Examination of Y Chromosome Linked Gene Expression in Healthy and Arrested Pre-implantation Bovine Embryos

By

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A Thesis
Presented To
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
In
Biomedical Science

Guelph, Ontario, Canada

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Abstract

Examination of Y chromosome linked gene expression in healthy and arrested pre-implantation bovine embryos

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University of Guelph, 2013

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Sexual dimorphisms in growth rate and metabolism have been observed in the early embryo suggesting a role for sex chromosome linked gene expression in the early embryo. It is hypothesized that Y chromosome gene expression is aiding male embryo development in vitro. Reverse transcription polymerase chain reaction revealed the expression of DDX3Y, EIF1AY, TSPY, USP9Y, ZFY, and ZRSR2 in blastocysts but not HSFY or SRY. Furthermore DDX3Y, USP9Y, and ZRSR2Y showed expression in 100% of individual male blastocyst analysed. USP9Y and ZFY showed expression as early as the 4-cell and 8-cell stage however not in embryos arrested at the 2- to 8-cell stage. RNA interference to knock down the transcripts of USP9Y resulted in a significant decreased the cleavage rate but no significant difference in the blastocyst rate. Given Y linked gene expression coincidence with embryonic genome activation, and the absence of USP9Y and ZFY transcripts in the arrested embryos it was concluded that these genes may be important for early male embryo development.
# Table of Contents

Acknowledgements..................................................................................................................... iv  

Declaration of Work Performed .................................................................................................... vi  

List of Figures ................................................................................................................................ vii  

List of Tables .................................................................................................................................. viii  

List of Abbreviations ..................................................................................................................... ix  

Introduction ..................................................................................................................................... 1  

Review of Literature ...................................................................................................................... 3  

Rationale .......................................................................................................................................... 26  

Materials and Methods .................................................................................................................. 29  

Results ........................................................................................................................................... 40  

Discussion ....................................................................................................................................... 53  

Summary & Conclusion ................................................................................................................... 65  

References ....................................................................................................................................... 66  

Appendix I ....................................................................................................................................... 84
Acknowledgements

I would like to thank everyone who helped me along the way.

A big thank you to Dr. Allan King who first took me on as an undergraduate student knowing very little about research and helped turn me into a graduate level researcher with his ideas and advice along the way. Thank you to Dr. Christine Hamilton who helped me with my first project and designed the basics for that research to continue into this thesis.

Technical support from Ed Reyes Monica Antenos, and Michelle Ross. A special thank you to Liz St. John who taught me IVF and was always there to help me. Thank you to Laura Favetta, Graham Gilchrist, and Tamas Reval who helped me learn and troubleshoot PCR.

Learning the tricky methods of micromanipulation was made easier with the help of Dr. Pavneesh Madan and Nayoung Rho.

I would like to thank Faz Ashkar, Jackie Ferris, Leslie Gonzalez Grajales, Kayla Perkel, Allison Tscherner, Stewart Russell for general help in the lab, and my office mates Carmon Co, Laurence Tessier, Sam Russell, Jacqueline Dynes, Tobi Oluwole, Soadad Saleh, Miyuki Kumagai, Larry Morgan for help with ideas or killing time between experiments.

I would also like to thank my committee members Dr. Pavneesh Madan and Dr. Tami Martino for helping with ideas for my thesis research as well as ideas for my written thesis.

I would like to thank my financial supporters who made this research possible: the OVC Scholarship, NSERC, and Canadian Research Chair programs.
Friends and family.

The biomedical science department, the OVC, and the University of Guelph.
Declaration of Work Performed

I declare that with the exception of the items below, all of the work reported in this thesis was performed by me.

Bovine ovaries and bovine kidney tissue was collected by Lalith Ranaweera and Pradeep Balaraju from the local abattoir. Bovine testis tissue was provided by Christine Hamilton. All media used for in vitro embryo production was made by Elizabeth St. John and Michelle Ross. The primers used during the Y chromosome gene analysis were designed by Christine Hamilton. The RT-PCR work for HSFY, SRY, TSPY, ZRSR2Y, and the RT-qPCR work for individual blastocysts was performed by Christine Hamilton. Alexandra McClenahan assisted in the sexing of the arrested embryos. Statistical analysis was performed by Leslie Gonzalez Grajales.
List of Figures

Figure 1. Y chromosome genes .................................................................................................................. 19

Figure 2. Microinjection set up for a single zygote .................................................................................. 38

Figure 3. X-homologues expression in female kidney tissue and male testis tissue ............................... 40

Figure 4. Expression of Y-linked genes in pooled bovine blastocysts ....................................................... 41

Figure 5. Results of DNA-based sexing and expression analysis in eight individual blastocysts ............. 44

Figure 6. USP9Y gene expression in pooled bovine embryos ................................................................. 45

Figure 7. ZFY gene expression in pooled bovine embryos ......................................................................... 46

Figure 8. Expression of ZFY and USP9Y in diluted blastocyst samples ................................................. 46

Figure 9. USP9Y gene expression in pooled bovine arrested embryos .................................................... 47

Figure 10. ZFY gene expression in pooled bovine arrested embryos ....................................................... 47

Figure 11. USP9X gene expression in pooled bovine arrested embryos and matured oocytes ............... 48

Figure 12. DNA-based sexing of 8 embryos arrested at the 2-8 cell stage .............................................. 48

Figure 13. Sexing of 100 arrested 2-cell, 4-cell, and 8-cell embryos ........................................................ 49

Figure 14. Total cleavage rates from siRNA injections ............................................................................. 51

Figure 15. Total blastocyst rates from siRNA injections .......................................................................... 51
List of Tables

Table 1 siRNA primer sequences .............................................................. 36
Table 2 Expression of Y-linked genes in sexed and unsexed individual bovine blastocysts........ 43
Table 3 Total cleavage and blastocyst rates from siRNA injections. ........................................ 50
Table 4 Total numbers from non injected group per injection run ........................................ 52
Table 5 Total numbers from scrambled-injected group per injection run ................................. 52
Table 6 Total numbers from knockdown-injected group per injection run ................................. 52
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>Anti-mullerian hormone</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>bFSH</td>
<td>Bovine follicle stimulating hormone</td>
</tr>
<tr>
<td>bLH</td>
<td>Bovine luteinizing hormone</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus-oocyte complexes</td>
</tr>
<tr>
<td>C&lt;sub&gt;q&lt;/sub&gt;</td>
<td>Quantification cycle value</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitylationing enzyme</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
</tr>
<tr>
<td>DFFRY</td>
<td>Drosophila fat facets related Y</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
</tr>
<tr>
<td>EIF1AY</td>
<td>Eukaryotic translation initiation factor 1A, Y-chromosomal</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HSFY</td>
<td>Heat shock transcription factor, Y chromosome</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>INF-t</td>
<td>Interferon-tau</td>
</tr>
<tr>
<td>IVC</td>
<td>In vitro culture</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>In vitro maturation</td>
</tr>
<tr>
<td>IVP</td>
<td>In vitro production</td>
</tr>
<tr>
<td>KD</td>
<td>USP9Y knockdown siRNA injected</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double minute 2 homolog</td>
</tr>
<tr>
<td>MSR</td>
<td>Male specific region</td>
</tr>
<tr>
<td>MZT</td>
<td>Maternal to zygotic transition</td>
</tr>
<tr>
<td>NI</td>
<td>Non injected</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>OH*</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;*</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SI</td>
<td>Scrambled siRNA injected</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex-determining region of the Y chromosome</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-interfering RNAs</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>TDF</td>
<td>Testis determining factor</td>
</tr>
<tr>
<td>TSPY</td>
<td>Testis specific protein Y-encoded</td>
</tr>
<tr>
<td>TWH</td>
<td>Trivers-Willard hypothesis</td>
</tr>
<tr>
<td>TALP</td>
<td>Tyrode’s albumin lactate pyruvate</td>
</tr>
<tr>
<td>UBP6</td>
<td>Ubiquitin-specific protease 6</td>
</tr>
<tr>
<td>UBP7</td>
<td>Ubiquitin-specific protease 7</td>
</tr>
<tr>
<td>UBP20</td>
<td>Ubiquitin specific peptidase 20</td>
</tr>
<tr>
<td>UBP33</td>
<td>Ubiquitin carboxyl-terminal hydrolase 33</td>
</tr>
<tr>
<td>USP9Y</td>
<td>Ubiquitin specific peptidase 9, Y-Linked</td>
</tr>
<tr>
<td>Yp</td>
<td>Y chromosome short arm</td>
</tr>
<tr>
<td>Yq</td>
<td>Y chromosome long arm</td>
</tr>
<tr>
<td>ZRSR2Y</td>
<td>Y-linked zinc finger gene</td>
</tr>
<tr>
<td>ZFY</td>
<td>Zinc finger protein, Y-linked gene</td>
</tr>
</tbody>
</table>
Introduction

The Canadian dairy industry, composed of 1.4 million head of cattle, was estimated to contribute $14 billion to the national economy in 2012 (Agriculture and AgriFood Canada, 2013). The milk production from last year was over 80 million hectolitres (Agriculture and AgriFood Canada, 2013). Due to the high genetic quality and demand for Canadian dairy cows, this industry has a $100 million net export of bovine embryos (Australia, Germany, Japan), semen (USA, Netherlands, Brazil), and live dairy cattle (USA, Russia, Kazakhstan) (Agriculture and AgriFood Canada, 2013).

