic and modeling studies of RNase III suggest a mechanism for dsRNA cleavage (Blaszczyk et al. 2001). Dicer not only processes dsRNA into siRNA, but also processes endogenous regulatory RNAs called micro RNAs (miRNAs). In *C. elegans*, RNAi pathway gene Rde-4 encodes a dsRNA binding protein that interacts during RNAi with RNA identical to the trigger dsRNA; Rde-4 protein also interacts with dicer and a conserved DExH-box helicase (Tabara et al. 2002). These and additional data obtained by the authors in the latter study suggest that Rde-1 and Rde-4 function together to detect, retain, and present dsRNA to Dicer for processing. Different domains of dicer have been identified including a dsRNA binding domain, an RNase III activity domain, a helicase activity domain and a PAZ domain (Piwi-Argonaut-Zwille domain, a region of a hundred amino acids, which could mediate interaction with argonaute proteins) (Bernstein et al. 2001). Mouse dicer is very similar to human dicer with a predicted size of 1,906 amino acids and molecular mass of 215 kDa, and contains a tandem repeat of RNase III catalytic domains, dsRNA binding region, a DExH/DEAH helicase motif and a PAZ domain (Nicholson and Nicholson 2002). The mouse dicer gene is located in chromosome 12 and the gene is widely expressed in cells throughout the body in embryonic and adult life.

2.3.2.2.2 Recognition and cleavage of the cognate mRNA

Once generated, the small 21-23 nt dsRNA fragments called siRNA are then recognized by RISC and used as a guide for the recognition and degradation of the target mRNA (Tuschl et al. 1999, Hammond et al. 2000, Zamore et al. 2000, Nykanen et al. 2001). Experiments in *D. melanogaster* showed that RISC is present as a precursor complex that can be activated by ATP to form a complex with endonuclease activity that can cleave endogenous mRNAs (Hammond et al. 2000, 2001, Nykanen et al. 2001). The specific components of the RISC are not known, but do include members of the Argonaute family (Hammond et al. 2001) that have been implicated in many processes previously linked to post-transcriptional silencing. Moreover, RISC should include

protein responsible for *endo*- and *exo*-nuclease activity and recently, RISC activity was studied in a human model. Two proteins of the Argonaute family, eIF2C1 and eIF2C2, were identified in the affinity-purified human RISC; the authors further showed that RISC used single-stranded siRNAs as a guide to cleave the endogenous mRNA. In their studies of the mechanism of RNAi in human cells, Chiu et al. (2002) provided evidence that the status of the 5′-hydroxyl terminus of the as strand of a siRNA determines RNAi activity, whereas blocking the 3′ terminus does not prevent RNAi. They found that an A-form helix structure was required for the formation of antisense-target RNA duplexes. Surprisingly, RNAi still occurred when the siRNA duplex was cross-linked by psoralen, suggesting that complete unwinding of the siRNA helix is not necessary for RNAi activity. Thus, it appears that amplification of RNA by RNA-dependent RNA polymerase (RdRP) is not essential for RNAi in mammal cells, because mammals most likely lack the RdRP ortholog. For this result RNAi in mammals also exhibits slower kinetics and lower efficiency compared to Drosophila and *C. elegans* (Svoboda et al. 2000, Ui-Tei et al. 2000). It is likely that additional proteins modify the different steps in the RNAi process. For example, recent experiments have shown that the Drosophila homolog of the fragile X mental retardation protein interacts with dicer and RISC suggesting a possible role in the RNAi machinery (Caudy et al. 2002, Ishizuka et al. 2002). The latter results also raise the possibility of a role of abnormalities in RNAi in various human diseases.

2.3.3 Application of RNA interference to establish developmental gene function

The most widely used RNAi technology has been in cell culture and *in vivo* studies aimed at understanding the function of an individual or multiple proteins. The complex and remarkably rapid chang that occurs during development of the fertilized oocyte or zygote into an adult organism remains a large mystery. There would appear to be a great potential for RNAi technology to unravel the cellular and molecular events that regulate development processes. Methods for silencing single or multiple selected genes in developing embryos *in vivo* and *in vitro* are beginning to reveal the functions of specific proteins in development processes (Table 2.2). The RNAi was used to demonstrate that siRNAs directed against the mRNA encoding Oct-3/4 and C-mos resulted in depletion

of the encoded proteins and phenotypes similar to those observed in Oct-3/4 and C-mos knockout mice (Kim et al. 2002). A key role for microtubule-associated protein-2 in the regulation of dendrite outgrowth in developing brain neurons was demonstrated using siRNAs (Krichevsky and Kosik 2002). The transcription factor Myc is known to play a fundamental role in the regulation of cell proliferation. A key role for the novel Myc target gene Mina53 in the regulation of cell proliferation by Myc was demonstrated using RNAi technology (Tsuneoka et al. 2002).

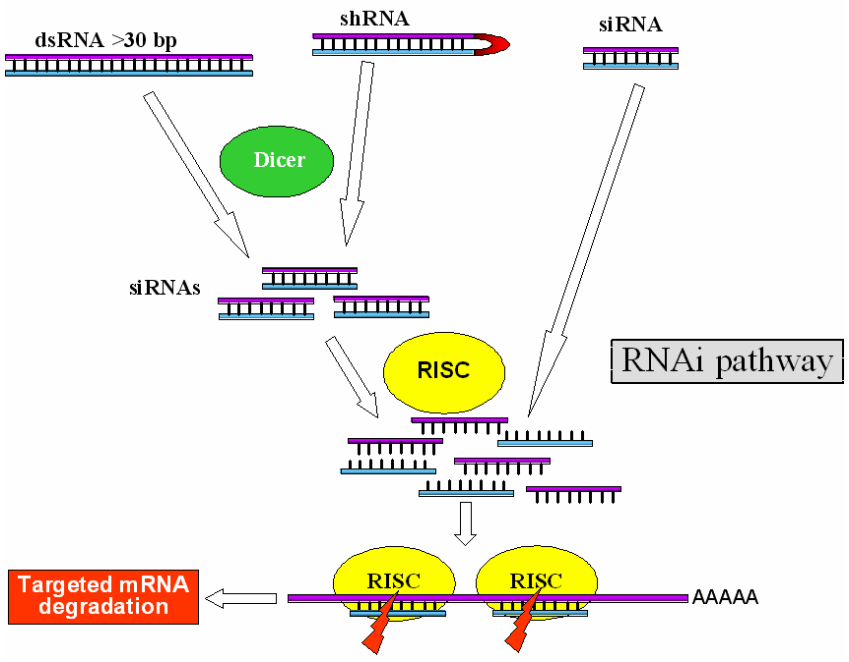

Figure 2.3: RNAi mechanism in mammals, starts with the processing of double-stranded RNA (dsRNA) or short hairpin RNA (shRNA) into small interfering RNA (siRNA) by the dicer emzyme. Small interfering RNA serves as guide sequences for RNA induced silencing complex (RISC), which recognizes and cleaves the cognate mRNA.

The application of RNAi in mammalian embryos was first reported in 2000 by 2 research groups (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000). Wianny and Zernicka-Goetz (2000) used this technique in mouse oocytes and preimplantation embryos. In this experiment, three genes were tested namely: MmGFP, C-mos, and E-cadherin. For the first gene, a mouse line was created in which carried the MmGFP

gene, was paternally inherited to prevent complications from maternal transcripts and translation products. Tests showed that when embryos were injected with dsRNA specific for MmGFP, the fluorescence was significantly diminished; indicating that expression of the gene had been blocked. Also, when the embryos were injected with dsRNA specific for C-mos or E-cadherin, no effect on the fluorescence occurred, although changes resulting from the blockage of these two genes were observed, which indicates that in mice, as in invertebrates, the interference effect is specific. A similar test was done with dsRNA specific for E-cadherin. The disruption of this gene leads to uncompaction, a severe preimplantation defect, which prevents the embryo from developing correctly (Larue et al. 1994, Riethmacher et al. 1995). Similar effects to the MmGFP study were found, dsRNA specific for E-cadherin resulted in uncompaction of the embryos and dsRNA specific for C-mos or MmGFP did not. The final test involved C-mos, a maternally inherited gene which arrests maturing oocytes at metaphase during the second meiotic division. The injection of dsRNA specific for C-mos caused 63% of the injected cells to fail to maintain arrest at MII, whereas 1-2% of the control group failed to maintain arrest (Wianny and Zernicka-Goetz 2000). This demonstrated that, unlike the knockout method, dsRNA can block expression of maternally provided gene products. RNA interference is important because it allows researchers to study the effects of genes loss of their function on developing embryos without the complications of the gene knockout method. The application of this mechanism to vertebrates and then to mammals is likely to provides better models for studying the effects of genes and inactivation of genes in livestock for example cattle, swine and poultry in additional to human. Also, dsRNA was used to investigate the possible role of Gdf-9 in mediating oocyte regulation of cumulus expansion (Gui and Joyce 2005). Fully-grown mouse oocytes injected with Gdf-9 dsRNA, Bmp15 dsRNA or injection buffer were cultured for 24 h and processed for measurement of Gdf-9 and Bmp-15 mRNA levels using real-time RT-PCR, and for measurement of Gdf-9 protein levels using western blotting and immunofluorescence staining techniques. Injection with Gdf-9 dsRNA knocked down Gdf-9 but not Bmp-15 mRNA expression in oocytes, and vice versa. Furthermore, Gdf-9 protein levels were reduced in the Gdf-9 dsRNA injected oocytes. To investigate the role of Gdf-9 in cumulus expansion, two endpoints genes were used to evaluate cumulus expansion namely: Has-2 and Ptgs-2. The mRNA levels were measured in

cumulus cells using real-time RT-PCR and assessment of cumulus expansion was undertaken morphologically. After 24 h of culture in the presence of 0.5 U/ml follicle stimulating hormone (FSH), cumulus shells co-cultured with buffer and Bmp-15 dsRNA injected oocytes exhibited a high degree of expansion, while cumulus shells co-cultured with Gdf-9 dsRNA injected oocytes exhibited only limited expansion. Supporting this observation, after 8 h of co-culture Has-2 and Ptgs-2 mRNA levels were lower in cumulus cells co-cultured with Gdf-9 dsRNA injected oocytes than in those co-cultured with buffer injected oocytes. These results strongly support the concept that Gdf-9 is a key mediator of oocyte-enabled cumulus expansion in mice. In bovine, RNAi represents a useful technique to study gene function in oocyte. The injection of Cyclin B1 dsRNA resulted in a decrease in Cyclin B1 mRNA and protein, while the Cyclin B2 mRNA remained unaffected. Furthermore, the injection of GFP dsRNA did not interfere with Cyclin B1 mRNA or protein with the ability of the oocyte to mature properly (Paradis et al. 2005). Moreover, the study conducted by Nganvongpanit et al. (2006a) has shown the E-cadherin transcripts and proteins were reduced after embryos were treated with E-cadherin dsRNA, and the blastocyte rates in those embryos was found to be lower as compared with that of the control group.

2.3.4 Methods of delivery

The classical methods for nucleic acid delivery, lipid-mediated or viral tranfection, electroporation, and microinjection were used in RNAi method (Table 2.3). Successful introduction of dsRNA molecules into cells using any of those methods depends on a number of factors including delivery reagent composition and stability, effective dsRNA concentrations, cell viability and cell growth characteristics. For this reason, delivery methods demand through and comprehensive optimization to achieve effective silencing while minimizing toxicity.

2.3.5 Methods for detecting gene silencing

Gene silencing can be detected using one of the three general techniques 1) quantitation of changes in the levels of the target mRNA, 2) direct analysis of changes in the

concentrations of the protein encoded by the target mRNA and 3) functional assays designed to examine one or more phenotypes affected by the target protein.

Table 2.2: The applications of RNAi in mammalian embryos

Species	Tissue	Gene	Molecule	Reference
Mouse	Oocytes	Bmp-15	dsRNA	Gui and Joyce 2005
		C-mos	dsRNA	Wianny and Zernica-Goetz 2000, Svoboda et al. 2000
		Plat	dsRNA	Svoboda et al. 2000
		PLCζ	shRNA	Knott et al. 2005
		Egfp	dsRNA	Wianny and Zernica-Goetz 2000, Stein et al. 2003b
		Gdf-9	dsRNA	Gui and Joyce 2005
		Itpr1	dsRNA	Xu et al. 2003
		Miss	dsRNA	Lefebyre et al. 2002
		Doc1r	dsRNA	Terret et al. 2003
		Bnc	dsRNA	Ma et al. 2002
		Ctcf	dsRNA	Fedoriw et al. 2004
		Msy2	dsRNA	Yu et al. 2004
	Embryo	Dicer1	siRNA	Svoboda et al. 2004
		E-cadherin	dsRNA	Wianny and Zernica-Goetz 2000, Sonn et al. 2004
		Egfp	siRNA	Haraguchi et al. 2004
		Nek2A	dsRNA	Sonn et al. 2004
		Oct-4	siRNA	Haraguchi et al. 2004
		Par3	dsRNA	Plusa et al. 2005
		aPKC	dsRNA	Plusa et al. 2005
Porcine	Oocyte	Plk1	dsRNA	Anger et al. 2004
	Embryo	Karyopherins α2, α3	dsRNA	Cabot and Prather 2003
Bovine	Oocyte	C-mos	dsRNA	Nganvongpanit et al. 2006b
		Cyclin B1	dsRNA	Paradis et al. 2005
	Embryo	E-cadherin	dsRNA	Nganvongpanit et al. 2006a
		Oct-4	dsRNA	Nganvongpanit et al. 2006a, 2006b

Table 2.3: The delivery methods used in RNAi technology

Delivery method	Molecules	Reference
Lipid-mediated transfection	siRNA	Elbashir et al. 2001a, Brummelkamp et al. 2002, Yu et al. 2002, Kim et al. 2004
	shRNA	Yu et al. 2002, Yu et al. 2003
	dsRNA	Caple et al. 2000, Billy et al. 2001
Viral transfection	siRNA	Tiscornia et al. 2003, Cao et al. 2005, Li et al. 2005
Electroporation	dsRNA	Grabarek et al. 2002, Mellitzer et al. 2002
	siRNA	Calegari et al. 2002, Weil et al. 2002, Oliveira and Goodell 2003, Djikeng et al. 2004
Microinjection	dsRNA	Fire et al. 1998, Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Cabot and Prather 2003, Stein et al. 2003b, Anger et al. 2004, Gui and Joyce 2005, Sonn et al. 2004, Paradis et al. 2005, Plusa et al. 2005, Nganvongpanit et al. 2006a, 2006b
	Long hpRNA	Stein et al. 2003b
	siRNA	Anantharam et al. 2003, Haraguchi et al. 2004

Quantitative real-time PCR allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples (Higuchi et al. 1992, Mandigers et al. 1998, Kammula et al. 2000). The suitability of this technique for the examination of gene expression in individual oocytes and embryos has been confirmed (Steuerwald et al. 2000, Hartshorn et al. 2002, Mohan et al. 2002). The application of this technique is also widely used to determine the quantitative expressed of mRNA in RNAi study (Gui and Joyce 2005, Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b).

Western blot analysis or immunoblotting is a common technique for quantitative and quantitative evaluation of protein levels and provides information about both the relative abundance and the size of the protein. This technique is a popular method for RNAi studies because the reduction in protein levels is the principle and represents the downstream effect of mRNA knockdown (Billy et al. 2001, Sui et al. 2002, Zhou et al. 2002, Amdam et al. 2003, Cabot and Prather, 2003, Kawasaki and Taira et al. 2003, Anger et al. 2004, Haraguchi et al. 2004, Gui and Joyce 2005, Sonn et al. 2004, Knott et al. 2005, Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b).

As mentioned previous, RNAi is a new technology which is used to study the function of the specific genes of interest. In addition to assays that quantify the effects of RNAi on mRNA and protein levels, more phenotypic changes have been developed. Such functional assays can be divided into different groups, including electrophysiological assay, growth and differentiation assays or viability assays. Specific biological assays can range from watching the development or morphology of the cell under the microscope (Calegari et al. 2002, Zhou et al. 2002, Stein et al. 2003b, Van De Wetering et al. 2003, Gui and Joyce 2005, Haraguchi et al. 2004, Sonn et al. 2004, Knott et al. 2005, Plusa et al. 2005, Nganvongpanit et al. 2006a, 2006b), or used enzyme activity determination specific gene functions (Ui-Tei et al. 2000, Billy et al. 2001, Yang et al. 2001, Anantharam et al. 2003, Kawasaki and Taira 2003, Anger et al. 2004, Knott et al. 2005).

3 Material and methods

3.1 Material

3.1.1 Embryos

For this study bovine embryos were obtained by *in vitro* production (IVP) technologies after *in vitro* maturation, fertilization and culture. The IVP embryos were produced from oocytes obtained from ovaries which obtained from local slaughter house.

3.1.2 Chemicals

Amersham Bioscience (Buckinghamshire, UK)	HRP-conjugated donkey anti-rabbit secondary antibody
Beckman Coulter (Krefeld, Germany)	Sample loading solution (SLS) Dye terminator cycle sequencing (DTCS)
Dynal Biotech (ASA, Oslo, Norway)	Dynalbeads Oligo (dT)25
Gibco BRL, Life Technologies (Karlsruhe, Germany)	BME (essential amino acids) MEM (non essential amino acids) Gentamycin
Invitrogen (Karlsruhe, Germany)	Superscript II reverse transcriptase 5X First-Stand buffer DTT 0.1 M
Kodak (Japan)	Autoradiography film (Kodak® Biomax XAR film)
MWG Biotech (Eberberg, Germany)	Oligonucleotide primers
Promega (Mannheim, Germany)	RNase free-DNase Ribo-nuclease inhibitor (RNasin) T4 DNA ligase and 2X rapid ligation buffer

Roth	5-bromo-4-chloro-3-indolyl-β-D-galactopyra-noside
(Karlsruhe, Germany)	(X-gal)
	Acetic acid
	Agar-Agar
	Anti-mouse IgG (whole molecule) FITC conjugated,
	Ampicillin
	Ammonium peroxydisulfate (APS)
	Boric acid
	Calcium chloride
	Chloroform
	dNTP
	Ethylenediaminetetraacetic acid (EDTA)
	Ethanol
	Ethidium bromide
	Formaldehyde
	Glycerin
	Isopropyl β-D-thiogalactoside (IPTG)
	Korsolin® FF Pepton
	Ponceau-S
	Proteinase K
	Sodium dodecyl sulfate (SDS)
	Sodium carbonate,
	Sodium chloride
	TEMED
	Tris-HCl
	T-octylphenoxypolyethoxyethanol (Triton X-100)
	Yest extract
Santa Cruz biotechnology	Donkey anti-goat IgG-HRP secondary antibody
(Heidelberg, Germany)	Oct-3/4 (N-19) goat polyclonal primary antibody
	Donkey anti-goat IgG-FITC secondary antibody
SERVA Electrophoresis	Acrylamide molecular biology grade
(Heidelberg, Germany)	Bisacrylamide

Sigma	10X Buffer for PCR
(Steinheim, Germany)	Agarose
	Albumin bovine
	Bisbenzemide (Hoechst H33528)
	Dulbecco´s Phosphate Buffer Saline (D-PBS)
	Heparin
	Hepes
	Hoechst 33258
	Hyaluronidase
	Hypotaurin
	Igepal
	Isopropanol
	L-Glutamin
	Megnesium chloride
	Medium 199
	Mineral oil
	Penicillin
	Polyvinyl pyrolidone (PVP)
	Propidium iodide
	Protease inhibitor cocktail
	Sodium hydrogen carbonate
	Sodium hydrogen sulphate
	Sodium lactate solution (60%)
	Sodium pyruvate
	Streptomycin sulfate
	SYBR® Green JumpStart™ *Taq* ReadyMix™
	Tag DNA polymerase
	Tween-20
Stratagene	DH5α *Escherichia coli* competent cells
(Amsterdam, NL)	
Stressgen	Rabbit anti-cMos Proto Oncogene Product (MOS)
(Victoria, Cannada)	Polyclonal Antibody

3.1.3 Reagents and media

Anode buffer I	Tris-HCl (pH 10.4)	300 mM
(100 ml)	Methanol	10 ml
	Water added to	100 ml
Anode buffer II	Tris-HCl (pH 10.4)	25 mM
(100 ml)	Methanol	10 ml
	Water added to	100 ml
Binding buffer	Tris-HCl (1 M pH 7.5)	1,000 µl
(50ml)	Lithium chloride (5M)	10 ml
	EDTA (0.005 M pH 8)	20 ml
	Water added to	50 ml
Blocking buffer	Polyvinyl pyrolidone	1 g
(100 ml)	TBST added to	100 ml
BSA (3 %)	Bovine serum albumin (BSA)	0.15 g
	PBS+PVA added to	5 ml
Capacitation medium	Sodium chloride	0.2900 g
(50 ml)	Potasium chloride	0.0115 g
	Sodium hydrogen carbonat	0.1050 g
	Sodium dehydrogen sulphate	0.0017 g
	Hepes	0.1190 g
	Magnisium chloride $6H_2O$	0.0155 g
	Calcium chloride	0.0145 g
	Sodiumlactate solution (60%)	184 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water add to	50 ml

Cathode buffer	Tris-HCl (pH 9.4)	25 mM
(100 ml)	Methanol	10 ml
	6-aminohexanoic acid	60 mM
	Water added to	100 ml
Culture medium;CR-1	Hemicalcium lactate	0.0273 g
(50 ml)	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potasium chloride	0.0112 g
	Sodium hydrogencarbonate	0.1050 g
	Sodium pyruvate	0.0022 g
	L-Glutamin	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl
DEPC-treated water	DEPC	1 ml
(1,000 ml)	Water added to	1,000 ml
Epinephrin solution	Sodiumdisulphate	0.0400 g
(40 ml)	Epinephrin	0.0018 g
	Water added to	40 ml
Fertilization medium	Sodium chloride	0.3300 g
(50 ml)	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.1050 g
	Sodium dihydrogen phosphate	0.0021 g
	Penicillin	0.0032 g
	Magnesium chloride hexahydrate	0.0050 g
	Calcium chloride dehydrate	0.0150 g
	Sodium lactate solution (60%)	93 µl
	Phenol red solution	100 µl
	Water added to	50 ml

Glycine+PBS	Glycine	0.02252 g
(30 mmol/ml)	PBS+PVA solution added to	10 ml
IPTG solution	IPTG	1.2 g
(10 ml)	Water added to	10 ml
LB-agar	Sodium chloride	8 g
(800 ml)	Pepton	8 g
	Yeast extract	4 g
	Agar	12 g
	Sodium hydroxide (40 mg/ml)	480 µl
	Water added to	800 ml
LB-broth	Sodium chloride	8 g
(800 ml)	Pepton	8 g
	Yeast extract	4 g
	Sodium hydroxide (40 mg/ml)	480 µl
	Water added to	800 ml
Lysis buffer	Igepal (0.8%)	0.8 µl
(100 µl)	RNasin	5 µl
	Dithiothreitol (DTT)	5 µl
	Water added to	100 µl
Modified parker medium	Sodium hydrogencarbonat	0.080 g
(110 ml)	Hepes	0.140 g
	Sodium pyruvat	0.025 g
	L-Glutamin	0.010 g
	Gentamycin	500 µl
	Medium 199	99 ml
	Hemicalcium lactate	0.06 g
	Water added to	110 ml

Paraformaldehyde, 16%	Paraformaldehyde	1.6 g
(10 ml)	Water added to	10 ml
PBS + PVA	Polyvinyl alcohol (PVA)	300 mg
(50 ml)	PBS added to	50 ml
Permeabilization solution	Tritonx-100	5 µl
(10 ml)	Glycine + PBS added to	10 ml
PHE medium	Physiological saline (0.9%)	16 ml
(30 ml)	Hypotaurin solution	10 ml
	Epinephrin solution	4 ml
Physiological saline	Sodium chloride	9 g
(1,000 ml)	Water added to	1,000 ml
Running buffer, 10X	Tris-HCl (0.25 M)	30.3 g
(1,000 ml)	Glycin (1.92 M)	144 g
	SDS	10.0 g
	Water added to	1,000 ml
Sample loading buffer, 4X	Tris-Hcl (1M pH 6.8)	13 ml
(50 ml)	SDS	6 g
	2-Mercaptoethanol	10 ml
	Glycerine	20 ml
	Bromophenol blue	10 mg
	Water added to	50 ml
Separating gel	Acrylamide (30%), bis-acrylamide (0.8%)	5 ml
(10% acrylamide)	Tris (1 M pH 8.8)	5.60 ml
	SDS (10%)	0.15 ml
	APS (20%)	30 µl

	TEMD	10 µl
	Water	4.25 ml
Stacking gel	Acrylamide (30%), bis-acrylamide (0.8%)	1.50 ml
(4% acrylamide)	Tris-HCl (1M pH 6.8)	1.30 ml
	SDS (10%)	0.15 ml
	APS (20%)	30 µl
	TEMD	10 µl
	Water	7.05 ml
TAE buffer, pH 8, 50X	Tris-HCl	242 mg
(1,000 ml)	Acetic acid	57.1 ml
	EDTA (186.1 mg/ml)	100 ml
	Water added to	1,000 ml
TBE buffer, 10X	Tris-HCl	108 g
(1,000 ml)	Boric acid	55 g
	EDTA	40 ml
	Water added to	1,000 ml
TBS	Tris-HCl	121.14 g
(1,000 ml)	Water added to	1,000 ml
TBST	Tween-20	1 ml
(1,000 ml)	TBS added to	1,000 ml
TE buffer, 1X	Tris-HCl (1M)	10 ml
(1,000 ml)	EDTA (186.1 mg/ml)	2 ml
	Water added to	1,000 ml
Washing buffer	Tris-HCl (1 M pH 7.5)	500 µl
(50 ml)	Lithium chloride (LiCl)	1,500 µl

	EDTA (0.005 M pH 8.0)	1,000 µl
	Water added to	50 ml
X-gal solution	X-gal	50 mg
	N,N´-dimethylformamide	1 ml

3.1.4 Kits

CEQ DTCS-Quick Start Kit	Beckman Coulter (CA, USA)
ECL Plus Western Blotting Detection	Amersham Biosciences (Buckinghamshire, UK)
GenElute™ Plasmid Miniprep Kit	Sigma (Steinheim, Germany)
QIAquick PCR purification kit	Qiagen (Hilden, Germany)
RiboMAX™ Large Scale RNA Production System/SP6 and T7	Promega (Medison, USA)
pGEM®-T vector	Promega (Medison, USA)

3.1.5 Software

ABI Prism® 7000 Sequence Detection System Software	Applied Biosystems (Foster City, CA, USA)
BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/)	National Center for Biotechnology Information (NCBI)
Image analysis	Bio-Rad Laser Sharp MRC-1024 CLS software
Primer Express® Software version 2.0	Applied Biosystems (Foster City, CA, USA)
Weight to Molar Quantity (for nucleic acids) (http://www.molbiol.ru/eng/scripts/01_07.html)	Molbiol, RU
SAS version 8.0	SAS Institute Inc. (NC, USA)

3.1.6 Equipments

ABI Prism® 7000 Sequence Detection System	Applied Biosystems (Foster City, CA, USA)
Carbon dioxide incubator (BB16)	Heraeus (Hanau, Germany)
Carbon dioxide incubator (MCO-17AI)	Sanyo (Japan)
Centrifuge	Heraeus (Hanau, Germany)
CEQ™ 8000 Series Genetic Analysis System	Beckman coulter (CA, USA)
CLSM LSM 510	Carl Zeiss (Germany)
Cryotube	Nunc (Roskilde, Germany)
Electrophoresis chamber	BioRad (Munich, Germany)
Epifluorescence microscope	Leica, Bensheim, Germany
Four-well dishe	Nunc (Roskilde, Germany)
Incubator (BB16)	Heraeus (Hanau, Germany)
Injection capillary (K-MPIP-3335-5)	Cook (Ireland)
Nitrocellulose transfer membrane (Protran®)	Schleicher & Schuell BioScience (Germany)
PCR thermal cycle (PTC 100)	MJ Research (USA)
Power Supply PAC 3000:	BioRad (München, Germany)
Power Supply Mini-Protan®	BioRad (Italy)
Slide	SuperFrost® Plus (Braunschweig, Germany)
Trans/Blot® Semi/Dry transfer Cell	BioRad (CA, USA)
Ultraspec 2100 pro spectrophotometer	Amersham Biosciences (Buckinghamshire, UK)

3.2 Methods

3.2.1 Experimentals design

3.2.1.1 The effect of dsRNA on *in vitro* bovine oocyte maturation

C-mos was used as candidate gene to study the effect of dsRNA on *in vitro* bovine oocyte maturation. The immature oocytes were categorized into three groups namely: those injected with C-mos dsRNA, water (RNase-free) and uninjected control. As shown in figure 3.1, the microinjection was performed at immature oocytes stage. Three to four hours after microinjection the number of oocytes was recorded for survival rate. Then, the microinjected oocytes were cultured for the desired period and tested for the RNAi effect. The first assay conducted with microinjected oocytes was phenotype assessment of matured oocytes. The culture was extended up to 24 h after maturation for further phenotype evaluation. Real-time PCR was performed to check whether the cognate mRNA had degraded. Moreover, independent maternal transcript Gdf-9 was quantified in all treatment groups to assess the specificity of mRNA suppression by the C-mos dsRNA. Western blot analysis was performed using samples of matured oocytes to evaluate C-mos protein expression.

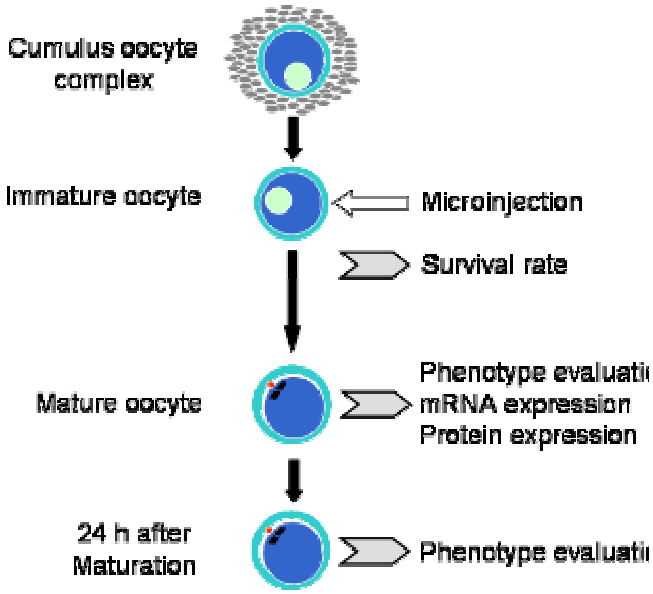

Figure 3.1: An outline of the first experimental design used in RNAi experiments in bovine oocytes. Immature oocytes were microinjected with dsRNA or water followed by *in vitro* culture for 24 hours and another 24 hours after maturation for phenotype change. Survival rate was observed 3-4 hour after microinjection procedure. The matured oocytes were used to study the effects on mRNA and protein expression. Phenotype evaluation was performed at maturation stage and 24 hours after maturation.

3.2.1.2 The effect of dsRNA on *in vitro* bovine embryo development

Oct-4 was used as candidate gene to study the effect of dsRNA on *in vitro* development of bovine embryos. The zygotes were categorized into three groups namely: those injected with Oct-4 dsRNA, water (RNase-free) and uninjected control. For this, the microinjection was performed at zygote stage. The number of zygote 3-4 h after microinjection was recorded for survival rate. Then, the zygotes were cultured for the desired period until testing for the RNAi effect is performed. The first assay conducted with microinjected zygotes was phenotype assessment including first cleavage, morula and blastocyst rate. Real-time PCR was performed to check whether the cognate mRNA had degraded at blastocyst stage. In this experiment, the E-cadherin transcript has been quantified in the three treatment groups to investigate the specificity of mRNA degradation by Oct-4 dsRNA. Moreover, the Fgf-4 gene which is reported to be co-expressed with Oct-4 gene (Nicholes et al. 1998), has been quantified in all treatment groups (in this experiment). Immunofluorescence and western blot analysis were performed using embryos at day 7 blastocyst stage for evaluating the Oct-4 protein expression (figure 3.2). Moreover, the differential cell staining was performed using embryos at day 8 blastocyst stage for evaluating the ICM and TE cells.

3.2.2 *In vitro* embryo production

3.2.2.1 Oocytes recovery and *in vitro* maturation

Bovine ovaries were obtained from the local slaughter house and transported to the laboratory within 4 h in a thermo flask (35 ºC) containing physiological saline (0.9% NaCl), supplemented with 0.5 ml Steptocombin® per liter. Before aspiration of cumulus oocyte complex (COCs), the ovaries were washed once with 70% ethanol followed by two times washing with 0.9% physiological saline to eliminate surface organisms and then dried with sterile paper to avoid contamination. Subsequently, COCs that have 2 to 8 mm diameter were aspirated from follicles using a 5 ml syringe attached with 18 G needles. This aspirated fluid was collected in sterilized 50 ml tubes kept at 35 ºC and was allowed to precipitate for 15 min. The competent COCs were

picked out using glass-pipette and washed three times in drops of modified parker medium (MPM) supplemented with 12% oestrus cow serum (OCS). The COCs were transferred in groups of 50 cells in 400 µl maturation medium under mineral oil (Sigma) in four well dishes (Nunc). Only oocytes with evenly granulated cytoplasm and surrounded by multiple layer of cumulus cells were used for *in vitro* maturation. During the IVM procedure, these competent COCs were culture in TCM-199 as basic medium at 24 ºC in incubator with humidified atmosphere of 5% CO_2 in air for 22-24 h.

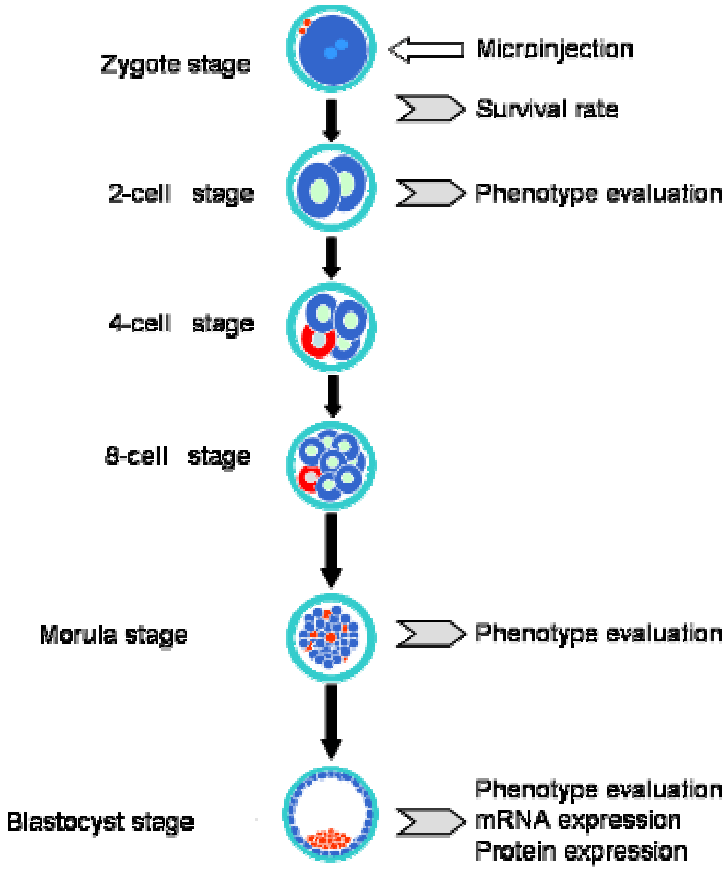

Figure 3.2: An outline of the second experimental design used in RNAi experiments in bovine embryos. Zygotes were microinjected with dsRNA or water followed by *in vitro* culture until blastocyst stage. Survival rate was determined 3-4 hours after microinjection procedure. The blastocysts (day 7) were used to study the effects on mRNA and protein expression. Phenotype evaluation was performed at 2-cell, morula and blastocyst stage.

3.2.2.2 Sperm preparation and capacitation

Base on the number of oocytes, 2-4 semen straws prepared from known breeding bull were thawed and motile spermatozoa were obtained by swim-up procedure (Parish et al. 1988). During the swim-up procedure frozen thawed sperm cell were incubated in a tube containing 5 ml capacitating medium supplemented with heparin for 50 min under 39 ºC in an incubator with humidified atmosphere of 5% CO_2 in air. The motile sperm cells found in the upper layer of the solution were transferred into new falcon tube. The sperm cells were pelleted by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed two times and finally resuspended in already prepared 3.5 ml capacitating medium and made ready to use for IVF.

3.2.2.3 *In vitro* fertilization

Matured oocytes were washed twice in the fertilization medium and transferred into a four-well dish containing 400 µl of fertilization medium supplement with 6 mg/ml bovine serum albumin (BSA), 2.2 mg/ml sodium pyruvate and 1 mg/ml heparin. Ten microliter of PHE medium was added to each well to initiate sperm motility and covered with mineral oil (Sigma). Motile spermatozoa selected by the above procedures were finally added to the fertilization medium to have a final concentration of $1x10^6$ spermatozoa/ml to add to a group of 50 oocytes in each well and co-cultured for 18 h at standard incubation conditions, 39 ºC and humidified atmosphere containing 5% CO_2 in air.

3.2.2.4 *In vitro* culture

After IVF, the presumptive zygotes were put into 15 ml falcon tube containing 1 ml of culture medium (CR-1aa) (Rosenkranz and First 1994) supplemented with 10% OCS, 10 µl/ml BME (essential amino acids) and 10 µl/ml MEM (non essential amino acids). The fertilized oocytes were gently vortexed to separate them from dead spermatozoa and the cumulus cells. The cumulus free zygotes were selected and washed two times with culture medium before being transferred in group of 50-60 cells into four-well

dish, each well containing a 400 µl culture medium cover with mineral oil. The first cleavage rate of the embryos is determined 48 h after fertilization followed by incubating for the consecutive days when different developmental stages were harvested at respective developmental times as shown in table 3.1.

3.2.3 Preparation of DNA template

3.2.3.1 Polymerase chain reaction

For all gene amplification studies, a pair of primers was designed according to bovine cDNA sequences found in GenBank (see Table 3.2 for details) using Primer Express® Software v2.0 (Applied Biosystems). These primers generated a PCR amplicon corresponding to the coding sequence. The identity of the product was confirmed by sequencing. The first round of PCR amplification was performed using *Taq* DNA polymerase (Sigma). At first, the sample was heated at 95 °C for 5 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at temperatures as indicated in table 3.2 for 30 s and extension at 72 °C for 1 min. Following the last cycle, a 10 min elongation step at 72 °C was performed.

Table 3.1: Average developmental time for embryos collection

Stage of embryos	Developmental time (hours post insemination; hpi)	
	Range	Average
2-cells	24-40	32
4-cells	40-52	46
8-cells	53-75	64
16-cells	80-110	96
Morula	110-135	120
Blastocyst	160-175	168

3.2.3.2 Isolation of DNA fragment from gel

The fragments of interest were carefully excised from 0.8% agarose gel put into 1.5 ml tube to be incubated at -20 °C overnight, or at -80 °C for 30 min and subsequently homogenised in 500 ml 1X TE buffer using 10 ml syringe and 18 G needle. Five

hundred milliters of phenol:chloroform:isoamyl (1:1:1 v/v) were added to homogenised solution and mixed well by vortexing. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C, then the aqueous phase of the solution was transferred to new 2.0 ml tube following add equal volume of chloroform. The mixture was centrifuged by 12,000 rpm for 10 min at 4 °C and the aqueous phase of the solution was transferred to a new 2.0 ml tube. Add 1:10 volume of sodium acetate (3 M, pH 5.2) and equal volume of 100% ethanol. The DNA was allowed to precipitate at -20 °C overnight. Thereafter, the pellet was recovered by centrifugation for 30 min at 4 °C. The pellet was two times washed with 75% ethanol and resuspended in 7 µl double distilled water and stored at -20 °C until further use.