To maintain production, dairy cows must become pregnant once a year which is usually done via artificial insemination. Given that the industry is driven by production by females, it is common practise to sex potential offspring. This is done via ultrasound after formation of the gonads or the pre-implantation embryo is flushed out and then sexed. Termination of males or reintroduction of only female embryos into the uterus allows for pregnancy with controlled offspring sex. Practises of this nature have essentially formed two separate industries: milk production which is exclusively female, and semen production which is exclusively male. During early embryogenesis bovine male embryos have been reported to develop faster than their female counterparts leading to the question of how the sex of an embryo may affect development and what the molecular mechanism behind it is. A better understanding of the Y chromosome therefore, which is unique to males, during pre-implantation embryo development will give insight into male embryo survival and viability in vitro. This will also
benefit embryo sexing (which is primarily done using DNA), and may introduce a new method of embryo sexing using RNA.

The increased importance of bovine *in vitro* embryo production has resulted from several factors: The increased availability of bovine embryos for use as an experimental model, the increased demand of IVF for agriculture purposes, and the similarity of bovine and human embryo development and the use as a model for human research (Thompson & Peterson, 2000). Since the first successful human IVF in 1978 and bovine in 1979, the technology has grown. In Canada in 2012 there was a combined 11,806 IVF treatment cycles resulting in 3,188 live births (Canadian Fertility and Andrology Society, 2013). Of these births 76% were singletons, 23% were twins, and 0.8% were triplets or more, representing a decrease in multiple births compared with 2009 (Canadian Fertility and Andrology Society, 2013). Multiple births are an issue during human IVF because it is common for the doctor to introduce multiple embryos to the uterus in the hopes that at least one will implant and result in a successful pregnancy. This is a common practise because of the lower chances of survival when dealing with embryos *in vitro*. With an increased understanding of why some embryos arrest development human IVF will be able to better select blastocysts for implantation and reduce the risks of pregnancy with multiple babies. Investigation into the causes of embryo arrest and embryo survival factors will benefit this procedure. Studying the Y chromosome and its impact on pre-implantation development as well as its possible impact on embryo arrest will provide the insight into this area of pre-implantation development.
Review of Literature

Gamete maturation and fertilization

The preparation of female gametes begins with differentiation of germ cells into oogonia, a process called oogenesis. As the germ cells multiply those that reach the prophase stage of meiosis are called primary oocytes. These primary oocytes remain at this stage until puberty where they must prepare the haploid nucleus (germinal vesicle) for fertilization via a series of meiotic arrests and prepare the cytoplasm for early embryonic development via stockpiling transcripts and proteins. As the oocyte develops within the follicle it is surrounded by epithelial cumulus cells and granulose cells. As the oocyte matures these cells thicken and help to secrete a glycoprotein barrier around the oocyte called the zona pellucida.

The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which acts on the pituitary gland to release follicle-stimulating hormone (FSH), and luteinizing hormone (LH).

The rising levels of FSH stimulate several oocytes to grow in size. The swollen germinal vesicle breaks down and the epithelial cumulus and granulosa cells around the oocyte begin to disaggregate and expand within the follicle. The granulosa cells secrete estradiol which stimulates the release of LH. The rising level of LH results in the resumption of meiosis from prophase I to metaphase II. Around 38 hours after a surge of LH ovulation occurs. The follicle ruptures to slowly allow the oocyte to exit the ovary and is guided by fimbriae into the oviduct where it survives with the capacity to be fertilized for up to 24 hours (reviewed by Schoenwolf et al., 2009; Senger, 2003).
The male gametes are produced after puberty when testosterone triggers spermatogenesis to begin. The non-mitotically active germ cells resume development into spermatogonia. The spermatogenesis cycle continuously drives development and maturation of spermatogonia into spermatozoa with a head of condensed nucleus, a midpiece of helical mitochondria, and a tail of microtubules for propulsion. Spermatozoa capacitation, the process by which sperm gain the ability to fertilize, occurs during the swim to fertilize where secretions from the oviduct modify the acrosome on the head of the sperm. If viable spermatozoa find the oocyte they begin to push through the cumulus cells. When the zona pellucida is reached the spermatozoa bind with a surface protein in a species specific interaction to a glycoprotein sperm receptor. The binding results in release of degradation enzymes from the acrosome on the spermatozoa head. After successful penetration of the zona pellucida the first sperm to reach the oocyte will fuse its membrane with the membrane of the oocyte. This fusing of the cell membranes result in two immediate events: a wave of calcium that spreads over the oocyte surface, and release of cortical granules into the space between the zona pellucida and oocyte membrane causing the zona pellucida to become impenetrable by additional spermatozoa. These events block fertilization from multiple spermatozoa (polyspermy).

The fertilized oocyte, now called a zygote, rapidly resumes meiosis. The nuclear membranes of the male and female pronuclei disappear as both maternal and paternal chromosomes replicate in preparation for the first cleavage (reviewed by Schoenwolf et al., 2009; Senger, 2003).

**Pre-implantation embryo development**
Within 24 hours of fertilization the zygote begins a rapid series of cellular divisions called cleavage with the daughter cells called blastomeres. This stage of development is not concerned with the blastomeres growing in size but on replicating the newly formed genome and increasing the number of cells. The timing of cleavage is species specific and in bovine the

*in vitro* 2-cell embryo is formed 1-2 days post fertilization, 4-cell embryo 3 days post fertilization, 8-cell embryo 4 days post fertilization, 16-cell embryo 5 days post fertilization, morula 6 days post fertilization, and the blastocyst forming 7-8 days post fertilization. The blastomeres of the blastocyst are organized into two groups: the inner cell mass which gives rise to the embryo proper, and the cells which surround it called the trophoblast which gives rise to the placenta. At this stage in embryonic development the embryo begins to grow in size as the blastocyst cavity or blastocoel (the cavity produced when the trophoblast cells separate from the inner cell mass) fills with fluid. Once the cavity fills the embryo is called an expanded blastocyst and is larger in size then previous stages. Once the blastocyst hatches from the zona pellucida nine days after fertilization, depending on the species, it is ready to implant into the uterine wall, however in an *in vitro* environment this is usually the final stage that can be easily studied. The developmental stage from fertilization to formation of a hatched blastocyst is called the pre-implantation embryonic stage (reviewed by Schoenwolf et al., 2009; Senger, 2003).

In preparation for pre-implantation development the oocyte must prepare the cytoplasm during oocyte maturation by accumulating maternal transcripts and proteins which will control early development (Telford et al., 1990; Memile et al., 1998; Wu et al., 2003). There is a period of time starting in late oogenesis where all transcription must be inactivated to
prepare the haploid nucleus for fertilization and development must rely on the pools of maternal mRNA stored in the cytoplasm (Verrotti & Strickland, 1997). This transcriptional silence remains until a major transition occurs when the newly formed embryonic genome becomes transcriptionally active and takes over control of development. This event is called the maternal to zygotic transition (MZT) or the embryonic genome activation (EGA) (Shultz, 2002). This genome activation occurs in the mouse by the 2-cell stage (Shultz, 1993), and in bovine, ovine, and human by the 4- to 8-cell stages (Telford et al., 1990; Nothias et al., 1995). Maternal transcripts ensure protein synthesis prior to embryonic genome activation and gradually degrade during early embryonic development (Paynton & Bachvarova 1994). In cattle the bulk of the embryonic genome is activated at the 4- to 8-cell stages however some embryonic transcription has been detected at the zygote and 2-cell stages (Plante et al., 1994; Memili & First, 2000). It is generally accepted that the embryonic genome activation is a gradual process where genes become active as development requires, however much about this process is still unknown such as the full picture of transcriptionally active genes or the actions of said genes during early embryonic development.

**Embryo development in vitro**

Proper pre-implantation development and survival is reliant on more than just the internal embryonic system. The environment (temperature, gases, metabolites, and more) has a great impact on in vivo embryonic development. In vitro culture systems attempt to mimic these conditions but cannot eliminate stressors such as light exposure and higher oxygen concentrations. Improvement and optimization of culture conditions is an ongoing process and
embryo loss is still common. In human *in vitro* fertilization 50% of embryos produced will arrest development during the first week (Hardy et al., 2001), and implantation occurs in only 25% of embryos transferred to patients (Dawson et al., 1995). In cattle less than half of the embryos produced *in vitro* reach the blastocyst stage (Xu et al., 1992) and furthermore many show issues with implantation after embryo transfer (Betts & King, 2001). While the exact reason(s) for embryo loss is unknown the leading theories are discussed below.

Dead and dying cells displaying the signs of apoptosis, a mechanism during healthy development for removal of unwanted cells, have been observed in both *in vitro* and *in vivo* arrested and healthy pre-implantation embryos and may play a role in embryo loss (Hardy, 1997; Matwee et al., 2000; Kamjoo et al., 2002). The indicators of apoptosis include nuclear membrane blebbing, chromatin condensation, cytoplasmic vacuoles as well as nuclear and cytoplasmic fragmentation. An example of healthy early apoptosis can be seen during blastocyst formation where cells exhibiting poor gap junction communication are excluded between the embryo and zona pellucida (Hardy et al., 1996). Apoptosis is a controlled method of maintaining proper cell population and in the pre-implantation embryo it seems to be developmentally regulated (Betts and King, 2001). However, cell death is increased when embryos are cultured in suboptimal conditions; using the in situ terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect fragmented DNA it was identified that bovine blastocysts cultured *in vitro* showed a higher incidence of apoptotic nuclei than those derived *in vivo* (Gjorret et al., 2003). Evidence also shows that cell death is regulated by survival factors from both the embryo and maternal reproductive tract (Hardy, 1997). These survival factors seem to be most important in the later stages of pre-implantation
development as no morphological or biochemical signs of apoptosis have been observed during the early cleavage stages (Byrne et al., 1999; Matwee et al., 2000, 2001; Betts and King, 2001; Hardy et al., 2001; Gjorret et al., 2003). Significant levels of apoptosis are not seen until the morula stage in humans (Hardy et al., 2001) and the blastocyst stage in mice (Handyside & Hunter, 1986; Brison & Shultz, 1997) and when 2- to 4-cell embryos exposed to agents to either depolarize mitochondria (releasing proteins which initiate the apoptotic cascade) or inhibit protein kinases (which respond to stress for maintenance of the cell) they were able to partially activate the apoptotic pathway showing signs of some caspase activation and limited DNA fragmentation (Matwee et al., 2000; Brad et al., 2007; Gjorret et al., 2007). It seems that the machinery for apoptosis is present but 2- to 4-cell embryos do not elicit this process due to immature mitochondria (Plante & King, 1994; Van Blerkom, 2004) and/or the presence of some form of apoptosis inhibition at this stage of development (Weil et al., 1996; Brad et al., 2007). Therefore the evidence shows that early pre-implantation embryos do not undergo apoptosis and it is not until the later stages where embryo loss from apoptosis is significant. However, it was observed that the level of cell death via apoptosis did not measure up to the high rate of embryo loss in vitro (Hardy et al., 2001), indicating that another form of embryo loss is occurring around the 2- to 4- cell stage. Hardy suggested that each embryo is programmed at the one-cell stage with a unique cell death/arrest rate based on internal factors. Zygotes with low cell death/arrest rates will survive and high cell death/arrest rates will not. Zygotes with an intermediate cell death/arrest rate will have the most variability and will mostly survive under optimal conditions but die or arrest under suboptimal conditions. If this model is true and developmental potential is determined at the one-cell stage then the most likely causes of
Embryo death or arrest would include chromosomal abnormalities (Hardy, 1999), which are seen in almost half of all arrested human embryos (Almeida & Bolton, 1998) and more prevalent in bovine arrested embryos produced in vitro than in vivo (Kawarsky et al., 1996; Viuff et al., 1999) or inadequate oocyte maturation which has been shown to be linked to embryonic failure in humans (Moor et al., 1998). Whether or not the fate of embryonic survival is decided at the one-cell stage is yet to be determined, but it has been observed that these early pre-implantation embryos do not follow the typical apoptotic pathway and seem to enter a permanent cell cycle arrest state (Favetta et al., 2004a,b), which is very similar to cellular senescence (Betts & Madan, 2008). Replicative senescence is a process where most proliferating somatic cell types stop dividing permanently after a certain number of population doublings (Hayflick & Moorhead, 1961). This process is thought to be a response to DNA replication stress and/or telomere disruption which both involve the p53 DNA damage signalling pathway (Bartek et al., 2007). Permanent replication arrest of cultured bovine somatic cells is affected by telomere shortening (Betts et al., 2008), increased levels of serine 20-p53 phosphorylation, and increased levels of oxidative damage (Favetta et al., 2004a). It is possible that mammalian embryos arrested at the 2- to 4-cell stage have been affected in a similar way (Betts & Madan, 2008).

Reactive oxygen species (ROS) have been shown to induce apoptosis (Stone and Yang, 2006), and cellular senescence (Passos et al., 2007) in somatic cells. Yang et al., 1998 confirmed a direct relationship between hydrogen peroxide (H₂O₂) concentration in human embryos and apoptosis by analyzing embryo fragmentation. ROS are by-products of embryo metabolism and the culture environment. The two main oxygen free radicals generated by embryos are the
superoxide anion ($O_2^-$) and the hydroxyl radical (OH*) with the intermediate being H$_2$O$_2$
(Guerin et al., 2001). It has been demonstrated that oxidative stress can damage cells through
lipid peroxidation (Nasr-Esfahani et al., 1990; Nasr-Esfahani and Johnson, 1992), protein
oxidation, and DNA strand breaks (Guerin et al., 2001; Orsi and Leese, 2001). However, low
oxygen culture conditions and antioxidant treatment has been shown to extend cell survival in
culture (Packer & Fuehr, 1997; Poot, 1991). Culturing embryos in reduced oxygen conditions
(5% rather than atmospheric 20%) has been shown to aid development to the blastocyst stage
(Batt et al., 1991; Nagao et al., 1994; Gardner and Lane, 1996 ) and is thought to be the result of
reduced levels of reactive oxygen species and therefore reduced oxidative stress (Guerin et al.,
2001). When cultured in 20% instead of 5% oxygen conditions the frequency of embryos
arrested at the 2- to 4- cell stage doubles (Favetta et al., 2007b). High levels of p66$^{shc}$ (a protein
known to help deal with oxidative stress) and intracellular ROS were found in permanently
arrested early embryos (Favetta et al., 2004b). This study also found that these arrested
embryos did not show any morphological or biochemical signs of apoptosis, suggesting that
oxidative stress levels influence the arrest of early embryo development through a gene
expression pathway. Other studies have also shown that bovine blastocysts alter gene
expression in response to different oxygen culture concentrations (Harvey et al., 2004).
Embryo loss seems to occur mostly via apoptosis and cell death or developmental arrest. Many
factors for both have been identified but it remains unclear exactly why one or the other
happens. There seems to be three outcomes for embryo development in vitro: developing
embryos will arrest development usually at the 2- to 4-cell stage, die via apoptosis, or survive to
the blastocyst stage.
Gonad development

Following implantation the embryo undergoes massive changes: Cell layers are formed giving rise to different body tissues, large cell migrations and invaginations form body axes, and development of physiological systems including the reproductive organs. The process of sexual development from determination to differentiation is outlined below.