3.2.3.3 Ligation

The PCR fragments were ligated with the pGEM®-T vector (Promega). The ligation reaction was performed in 5µl reaction volume containing 2.5 µl ligation buffer, 0.5 µl vector, 0.5 µl T4 DNA ligase (3 U/µl) and 1.5 µl DNA template. The reaction was incubated at 4 °C overnight or 20 °C for 2 h.

Table 3.2: Detail of primers used for PCR

Gene (genbank accession number)	Primer sequences	Annealing temperature (°C)	Product size (bp)
C-mos (AY630920)	5'-GTTCCATCGACTGGGAGCAGGT-3' 5'-TGCTTTGCGCGTGGAGGAACAG -3'	65	435
E-cadherin (AY508164)	5'-GTACACCTTCATCGTCCAGAGCTAA-3' 5'-GCTCTTCAATGGCTTGTCCATTTGA-3'	60	496
Fgf-4 (AF170490)	5'-GGCTCTCTGGCTTTGATCGTG-3' 5'-GAACTGTCGGGCCAGAGGAA-3'	60	129
Gdf-9 (NM174681)	5'- GATTGAGATTGATGTGACAGCTCCT -3' 5'- TTGTCCCACTTCAGTTGACTAAAGC -3'	60	471
Histone 2a (NM178409)	5'-CTCGTCACTTGCAACTTGCTATTC-3' 5'-CCAGGCATCCTTTAGACAGTCTTC-3'	60	148
Oct-4 (AY490804)	5'-CCCAGGACATCAAAGCTCTTCAG-3' 5'-GAACATGCTCTCCAGGTTGCCT-3'	60	341

3.2.3.4 Transformation

For cloning of PCR fragments, 3 µl of the ligation reaction was co-incubated with 60 µl DH5α *E. coli* competent cells (Stratagen) for 30 min on ice. The mixture was heat shocked by putting it into a 42 °C water bath for 90 s and immediately transferred on ice for 2 min. LB-broth (750 ml) was added to the bacteria solution and shook at 150 rpm at 37 °C for 90 min. Each bacterial suspension was plated on two ampicillin containing LB-agar plates. The medium was containing 20 µl X-gal and IPTG solutions and incubated at 37 °C overnight.
Colonies were differentiated by the activity of β-galactosidase as white and blue for the presence of inserted DNA fragment. Due to the activation of LacZ gene on the vector, colonies containing the insert target DNA appear as white colonies and those with active LacZ gene without insert DNA formed blue colonies.

3.2.3.5 Screening of insert DNA fragments using PCR

To identify and screen insert target DNA fragment, two white colonies were picked up from each plate and suspended in 30 µl 1X PCR buffer (Sigma). One blue colony was picked up as a control standard to differentiate the presence of the target insert by comparing the length of amplified DNA fragments from white and blue colonies.

3.2.3.6 Sequencing of DNA fragments

The positive clones were sequenced using CEQ^{TM} 8000 Series Genetic Analysis System (Beckman coulter). Briefly, PCR product of positive clones were resolved on 1% agarose gel and visualized by ethidium bromide staining, and then quantified and purified using QIAquick PCR purification kit (Qiagen). Sample was excised from gel, record the weight, and put in the spin column. The samples were incubated with 3 volume of QG buffer (Qiagen) at 50 °C for 10 min until the gel is completely melted. Add 1 volume of 100% 2-propanol (Roth) and mix by inverting. The spin column was placed into collection tube, following by centrifuged at 18,000 rpm under 20 °C for 1 min. After the flow-through liquid was discarded, this cleaning up procedure was

repeated two times and the buffer was replaced by 500 µl QG and 750 µl PE buffer (Qiagen), respectively. The spin column containing sample was moved to the new collection tube. Fifty microliters of double distilled water was added into the column and incubated at room temperature for 5 min. Centrifugation by 18,000 rpm at 20 °C for 1 min was done to collect the dissolved sample. Dehydration was performed and the sample was diluted by adding 10 µl of double-distilled water. For sequencing PCR, five microliters of purified sample was used for cycle sequencing, with specific primers (Table 3.2), and Dye Terminator Cycle Sequencing (DTCS) (Beckman Coulter). Twenty microliter of sequencing PCR was performed for each primer. Three molar NaOAc, 100 mM EDTA, and glycogen were added after PCR was finished. Followed by, added 60 µl of 100% ethanol and mixed well by vortex, then centrifuged by 18,000 rpm at 4 °C for 15 min. All liquid was removed and washed 2 times with 200µl 70% ethanol without mixing and centrifuge. Finally, the ethanol was then removed and the sample was air dried. The sample was then resuspended in 40 µl of sample loading solution (SLS) (Beckman Coulter). Samples were transferred to a CEQ sample plate and overlaid with mineral oil and then sequenced using CEQ™ 8000 Genetic Analysis System.

3.2.3.7 Sequence analysis

The completed sequence of the fragment was utilized to search for homologus sequences in National Center for Biotechnology Information (NCBI) non redundant DNA sequence data base using BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence similarities were considered to be significant when identity percentage was ≥ 90%.

3.2.4 Plasmid isolation

For plasmid isolation the transformated colonies containing inserts were cultured overnight in 5 ml LB-broth containing ampicillin. Plasmids were isolated using GenElute™ Plasmid Miniprep Kit (Sigma) following the manufacturer's instruction. Briefly, the cells were harvested by centrifugation at 12,000 rpm for 1 min and the

supernatant was discarded. The pellet was resuspended in 200 µl resuspension solution and vortexed. The resuspended cells were lysed by adding 200 ml lysis solution and mixed gently. After that, cell debris was precipitated by adding 350 µl neutralization solutions, mixed gently and centrifuged at 12,000 rpm for 10 min. The cleared lysate was transferred to the previously prepared GenElute Miniprep binding column and centrifuged at 12,000 rpm for 1 min. The flow-through liquid was discarded. The column was washed by adding 750 µl diluted wash solution and centrifuged at 12,000 rpm for 1 min. To elute the DNA, the column was transferred to a new tube, 50 µl of double-distilled water was added and centrifuged at 12,000 rpm for 1 min then the column was discarded. The isolated plasmids were subjected to be kept at -20 °C until further use.

3.2.5 Double-stranded RNA sythesis

3.2.5.1 Preparation of DNA template for *in vitro* transcription

The PCR conditions have been used generated DNA template for *in vitro* transcription using plasmid as template (see 3.2.3.1). However, in this PCR amplification using T7 promoter (GTAATACGACTCACTATAGGG) attached to the 5´-end of each primer to generate in vitro transcription template (Table 3.3).

Table 3.3: Primers with T7 promoter used to prepare DNA templates for *in vitro* transcription

Gene	Primer sequences
C-mos	5’-GTAATACGACTCACTATAGGGGTTCCATCGACTGGGAGCAGGT-3’
	5’-GTAATACGACTCACTATAGGGTGCTTTGCGCGTGGAGGAACAG-3’
Oct-4	5’-GTAATACGACTCACTATAGGGCCCAGGACATCAAAGCTCTTCAG-3’
	5’-GTAATACGACTCACTATAGGGGAACATGCTCTCCAGGTTGCCT-3’

3.2.5.2 *In vitro* transcription and annealing step

3.2.5.2.1 Generating dsRNA in separate reaction

Two different templates for *in vitro* transcription to produce sense and antisense RNA strands were synthesized in separate reaction (Wianny and Zernicka-Goetz 2000, Nganvongpanit et al. 2006b) as shown in figure 3.3. These C-mos and Oct-4 specific templates were purified using the QIAquick PCR Purification Kit (Qiagen). The DNA templates coupled with T7 promoter were *in vitro* transcribed using RiboMAX^{TM} Large Scale RNA Production T7 System (Promega) (Svoboda et al. 2000, Amdam et al. 2003, Plusa et al. 2004, Nganvongpanit et al. 2006a, 2006b) by which sense and antisense strands were transcribed from DNA template in separate reaction. The appropriate reaction for T7 RNA polymerase was set up at room temperature. The reaction components were added in the order shown in table 3.4, being careful to dissolve the DNA template in RNase-free water before addition to the reaction mixture. The reaction mixture was gently pipeted in order to mix it and incubated at 37 °C for 2-4 h. After *in vitro* transcription, the DNA template was removed by digestion with RNase-free DNase at 37 °C for 15 min. Subsequently, annealing of equal volume of sense and antisense RNA strands was performed by incubating the reaction at 37 °C for 4 h after heating to 68 °C for 10 min to produce the dsRNA (Wianny and Zernicka-Goetz 2000, Nganvongpanit et al. 2006a, 2006b).

3.2.5.2.2 Generating dsRNA in same reaction

The dsDNA templates coupled with T7 promoter at both 5´-end were *in vitro* transcribed using RiboMAX^{TM} Large Scale RNA Production T7 Systems (Promega) by which sense and antisense strands were transcribed from DNA template in same reaction (Amdam et al. 2003, Nganvongpanit et al. 2006a). Briefly, the appropriate reaction for T7 RNA polymerase was set up at room temperature. The reaction components were added in the order shown in table 3.4, being careful to dissolve the DNA template in water before adding it into the reaction mixture. The reaction mixture was gently pipeted in order to mix and then incubate at 37 °C for 2-4 h. After *in vitro* transcription, the DNA template was removed by digestion with RNase-free DNase at 37 °C for 15 min. Subsequently, the re-annealing of sense and antisense RNA strands was performed by incubating the reaction at 37 °C for 4 h after heating to 68 °C for 10

min to produce the dsRNA (Wianny and Zernicka-Goetz 2000, Nganvongpanit et al. 2006a, 2006b).

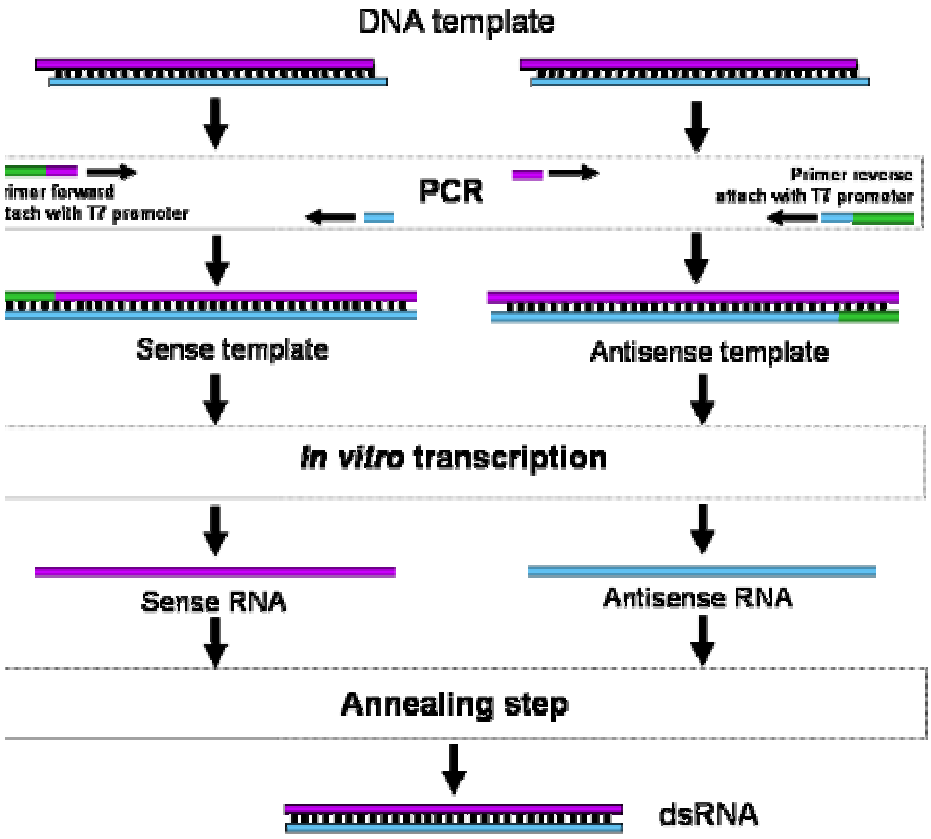

Figure 3.3: Synthesis of dsRNA in separate reaction. PCR was performed to generate sense and antisense templates, which were used for *in vitro* transcription in separate reaction. Finally, both sense and antisense strands were annealed to give rise to dsRNA.

Table 3.4: Components of *in vitro* transcription reaction

Reaction components	Volume (µl)
T7 Transcription 5X Buffer	4
rNTPs (25mM ATP, CTP, GTP, UTP)	6
DNA template	8
T7 Enzyme Mix	2
Total	20

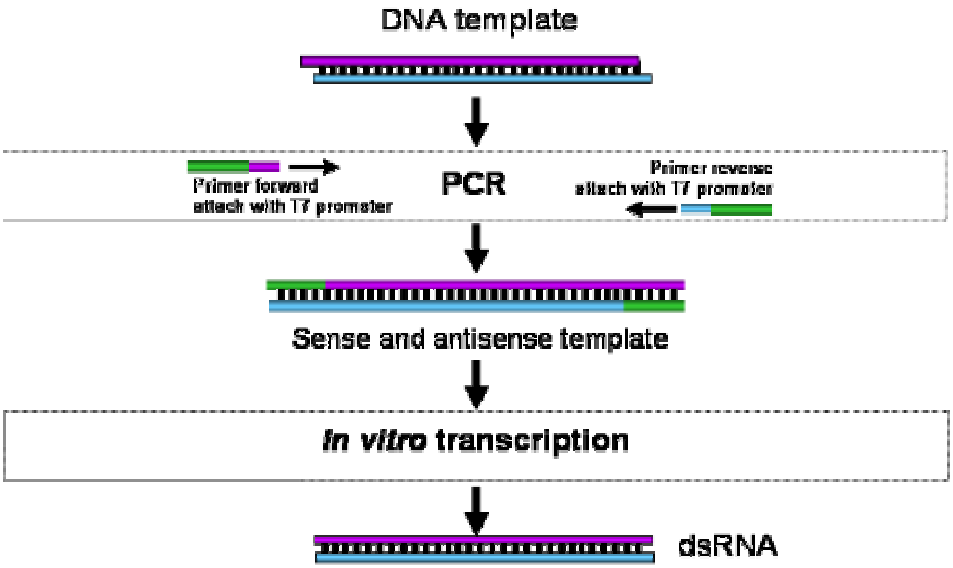

Figure 3.4: Synthesis of dsRNA in same reaction. PCR was performed to generate template with T7 promoter at both 5´-end. These templates were used for *in vitro* transcription to generate dsRNA.

3.2.5.3 Purification of dsRNA

After *in vitro* transcription and annealing step, dsRNA was purified using phenol-chloroform extraction (Nganvongpanit et al. 2006a, 2006b). The samples were extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) by vortexing for 1 min and spin at 15,000 rpm for 2 min. The aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1 v/v) was added. After vortexing, sample was centrifuged at 15,000 rpm for 2 min. The upper aqueous phase was transferred to a fresh tube. The RNA was precipitated using 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol or 1 volume of isopropanol. The sample was centrifuged at 15,000 rpm at 4 °C for 10 min. The resulting pellet was washed 2 times with 70% ethanol. Finally, the dsRNA pellets were resuspended with diethylpyrocarbonat (DEPC) treated water and store at -80°C until used.

3.2.5.4 Determination of dsRNA concentration

The dsRNA concentration was measured by ultraviolet light absorbance using Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). A 7:700 dilution of the dsRNA is prepared and the absorbance was read at a wavelength of 260 nm. And then, 2 µl of the dsRNA and the corresponding dsDNA template (20 µl) were also resolved by electrophoresis on a 2% agarose gel to evaluate the size and purity of the dsRNA (Figure 4.1- 4.3).

3.2.6 Microinjection

3.2.6.1 Microinjection of oocytes

The COCs were stripped off their cumulus cells by vortexing 3 min in 500 µl phosphate saline buffer (PBS) without calcium and magnesium supplemented with 1 mg/ml hyaluronidase (H-2251, Sigma). Selection was based on cumulus aspects (Leibfried and First 1979) and cytoplasmic aspects. Once the oocytes were selected, the cumulus cells were partially removed (Figure 4.5, A) by vortexing to avoid technical difficulties during microinjection of dsRNA or water in the cytoplasm of the oocytes. Then, oocytes were held in a tissue culture medium (TCM) 199 supplemented with 0.1% BSA (A-3311, Sigma), 0.2 mM pyruvate and 50 µg/ml gentamycin sulphate (Sigma) until use in a humidified atmosphere with 5% CO_2 at 39 °C. Prior to injection, immature oocytes were incubated for 20 min in TCM-199 medium supplemented with cytochalasin B (8 µg/ml) in order to reduce mechanical damage during injection (Paradis et al. 2005). As mentioned above, the immature oocytes were categorized into 3 groups namely: C-mos dsRNA injected, water (RNase-free) injected and uninjected control. Microinjection was performed on an inverted microscope (Leica DM-IRB) at 200x magnification. The group of 50-60 immature oocytes were placed in a 10 µl droplet of Hepes-buffered tissue culture medium 199 (H-TCM) supplemented with 8 µg/ml cytochalasin B under mineral oil. The C-mos dsRNA or water was placed in a 1 µl droplet near to the droplet containing the oocytes. Injection was performed by aspiration of the dsRNA into the injection capillary (Cook, Ireland, K-MPIP-3335-5). The inner diameter of the injection

capillary was 5 µm. The injection volume of ~7 pl was estimated from the displacement of the minisque of mineral oil in the capillary. The different experimental groups were injected one after the other, every time preparing a new dish with fresh medium. Subsequently, in three experimental replications, a total of 935 immature oocytes were categorized into 3 groups namely: C-mos dsRNA injected (n=327), water injected (n=303) and uninjected control (n=305). After microinjection all groups of ooytes were washed twice in TCM-199 and set back into culture (in 3.2.2.1).

3.2.6.2 Microinjection of zygotes

Two groups of 50-60 zygotes (from 3.2.2.3) were placed into injection medium (H-TCM) for injection with Oct-4 dsRNA or water (RNase-free). Microinjection was performed on an inverted microscope at 200x magnification. Zygotes to be injected were placed in a 10 µl droplet of H-TCM under mineral oil. Injection was performed by aspiration the dsRNA into the 5 µm diameter injection capillary. The injection volume of ~7 pl was estimated from the displacement of the minisque of mineral oil in the capillary. After injection all groups of zygotes were washed twice in CR1aa medium and set back into culture (in 3.2.2.4). The zygotes were checked for survival 3-4 h after injection. For this experiment, a total of 1,437 zygote stage bovine embryos were produced above and categorized into three groups namely: those injected with Oct-4 dsRNA (n=439), those injected with water (n=427) and uninjected control (n=571).

3.2.7 Oocytes and embryos collection

In order to assess the effect of sequence-specific dsRNA in oocytes and embryos on mRNA transcript abundance and protein expression, oocytes and embryos were collected at specific time after treatment for mRNA and protein analysis using real-time quantitative PCR and western blotting analysis, respectively. In experiment 1 (in 3.2.1.1), immature oocytes were cultured for 48 h after treatment to allow any parthenogenic development, those used for transcriptional and protein expression analysis were collected at 24 h after microinjection and subsequent maturation. In experiment 2 (in 3.2.1.2), zygotes injected with Oct-4 dsRNA or water and uninjected

controls were cultured *in vivo* until day 8 blastocyst stage to assess development and resulting blastocysts from each treatment group were used for both transcription and protein analysis. Prior to freezing, all oocytes and embryos were washed two times with PBS (Sigma) and treated with acidic Tyrode pH 2.5-3 (Sigma) to dissolve the zona pellucida. The zona free embryos were further washed two times in drops of PBS and frozen in cryo-tubes containing minimal amounts of lysis buffer. Embryos for western blot analysis were additionally treated with protease inhibitor (Sigma). Until used for RNA isolation (in 3.2.8) or western blotting (in 3.2.11), all frozen embryos were stored at -80 ^{o}C.