Mammalian sex determination is controlled by the two sex chromosomes, X and Y. The process of male sexual differentiation is initiated by the Y chromosome to produce gonads which then propagate the rest of development by endocrine mechanisms. Normal females have two X chromosomes and normal males have one X chromosome and one Y chromosome. In this method of sex determination the haploid oocyte will always contribute one X chromosome and the haploid spermatozoa will contribute either one X chromosome or one Y chromosome. Zygotes with a normal Y chromosome will develop as male regardless of the number of X chromosomes (Jacobs & Strong, 1959; Cattanach, 1961) while zygotes containing no Y chromosome and at least one healthy X chromosome will develop as female (Ford et al., 1959; Welshons & Russell, 1959). Therefore the default sex in mammalian development is female and only in the presence of a Y chromosome does a male develop. Gonad formation is initiated during week 5 of gestation with the migration of primordial germ cells from the yolk sac to an area on the posterior body wall close to the developing kidney to form a pair of genital ridges. These genital ridges are thought to be sexually indifferent at this time (Gillman, 1948; Jost et al., 1981). During week 6 of gestation somatic support cells aggregate within these ridges to prevent the degradation of the germ cells. Also at this time the mullerian ducts begin to form. The initiation of sexual differentiation begins with the transcription of the sex-determining
region of the Y chromosome (SRY). The SRY protein causes the somatic support cells to differentiate into Sertoli cells which envelope the germ cells and secrete anti-mullerian hormone (AMH). Without the actions of SRY the somatic support cells will differentiate into ovarian follicle cells. Signals from the Sertoli cells cause migration of mesenchymal cells to the genital ridge which differentiate into Leydig cells and secrete testosterone. Around week 7 of gestation in humans androgen receptors are expressed and are required for the development of the epididymis, vas deferens, and seminal vesicle in the male, and the oviduct, cervix, and upper vagina in the female (Rey and Picard 1998). Testosterone drives the development of the male phenotype during fetal life and again at puberty. In the developing female, lack of SRY and therefore no Sertoli cells to secrete AMH or Leydig cells to secrete testosterone results in continued development as female. While female development may be referred to as the default developmental pathway it is still an active and complex process. Hormonal processes control sexual development to form a mature adult where sexual dimorphism is obvious. However, sexual dimorphism has also been observed in early development before the formation of the gonads, and is therefore not the result of gonadal hormone action.

**Sexual dimorphism in embryo**

Sexual dimorphism in early development has been observed as weight differences in day 12 rat embryos (Scott and Holsen, 1997), body size and head diameter in first trimester of human fetuses (Pedersen, 1980), gonad volume on day 13.5 in the rat (Mittwoch et al., 1969), higher number of somites in day 9 male mice embryos (Seller and Perkins-Cole, 1987). Evidence like this suggests that pre-gonadal sexual dimorphism is the result of genetic mechanisms and
lead to the examination of potential sexual differences in the early embryo. Embryos developing *in vitro* are not identical and develop at different rates. Mice embryos were separated based on the time of blastocoel formation into groups with each group representing a different developmental rate. These embryos were then transferred into the oviduct to allow live births. It was observed that the fast cleaving embryos had a higher percentage of males and the slow cleaving embryos had a higher percentage of females (Tsundo et al., 1985). The same was observed in bovine embryos (Avery et al., 1989; Avery et al., 1991; Xu et al., 1992) ovine embryos (Bernardi & Delouis, 1996; Catt et al., 1997), and human embryos (Pergament et al., 1994). This phenomenon of advanced male development is an example of sexual dimorphism in the first week of development.

Glucose is also an area of study when it comes to sexual dimorphism in the embryo. When bovine embryos were cultured *in vitro* it was found that the presence of glucose appears to inhibit the development of female embryos more than that of male embryos (Gutierrez-Adan et al., 2001). Similarly, studies on mice for human diabetes research found that an increase in circulating glucose resulted in a male-biased sex ratio (Machado et al., 2001), and human mothers with insulin dependency (and therefore low glucose availability) show a sex ratio biased toward daughters (Rjasanowski et al., 1998).

**Sex ratios**

When examining sex as a group it is possible to determine the sex ratio. The expected sex ratio is 50% male and 50% female, or a 1:1 ratio, and any deviation from this is considered a
skew in sex ratio. Sex is determined when the male and female gamete union forms a new genome, either XX or XY.

It is thought that X-bearing sperm and Y-bearing sperm are present in equal proportions and have an equal chance at fertilization; however some evidence shows that Y-bearing sperm populations vary at a range of 37% to 58% (Checa. 2002). Furthermore, during the long swim to the oocyte X- and Y-bearing sperm can be selected for or against (Grant & Irwin, 2005) indicating that sex determination may not be a random process.

An often cited hypothesis for sex ratio changes is the Trivers-Willard hypothesis (TWH) which theorized that mothers in good living conditions would have the advantage of producing more offspring with the most reproductive success and mothers in poor living conditions would have the advantage of producing more offspring with reproductive stability (Trivers & Willard, 1973). In a polygynous species males show more reproductive success with the ability to father more offspring then even the most successful female who is limited to one or two offspring a year. However, female offspring have more reproductive stability by producing at least a few offspring even in poor conditions, whereas unsuccessful males in polygynous species may never breed. This hypothesis has been cited by hundreds of papers with significant results both for and against. A meta-analysis was performed using papers that referenced TWH or ‘sex ratio’ and focused on mammals and concluded that sex-ratio adjustment occurs at or near implantation (Cameron, 2004).

Interestingly it was found that bovine embryos show sexual dimorphism in the expression of interferon-tau (INF-t) (a secretory product of the cattle, sheep, and deer
blastocyst which is responsible for maternal recognition of pregnancy (Bazer et al., 1997))
alerting mothers of embryo sex early in development (Larson et al., 2001). This is one potential
mechanism for early embryo selection and sex ratio adjustment.

Sex ratios were examined in dairy cows comparing in vitro produced embryos (IVP),
artificial insemination (AI), and natural breeding. Compared to the 1:1 sex ratio it was found
that IVP increased the male offspring amount to 76%, while there was no difference from 1:1 in
AI and natural breeding (Camargo et al., 2010).

Mittwoch (1989) proposed that sexual dimorphism might be initiated at the time of
fertilization. At a point this early in development prior to gonad formation all differences must
be controlled by genetics, however it is not known whether the advanced developmental rates
seen in male embryos is due to the actions of the Y chromosome or to the single state of the X
chromosome, or both factors combined (Mittwoch, 1989).

**X-chromosome vs. Y-chromosome actions on development**

It has been proposed that the presence of two X chromosomes may have a retarding
affect on female pre-implantation embryos. It has been seen that total glucose uptake tends to
be higher in female embryos (Gardner & Leese, 1987), as well as the activity of the pentose
phosphate pathway (PPP) (Tiffin et al., 1991). The first step of the PPP is catalyzed by the
enzyme glucose-6-phosphate dehydrogenase (G6PD) which is encoded by a gene on the X
chromosome (Chapman & Shows, 1976). There would therefore be two copies of this gene in
female cells but only one in male cells. Early in development one of the X chromosomes in
females is inactivated (Lyon, 1961) thereby eliminating the effect of double X gene actions.
However there is a time during early embryonic development where the embryonic genome is activated but X chromosome inactivation has yet to occur and potentially both X chromosomes are active giving female embryos a double dose of several X-linked enzymes (Epstein et al., 1978). It was demonstrated that G6PD transcripts were increased 2-fold in in vitro produced female embryos compared with males, indicating that in female embryos both X chromosomes were active (Gutierrez-Adan et al., 2000; Lonergan et al., 2000; Wrenzycki et al., 2002).

Furthermore when different glucose levels were examined in culture it was found that a high glucose level affects embryo development, especially in females, showing arrested development at morula to blastocyst stage (Gutierrez-Adan et al., 2001). Media high in glucose produced more male blastocysts while media lacking glucose produced equal numbers of male and female blastocysts (Gutierrez-Adan et al., 2001). Given the negative effects of glucose on female embryo development it is possible that the presence of two active X chromosomes leads to imbalances in carbohydrate metabolism and toxicity that is female specific (Gutierrez-Adan et al., 2001). However, in cattle it was found that regardless of the presence or absence of glucose, male embryos develop faster than female embryos during the pre-implantation stage (Gutierrez-Adan et al., 2001). While glucose has been shown to affect development of embryos in vitro, especially females, it may not be the reason for advanced male embryo development. A study done on mice by Burgoyne in 1993 demonstrated that the advanced development of male embryos is due to the presence of the Y chromosome. He crossed several mice strains to compare healthy female embryos with healthy male embryos and male embryos with a large section of their Y long arm deleted to remove over half of the Y-specific genes. When he compared healthy male and female embryo development the male embryos showed a clear
difference in developmental rates first demonstrated by Tsundo and colleagues (1985). However, when he compared male Y deleted embryos to healthy female embryos there was no observed difference in development. It was concluded that the Y chromosome is responsible for the increased rate of male embryo development.