3.2.8 Isolation of RNA

A total of three pools of each containing 20 matured oocytes or 10 blastocyst stage embryos from each treatment groups were used for mRNA isolation using oligo (dT)25 attached magnetic beads (Dynal, Norway, Oslo) following manufacturers instruction. Briefly, embryos in lysis buffer were mixed with 40 µl binding buffer (20 mM Tris-HCl with pH 7.5, 1 M LiCl, 2 mM EDTA with pH 8.0) and incubated at 70 ^{o}C for 5 min to obtain complete lysis of the embryo and release of RNA. Ten microliters of oligo(dT)25 magnetic bead suspension was added to the samples, and incubated at room temperature for 30 min. The hybridized mRNA and Oligo (dT)25 magnetic beads were washed three times with washing buffer (10 mM Tris-HCl with pH 7.5, 0.15 mM LiCl, 1 mM EDTA with pH 8.0). Finally, mRNA samples were eluted in 12 µl DEPC-treated water used for reverse transcription procedure.

3.2.9 Complementary DNA synthesis

All RNA sample (from 3.2.8) were reverse transcribed in 20 µl reaction volume containing 2.5 µM oligo (dT)12N (where:N = G, A or C) primer, 4 µl of 5X first stand buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris-HCl with pH 8.3), 2.5 mM of each dNTP, 10 U RNase inhibitor (Promega) and 100 U of SuperScript II reverse transcriptase (Invitrogen). In terms of the order of adding reaction components, mRNA and oligo(dT) primer were mixed first, heated to 70 ^{o}C for 3 min, and placed on ice until

the addition of the remaining reaction components. The reaction was incubated at 42 ^{o}C for 90 min, and terminated by heat inactivation at 70 ^{o}C for 15 min. Finally, the cDNA were stored at -20 ^{o}C until used (in 3.2.10.4).

3.2.10 Quantitative real-time PCR

The ABI Prism® 7000 apparatus (Applied Biosystems) was used to perform the quantitative analysis using SYBR® Green JumpStart™ *Tag* ReadyMix™ (Sigma) incorporation for dsDNA-specific fluorescent detection dye. The amount of DNA present in a sample was measured as a function of how quickly a fluorescent signal is first observed above threshold (C_T value) during the process of sequence amplification. Threshold cycle (C_T) is the point at which the fluorescence values are recorded during every cycle and represent the amount of the product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, a fewer number of cycles it takes to reach this point.

3.2.10.1 Plasmid serial dilutions preparation

The concentration of the plasmids which carry the target fragments have been measured by reading the absorbance at 260 nm using Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). Plasmid concentration was converted into number of copies (molecules) using program that is available by www.molbiol.ru. The plasmid solution was diluted several folds to be at a concentration range similar to the target in the embryos. Serial dilutions were freashly prepared for real-time PCR from 10^1 to 10^8 copy numbers in 50 µl volume.

3.2.10.2 Dissociation curve generation analysis

The SYBR® Green dry can bind to any DNA and generate fluorescence. Therefore an additional verification was achieved by plotting fluorescence as a function of temperature to generate a melting or dissociation curve of the amplicon which is sequence specific. This curve has been used to distinguish between the amplicon and

non-specific DNA. The dissociation curve was produced at the end of PCR by monitoring fluorescence continuously while slowly heating the sample from 60 °C to 95 °C at 0.2 °C intervals to observe the loss of fluorescence at the denaturing temperature. As the products specificity is determined entirely by its primers, an optimization step has been done for each primer pair before quantification.

3.2.10.3 Optimization of the real-time PCR conditions

Optimization of the primers concentration has been done to determine the minimum primer concentrations give the lowest threshold cycle without primer dimmer formation. For each primer pair, 9 reaction of difference condition (Table 3.5) with a total 20 µl were carried out for both the template (DNA) and non-template as control. At the end of the run, the dissociation curves were generated to check the absence of non specific amplification and subsequent confirmation by analysis of the PCR products by agarose gel electrophoresis. After analysing the dissociation curves of these different combinations, the optimum primer combination was selected to use for target quantification.

Table 3.5: Primer optimization used for real-time PCR

Forward primer	Reverse primer		
	100	200	400
100	100/100	100/200	100/400
200	200/100	200/200	200/400
400	400/100	400/200	400/400

3.2.10.4 Quantification of the real-time PCR reaction

Quantification of C-mos, Oct-4 and H2a as endogenous control, mRNA in the oocytes/embryos of each treatment group was assessed by real-time quantitative PCR. Moreover, idependent maternal transcript Gdf-9 had been quantified in the three treatment groups of the experiment 1 to assess the specificity of mRNA suppression by the C-mos dsRNA. Similarly, the E-cadherin transcript had been quantified in the three treatment groups of the experiment 2 to investigate the specificity of mRNA transcript

inhibition by Oct-4 dsRNA. Moreover, the Fgf-4 which is reported to be co-expressed with Oct-4 gene (Nicholes et al. 1998), was quantified in the treatment groups of the experiment 2. The ABI Prism® 7000 apparatus was used to perform the quantitative analysis using SYBR® Green JumpStart™ *Tag* ReadyMix™ (Sigma) incorporation for dsDNA-specific fluorescent detection dye. Quantitative analyses of all studied transcripts were performed in comparison with H2a as an endogenous control (Robert et al. 2002), and were run in separate wells. The PCR was performed by using 2 µl of each sample cDNA and specific primers which amplify. The primer sequences were designed for PCR amplification according to the bovine cDNA sequence (Table 3.6) using Primer Express® Software v2.0 (Applied Biosystems). Standard curves were generated for both target and endogenous control genes using serial dilution of plasmid DNA (10^1 – 10^8 molecules). The PCRs were performed in 20 µl reaction volume containing of 10.2 µl SYBR® Green JumpStart™ *Tag* ReadyMix™ optimal levels of forward and reverse primers and 2 µl of embryonic cDNA. During each PCR reaction samples from the same cDNA source were run in duplicate to control the reproducibility of the results. A universal thermal cycling parameter (initial denaturation step at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 15 s and 60 °C for 60 s) was used to quantify each gene of interest. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60 °C and taking measurements every 7 s interval until the temperature reached 95 °C.

3.2.11 Western blotting analysis

3.2.11.1 Protein isolation

Protease inhibitor was used to stop protease enzymes from denaturing proteins in embryo samples. Sample loading buffer (1X) was used to lyse cells (causes cell membranes to break and proteins to be released into buffer). Groups of 120 matured oocytes and 50 embryos at day 7 blastocyst stage were used from each treatment group, which include C-mos or Oct-4 dsRNA injected, water injected and uninjected control. In order to assess the amount of protein available before treatment in immature oocytes and zygotes, equal amount of immature and matured oocytes were used for protein

analysis prior to treatment. The oocytes and embryos were homogenized in sample loading buffer by heat at 95 °C for 5 min.

Table 3.6: Detail of primers used for quantitative real-time PCR

Gene (genbank accession number)	Primer sequences	Annealing temperature (°C)	Product size (bp)
C-mos	5'- GGGCAATATCACCTTGCACCA -3'	60	113
(AY630920)	5'- CGCTGACCACGTCTAGGGAGTA -3'		
E-cadherin	5'-GTACACCTTCATCGTCCAGAGCTAA-3'	60	496
(AY508164)	5'-GCTCTTCAATGGCTTGTCCATTTGA-3'		
Fgf-4	5'-GGCTCTCTGGCTTTGATCGTG-3'	60	129
(AF170490)	5'-GAACTGTCGGGCCAGAGGAA-3'		
Gdf-9	5'- GATTGAGATTGATGTGACAGCTCCT -3'	60	471
(NM174681)	5'- TTGTCCCACTTCAGTTGACTAAAGC -3'		
Histone 2a	5'-CTCGTCACTTGCAACTTGCTATTC-3'	60	148
(NM178409)	5'-CCAGGCATCCTTTAGACAGTCTTC-3'		
Oct-4	5'-CCCAGGACATCAAAGCTCTTCAG-3'	60	341
(AY490804)	5'-GAACATGCTCTCCAGGTTGCCT-3'		

3.2.11.2 Preparation of the SDS-PAGE gel

Appropriate percentage SDS-PAGE gel was used for protein of interest. Typically 10-12% acrylamide gels were used for high molecular weight proteins (>50 kDa), 15% gel for mid range molecular weight proteins (15 - 50 kDa) and 20% gel for low molecular weight proteins (<15 kDa). In this study, 14% acrylamide gel was used because the molecular weight of C-mos is 39 kDa and Oct-4 is 43 kDa. SDS is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone, confers a negative charge to the polypeptide in proportion to its length.

Two solutions were prepared namely: separating gel and stacking gel. Separating gel is used to separate proteins into their respective sizes allowing sharp bands to be seen, whereas the stacking gel organizes proteins before they enter the separating gel. Glass plates were cleaned thoroughly using soap and distilled water and finally cleaned using 70% ethanol. The sandwich glass plates were introduced into the support piece and placed on a flat surface.

First, the separating gel (30% acrylamide, 0.8 % bis acrylamide, 1M Tris-HCl with pH 8.8, 10% SDS, 20% APS, TEMD) was prepared, quickly mixed and poured in 3/4 volume of each sandwich and the remaining space (1/4 volume) was filled with isopropanol to ensure that no air bubbles would be formed at the surface of the gel during gel polymerization. The isopropanol was poured off after polymerization had occurred. Stacking gel (30% acrylamide, 0.8% bis acrylamide, 1M Tris-HCl with pH 6.8, 10% SDS, 20% APS, TEMD) was added on the top of separating gel. A 10 well comb was inserted and then stacking gel was filled once again.

3.2.11.3 Running the SDS-PAGE gel

Two support pieces of the gel sandwiches were mounted with the U-shape rubber piece and then put into the chamber. Upper reservoir was filled with running buffer and then the samples were loaded in the prepared wells. A standard vertical gel electrophoresis apparatus (BioRad) at 10 mA was used for each 0.75 mm gel. The SDS-PAGE was run one hour or until the blue line has run out of the bottom gel.

3.2.11.4 Transferring proteins to the membrane

Proteins were then transferred into nitrocellulose transfer membrane, pore size 0.45 µm Protran® (Schleicher&Schuell, BioScience) using Trans-Blot Semi-Dry Transfer Cell (BioRad). Transfer membrane was prepared by soaking in wetting solution for a few seconds. Three pieces of filter paper were wetted in anode buffer I and placed on anode plate of the blotter. Three piece of filter paper were soaked in anode buffer II and placed on top of filter papers previously placed on electrode. Membrane was equilibrated in water for 5 min, and then removed from water and placed on top of filter paper stack. Gel was placed on top of transfer membrane and finally, three pieces of filter paper were socked in cathode buffer and placed on top of gel. Time of transfer was 1 h using 100 mA per each gel (1.75mA/cm^2/h). After transfer was completed, the blot membrane was washed 2 times with water and subsequently the blot membrane was stained with ponceau-S (Roth) to evaluate the transfer quality. The blot membrane was washed 2 times in TBST (10 min/time).

3.2.11.5 Immunostaining

The blot membrane was blocking unoccupied protein binding sites on membrane by placing blot membrane in blocking buffer (1% PVP). It was incubated on the shaker at room temperature for 1 h to prevent a non-specific absorption of the immunological reagent. The blocking solution was poured off and 10 ml of primary antibody in 0.1% blocking buffer was added to the blot membrane. Both anti-rabbit C-mos primary antibody (Stressgen) and Oct-3/4 goat polyconal primary antibody (Santa Cruz biotechnology) were used at a dilution of 1:500. It was incubated overnight on shaker at 4 °C. The primary antibody was poured off and then blot membrane was washed for 10 min in 10 ml washing buffer. Washing step was repeated for 6 additional times. HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Bioscience) for C-mos primary antibody and donkey anti-goat IgG-HRP secondary antibody (Santa Cruz biotechnology) for Oct-4 primary antibody were used a dilution of 1:50,000 in 0.1% blocking buffer. Blot membrane was finally incubated with 10 ml of secondary antibody on shaker at room temperature for 1 h. The blot membrane was washed for 10 minutes in washing buffer and washing was repeated 6 additional times.

3.2.11.6 Detection

The ECL Plus Western Blotting Detection (Amersham Biosciences) was employed using manufacturer's protocol. Briefly, detection solution A and solution B were mixed in a ratio of 40:1 and the final volume of detection reagent required was 0.1 ml/cm^2. The mixed detection reagent was added on to the blot membrane following incubation for 5 min at room temperature, and then the blot membrane was placed on to a fresh piece of saran wrap. Place the wrapped membrane side up in an x-ray film cassette. A sheet of autoradiography film (Kodak® Biomax XAR film, Kodak) was placed on top of membrane. The cassette was closed and expose for 15, 30, 60 and 180 s in the dark room.

3.2.12 Immunofluorescence

3.2.12.1 Pre-treatment of sample

Fixation is the critical step in the preparation of histological specimens (Kurth 2003). For immunocytochemistry, fixation has to yield preservation of the structure and antigenicity. Day 7 blastocyst of Oct-4 dsRNA injected (n=10), water injected (n=10) and uninjected control (n=10) groups were washed three times in PBS, fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS overnight at 4 °C. The fixed specimens were washed twice in glycine-PBS (0.3 mmol/l) supplemented with PVA then, permeabilized by incubation in PBS containing 0.5% (v/v) Triton-X100 for 2.5 h at room temperature. Non-specific immunoreactions were avoided by incubating embryos in 3% (w/v) BSA freshly added in PBS plus PVA for 1 h and then washed three times in PBS solution.

3.2.12.2 Incubation with specific Oct-4 antibody

The antibody was removed from -20 °C, thawed and diluted at the corresponding factor (determined after a serial dilution) in BSA solution shortly before use (Table 3.7). Indeed, BSA treatment reduces non-specific binding reactions. In drops (100 µl) of prepared Oct-3/4 (N-19) goat polyclonal primary antibody (Santa Cruz biolechnology) in a 96 well petri-dish smeared with mineral oil the embryos were kept at 39 °C in incubator without CO_2 for 1 h. The specimens were washed three times 10 min each in PBS. Omitting primary antibody treatment before incubation with secondary antibody was used to determine the specificity of the first antibody.

3.2.12.3 Identification of antigen-antibody complex by FITC secondary antibody

The antigen-antibody recognition was made possible with help of fluoroscein isothiocyanate (FITC) conjugated to donkey anti-goat IgG antibody (Santa Cruz biotechnology). Fluorescein is a small organic molecule, conjugated to proteins via primary amines (lysines), excited by the 488 nm line of an argon laser, and emission is

collected at 530 nm. As for the primary antibody, prior to use the aliquot of secondary antibody was diluted 1:50 in BSA solution (Table 3.7). The embryos are incubated in 100 µl of prepared antibody smeared with mineral oil at 39 °C for 1 h in dark. They were washed three times 10 min. each in PBS again.

Table 3.7: Daily working solutions used for immunoflurescence staining

Chemicals	Volume
Paraformaldehyde 4%	250 µl stock solution
	750 µl CB buffer
Glycine-PBS 0.3 mmol/l	100 µl stock solution
	9.9 ml PBS/PVA
Propidium iodide 0.5 µg/ml	1 µl stock solution
	399 µl PBS/PVA

3.2.12.4 Propidium iodide staining

In order to visualize cell nucleus, propidium iodide was used. Propidium iodide stain is an intercalating dye that appears red at 488 nm and used in flow cytometry to analyze cellular DNA content. The samples were incubated in well containing propidium iodide (0.5 µg/ml) for 25 min in dark at room temperature. After an ultimate three times wash in PBS the embryos were mounted on glass slides (SuperFrost®Plus) in a drop of Vectashield mounting medium (Vector®) protected by cover slip sealed with nail polish, stored at 4 °C till microscopic analysis as fast as possible to reduce any fluorescence damage.

3.2.12.5 Image capture and analysis

Observing thick biological specimens using a conventional light microscope is often an unrewarding experience, the structures above and below the plane of focus being examined usually badly obscure the image (White and Dixon 2003). The last decade confocal imaging has gained favour as a method for fluorescence microscopy, allowing direct visualisation within thick, fluorescently labelled tissue. The big advantage of confocal microscopy is the possibility to collect light exclusively from a single plane.

The laser scanning microscope scans the sample sequentially point by point, line by line and assembles the pixel information to one image with high contrast and resolution. Fluorescence of FITC was visualized by excitation at 488 nm with the argon laser on a confocal laser scanning microscope (CLSM LSM-510; Carl Zeiss, Oberkochen) equipped with Bio-Rad Laser-Sharp MRC-1024 confocal laser scanning software. Fluorescent signals approach to analyse protein localization.

3.2.13 Differential cell staining

Day 8 blastocysts from Oct-4 dsRNA injected (n=25), water injected (n=27) and uninjected control (n=26) groups were collected from culture media and incubated in freshly prepared 1% Triton X-100 and 1 μg/μl propidium iodide in PBS without calcium and magnesium containing 1 mg/ml BSA for 50 s, and immediately washed twice in PBS-BSA medium. Embryos were then transferred into ethanol containing 0.03μg/ml bisbenzemide (Hoechst 33258; Hoechst, Sigma), incubated for 4 min on ice, and washed twice in PBS-BSA medium. Embryos were immediately mounted on glass slides after washed and examined under an epifluorescence microscope (DM-IRB, Leica, Bensheim, Germany). The standard filter was employed, DAPI filter (emission wavelength: 425 nm) to determine the number of ICM and TE cells. The total number of cells was counted.

3.3 Statistic data analysis

The mRNA expression analysis for studied genes in all treatment groups and the bovine preimplantation embryos was analysed based on the relative standard curve method. The relative expression data were analysed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc.) software package. Differences in mean values between two or more experimental groups or developmental stages were tested using ANOVA followed by a multiple pair wise comparisons using *t*-test. Differences of $P \leq 0.05$ were considered to be significant.

4 Results

4.1 Generated dsRNA

To generate dsRNA in *in vitro* transcription, the targeted fragments were cloned to vector. As mentioned in material and methods, 2 strategies were used: 1) sense and antisense strands were transcribed from DNA template in a separate reaction and 2) sense and antisense strands were transcribed from DNA template in same reaction. Figure 4.1 showed the product of ssRNA, asRNA compared to dsRNA, while figure 4.2 showed dsRNA band produced from different methods which did not show any difference.

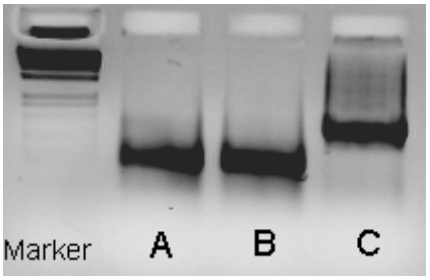

Figure 4.1: Three percent agarose gel stained with ethidium bromide showing the generation of sense (A) and anti-sense (B) RNA from *in vitro* transcription as compared with double-stranded (C), which was generated after annealing step.

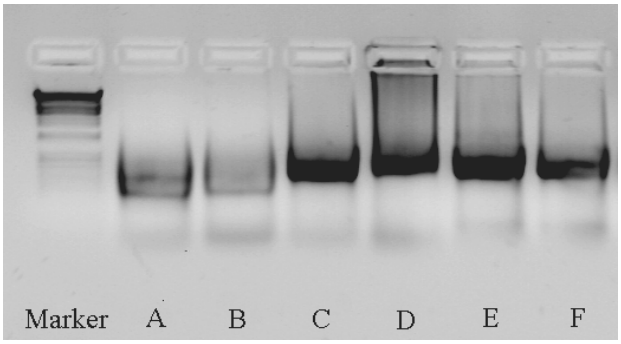

Figure 4.2: Three percent agarose gel stained with ethidium bromide showing the generation of sense (A) and anti-sense (B) RNA from *in vitro* transcription as compared with double-stranded, which was generated in separate (C) and same reaction (D,E) compared with DNA template (F).

Finally, the products of dsRNA (C-mos and Oct-4) were confirmed using electrophoresis in comparison with DNA template. Gel electrophoresis revealed a transcript of approximately 435 bp of C-mos dsRNA and 341 bp of Oct-4 dsRNA as compared with DNA template as shown in figure 4.3.

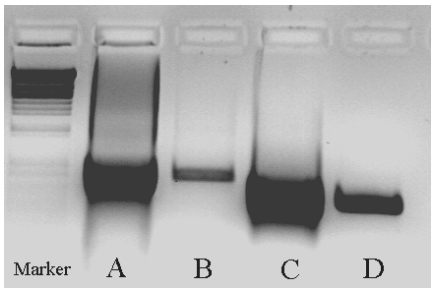

Figure 4.3: Two percent agarose gel stained with ethidium bromide showing the generation of C-mos (435 bp) and Oct-4 (341 bp) dsRNA compared with DNA template used for *in vitro* transcription. (A; C-mos dsRNA, B; C-mos DNA, C; Oct-4 dsRNA; D; Oct-4 dsRNA)

4.2 Effect of microinjection procedure on embryos survival rate

The survival rate of oocytes and embryos due to injury during microinjection has been determined 3-4 h after microinjection. As indicated in figure 4.4, about 10-12% of oocytes and 15-18% of zygotes did not survive the microinjection procedure due to physical injuries. However, within the injected groups with dsRNA (C-mos or Oct-4) or water were not significantly different ($P > 0.05$). Only those embryos which survived the microinjection procedure were considered for future development data collection.

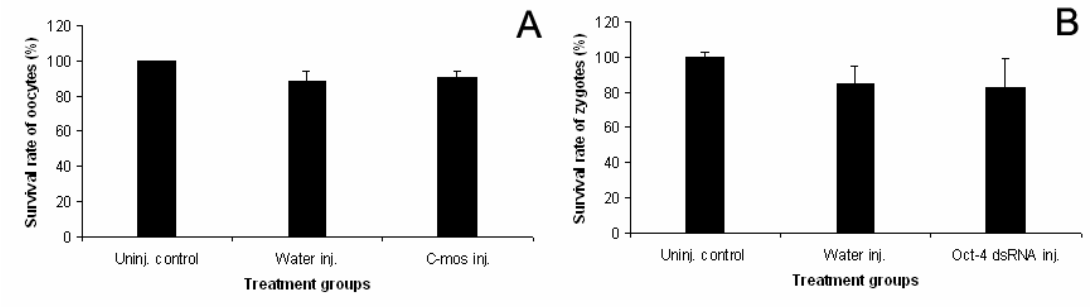

Figure 4.4: The survival rate of bovine oocytes (A) and zygotes (B) after microinjection procedure.

4.3 Effect of C-mos dsRNA on oocytes maturation, mRNA and protein expression

4.3.1 Effect of C-mos dsRNA on *in vitro* oocytes maturation

About 60 % of oocytes injected with C-mos dsRNA showed extrusion of the first polar body, while only 50% of water injected and 44% of uninjected controls extruded their first polar body as shown in table 4.1. Moreover, in this study, it was found that about 2.5% of the oocytes injected with C-mos dsRNA developed parthenogenetically to 2-cell stage, while no parthenogenetic development was observed in water injected and non injected control (Figure 4.5).

Table 4.1: The phenotypes of embryo development following treatment with C-mos dsRNA and water compared to the uninjected control groups

Treatment group	No. of oocytes	First polar body 48 h after microinjection (%)	Parthenogenetic embryos (%)
C-mos dsRNA injected	327	59.62 ± 9.30^{a}	2.42 ± 0.39^{a}
Water injected	303	$49.50 \pm 14.88^{a,b}$	0^{b}
Uninjected control	305	44.05 ± 17.20^{b}	0^{b}

Different letters of superscripts (a,b) indicate significant difference within the same column ($P \leq 0.05$).

4.3.2 Effect of C-mos dsRNA on targeted mRNA expression

In order to get an insight on temporal expression pattern of maternal transcripts (C-mos and Gdf-9), a real-time PCR analysis was conducted throughout the preimplantation developmental stages of *in vitro* produced bovine embryos (Figure 4.6). The C-mos and Gdf-9 were detected at higher level between immature oocyte and 4-cell stage and down-regulated or not detected in the late developmental stages.

To assess the effect of C-mos dsRNA on the target mRNA, the relative expression level of this transcript was investigated between the treatment groups. Moreover, the selective suppression efficiency of C-mos dsRNA was assessed by analysing the expression level of other maternal transcript (Gdf-9) in the three treatment groups. The result of this mRNA quantification shows that the injection of the C-mos dsRNA triggered a remarkable suppression in the amount of C-mos mRNA in oocytes.

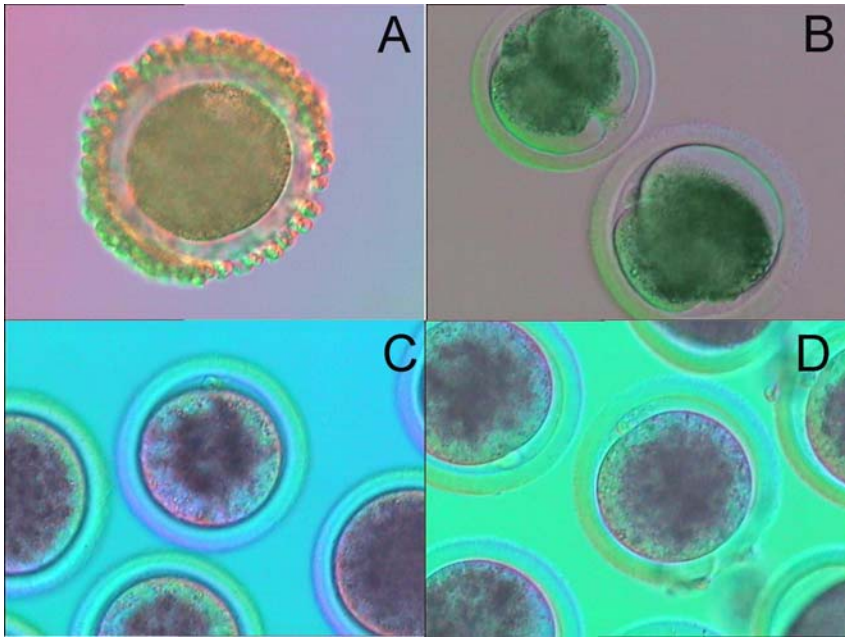

Figure 4.5: Representative picture of oocytes, whose cumulus cells are partially removed before any treatment (A); some parthenogenetically developed oocytes after injection with C-mos dsRNA (B); those oocytes injected with water (C) and uninjected controls (D).

As shown in figure 4.7, the relative expression level of C-mos transcript at the matured oocyte stage was found to be reduced by 70% compared to water injected and uninjected control group ($P < 0.01$). However, no significant differences were observed in the relative abundance of this transcript in water injected and uninjected controls. No differences were observed in the relative abundance of Gdf-9 transcript between the three treatment groups ($P > 0.05$). This shows neither the injection of water nor C-mos dsRNA did affect the expression of Gdf-9 mRNA in the treated oocytes.

4.3.3 Effect of C-mos dsRNA on protein expression

To determine the effect of C-mos dsRNA on C-mos protein expression, western blot analysis was performed using proteins extracted from matured oocytes of the three treatment groups. Moreover, proteins extracted from immature oocytes and bovine muscles were used to assess control of C-mos protein. As shown in figure 4.8, there is a

decrease in the intensity of C-mos protein band (39 kDa) in C-mos dsRNA injected group, while the C-mos protein band in water injected group was similar with uninjected controls and proteins from muscle. Injection of water did not affect the amount of C-mos protein, which is similar with the amount of C-mos protein present in uninjected control groups.

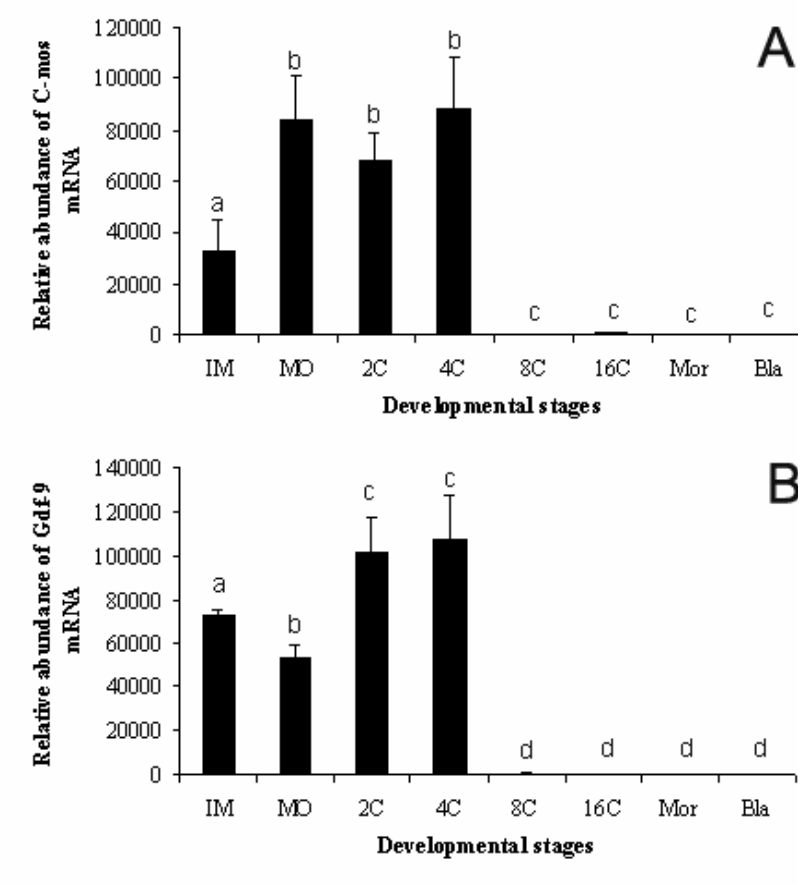

Figure 4.6: Relative abundance of C-mos (A) and Gdf-9 (B) mRNA in *in vitro* bovine preimplantation stage embryos, immature oocyte (IM), mature oocytes (MO), 2-cell (2C), 4-cell (4C), 8-cell (8C), 16-cell (16C), morula (Mor) and blastocyst (Bla). The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (blastocyst stage) which is set as 100. Bars show the treatment mean ± SD. Values with different superscripts (a,b,c,d) are significantly different ($P \leq 0.05$).

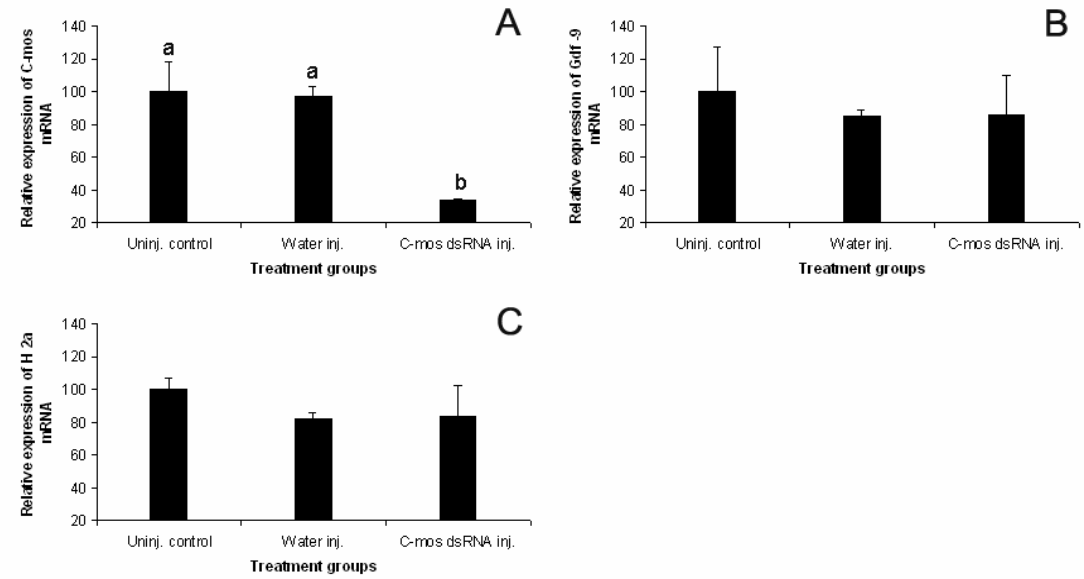

Figure 4.7: Relative abundance of C-mos (A), Gdf-9 (B) and H2a (C) transcripts at mature oocyte stage in the three treatment groups. The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (uninjected control) which is set as 100. Bars show the mean ± SD. Values with different superscripts (a,b) are significantly different ($P \leq 0.05$).

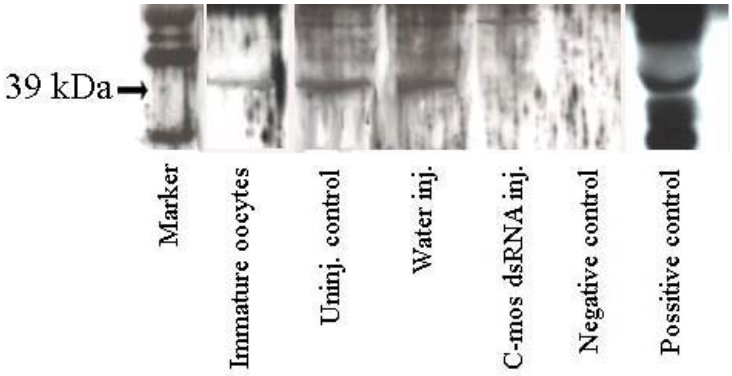

Figure 4.8: Western blot analysis for the presence of C-mos protein (39 kDa) in bovine oocytes following C-mos dsRNA injected, water injected compared to uninjected control and proteins extracted from immature oocytes and bovine muscle as positive control.

4.4 Effect of Oct-4 dsRNA on embryos development, mRNA and protein expression

4.4.1 Effects of Oct-4 dsRNA on *in vitro* development of bovine embryos

As shown in table 4.2, the first cleavage rate after microinjection was 70, 81 and 80% for embryos injected with Oct-4 dsRNA, water injected and uninjected control group, respectively. However, these differences were not statistically significant ($P > 0.05$). Similary, the day 5 morula rate was not significantly different between the three embryo groups i.e. 37.3 ± 3.2% in Oct-4 dsRNA injected, 40.2 ± 9.5% in water injected and 42 ± 4.5% in uninjected control group ($P > 0.05$). There is a considerable variation in the number of blastocysts appeared from each treatment group at each day of development between days 6-8 (Table 4.3). Even though the overall blastocyst rate was lower in Oct-4 dsRNA injected groups (35.8 ± 1.5%) as compared to the water injected (39.7 ± 2.6%) and uninjected controls (41.6 ± 4.2%), these differences were not significant ($P > 0.05$). However, the day 7 blastocyst rate was significantly lower in Oct-4 dsRNA injected group (18.5 ± 2.5%) compared to water injected (20.2 ± 6.3%) and uninjected control (26.4 ± 3.9%). But, the day 8 blastocyst rate was significantly lower in uninjected control (2.3 ± 0.9%) compared to water (8.7 ± 1.4%) and Oct-4 dsRNA (9.6 ± 4.5%) injected group (Table 4.3).

Table 4.2: The phenotypes of embryo development following treatment with Oct-4 dsRNA and water injected compared to the uninjected control groups

Treatment group	No. of embryos	First cleavage rate (%)	Morula rate (%)	Total Blastocyst (%)	Bla/ Mor
Oct-4 dsRNA injected	365	69.67 ± 20.73	37.27 ± 3.25	35.85 ± 1.53	0.96
Water injected	375	80.04 ± 7.07	40.17 ± 9.48	39.69 ± 2.57	0.99
Uninjected control	541	80.50 ± 7.65	41.95 ± 4.51	41.57 ± 4.15	0.99

Table 4.3: The blastocyst rate of embryos in different day following treatment with Oct-4 dsRNA and water injected compared to the uninjected control groups

Treatment group	Blastocyst rate (%)			
	Day 6	Day 7	Day 8	Total
Oct-4 dsRNA injected	7.74 ± 5.46	18.48 ± 2.46 [a]	9.64 ± 4.50 [a]	35.85 ± 1.53
Water injected	10.81 ± 2.57	20.20 ± 6.32 [a,b]	8.68 ± 1.40 [a]	39.69 ± 2.57
Uninjected control	12.80 ± 9.09	26.45 ± 3.95 [b]	2.32 ± 0.89 [b]	41.57 ± 4.15

The different letter superscripts (a,b) indicate significant difference within column ($P \leq 0.05$).

4.4.2 Effects of Oct-4 dsRNA down-regulation in the number of ICM and TE cells of blastocysts

Differential cell staining (Figure 4.9) of a representative number of blastocysts from the three treatment groups showed that the number of ICM cells was significantly lower in Oct-4 dsRNA injected embryos (27.4 ± 7.3) compared to the other two groups (Table 4.4). However, no differences were observed in the number of TE cells between the three groups. Consequently, the ration of ICM:TE cells was lower ($P \leq 0.05$) in Oct-4 dsRNA injected group than in the other two groups. The total cell number of blastocysts was consequently lower in the Oct-4 dsRNA injected group (122.5 ± 16.5) compared to the water injected group (134.4 ± 6.8) and uninjected controls (140.2 ± 18.4).

Table 4.4: The number of inner cell mass (ICM), trophectoderm cell (TE) and total cells of day 8 blastocysts derived from the three treatment groups

Treatment group	No. of embryos	ICM	TE	Total	ICM:TE
Oct-4 dsRNA injected	25	27.40 ± 7.30[a]	95.10 ± 13.20	122.50 ± 16.50 [a]	0.29 ± 0.12 [a]
Water injected	27	40.90 ± 8.20 [b]	94.40 ± 8.90	134.30 ± 6.80 [b]	0.42 ± 0.18 [b]
Uninjected control	26	410.00 ± 4.80 [b]	99.20 ± 18.60	140.20 ± 18.40 [b]	0.43 ± 0.17 [b]

Columns with different letters of superscript are significantly different ($P \leq 0.05$).

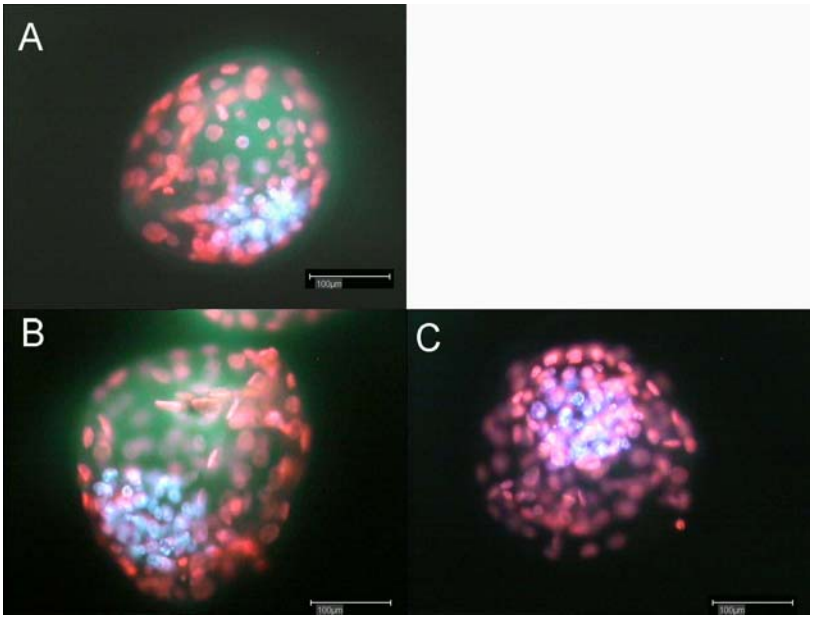

Figure 4.9: A representative picture showing differential cell staining of inner cell mass (red) and trophoectoderm cell (blue) (A, B, and C represent Oct-4 dsRNA injected, water injected and uninjected control groups respectively). Scale bar represents 100 μm.