Three theories as to how the Y chromosome is impacting development are discussed here. The first theory states there may be a possibility of earlier fertilization by Y-bearing sperm than X-bearing sperm. Burgoyne compared early development rates of XO and OY embryos and compared them to Tsundo’s findings and saw that XX-XY differences are not present at fertilization but increase as development progresses. This is supported by findings from Avery et al., (1991) and Xu et al., (1992) where males showed advanced development despite fertilization occurring in vitro at the same time. The second theory is that with reduced DNA content of the Y, male embryos may be able to replicate their entire DNA faster and therefore advance through cleavage faster. However, even with a large deletion of the Y chromosome male embryos did not show advanced development but actually the opposite (Burgouyne, 1993). The final, and currently leading, theory is that there must be some kind of Y gene(s) expressed by the Y chromosome that speeds up the rate of cell division (Burgouyne, 1993).

The Y chromosome

The Y chromosome is the smallest and least understood chromosome representing 2-3% of the human haploid genome (Singh et al., 2005). The Y chromosome along with the X chromosome is thought to have originated from an autosomal pair of chromosomes among reptiles around 300 million years ago, before mammals evolved (Foster & Graves, 1994). The Y
chromosome has a short arm (Yp) which is euchromatic and a long arm (Yq) where the proximal portion is euchromatic and the distal portion is heterochromatic. At either end of the Y chromosome there is a small pseudoautosomal region which has sequences identical to the X chromosome allowing pairing during meiosis. The rest of the Y chromosome is part of the non-recombining region or male specific region (MSR) made up of sequences either homologous to regions on the X chromosome or Y specific. Determining the functions of Y chromosomal genes is difficult because of its non-recombining nature (Jobling & Smith, 2003). The only way to fully know the action(s) of a gene is to observe the result when it is deleted, and since single gene deletions are rare, the Y chromosome still remains mysterious (Singh et al., 2005).

**Short arm genes**

The Yp is a well studied region of the Y chromosome when it comes to the male phenotype as this is where the testis determining factor (TDF) is located (Vergnaud et al., 1986). The TDF is a gene located on the Y chromosome that initiates the process of male sexual differentiation beginning with the formation of the testes from the undifferentiated genital ridges (Goodfellow & Darling, 1988). Through analysis of XX males and XY females the position of the TDF was localized to the distal Yp region (Vergnaud et al., 1986). An early candidate for the TDF was the zinc finger protein, Y-linked gene (ZFY) because it is located in the distal Yp, it is expressed in testis tissue, and it encodes a transcription factor with multiple zinc-finger motifs (Page et al., 1987; Mardon & Page, 1989).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Location on Y Chromosome</th>
<th>Gene Function</th>
<th>Gene Function in the embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRY</td>
<td>Short arm</td>
<td>Transcription factor – testis formation (TDF)</td>
<td>Unknown</td>
</tr>
<tr>
<td>ZFY</td>
<td>Short arm</td>
<td>Transcription factor- testis development</td>
<td>Unknown</td>
</tr>
<tr>
<td>TSPY</td>
<td>Long arm</td>
<td>Suggested role in spermatogenesis</td>
<td>Unknown</td>
</tr>
<tr>
<td>USP9Y</td>
<td>Long arm</td>
<td>Enzyme- spermatogenesis</td>
<td>Unknown</td>
</tr>
<tr>
<td>DDX3Y</td>
<td>Long arm</td>
<td>RNA metabolism-spermatogenesis</td>
<td>Unknown</td>
</tr>
<tr>
<td>HSFY</td>
<td>Long arm</td>
<td>Transcription factor – spermatogenesis</td>
<td>Unknown</td>
</tr>
<tr>
<td>EIF1AY</td>
<td>Long arm</td>
<td>Translation initiation factor – spermatogenesis</td>
<td>Unknown</td>
</tr>
<tr>
<td>ZRSR2Y</td>
<td>Long arm</td>
<td>Suggested RNA and nucleotide binding</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Figure 1. Diagram of the protein coding genes on the Y chromosome (left) (modified from Jobling & Tyler-Smith, 2003), and functions of the Y linked genes (right).

However further investigation and localization of the smallest amount of the TDF region revealed the absence of ZFY (Palmer et al., 1989). This led to the recognition of a new transcription factor gene 35 kb proximal to the pseudoautosomal boundary called sex-determining region Y (SRY) (Sinclair, et al., 1990). Of 50 consecutive sequences analyzed in the search for the TDF only one was found to be present in all XX males and conserved in all eutherian mammals tested, its homologue in the mouse was found in minimum portion of the Y chromosome known to contain the TDF, and its transcripts were detected in the embryonic
gonad during Sertoli cell differentiation making SRY the best candidate for the TDF (Gubbay et al., 1990). Although ZFY is not the TDF it still has a role in male development. ZFY is a single copy gene with a ubiquitous expression pattern encoding a zinc finger-containing protein that functions as a transcription factor and a deletion of this gene will result in sex reversal (Jobling & Tyler-Smith, 2003).

**Long arm genes**

Much like the search for the TDF (a single gene responsible for initiating the male phenotype) another search is currently underway on the long arm for the factor responsible for male fertility. It may be a single gene responsible for initiating spermatogenesis or a group of genes imperative for spermatogenesis to function. This search began with the discovery that deletions of genes on the Yq is associated with spermatogenic failure (Tiepolo & Zuffardi, 1976), and are the most common mutations in infertile males, occurring in 10-15% of male infertility cases (Foresta et al., 2001). Tiepolo & Zuffardi were the first to link Y gene deletions with male infertility giving rise to the three azoospermia factors AZFa, AZFb, and AZFc. Deletions of any one of these regions affects spermatogenesis and causes oligospermia (semen with a low concentration of sperm) and/or azoospermia (semen with no measurable sperm) resulting in male infertility (Tiepolo & Zuffardi, 1976; Ma et al., 1992; Reijo et al., 1995; Vogt et al., 1996).

The AZFa region contains single copy genes with homology to the X chromosome (Vogt et al., 1996; Qureshi et al., 1996; Pryor et al., 1997). The first gene discovered in this region was ubiquitin specific peptidase 9, Y-Linked (USP9Y), previously named Drosophila fat facets related
Y (*DFFRY*) (Foresta et al., 2001). It shows ubiquitous expression and encodes a C-terminal ubiquitin hydrolase (Lahn & Page, 1997; Brown et al., 1998). *USP9Y* has a role in spermatogenesis and deletions result in azoospermia or severe oligospermia (Sun et al., 1999; Brown et al., 1998). The majority of infertile males show deletions in the AZFa region, however *USP9Y* is not the only gene in this region and it has been proposed that it works together with another gene (Huynh et al., 2002). Another major gene involved in spermatogenesis from this region is DEAD-box polypeptide 3, Y linked (DDX3Y also called DBY); it shows specific expression in testis and encodes a DEAD box RNA helicase (Lahn & Page, 1997; Foresta et al., 2000). This gene is more frequently deleted than *USP9Y* and it has been suggested that deletions of *USP9Y* resulted in male infertility and further deletions of DDX3Y manifested this condition (Foresta et al., 2000). However the exact function of these genes still remains unknown.

The second of the azoospermia factors is the AZFb and a deletion in this region is most often associated with azoospermia (Vogt et al., 1996). This region contains the eukaryotic translation initiation factor 1A, Y–chromosomal (*EIF1AY*). This single copy gene encodes a Y isoform of eif-1A, a ubiquitously expressed translation initiation factor; its X homolog encodes an essential initiation factor and shows 97% amino acid sequence identity with *EIF1AY* (Lahn & Page, 1997). Its exact function in spermatogenesis will not be completely understood until a specific deletion of this gene is reported. Like the AZFa, genes of this region may act together in spermatogenesis. Another gene in this region is the heat shock transcription factor, Y chromosome (*HSFY*). This gene has three transcripts which are differentially expressed in testis (Tessari et al., 2004). It belongs to the heat shock factor family of transcription factors that has been shown to have a role in spermatogenesis (Neuer et al., 2000). The three proteins of *HSFY*
may have different roles suggested by the absence of DNA-binding domains in transcripts 2 and 3 which suggest they do not act as transcription factors (Tessari et al., 2004).