4.4.3 Effect of Oct-4 dsRNA on targeted mRNA expression

In order to get an insight on temporal expression pattern of embryonic transcripts (Oct-4 and E-cadherin) a real-time PCR analysis was conducted throughout the preimplantation developmental stages of *in vitro* produced bovine embryos (Figure 4.10). The E-cadherin mRNA transcript was detected at higher level at immature and matured oocytes, morula and blastocyts stages of development. However, transcript abundance was lower between 2- and 16-cells developmental stages. The Oct-4 transcript was found to be highly abundant at early developmental stages (between immature oocytes and 4-cell stages) and further down-regulated between 8-cell and morula stages. Relatively higher transcript abundance was detected at the blastocyst stage. The Fgf-4 transcript was highly abundant only at morula and blastocyst stages, while could not be detected from immature oocytes to 16-cell stage.

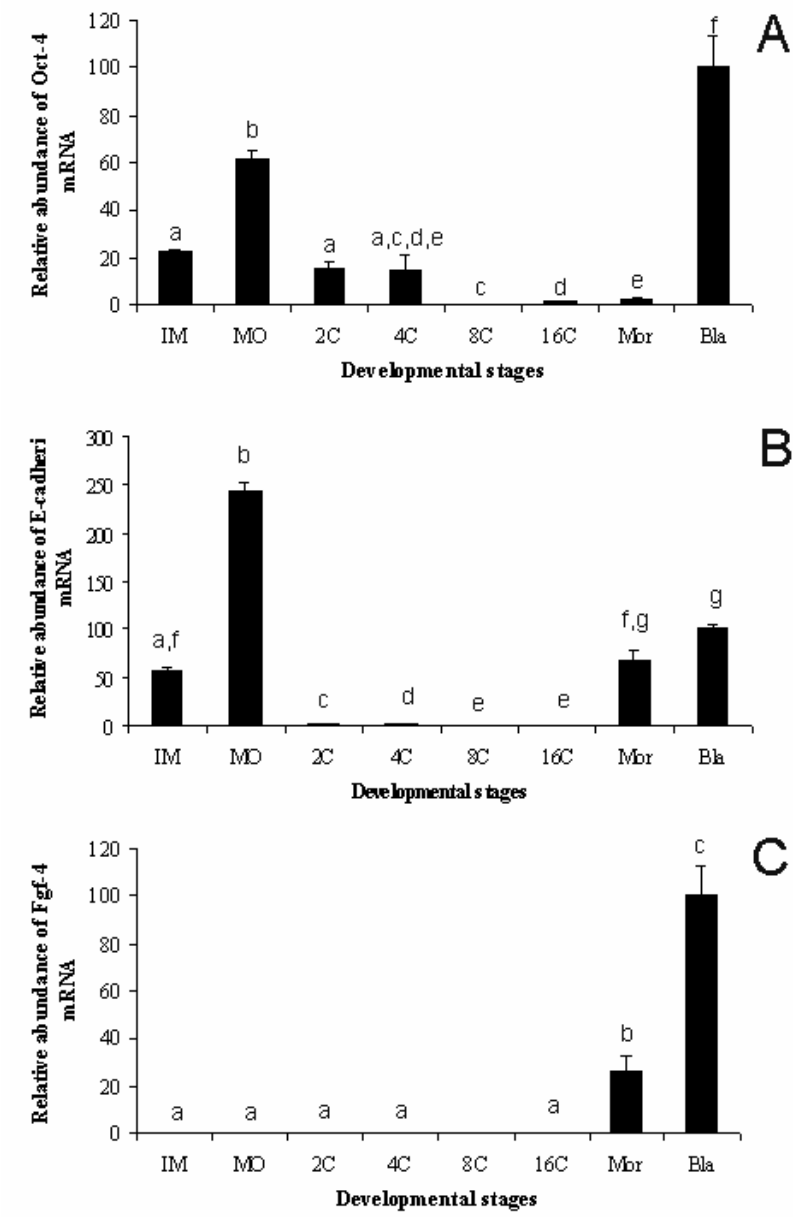

Figure 4.10: Relative abundance of Oct-4 (A), E-cadherin (B) and Fgf-4 (C) mRNA in *in vitro* bovine preimplantation stage embryos, immature oocyte (IM), mature oocytes (MO), 2-cell (2C), 4-cell (4C), 8-cell (8C), 16-cell (16C), morula (Mor) and blastocyst (Bla). The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (blastocyst stage) which is set as 100. Bars show the treatment mean ± SD. Values with different superscripts (a,b,c,d,e,f,g) are significantly different ($P \leq 0.05$).

To assess the effect of Oct-4 specific dsRNA on Oct-4 mRNA abundance, relative expression level of this transcript was investigated in blastocyst stage using real-time PCR. The specificity of Oct-4 dsRNA was determined by analyzing the expression of E-cardherin and Fgf-4 transcript. The results of mRNA transcription in all treatment groups (Figure 4.11) showed that, the relative abundance of Oct-4 mRNA was down-regulated by 72% in Oct-4 dsRNA injected groups compared to the water injected and noninjected control group ($P < 0.01$). In order to investigate the specificity of the Oct-4 dsRNA, E-cadherin was quantified in all treatment groups. No differences were found in the relative abundance of the E-cadherin transcripts in all treatment groups. Moreover, the relative abundance of Fgf-4 transcript responded to suppression of Oct-4 transcript through Oct-4 dsRNA injection at blastocyst stage. The relative abundance of Fgf-4 in Oct-4 dsRNA injected group was found to be significantly down-regulated (by 70%) compared to the other groups ($P < 0.01$).

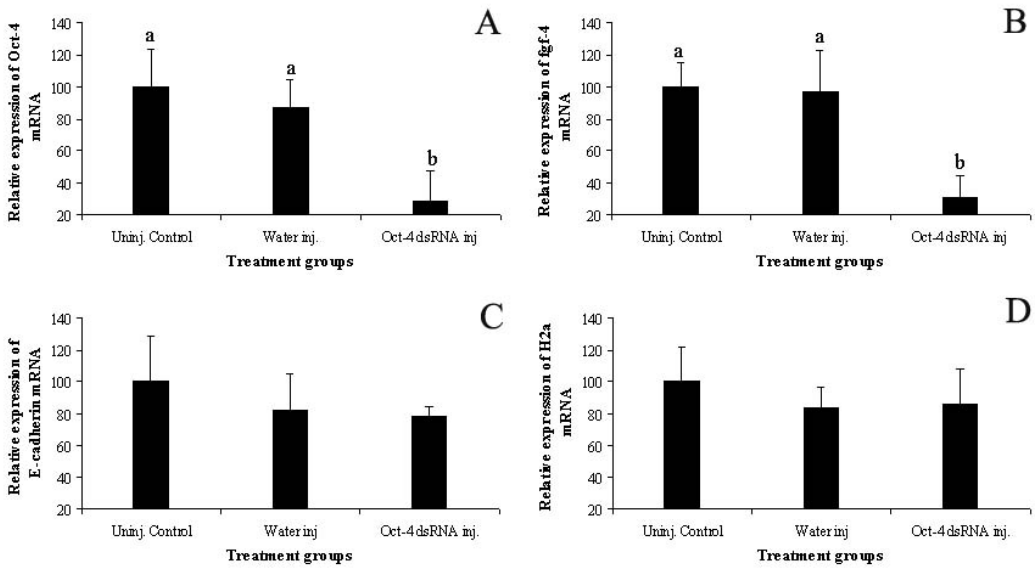

Figure 4.11: Relative abundance of Oct-4 (A), Fgf-4 (B), E-cadherin (C) and H2a (D) mRNA at blastocyst stage in the three treatment groups. The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (uninjected control) which is set as 100. Bars show the mean ± SD. Values with different superscripts (a,b) are significantly different ($P \leq 0.05$).

4.4.4 Effect of dsRNA on protein expression

4.4.4.1 Effect of dsRNA on protein expression using immunofluorescence

Immunofluorescence staining was performed using embryos at day 7 bastocyst stage to determine the effect of Oct-4 dsRNA injection on Oct-4 protein expression and to localize this protein in *in vitro* bovine blastocyst stage. In early blastocyte as shown in figure 4.12, intensity signal was associated with nuclei of inner cell mass. But a weak diffuse signal was also visible in trohectoderm cells. Moreover, the reduction of the fluorescent signal was found in Oct-4 dsRNA injected group when compared to the other groups.

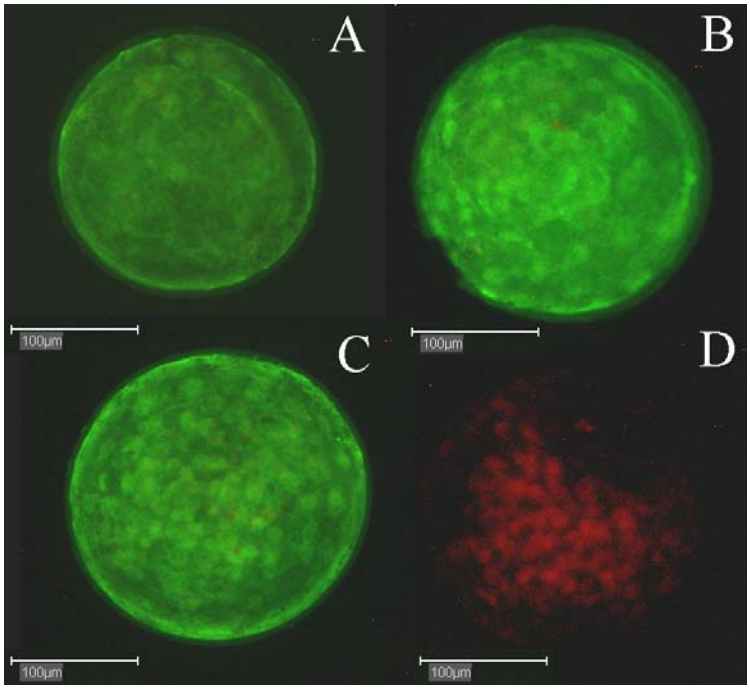

Figure 4.12: Representative picture of embryos stained with immunofluorescence in Oct-4 dsRNA injected (A) compared with water injected (B) and uninjected control (C) groups. While embryo in D served as negative control with out anti Oct-4 antibody. Reduction of fluorescent signal by Oct-4 dsRNA was observed in Oct-4 dsRNA injected group. Scale bar represents 100 µm.

4.4.4.2 Effect of dsRNA on protein expression using western blot

To determine the effect of Oct-4 dsRNA on the amount of Oct-4 protein expression, western blot analysis was performed using proteins extracted from the embryos at blastocyst stage of the three treatment groups. Moreover, *in vitro* bovine matured oocytes before any treatment were used to assess maternal produced Oct-4 protein. While, proteins extracted from bovine brain were as positive control. As shown in figure 4.13, there is a decrease in the intensity of Oct-4 protein band (43 kDa) in Oct-4 dsRNA injected group, while the Oct-4 protein band in water injected group was similar with uninjected controls and matured oocytes. Injection of water did not affect the amount of Oct-4 protein, which is similar with the amount of Oct-4 protein present in uninjected control groups and mature oocytes. Moreover, the Oct-4 protein expression was lower in Oct-4 dsRNA injected embryos compared to maternal protein in mature oocytes before any treatment.

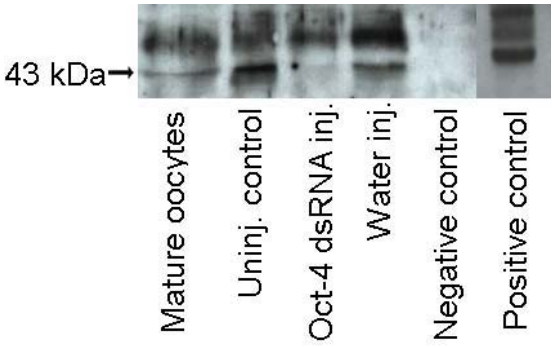

Figure 4.13: Western blot analysis for the presence of Oct-4 protein (43 kDa) in bovine embryos following Oct-4 dsRNA injected, water injected compared to uninjected control, mature oocytes and proteins extracted from bovine brain.

5 Discussion

Bovine preimplantation embryogenesis is supported by transcripts activated from both maternal and embryonic genome. Despite enormous advances in the identification and temporal expression profiling of bovine preimplantation genes, the specific function of the majority of transcripts is not yet known in bovine embryogenesis. Till recently, the function of a specific gene in bovine species has been predicted using knockout experiments conducted in mouse (Larue et al. 1994, Riethmacher et al. 1995), which is extremely laborious and needs long time to see the effects. To overcome this, the RNAi approach through introduction of sequence specific dsRNA into the cells has been reported for various vertebrates and non vertebrates as an effective tool to study gene function (more detail see table 2.1). Consequently, this study has demonstrated that the injection of sequence specific dsRNA into the cytoplasm of bovine oocytes and zygotes induced suppression of maternal and embryonic transcript abundance, respectively and results in subsequent decrease in protein synthesis and distinct phenotype.

5.1 Expression profile of C-mos transcript in bovine preimplantation embryos

The expression pattern of C-mos in bovine preimplantation embryos has not been reported so far. This study demonstrated the C-mos transcript to be abundant at highest level in oocyte and down-regulated from 4-cell to blastocyst stage of the embryos. This shows the C-mos to be a maternal transcript failed to be abundant during the major embryonic activation, which is beyond the 8- to 16-cell stage in bovine. Similarity, the study in mouse embryos by Alizadeh et al. (2005) which showed the expression of C-mos was down-regulated after IVF. In year 1988, Sagata et al., showed that C-mos transcript was required for activation of MPF in G2 arrested xenopus oocytes. And the study done by Roy et al. (1990) on xenopus species also showed that injecting C-mos mRNA into the oocytes induced oocytes maturation, while inhibiting C-mos transcript prevented oocytes maturation. The C-mos gene is reported to play an important role in control of the oocytes arrest at MII in mouse (O'Keefe et al. 1989, Araki et al. 1996, Choi et al. 1996, Sovoboda et al. 2000, Wianny and Zernicka-Goetz 2000). $Mos^{-/-}$ oocytes undergo GVBD and progress through the first meiosis but fail to arrest in MII.

Instead, they undergo spontaneous parthenogenetic activation (Colledge et al. 1994, Hashimoto et al. 1994).

5.2 Expression profile of Gdf-9 transcript in bovine preimplantation embryos

In this study, the relative abundance of Gdf-9 transcript was detected throughout preimplantation developmental stage. However, highest level of expression was observed from immature oocyte to 4-cell stage. This result was similar with previouse studies in bovine preimplantation embryo using RT-PCR technique (Sendai et al. 2001, Pennetier et al. 2004). However, most studies of Gdf-9 transcript expression were done in different tissues such as ovary, follicular cells, granulosa cells, testis or liver (Bodensteiner et al. 1999, Pennetier et al. 2004, Xu et al. 2004, Johnson et al. 2005). Gdf-9 is a member of the transforming growth factor β (TGF-β) super-family. TGF-β super-family members are pivotal in controlling cellular growth and differentiation during fatal and adult life (Heldin et al. 1997, Ten Dijke et al. 2000). In 1999, using recombinant form of Gdf-9 demonstrated that this protein promoted cumulus expansion in *in vitro* produced mouse oocytes (Evin et al. 1999). They further proposed that Gdf-9 to be a cumulus expansion-enabling factor. Supporting this, Van Derhyden et al. (2003) found that unlike wild-type oocytes, oocytes derived from Gdf-9 null mice are unable to promote cumulous expansion *in vitro*.

5.3 Expression profile of E-cadherin transcript in bovine preimplantation embryos

E-cadherin transcript as both maternal and embryonic origin was also detected throughout preimplantation developmental stage of IVP bovine embryos. E-cadherin transcript was detected at immature and matured oocytes stage and after the activation of embryonic genome at morula and blastocyst stages. So far, the expression of E-cadherin transcript had not been studied in bovine preimplantation embryos. It has been established that maternal E-cadherin is present in all stage of mouse embryo (Sefton et al. 1992) and also, found on the cell surface of unfertilized and fertilized egg but it is not synthesized in these cells (Van Eijk et al. 1987). Kawai et al. (2002) have shown the location of E-cadherin protein in embryos using a laser scanning confocal microscropy,

where E-cadherin protein was distributed uniformly through the cell surface in normal 2-cell embryos. Thus, it is likely that the E-cadherin protein functions in the compaction of the morula by cell to cell adhesion.

5.4 The expression profile of Fgf-4 transcript in bovine preimplantation embryos

This study is the first to show the expression profile of Fgf-4 transcripts in IVP bovine. This transcript was detected at higher level at morula and blastocyst stage, while it could not be detected before morula stage (immature oocyte, mature oocyte, 2-, 4-, 8- and 16-cell stage). This pattern is different when compared with the expression profile mouse preimplantation embryos. In mouse, Fgf-4 was expressed throughout preimplantation developmental stages (1-cell to blastocyst stage) (Rappolee et al. 1994). The role of Fgf-4 has not been studied so far in early stages of mammalian development so, especially in bovine. In mouse, a study suggested that Fgf-4 is involved in trophoblast proliferation and in the maintenance of the ICM (Tanaka et al. 1998). Moreover, inhibition of Fgf signalling by abrogating Fgf-receptor function through a dominant negative mutation blocks development at the fifth cleavage division (Chai et al. 1998).

5.5 The expression profile of Oct-4 transcript in bovine preimplantation embryos

In this study, the relative abundance of Oct-4 transcript was detected at higher level in all developmental stages except minimum transcript abundance between 8-cell and morula stages. This result was similar with previous studies in bovine embryos using semi-quantitative PCR (Kurosaka et al. 2004) and real-time PCR (Vigneault et al. 2004). In bovine, Oct-4 transcript was detected throughout preimplantation stages (Van Eijk et al. 1987, Daniels et al. 2000, Kurosaka et al. 2004, Vigneault et al. 2004) but in oocyte and early developmental stage expression was relatively lower level compared to post-compaction stage (Kurosaka et at. 2004, Vigneault et al. 2004), while it was not detected at 2- and 4-cell stages and this transcript was up-regulated from 8-cell to blastocyst stage in mouse (Kurosaka et al. 2004). The Oct-4 transcript was detected at highest level at blastocyst stage in both bovine and mouse, because the blastocsyts were

composed of trophectoderm cells and ICM cells whereby Oct-4 plays a central role during cell differentiation at this stage (Nichols et al. 1998, Kirchof et al. 2000). Moreover, it is likely that Oct-4 is responsible for various functions at different embryonic stages, and Oct-4 must occupy a pre-eminent position within the regulatory hierarchy of genes controlling preimplantation development (Ovitt and Schöler 1998). Also, because placental abnormalities are frequently observed in cloned animals, analysis of Oct-4 expression in respect to its interacting protein partners, particularly those involved in extraembryonic lineage differentiation may provide important information concerning the abnormalities in somatic cell clones (Kurosaka et al. 2004).

5.6 Microinjection as a technique to introduce dsRNA into bovine oocytes and embryos

The efficient introduction of dsRNA is an important step in getting successful suppression of transcripts through RNAi. In the present study microinjection was the technique used to introduce dsRNA into the immature oocyte and embryos. This system has the advantage of being relatively easy and allows for better control of the amount of dsRNA molecules introduced into the embryo (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Kim et al. 2002, Anger et al. 2004, Sonn et al. 2004, Gui and Joyce 2005, Paradis et al. 2005, Nganvongpanit et al. 2006a, 2006b), compared with electroporation (Grabarek et al. 2002, Mellitzer et al. 2002) and transfection technique (Billy et al. 2001, Siddall et al. 2002, Lazar et al. 2004, Cao et al. 2005, Li et al. 2005). However, after microinjection, embryos are subjected to a physical injury or stress and some die following injection. Consequently, in the present study, 10-12% of oocytes and 15-18% of zygotes did not survive the microinjection procedure but this remains the same between the dsRNA and water injected groups. Moreover, the large volume of dsRNA injected and longer microinjection time resulted in lower survival rate. In the present microinjection of ~7 pl of dsRNA or water was performed into each oocyte or embryo. This volume is equivalent to 0.7% of total volume of bovine oocyte (based on a bovine oocyte volume of 900 pl). While similar study in bovine showed injection ~15 pl which is equal to 1.7% of total oocyte volume (Paradis et al. 2005). However, studies in mouse showed that injection of higher volume was still effective. Oocytes were injected with 5 pl dsRNA which is equal to 2% of total volume of mouse oocyte, this is base on

a mouse oocyte volume of 250 pl (Svoboda et al. 2000, Haraguchi et al. 2004), or injection of 10 pl is equal to 2.5% of total oocyte volume (Wianny and Zernicka-Goetz 2000, Kim et al. 2002, Xu et al. 2003, Sonn et al. 2004, Gui and Joyce 2005). In this study, embryos were injected about 50-60 cells at each time, to avoid long time injection. Moreover, since the effect of microinjection on the survival of the embryos after injection was similar over the whole injected groups either with dsRNA or water, influences by physical injuries during further development were ruled out.

5.7 The use of dsRNA to study genes function

It had been demonstrated that the mechanisms of RNAi are limited at post-transcriptional level by degrading the sequence-specific mRNA or blocking the activity of rRNA, which leads to loss of function in mouse (Sovoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Grabarek et al. 2002, Siddall et al. 2002, Sovoboda 2004). In *C. elegans*, dsRNA molecules, 300-600 bp in length, are degraded by dicer into siRNA, 21-23 nt in length (Ketting et al. 2001). These siRNAs interact with homologous regions in target mRNA molecules and trigger their degradation. Moreover, these siRNA molecules appear to be self-replicating and eliminate target mRNA over several generations in *C. elegans* (Hunter 1999). But this mechanism was not found in mammals because in mammalian cell inefficiency of an RdRp component (Stein et al. 2003a, Svoboda 2004). However the RNAi still widely use to study the function of genes in mammalian cells. It had been reported that molecules such as dsRNA (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Anger et al. 2004, Gui and Joyce 2005, Parradis et al. 2005, Nganvongpanit et al. 2006a, 2006b), siRNA (Anantharam et al. 2003, Djikeng et al. 2004, Kim et al. 2004, Li et al. 2005), shRNA (Yu et al. 2002, Yu et al. 2003) or expression vector (Haraguchi et al. 2004) are used to induce RNAi in mammalian cells. Most RNAi studies used dsRNA molecules to induce RNAi mechanism in mammalian embryos (more detail see table 2.2). Even in mammalian cells, the presence of dsRNA molecules triggers an interferon-mechanism response, resulting in an overall reduction in cellular mRNA levels and block in message translation (Stark et al. 1998, Elbashir et al. 2001a, Svoboda 2004). Up to now, 300-1,500 bp long dsRNA (more detail see table 5.1) could induce efficient RNAi but

does not trigger apoptosis in mammalian oocytes (Svoboda et al. 2000, Wianny and Zernicka-Goetz 2000, Xu et al. 2003, Anger et al. 2004, Gui and Joyce 2005, Lazar et al. 2004, Parradis et al. 2005, Nganvongpanit et al. 2006b) and embryos (Wianny and Zernicka-Goetz 2000, Cabot and Prather 2003, Sonn et al. 2004, Plusa et al. 2004, Nganvongpanit et al. 2006a, 2006b). The dsRNA were randomly digested into siRNA by dicer enzyme. In this process, one molecule of dsRNA was supplies a lot of siRNA molecules to induced RNAi in cells (Figure 5.1). It should give better result from suppression of cognate mRNA, when compared with siRNA molecule. However, the study need to be designed to compare the gene silencing effects between dsRNA and siRNA, and to find out the best molecules used for inducing RNAi mechanism in mammalian cells without induction of apoptosis response.

In choosing a method for targeted knockdown of gene expression, an important consideration has to be the potential for off-target (non-sequence specific effects) and side effects. Jackson et al. (2003) used gene expression profiling to characterize the sepecificity of gene silencing by siRNAs in cultured human cell. Transcript profiles revealed siRNA-specific rather than target-specific signatures, including direct silencing of non-targeted genes containing as few as eleven contiguous nucleotides of identical to the siRNA. These results demonstrate that siRNAs may cross-react with targets of limited sequence similarity. The other potential issue of importance in sequence-specific knockdown of mRNA is unanticipated side effect. At lest 2 reports suggest that siRNA and shRNA can activate arms of the interferon response pathways, which could lead to non-specific inhibition of protein synthesis and general RNA degradation (Bridge et al. 2003, Sledz et al. 2003). Sledz et al. (2003) found that transfection of siRNAs results in interferon-mediated activation of the Jak-Stat pathway and global up-regulation of interferon-stimulated genes. This effect is mediated by the dsRNA-dependent protein kinase, which is activated by siRNAs and required for up-regulation of interferon-β in response to siRNAs. In the same study, it has been show that the RNAi mechanism itself is independent of the interferon system by using cell lines deficient in specific component. Thus, siRNAs have broad and complicated effects beyond the selective silencing of target genes when introduced into cells. However, in the present study we tested the specificity of dsRNA by analysing the expression of other independent genes. For this, Gdf-9 transcript which is known as maternal transcript was used to study the

specificity of C-mos dsRNA, while E-cadherin transcript was used study the specificity of Oct-4 dsRNA. Both C-mos and Oct-4 dsRNA did not affect the expression of other independent genes. However to get insight into the effect of suppression of genes express mRNA level, experiment need to be conducted to investigate the global gene expression using micrroarry technique.

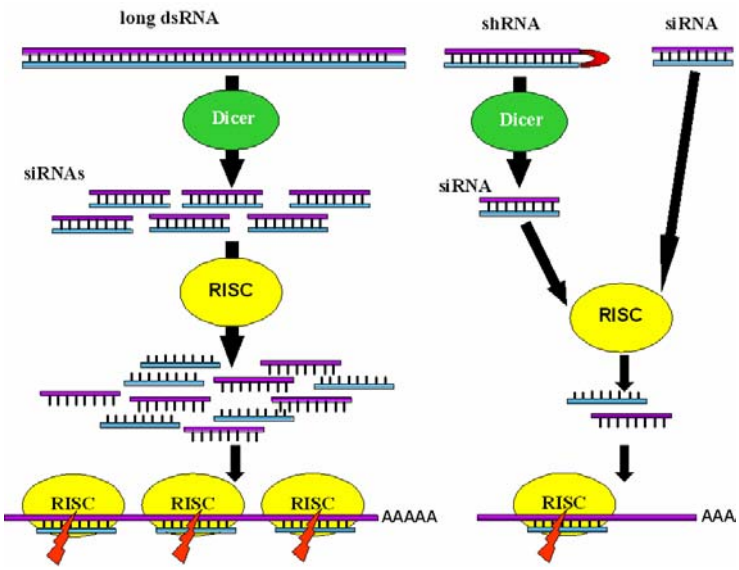

Figure 5.1: The process of dsRNA and siRNA molecules to induce RNAi in cells. Previously attached by RNA induced silecing complex (RISC), both dsRNA and shRNA was digested by dicer into endogenous siRNAs, while exogenous siRNA molecules can directly be attached to RISC. One long dsRNA can be digested into a lot of siRNAs which increase the opportunity of siRNAs to attach with cognate RNA and processed mRNA degradation.

5.7.1 Effects of dsRNA on oocyte maturation

This study has shown that the injection of dsRNA of oocyte or zygote specific transcripts induced sequence specific mRNA degradation and subsequent protein synthesis during bovine oocytes maturation. In the studies conducted in mouse, C-mos is known to play a role as an essential component of cytostatic factor, which is responsible for arresting the maturing oocytes at metaphase in the second meiotic division (Wianny and Zernicka-Goetz 2000).

Table 5.1: The length and concentration of dsRNA used to study the function of genes in mammalian oocytes and embryos

Species / tissue	Gene	dsRNA		Effects	References
		Length (bp)	Concentration (µg/µl)		
Mouse					
Oocyte	Bmp-15	528	-	78 % mRNA reduction	Gui and Joyce 2005
	Cyclin B1	650	33 ng/µl (transfec.)	>50% mRNA and protein reduction	Lazar et al. 2004
	Doc1r	~400	0.5	aberrant MII spindle	Terret et al. 2003
	Gdf-9	589	-	89.2 % mRNA reduction	Gui and Joyce 2005
	Gfp	750	2	loss of fluorescence	Wiannz and Zemicka-Goety 2000
	Itpr1	686	1-5 x 10^6 molecules	90% mRNA reduction	Xu et al. 2003
	Mos	650	0.2	95% reduction of activity	Svoboda et al. 2000, 2004
		~500	100 µg/ml (microinj.) 50 µg/ml (electro.)	show null phenotype	Grabarek et al. 2002
	Plat	650	0.2	90% mRNA reduction	Svoboda et al. 2000
	Dicer	~1,500	-	90% mRNA reduction	Svoboda et al. 2004
Embryo	E-cadherin	580	2	70% null phenotype	Wiannz and Zemicka-Goety, 2000
		330	2-4	~55-60% null phenotype	Sonn et al. 2004
	Nek2	501	2-4	mRNA and protein reduction, loss of function	Sonn et al. 2004
	Par3	~600	1-8	function reduction	Plusa et al. 2005
	aPKR	~600	1-8	function reduction	Plusa et al. 2005
Porcine					
Embryo	Karyopherins -α2	721	1.0	loss of function	Cabot et al. 2003
	Karyopherins -α3	603	1.0	loss of function	Cabot et al. 2003
	PLK1	543	1	50% mRNA reduction	Anger et al. 2004

Table 5.1: The length and concentration of dsRNA used to study the function of genes in mammalian oocytes and embryos (cont.)

Species / tissue	Gene	dsRNA		Effects	References
		Length (bp)	Concentration (µg/µl)		
Bovine					
Oocyte	Cyclin B1	297	0.2	90% mRNA reduction	Paradis et al. 2005
	C-mos	435	10	70% mRNA reduction	Nganvongpanit et al. 2006b
Embryo	E-cadherin	496	25	80% mRNA reduction	Nganvongpanit et al. 2006a
	Oct-4	341	25	60% mRNA reduction	Nganvongpanit et al. 2006a
				72% mRNA reduction	
			10		Nganvongpanit et al. 2006b

electro. = electroporation, microinj. = microinjection, trans. = transfection

In the present study the injection of C-mos dsRNA at immature oocyte stage resulted in 72% reduction in amount of C-mos mRNA after maturation compared to the water injected and uninjected controls. This result is comparable with the results reported in mouse oocytes, where a suppression of 80-95% of C-mos mRNA was achieved by microinjection of C-mos dsRNA (Svoboda et al. 2000, Grabarek et al. 2002, Stein et al. 2003b, Svoboda et al. 2004). Similar studies in mouse which targeted oocyte specific maternal transcripts namely: Gdf-9 and Bmp-15 have shown the suppression of 89% and 78% in mRNA transcript abundance, respectively (Gui and Joyce 2005). Moreover, up to a level of 90% suppression in transcript abundance had been attained for Plat (Sovoboda et al. 2000), ITPRT (Xu et al. 2003) and BNC (Ma et al. 2002) genes in mouse oocytes. A complete degradation of Cyclin B1 mRNA had been achieved in the work of Lazar et al. (2004) in oocytes treated with Cyclin B1 dsRNA. A recent report from Paradis et al. (2005) has shown suppression of transcripts by 90% in bovine oocytes. The efficiency of targeted suppression of transcripts in mammalian oocytes seems to determine the extent of change in developmental phenotype. This variation in the efficiency of suppression of mRNA and protein synthesis and the expected developmental phenotype using dsRNA may be associated with the concentration or sequence of dsRNA introduced. This has been evidenced by Wianny and Zernicka-

Goetz (2000), who found that 50% of the oocytes injected with 2 mg/ml C-mos dsRNA showed spontaneous activation, while only 36% of the oocytes injected with 0.1 mg/ml C-mos dsRNA developed parthenogenetically to cleavage stage embryos. Holen et al. (2002) had demonstrated siRNAs targeting different positions on mRNA thus differed in activity. Moreover, despite the minimal sequence and position differences between these siRNAs, they displayed a wide range of activities (Holen et al. 2002). Studies in C-mos$^{-/-}$ knockout mouse have shown a reduced fertility because of the failure of matured eggs to arrest during meiosis (Colledge et al. 1994). The C-mos$^{-/-}$ oocytes undergo germinal vesicle breakdown and extrusion of both polar bodies followed in some cases by progression into cleavage. In the present study, despite significant reduction in the transcript abundance and protein synthesis, the proportion of oocytes undergo spontaneous activation after treatment with C-mos dsRNA which was much lower compared to the studies in mouse (Wianny and Zernicka-Goetz 2000, Grabarek et al. 2002, Stein et al. 2003b, Svoboda et al. 2004). In the present study 60% C-mos dsRNA injected oocytes showed extrusion of first polar body of which 2.5% showed spontaneous activation and development to 2- to 4-cell stage. However, while only 44-50% of the oocytes showed first polar body extrusion in water injected and uninjected controls, no spontaneous activation and parthenogenetic development has been observed in these treatment groups. The reason for lower percentage of spontaneous activation in C-mos dsRNA injected groups compared to comparable studies in the mouse can not be explained at this level of the study.

However, this study has demonstrated that the injection of C-mos dsRNA leads to the specific degradation of the C-mos mRNA without affecting the expression of other genes (Gdf-9 and H2a). These results are consistent with the results obtained in mouse, where the injection of dsRNA directed towards C-mos mRNA resulted in the suppression of the targeted mRNA without affecting the untargeted transcript (Svoboda et al. 2000). Including the results reported by Gui and Joyce (2005), injection of Gdf-9 or Bmp-15 dsRNA leads to specific degradation of cognate mRNA without affecting the expression of other genes. Previous reports in bovine oocytes also showed that the suppression of Cyclin B1 had no affect on the expression of housekeeping gene (β-actin) or Cyclin B2, as member of Cyclin B family (Paradis et al. 2005). Moreover, this study has demonstrated that degradation of C-mos mRNA has resulted in a consequent

reduction of C-mos protein synthesis as it is evidenced by western blot analysis, but the water injection had no effect on C-mos protein synthesis. These results are also consistent with the results obtained in mouse, where the injection of Gdf-9 dsRNA directed towards Gdf-9 protein resulted in the reduction of the targeted protein but the reduction did not occur in buffer injected group (Gui and Joyce 2005).

5.7.2 Effects of dsRNA on embryonic development

Bovine embryogenesis in the early preimplantation stages is supported by mRNA and protein transcribed from maternal and embryonic genome. Until the major round of embryonic transcription during the 8- to 16-cell stage in bovine embryos, development is largely dependent on the transcripts and protein formed by the oocyte (Memili and First 2000). Oct-4 is the earliest expressed transcription factor that is known to be crucial in murine preimplantation development (Okamoto et al. 1990, Rosner et al. 1990, Schöler et al. 1990, Nichols et al. 1998). The mRNA and protein of Oct-4 had been found in murine oocytes and in the nuclei of subsequent cleavage stage embryos (Rosner et al. 1990, Schöler et al. 1990, Palmieri et al. 1994), while in the expanded murine blastocyst stage both mRNA and protein were predominantly found in the ICM (Palmieri et al. 1994, Pesce et al. 1998, Kirchhof et al. 2000). However, even in fully expanded bovine and porcine blastocysts both ICM and TE cells were found to be positive for Oct-4 protein (Kirchhof et al. 2000). The quantitative expression profiling results throughout the preimplantation embryonic stages in the present study evidenced that Oct-4 is activated from both maternal and embryonic genome. Transcript abundance sharply increases after maturation and down-regulated until 4-cell stage. The detectable amount of Oct-4 transcript was very low between 8-cell and morula stages, after which it was up-regulated at the blastocyst stage. Therefore, injection of Oct-4 dsRNA is targeting transcripts starting the minor embryonic activation at the 2- to 4-cell stages and in the major embryonic activation after 16-cell stage. Consequently, injection of Oct-4 dsRNA at the zygote stage has resulted in 72% reduction at the blastocyst stage compared to the uninjected controls. Despite slight variations in the relative abundance of Oct-4 transcript between water injected and uninjected control groups, differences are not significant. Similar studies in mouse have reported that suppression of about

90% has been achieved using sequence specific dsRNA (Svoboda et al. 2004). Previous reports using dsRNA in bovine embryos also showed that the suppression of the relative abundance of E-cadherin mRNA in the E-cadherin dsRNA injected morula stage embryos was reduced by 80% compared to the control group (Nganvongpanit et al. 2006a). Moreover, the western blot analysis also showed a significant decrease in the E-cadherin protein in E-cadherin dsRNA injected embryos compared to the other three groups and microinjection of E-cadherin dsRNA has resulted only 22% blastocyst rate compared to 38-40% in water injected and uninjected controls.

Oct-4, as transcription factor protein is known to bind to DNA and activate or repress transcription of several genes expressed during early embryonic development (Nichols et al. 1998, Shin et al. 2005). In the present study suppression of Oct-4 transcript in bovine embryogenesis using dsRNA has resulted in co-suppression of Fgf-4 gene at a level of 70%, while transcript remained unaffected in water injected and uninjected controls. This is in agreement with the observation made in Oct-$4^{-/-}$ mouse embryos, where Fgf-4 transcript abundance has been reduced (Nichols et al. 1998). Moreover, the expression of Fgf-4 transcript was found to be down-regulated after targeted suppression of Oct-4 using siRNA expression vector in mouse (Haraguchi et al. 2004). The Fgf-4 gene is an octamer-containing enhancer in its 3´-noncoding region and has been demonstrated to respond to Oct-4 gene (Yuan et al. 1996, Ambrosetti et al. 1997, Daniels et al. 2000). Studies in mouse have shown that this gene is co-expressed with Oct-4 in the ICM and epiblast (Ma et al. 1992, Niswander and Martin 1992). Recently, the effect of down-regulation of Oct-4 transcript using dsRNA on the expression of other genes in mouse embryos had been investigated using annealing control primer technique (Shin et al. 2005), whereby of the 10 genes, 8 (Atp6ap2, GK003, Ddb1, hRscp, Dppa1, Dpp3, Sap18, and Rent1) were down-regulated and 2 (Rps14 and ETIF2B) were up-regulated in Oct-4 dsRNA-injected blastocysts. The specificity of Oct-4 dsRNA on targeted mRNA has been investigated by quantitative expression analysis of other blastocyst transcript (E-cadherin) and a house keeping gene (H2a).