There is a gene family located on the Yq but not within one of the AZF regions. Testis specific protein Y-encoded (TSPY) is a gene family located immediately adjacent to the centromere on the Yq whose expression is localized to the testis (Arnemann et al., 1987). This gene has the highest copy number at 50-200 copies in cattle with copy number variation both within and between breeds (Hamilton et al., 2009). Its suggested role is in spermatogenesis and the proliferation of germ cells (Schnieders et al., 1996; Chai et al., 2001; Ozbun et al., 2001; Vogel et al., 1998), and fertility (Hamilton et al., 2012).

The Y chromosome is the only chromosome that remains unsequenced in the cattle genome project (Elsik et al., 2009) and as a result new genes are still being discovered. The Y-linked zinc finger gene (ZRSR2Y) was not identified until 2010 by Bermejo-Alvarez and colleagues. This gene is located in the non-recombining region of the Y-chromosome and may have a function in sex determination since its X-homolog ZRSR2 encodes an essential splicing factor (Tronchere et al. 1997) however the exact function of this gene is unknown.

The Y chromosome is unlike any other chromosome since it itself is not essential for life of an individual. Furthermore the inability to fully identify specific gene actions makes it mysterious. It was generally accepted that the Y chromosome had two functions: To create a male by first creating testis and to create a fertile male through spermatogenesis. Therefore the Y chromosome was expected to be silent until the formation of the testis during the 6th week of development. However that is not the case.
Y chromosome expression in embryos

A study done on mice pre-implantation embryos found that Sry and Zfy are expressed from the 2-cell stage and on (Zwingman et al., 1993). Shortly after it was discovered that human embryos are also positive for SRY mRNA as early as the one-cell stage and on to the blastocyst stage (Fiddler et al., 1995). Since any Y chromosome activity must come from the embryonic genome this raised some concerns as Fiddler reported embryonic genome transcription prior to the accepted time for embryonic genome activation at the 4- to 8-cell stages (Telford et al., 1990; Nothias et al., 1995). To confirm these findings Fiddler and colleagues also analysed sperm and oocytes for SRY mRNA and found all were negative. This study showed that not only are Y-linked genes expressed in the pre-implantation embryo, they may not follow a typical pre-implantation expression pattern. SRY expression has been confirmed in bovine pre-implantation embryos from the 4- to 8-cell stages through to the blastocyst (Gutierrez-Adan et al., 1997). Studies have shown ZFY expression in pre-implantation embryos of murine (Zwingman et al., 1993), human (Ao et al., 1994), ovine (Bernardi et al., 1996), and bovine (Peippo et al., 2002). These two genes (ZFY and SRY) were popular Y chromosome genes to study as they were/are both candidates for the testis determining factor, and it wasn’t until recently that other Y-linked genes were analysed in the pre-implantation embryo. YZRSR2 and DDX3Y were found to be expressed in bovine blastocysts (Bermejo-Alvarez et al., 2010).

It is now known that there is expression of Y-linked genes in the pre-implantation embryo. Many of these genes act as transcription factors for either the development of testis or spermatogenesis but it is obvious that those actions are not happening within the early
embryo. In fact it is currently unknown if these genes have any effect on embryonic development, or even if translation is occurring. However there is a method to determine if the transcripts are impacting early development introduced 15 years ago.

**RNA interference**

The first injection of double stranded RNA (dsRNA) into tissue led to post transcriptional silencing of genes with homology to the delivered dsRNA (Fire et al., 1998; Hamilton & Baulcombe, 1999). This lead to a new method of studying gene knock out which allowed selective post transcriptional silencing and proved to be a better method than alternative gene knockouts or antisense technology by working more often and showing a more successful decrease in gene expression (Bass, 2001). This new method of dsRNA mediated post transcriptional gene silencing was given the name RNA interference (RNAi). The use of RNAi was first demonstrated in plants but has since been used successfully in Drosophila (Kennerdell & Carthew, 2000), mammalian tissues (Elbashir et al., 2001), and the mouse oocyte (Stein et al., 2003), as well as several bovine embryo experiments mentioned below.

The process has two steps: The initiation step where dsRNA is cleaved into small-interfering RNAs (siRNA) of about 21-23 nucleotides by an RNase-III-like dsRNA-specific ribonuclease called DICER. The effector step is where the double stranded siRNAs are unwound and incorporated into the RNA-induced silencing complex (RISC). This activated RISC uses a single stranded siRNA as a template to identify complementary RNA where an endoribonuclease will cleave the target to be degraded by exoribonucleases (Cerutti, 2003).
The use of siRNA and microinjections has been used in bovine embryos to demonstrate the importance of several factors during development. It has been shown that the JY-1 gene encodes an oocyte specific protein with important regulatory roles in granulosa cell function (Bettegowda et al., 2007). It has also been demonstrated that follistatin (a safeguard against uncontrolled cellular proliferation) is present in oocytes at the time of fertilization and influences cleavage timing and survival to blastocysts (Lee et al., 2009). This method has also shown that p66shc (a protein known to help deal with oxidative stress) is involved in the regulation of early cleavage, embryo arrest and development to the blastocyst stage. (Favetta et al., 2007a). This technique of siRNA gene silencing is an effective approach aimed at studying protein action, and may lead to the understanding of any gene function during embryo development.
Rationale

Over half of embryos fertilized in vitro do not survive to the blastocyst stage. Embryo loss in vitro is usually associated with sub-optimal culture conditions. Oxidative stress results in failure of embryo development through apoptosis and embryo arrest (Hardy, 1997; Betts & King, 2001; Favetta et al., 2007b). Developmental arrest seen in early pre-implantation embryos may be the result of stress from the in vitro environment where embryos less able to cope with this stress enter a senescence like state. The advanced development of male embryos (Tsundo et al., 1985; Avery et al., 1989; Avery et al., 1991; Xu et al., 1992; Bernardi & Delouis, 1996; Catt et al., 1997; Pergament et al., 1994), the ability to survive better in vitro when cultured under glucose stress (Gutierrez-Adan et al., 2001; Machado et al., 2001; Rjasanowski et al., 1998), and the higher survival rates in vitro (Camargo et al., 2010) indicate that something unique to male embryos is aiding development in vitro.

It has been observed that the Y chromosome is active much earlier than expected showing expression of some genes during pre-implantation embryo development across several species (Zwingman et al., 1993; Fiddler et al., 1995; Gutierrez-Adan et al., 1997; Ao et al., 1994; Bernardi et al., 1996; Peippo et al., 2002; Bermejo-Alvarez et al., 2010). The Y chromosome is active in development during the 6th week of gestation (reviewed by Schoenwolf et al., 2009; Senger, 2003), however shows expression during the 1st week of gestation with an unknown impact. These genes have shown involvement in either testis formation or spermatogenesis but the complete function of these genes is unknown.
The hypothesis for this thesis is that Y-linked genes are expressed by the pre-implantation bovine embryo and are important for development. This hypothesis will be examined by research with the following objectives:

**Objective 1:** Determine the expression of eight Y chromosome linked genes (*DDX3Y, EIF1AY, HSFY, SRY, TSPY, USP9Y, ZFY, and ZRSR2Y*) in pre-implantation bovine blastocysts and determine the expression of two of these genes (*USP9Y* and *ZFY*) in healthy pre-implantation bovine embryos from the 2-cell stage to blastocyst using reverse transcriptase polymerase chain reaction.

**Objective 2:** Determine the expression of two Y chromosome linked genes (*USP9Y* and *ZFY*) and the sex ratio in pre-implantation bovine embryos that have arrested development at the 2- to 8-cell stage using reverse transcriptase polymerase chain reaction.

**Objective 3:** Determine if *USP9Y* has an effect on male pre-implantation bovine embryo development by silencing its transcripts using RNA interference.

Analyzing a large group of Y chromosome genes will establish the basis for this thesis by confirming the expression of some Y linked genes already known to be expressed in bovine pre-implantation embryos [*SRY* (Gutiérrez-Adán et al., 1997), *ZFY* (Peippo et al., 2002), *ZRSR2Y* (Bermejo-Alvarez et al., 2010), *DDX3Y* (Bermejo-Alvarez et al., 2010)] and investigating new genes (*EIF1AY, TSPY, USP9Y, HSFY*) with unknown expression in the pre-implantation bovine embryo. This will aid in understanding how active the Y chromosome is at this stage of development. Determining when *USP9Y* and *ZFY* become expressed will allow a better understanding of when these genes begin to impact male development and allow comparison
to arrested embryos. Analyzing arrested embryos will give an initial indication as to how important these genes are for development and developmental arrest.

The final objective will determine the importance of *USP9Y*, and therefore the Y chromosome, in developing pre-implantation bovine embryos by establishing if survival *in vitro* is possible without or with reduced levels of transcripts of this gene.
Materials and Methods

*In vitro* embryo production and embryo harvesting

*In vitro* maturation (IVM) the process of maturing oocytes, *in vitro* fertilization (IVF) the process of introducing live sperm cells to the oocytes, and *in vitro* culture (IVC) the process of allowing the presumptive zygotes to grow, were accomplished as described previously (Ashkar et al., 2010). In brief, ovaries were collected from slaughtered cows (Cargill Meat Solutions, Guelph, ON, Canada) and cumulus-oocyte complexes (COC) were aspirated from visible ovarian follicles into HEPES-buffered Ham’s F-10 plus 2% steer serum (Cansera, Rexdale, ON, Canada). Oocytes were selected based on morphology and those with layered cumulus cells and homogenous cytoplasm were chosen to be matured in groups of 15 in 80 μL drops of TCM199 medium + 2% steer serum supplemented with 1 μg/mL of estradiol, 0.5 μg/mL of bovine FSH (bFSH), and 1 μg/mL of bovine LH (bLH) (NIH, Washington DC, USA) covered in silicone oil (Paisley Products, Scarborough, ON, Canada) at 5% CO₂ and 38.5°C for 22 to 23 hours. Sperm was co-incubated in 80 μL drops of IVF Tyrode’s albumin lactate pyruvate (TALP) under silicone oil for 18 h in an atmosphere of 5% CO₂ at 38.5 °C. The frozen-thawed sperm was prepared by a swim-up method in sperm TALP as previously described (Ashkar et al., 2010) and oocytes were inseminated with $1 \times 10^6$ motile frozen-thawed sperm/mL (Gencor, Guelph, ON, Canada). Presumptive zygotes were stripped of remaining cumulus cells via vortex after 18 to 20 h post-insemination and cultured in groups of 30 in 30 μL drops of culture medium previously described (Ashkar et al., 2010). Embryo quality was assessed according to IETS standards (IETS Manual, 1998). Two-cell embryos were harvested two days post-insemination and grouped into two pools of 30, 4-cell embryos were harvested three days post-insemination and grouped into
two pools of 30, 8-cell embryos were harvested four days post-insemination and grouped into
two pools of 30, 16-cell embryos were harvested five days post-insemination and grouped into
two pools of 30, morula were harvested six days post-insemination and grouped into two pools
of 30, expanded blastocysts were harvested eight days post-insemination and grouped into
three pools of 10, one pool of 20, and 50 were harvested individually. Arrested embryos, those
which remained at 2-cell, 4-cell, or 8-cell stage eight days post-insemination were harvested in
two pools of 30 for each stage (2-cell, 4-cell, 8-cell) or harvested individually for sexing. To
eliminate false positives due to residual sperm attached to the zona pellucida during embryo
sexing, embryos were washed in PBS containing 0.1% polyvinyl alcohol and exposed to 0.2%
pronase (Pronase E, Sigma-Aldrich, St. Louis, MO, USA) for two to three minutes at room
temperature to digest the zona pellucida and then were washed three times in PBS.
Immediately after harvesting all embryos were snap-frozen in liquid nitrogen and stored at -80°C.

RNA extraction and reverse transcription

Messenger RNA was extracted from embryo pools (groups of 10, 20 and 30) as well as
individual embryos using the Arcturus PicoPure RNA Isolation Kit (Life Technologies, Burlington,
ON, Canada) and treated with DNase I (Qiagen, Inc., Mississauga, ON, Canada) according to the
manufacturer’s instructions to eliminate any residual DNA. Messenger RNA was also isolated
from male testis and female kidney tissues obtained from slaughtered animals (Semex Alliance,
Guelph, ON, Canada). 30 mg of each tissue was homogenized with a Powergen 700
homogenizer (Fisher Scientific, Ottawa, ON, Canada). Using an RNeasy Mini Kit (Qiagen, Inc.)
total RNA was extracted from samples and treated with DNase I (TURBO DNA-free, Ambion, Inc., Austin, TX, USA) according to the manufacturers' instructions. The RNA was then stored at −80°C until reverse transcription. All reverse transcription reactions were carried out using Superscript II reverse transcriptase, Oligo(dT)$^{12-18}$ primers (Life Technologies, Burlington, ON, Canada) and Rnasin ribonuclease inhibitor (Promega, Madison, WI, USA) according to the following protocol: The Oligo(dT)$^{12-18}$ primers were added to the RNA samples to anneal by denaturing at 72°C for 2 min. A mixture of 4 μL RT buffer, 2 μL 0.1M dithiothreitol, 1 μL 10mM dNTP mix, 0.5 μL Rnasin (40 U/μL) and 1 μL of Superscript II (200 U/μL) was added and incubated at 42°C for 1 hour. The reaction was completed with denaturing at 70°C for 30 minutes. The cDNA was stored at -20°C until it was used.

Gene expression analysis

The following genes were analysed for expression in bovine embryos: DDX3Y, EIF1AY, HSFY, SRY, TSPY, USP9Y, ZFY, and ZRSR2Y. The X-homologous genes, DDX3X, EIF1AX, TSPYL2, USP9X, ZFX, and ZRSR2 were also analysed to confirm their expression in the male testis and female kidney controls, and that a negative signal for the female sample indicated that the Y-linked primers were not amplifying these X-homologs unintentionally. Testicular tissue was used as the positive control because most of the Y-linked genes have shown previous expression in bovine testis tissue except for ZRSR2Y which has not been characterized yet (Hamilton et al., 2011; Liu et al., 2009; Brown, et al., 1998; Lahn & Page, 1997; Palmer et al., 1990; Daneau et al., 1995; Vogel T et al., 1997; Jakubiczka et al., 1993). The gene GAPDH was used as a positive control as it has previously been shown to be expressed in bovine embryos.
(Lequarre et al., 1997). Two primer sets were designed for each male-specific gene and used in the analysis. Whenever possible, at least one of the primers spanned an intron to avoid residual DNA amplification. The primers were designed with Primer3 software version 4.0 (Rozen & Skaletsky, 2000) unless otherwise indicated under the “Source” column of appendix I. Because TSPY and GAPDH were used for both the expression analysis and the DNA-based sexing experiments, the primers that were used for amplifying its expression were labeled as TSPY RNA and GAPDHRNA, and the primers for DNA-based sexing were labelled TSPYDNA and GAPDHDNA. All primers used in the expression analyses for genes with X-homologs were designed to target regions of the Y-linked genes that have sequence mismatches with their X-homologs to ensure that only the Y-linked genes were being amplified. Sequence alignments of the Y-linked genes and their X-homologs were compared using ClustalW2 Multiple Sequence Alignment (Thompson et al., 1994). The techniques used for gene expression analysis were reverse transcription polymerase chain reaction (RT-PCR) and reverse transcriptase quantitative PCR (RT-qPCR). The embryo pools (groups of 10 and 20 for blastocysts and groups of 30 for earlier stages) were analysed using RT-PCR and the individual blastocysts were analysed using RT-PCR and RT-qPCR. For the pooled embryos each RT-PCR reaction used one-tenth of the isolated RNA (2 μL cDNA from a total of 20 μL), 1 μL 1mg/μL forward/reverse primer, 5 μL GeneAmp 10× PCR Buffer II (Life Technologies, Burlington, ON, Canada), 4 μL 25mM MgCl₂, 1 μL 10mM dNTP Mix (Life Technologies, Burlington, ON) and 0.5 μL Amplitaq gold (Life Technologies, Burlington, ON, Canada). All of the RT-PCR reactions were carried out on an MJ Research PTC-200 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following protocol: denaturation at 95°C for 10 minutes, followed by 40-45 cycles of denaturation at 94°C
for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, followed by a final elongation at 72°C for 10 minutes. All RT-PCR products were visualized with gel electrophoresis (15 μL product, 2% agarose gel, 100 volts for 25 minutes), excised with QIAquick Gel Extraction kit (Qiagen, Inc.) and sequenced to ensure primer specificity (University of Guelph Laboratory Services, Guelph, ON, Canada). For the individual embryos, each RT-qPCR reaction included 1/20 of the isolated RNA and was carried out as described previously (Hamilton et al., 2009). Briefly, each RT-qPCR reaction was performed in a LightCycler 1.5 apparatus using a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mississauga, ON, Canada) and 3 mM of MgCl₂ and 0.5-μM primer were used for each reaction in a total volume of 10 μL. The RT-qPCR program included a denaturation step for 10 min at 95 °C, followed by 50 cycles of denaturation at 95 °C for 10 sec (20 °C/sec); annealing at 66 °C for 10 sec (20 °C/sec) and an elongation step at 72 °C for 10 sec (2 °C/sec). A melting curve analysis began at 72 °C with measurements being taken every 0.1 °C until 95 °C. Only embryos which had a quantification cycle (Cₚ) value for GAPDH of 30 or less were included in this study to ensure that a relatively high level of RNA was isolated from the embryo. This restriction was included to decrease the potential for false negatives in our analysis (i.e., the gene transcript is present, but not in detectable amounts).