This study has demonstrated that degradation of Oct-4 mRNA resulted in consequent reduction in protein synthesis and resulted in developmental aberrations. Oct-4 dsRNA injection has affected the cleavage rate of zygotes to develop to the 2-cell stage. Even though the day 5 morula rate was lower in the Oct-4 dsRNA injected group compared to

the water injected and uninjected controls, these differences were not significant. In order to investigate the effect of Oct-4 suppression on the rate of embryo development, we have investigated the blastocyst rate from day 6-8. Most of the blastocysts from Oct-4 dsRNA injected groups appeared at day 7 and 8 while only few blastocysts were found at day 6 of development. However, comparable developmental rate with respect to blastocysts rate between days 6-8 has been observed in water injected and uninjected controls. The overall blastocyst rate was lower in Oct-4 dsRNA injected embryos compared to the water injected and uninjected controls but differences are not significant. While the Oct-$4^{-/-}$ mouse showed a postimplantation lethality before egg cylinder formation, Oct-4 deficient mouse embryos developed normally up to blastocyst stage but the ICM were not pluripotent and divert to a trophoblast fate when placed in embryonic stem cell culture conditions (Nichols et al. 1998). Marked differences had been observed in Oct-4 mRNA and protein expression in mouse, murine and bovine species (Kirchhof et al. 2000). As opposed to the study in mouse where Oct-4 expression was correlated with the undifferentiated cell types suggesting that Oct-4 was a marker for pluritency and its expression was restricted to ICM (Ovitt and Schöler 1998), Even, higher level of Oct-4 protein expression had been detected in the ICM as opposed to the trophectoderm cells of human blastocysts (Hansis et al. 2000), but in bovine and porcine blastocyst, immunocytochemical analysis has detected Oct-4 protein in both ICM and TE cells (Kirchhof et al. 2000). This could indicate that it may be the biological activity of the Oct-4, and not simply its presence, that correlates with the embryonic stem cell type (Kirchhof et al. 2000). In this present study, Oct-4 dsRNA injected zygotes resulted in blastocysts of lower cell number compared to the water injected and uninjected control groups. This was significantly evident in the number of ICM cells which were found to be reduced due to down-regulation of Oct-4 transcript. The optimal level of Oct-3/4 is reported to determine the fate of embryonic stem cells (Niwa et al. 2000), in which less than a two-fold increase from the normal expression level causes differentiation into ectoderm and mesoderm, whereas a lower level leads to dedifferentiation into TE. However, due to absence of differences in the number of TE cells between the three groups, migration of cells to TE cells can not be evidenced in the present study.

5.8 Future prospects

RNAi is a revolution in the field of animal molecular genetics that it has enormous potential for engineering control of gene expression, as well as for the use of a tool in functional genomics. The ability to manipulate RNAi has a wide variety of practical applications of biotechnology ranging from molecular biology to gene therapy. The use of RNAi as a method to alter gene expression in mammalian embryo has been attempted in a diverse rate of success as previous mentioned. However, RNAi seems to be an effective, specific and valuable tool for reverse genetics. Moreover, RNAi is a straight forward technique which is used to rapidly assess gene function and reveal null phenotypes in verity of species such as nematode, insect, mouse, swine and bovine. Analysis of more genes using RNAi in bovine including other mammalian species will help researchers to better understand what genes are suitable for RNAi targeting and function of those genes. However, various RNAi approaches need to be compared and standard protocols must be developed for a better use. The RNAi transgenic approach is very attractive for studies of early mammalian development. Because, this technique could solves the problem of elimination of the maternal pool of targeted protein as well as the problem with the need for extensive microinjection. Moreover, RNAi usually does not totally eliminate gene function; this would provide a range of phenotypes depending on the level of interference. But, it might be an advantage uses as it provides with relevant information about threshold affects that can not be obtained by the classical gene knockout experiments. Better understanding of the RNAi mechanism in mammals will also lead to an attempt to modify and enhance the RNAi response. And as RNAi emerges as a useful silencing tool for studies in mammals, its therapeutic use can be assessed, most likely in fields using tissue cultures extensively, such as cancer disease, viral disease or in treatment of parasitic infections.

6 Summary

RNAi has been used for selective degradation of mRNA transcript or inhibits its translation to functional protein at the post transcriptional state in various species. This work aimed to apply the RNAi approach to suppress the expression of the maternal transcript C-mos (AY630920) and embryonic transcripts Oct-4 (AY490804) during *in vitro* development of bovine embryos using microinjection of sequence-specific dsRNA. For this, 435 and 341 bp long dsRNA specific to the coding sequences of C-mos and Oct-4 transcripts, respectively, were synthesized and used for microinjection. While C-mos dsRNA was injected at the immature oocyte stage, Oct-4 dsRNA was injected at the zygote stage.

In experiment 1, 935 good quality immature oocytes were categorized into three groups namely: those injected with C-mos dsRNA (n = 327), water (RNase-free) (n = 303) and uninjected controls (n = 305). Oocytes were held in a TCM-199 medium supplemented with 0.1% BSA, 0.2 mM pyruvate and 50 µg/ml gentamycin sulphate until use in a humidified atmosphere with 5% CO_2 at 39 °C. Prior to microinjection, theses immature oocytes were incubated for 20 min in TCM-199 supplemented with 8 µg/µl cytochalasin B in order to stabilize the cytoskeleton during injection. In experiment 2, *in vitro* produced zygotes (n = 1,437) were categorized into three groups namely, those injected with Oct-4 dsRNA (n = 439), water (RNase-free) (n = 427) and uninjected controls (n = 571). In both experiments, microinjection was performed on an inverted microscope at 200x magnification. During microinjection a group of 50-60 immature oocytes or zygotes were placed in a 10 µl droplet of H-TCM under mineral oil and the dsRNA or water was placed in a 1µl droplet near to the droplet containing the oocytes or zygotes. H-TCM medium was supplemented with cytochalasin B during injection of immature oocyte to improve the survival rate of the oocytes after microinjection. The injection volume of ~7 pl was applied using injection capillary with 5 µm in diameter.

In order to assess the effect of sequence specific dsRNA in oocytes and embryos on mRNA transcript abundance and protein expression, oocytes and embryos were collected at specific times after treatment while the phenotype was observed throughout the developmental stages. In experiment 1, immature oocytes were cultured after microinjection with C-mos dsRNA and water until 48 hpi. While ooctes were being

cultured for 48 h after treatment to allow any parthenogenetic development, oocytes were collected for transcription and protein expression analysis using real-time PCR and western blot analysis, respectively. In experiment 2, zygotes injected with Oct-4 dsRNA or water and non injected controls were culture *in vitro* until day 8 blastocyst stage. The blastocyst stage embryos from each treatment group were used for both transcription and protein analysis.

First, in order to get an insight on normal temporal expression pattern of the studied transcripts (C-mos, Gdf-9, Oct-4, Fgf-4 and E-cadherin), a real-time PCR analysis was conducted throughout the preimplantation development stage of *in vitro* produced bovine embryos. The C-mos and Gdf-9 were detected at higher level between immature oocytes and 4-cell stage and, down-regulated in the later developmental stages. The Oct-4 transcript was found to be highly abundant at early developmental stages (between immature oocyte and 4-cell stage) and further down-regulated between 8-cell and morula stage. Relatively higher transcript abundance was detected at the blastocyst stage. The E-cadherin mRNA transcript was detected at higher level at immature and matured oocytes, morula and blastocyst stages of development. However, transcript abundance was lower between 2-cell and 16-cell developmental stages. The Fgf-4 transcript was highly abundant at morula and blastocyst stages, while it could not be detected in earlier developmental stages (from immature oocyte up to 16-cell stage).

Microinjection of C-mos dsRNA has resulted in 70% reduction of C-mos transcript abundance after maturation compared to the water injected and uninjected controls ($P < 0.01$). Similarly, microinjection of Oct-4 dsRNA at the zygote stage has resulted in 72% reduction in transcript abundance at the blastocyst stage as compared to the uninjected controls ($P < 0.01$). In order to investigate the specificity of the dsRNA, two independent genes, Gdf-9 (maternal) and E-cadherin (embryonic) were quantified in all treatment groups and no differences were observed. The reduction on those transcripts led to reduce the protein synthesis. The intensity of C-mos and Oct-4 protein band was decreased in C-mos and Oct-4 dsRNA injected groups, respectively, while strong reactive bands were detected in water injected and uninjected control groups. Moreover, the relative expression of Fgf-4 gene, which is known to be co-expressed with Oct-4 gene, was found to be significantly down-regulated by 70% in Oct-4 dsRNA injected group compared to the other groups.

First, the survival rate of the oocytes and embryos due to the physical injury during microinjection has been determined 3-4 h after microinjection. No significant differences were observed in survival rate of oocytes and embryos injected with dsRNA and water. Compared to uninjected controls about 10-12% of oocytes and 15-18% of zygotes did not survive the microinjection procedure. From oocytes injected with C-mos dsRNA, about 60% showed the extrusion of first polar body compared to the water injected (50%) and uninjected controls (44%). Moreover, 2.5% of the oocytes injected with C-mos dsRNA developed parthenogenetically to the 2-cell stage. The first cleavage after microinjection was 70, 80 and 81% for embryos injected with Oct-4 dsRNA, water and uninjected controls, respectively. The day 5 morula rate in Oct-4 dsRNA injected group (37%) was not significantly different compared to water injected (40%) and uninjected control (42%) groups. However, there is a considerable variation in the number of blastocysts from each treatment group at each day of development between days 6-8. Most of the blastocyst from Oct-4 dsRNA injected groups appeared at the day 7 (18%) and day 8 (10%) while only few blastocysts were found at day 6 (8%) of development. However, the over all blastocyst rate was lower in Oct-4 dsRNA injected embryos (36%) compared to the water injected (40%) and uninjected control (42%) but differences are not significant. Moreover, Oct-4 dsRNA injected embryos resulted in lower cell number compared to the other two groups. This was evident in the number of ICM cells, they were found to be reduced due to down-regulation of Oct-4 transcript.

In conclusion, the present study has gives evidence that the use of sequence specific dsRNA to induce RNAi in bovine oocytes and embryos to suppress maternal and embryonic transcripts leads to a subsequent reduction in functional protein expression and a distinct developmental phenotype. Moreover, these results demonstrated that sequence specific dsRNA can be used to knockdown maternal or embryonic transcripts in bovine embryogenesis and used as a tool to study the function of genes.

7 Zusammenfassung

RNAi wird für die selektive Degradierung von mRNA Transkripten verwendet oder verhindert ihre Translation zu einem funktionierenden Protein nach der Transkription in verschiedenen Spezies. Diese Arbeit zielt darauf, den RNAi Ansatz anzuwenden, um die Expression des maternalen Transkriptes C-mos (AY630920) und des embryonales Transkriptes Oct-4 (AY490804) während der in vitro Entwicklung boviner Embryonen durch Mikroinjektion von sequenz-spezifischer dsRNA zu verhindern. Dazu wurde eine 435 bp und 341 bp lange dsRNA, die spezifisch für die jeweils kodierenden Sequenzen der C-mos und Oct-4 Transkripte ist, synthetisiert und für die Mikroinjektion verwendet. Während C-mos dsRNA in Oozyten im unreifen Stadium injiziert wurde, wurde Oct-4 dsRNA im Zygotenstadium injiziert.

In Experiment 1 wurden 935 unreife Oozyten einer guten Qualität in drei Gruppen eingeteilt: solche mit C-mos dsRNA injizierten (n = 327), mit Wasser (RNase-freiem) injizierten (n = 303) und Kontrollen ohne Injektion (n = 305). Die Oozyten wurden in TCM-199 mit 0,1% BSA, 0.2 mM Pyruvat und 50 µg/ml Gentymycinsulfat in einer Atmosphäre 5% CO_2 bei 39 °C kultiviert. Vor der Mikroinjektion wurden diese ungereiften Oozyten 20 min in TCM-199 mit 8 µg/µl Cytochalasin B inkubiert, um das Zytoskellet während der Injektion zu stabilisieren. In Experiment 2 wurden in vitro produzierte Zygoten (n = 1.437) in drei Gruppen eingeteilt, mit Oct-4 dsRNA (n = 439) und mit Wasser (RNase-freiem) (n=427) injizierte, sowie nicht injizierte Kontrollen (n = 571). In beiden Experimenten wurde die Mikroinjektion auf einem inversen Mikroskop bei 200 facher Vergrößerung durchgeführt. Während der Mikroinjektion wurde eine Gruppe von 50-60 ungereiften Oozyten oder Zygoten in einen 10 µl Tropfen H-TCM unter Mineralöl platziert. Die dsRNA beziehungsweise das Wasser wurden in einem 1µl Tropfen neben dem Tropfen mit den Oozyten oder Zygoten platziert. H-TCM Medium wurde während der Injektion der unreifen Oozyten mit Cytochalasin B versetzt, um die Überlebensrate der Oozyten nach der Mikroinjektion zu erhöhen. Das Injektionsvolumen von ~7 pl wurde mit einer Injektionskapillare mit einem Durchmesser von 5 µm verabreicht.

Zur Beurteilung des Effektes der sequenz-spezifischen dsRNA in Oozyten und Embryos auf die Menge an mRNA Transkripten und Protein Expression wurden Oozyten und

Embryonen zu spezifischen Zeitenpunkten nach der Behandlung gesammelt, während der Phänotyp in allen Entwicklungsstadien beobachtet wurde. In Experiment 1 wurden die unreifen Oozyten nach der Mikroinjektion mit C-mos dsRNA oder Wasser bis 24 hpi kultiviert. Während die Oozyten nach der Behandlung 48 h kultiviert wurden, um jegliche parthenogenetische Entwicklung zu ermöglichen, wurden die Oozyten für Transkriptions- und Proteinexpressionsanalyse mit Hilfe von Real-Time PCR beziehungsweise Western Blot Analyse gesammelt. In Experiment 2 wurden die mit Oct-4 dsRNA oder Wasser injizierten und nicht injizierten Kontrollen in vitro bis zum Tag 8 des Blastozystenstadiums kultiviert. Die Embryonen im Blastozystenstadium wurden von jeder behandelten Gruppe für Transkriptions- und Proteinanalyse verwendet.

Um einen Einblick in das normale zeitliche Expressionsmuster der untersuchten Transkripte (C-mos, Gdf-9, Oct-4, Fgf-4 und E-cadherin) zu erhalten, wurde durchgehend im präimplantativen Entwicklungsstadium der in vitro produzierten Rinderembryonen eine Real-Time PCR Analyse durchgeführt. Bei den C-mos und Gdf-9 Transkription wurde ein höherer Spigel im Vergleich der unreifen Oozyten und des 4-Zellstadiums festgestellt. In den späteren Entwicklungsstadien nahm die Expression ab. Es wurde festgestellt, dass das Oct-4 Transkript in den frühen Entwicklungsstadien (zwischen unreifen Oozyten und 4-Zellstadium) in hohen Mengen vorhanden ist und später zwischen dem 8-Zell und Morulastadium wieder herunter reguliert ist. Eine relativ höhere Transkriptmenge wurde im Blastozystenstadium festgestellt. Das E-cadherin mRNA Transkript wurde in höheren Niveaus bei unreifen und reifen Oozyten, Morula- und Blastozystenstadien der Entwicklung gefunden. Jedoch war die Transkriptmenge niedriger zwischen den 2-Zell und 16-Zell Entwicklungsstadien. Die Fgf-4 Transkripte waren in den Morula- und Blastozystenstadien reichlich vorhanden, während es in den früheren Entwicklungsstadien (von unreifen Oozyten bis zum 16-Zellstadium) nicht gefunden wurde.

Die Mikroinjektion von C-mos dsRNA führte zu einer 70%igen Verringerung der C-mos Transkriptmenge nach der Reifung, verglichen mit den Wasser injizierten und den nicht injizierten Kontrollen ($P < 0.01$). Ähnlich ergab auch die Mikroinjektion von Oct-4 dsRNA im Zygotenstadium eine 72%igen Reduktion der Transkriptmenge im Blastozystenstadium ($P < 0.01$). Um die Spezifität der dsRNA-wirkung zu untersuchen,

wurden zwei unabhängige Gene, Gdf-9 (maternal) und E-cadherin (embryonal), in allen behandelten Gruppen quantifiziert, wobei keine Unterschiede festgestellt wurden. Die Reduktion der Transkripte C-mos und Oct-4 führt zu einer Reduktion der jeweiligen Proteinsynthese. Die Intensität der C-mos und Oct-4 Proteinbanden waren in den C-mos und Oct-4 dsRNA injizierten Gruppen jeweils verringert, während stark experimiert Banden in den Wasser injizierten und nicht injizierten Kontrollgruppen festgestellt wurden. Darüber hinaus wurde festgestellt, dass die relative Expression des Fgf-4 Gens, welches mit dem Oct-4 Gen co-exprimiert wird, signifikant mit 70% in der Oct-4 dsRNA injizierten Gruppe im Vergleich mit den anderen Gruppen herunter reguliert ist.
Zunächst wurde die Überlebensrate der Oozyten und Embryonen auf Grund der physischen Beschädigung während der Mikroinjektion 3-4 h nach der Mikroinjektion festgestellt. Es wurden keine signifikanten Unterschiede der Überlebensrate der Oozyten und Embryonen nach Injektion mit dsRNA oder Wasser festgestellt. Verglichen mit den nicht injizierten Kontrollen überlebten 10-12% der Oozyten und 15-18% der Zygoten nach der Mikroinjektion nicht. Von den mit C-mos dsRNA injizierten Oozyten zeigten im Vergleich zu den mit Wasser injizierten (50%) und nicht injizierten Kontrollen (44%) 60% eine Extrusion des ersten Polarkörpers. Weiterhin entwickelten sich 2.5% der mit C-mos dsRNA injizierten Oozyten parthenogenetisch zum 2-Zellstadium. Die erste Teilung nach der Mikroinjektion betrug 70, 80 und 81% der jeweils mit Oct-4 dsRNA injizierten, Wasser injizierten und nicht injizierten Embryonen. Die Tag 5 Morula entwicklungsrate in der mit Oct-4 dsRNA injizierten Gruppe (37%) war nicht signifikant unterschiedlich von der mit Wasser injizierten (40%) und der nicht injizierten (42%) Kontrollgruppe. Jedoch gibt es eine erhebliche Variation der Anzahl Blastozysten, welche aus jeder Gruppe in den Entwicklungsstadien zwischen Tag 6-8 stammten. Die meisten Blastozysten der Oct-4 dsRNA injizierten Gruppen erschienen am Tag 7 (18%) und Tag 8 (10%), während einige Blastozysten am Tag 6 (8%) der Entwicklung gefunden wurden. Die gesamte Blastozystenrate war niedriger in den Oct-4 dsRNA injizierten Embryonen, was im Vergleich zu den anderen zwei Gruppen zu einer niedrigeren Zellzahl führte. Dies war ein Beweis, dass die gefundene Anzahl ICM Zellen aufgrund der verringerten expression des Oct-4 Transkriptes reduziert ist.

Zusammenfassend gibt die vorliegende Untersuchung einen Beweis, dass die Verwendung der sequenz-spezifischen dsRNA zur Induzierung von RNAi in bovine Oozyten und Embryos zur Herunterregulation der maternalen und embryonalen Transkripte zu einer anschließenden Verringerung der funktionellen Proteinexpression und einem unterschiedlichen Entwicklungsphänotyp führt. Dieses System kann damit als Werkzeug zur Untersuchung der Funktion von Genen verwendet werden kann.

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Publications

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Oral presentations

1. Selective suppression of genes in in vitro produced bovine preimplantation embryo using RNA interference technology. The 12th International Congress on Biotechnology in Animal Reproduction. August, 4th-6th 2005, Chiang Mai, Thailand.
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Poster presentations

1. **Nganvongpanit K**, Rings F, Jennen D, Bauch K, Gilles M, Tholen E, Hölker M, Schellander K, Tesfaye D. Selective suppression of genes in in vitro produced bovine preimplantation embryo using RNA interference technology. The 12th International Congress on Biotechnology in Animal Reproduction. August, 4th-6th 2005, Chiang Mai, Thailand.
2. **Nganvongpanit K**, Tesfaye D, Müller H, Chomdej S, Gilles M and Schellander K. Targeted suppression of C-mos mRNA level during bovine oocyte maturation using RNA interference technology. The European Society for Domestic Animal Reproduction 9th Annual Conference. September, 1st-3rd 2005. Faculty of Veterinary, University of Murcia, Murcia, Spain.
3. Tesfaye D, **Nganvongpanit K**, Rings F, Gilles M, Jennen D, Hölker M, Tholen E and Schellander K. Targeted suppression of the expression of maternal and embryonic genes during in vitro development of bovine embryos. The 32nd Annual Conference of the International Embryo Transfer Society. January 8th-10th, 2006. Caribe Royale, Orlando, Florida, USA.

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