Embryo sexing

A total of 32 of the individually harvested blastocysts were used for DNA-based sexing. The snap-frozen embryo material was divided into two parts, 0.5 μL underwent cell lysis, and the rest of the embryo/PBS mixture underwent RNA extraction and reverse transcription as
described above. A lysis buffer of (7.8 μL nuclease-free water, 2 mM MgCl₂, 1 μL PCR buffer [GeneAmp 10× PCR buffer II, Applied Biosystems, Canada] and 2 μg protein kinase [Fisher Scientific, Ottawa, ON, Canada]) was added to the blastocysts which were then processed by the following program in a MJ Research PTC-200 Thermo Cycler: 37°C for 1 h, followed by 95°C for 15 min. The lysed embryonic cells were then sexed by DNA-based sexing methods using both RT-PCR and RT-qPCR which were carried out as described above.

All of the individually snap-frozen arrested embryos were subjected to cell lysis. A lysis buffer of (7.8 μL nuclease-free water, 1 μL 10x ThermoPol Reaction Buffer [New England Biolabs, Ipswich, MA, USA], 1.2 μL mg/μL protein kinase [Fisher Scientific, Ottawa, ON, Canada] was added to the embryos and then processed as described above. The lysed embryonic cells were then processed for DNA-based sexing method using reverse transcriptase polymerase chain reaction RT-PCR. Each RT-PCR reaction used half of the lysed embryonic material (5 μL from a total of 10 μL). For GAPDH primers a mixture of 5 μL GeneAmp 10× PCR Buffer II (Life Technologies, Burlington, ON, Canada), 8 μL 25mM MgCl₂, 1 μL 1mg/μL forward/reverse primer, 1 μL 10mM dNTP Mix (Life Technologies, Burlington, ON, Canada) and 0.5 μL Amplitaq gold (Life Technologies, Burlington, ON, Canada) was used. For TSPY primers a mixture of 5 μL 10x ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA, USA), 1 μL 10mM dNTP Mix (Life Technologies, Burlington, ON, Canada), 1 μL 1 mg/μL forward/reverse primer, Taq DNA Polymerase with ThermoPol Buffer (New England Biolabs, Ipswich, MA, USA) was used. The RT-PCR was carried out on an MJ Research PTC-200 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA, USA) according to the following protocol: denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for
1 min, followed by a final elongation step of 72°C for 10 min. All RT-PCR products were visualized with gel electrophoresis (15 μL PCR product, 2% agarose gel, 100 V for 25 min). The male-specific gene TSPY was used for DNA-based sexing during RT-PCR for both blastocysts and arrested embryos using the TSPYDNA primer set. The male specific-gene SRY was used for the RT-qPCR experiments using the SRY-1 primer set. The autosomal gene ZAR1 was used as the positive control for both RT-PCR and RT-qPCR for the blastocyst sexing as it is a single-copy gene amplified in both male and female genomic DNA (Uzbekova et al., 2006). The autosomal gene GAPDH using primer set GAPDHDNA was used as the positive control during RT-PCR for the arrested embryos as it has previously showed expression in bovine embryos (Lequarre et al., 1997). An odds ratio was performed to determine if the resulting sex ratio was significantly different from the expected 1:1 ratio.

The injected blastocysts underwent lysis with a buffer of (7 μL nuclease free water, 1 μL prepGEM, 1 μL 10X buffer [ZyGem Corp. Ltd., New Zealand]) added to each embryo and processed with the following program: 75°C for 15 minutes and 95°C for 5 minutes. The lysed embryonic cells were then processed for DNA-based sexing method using reverse transcriptase polymerase chain reaction RT-PCR. Each RT-PCR reaction used half of the lysed embryonic material (5 μL from a total of 10 μL). TSPY was used as the male indicator and ZAR1 was used as the autosomal control. A mixture of 2 μL GeneAmp 10× PCR Buffer II (Life Technologies, Burlington, ON, Canada), 1.6 μL 25mM MgCl₂, 0.8 μL 1mg/μL forward/reverse primer, 1 μL 10mM dNTP Mix (Life Technologies, Burlington, ON, Canada) and 0.3 μL Amplitaq gold (Life Technologies, Burlington, ON, Canada) was used. The RT-PCR was carried out on an MJ Research PTC-200 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA, USA) according to the
following protocol: denaturation at 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min, followed by a final elongation step of 72°C for 10 min. All RT-PCR products were visualized with gel electrophoresis (15 μL PCR product, 2% agarose gel, 100 V for 25 min).

**Microinjection of siRNA**

Due to poor quality of *in vitro* produced embryos, oocytes showing poor morphology (non-homogenous cytoplasmic or weakly layered cumulus) were co-cultured with granulosa cells during the IVM process. Granulosa cells were collected from the aspiration media once the oocytes were removed and cultured for four days in Dulbecco’s Modified Eagle Medium (DMEM) + 10% fetal bovine serum. After aspiration oocytes were co-cultured with granulosa cells in TCM199 medium + 2% steer serum supplemented with estradiol, bFSH, bLH at 5% CO₂ and 38.5°C for 22 to 23 hours as described above. Presumptive zygotes were stripped of remaining cumulus cells via vortex 16 hours post-insemination and randomly separated into three groups: Non injected (NI), scrambled siRNA injected (SI), and *USP9Y* knockdown siRNA injected (KD). siRNA was designed using Invitrogen custom primer software (Life Technologies Pleasanton, CA, USA) with no homology to the X homolog *USP9X*.

Table 1. siRNA primer sequences used for microinjection.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Invitrogen Primer number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>USP9Y</em> siRNA knockdown</td>
<td>UAAGACCACUGAAGUAUGC</td>
<td>224261A01</td>
</tr>
<tr>
<td>Primer name</td>
<td>Primer sequences</td>
<td>Invitrogen Primer number</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>USP9Y siRNA knockdown</td>
<td>GCAUACUUCAGUGGUCUUA</td>
<td>224261A02</td>
</tr>
<tr>
<td>siRNA scrambled</td>
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</tr>
<tr>
<td>siRNA scrambled</td>
<td>GCAUUGCACGGUCUUAUAUAUAUA</td>
<td>224261A04</td>
</tr>
</tbody>
</table>

The non injected group was cultured in groups of 30 in 30 μL drops of culture medium previously described (Ashkar et al., 2010). The other two groups immediately underwent the microinjection process performed with an inverted microscope (Leica DMIRE2) in 10 μL HEPES TALP. 30-60 zygotes were injected at a time to reduce exposure to sub-optimal conditions. Micromanipulation was performed using an Eppendorf manipulator (Hamburg, Germany). Micromanipulation holding pipette and injection needle were controlled using TransferMan NK2 micromanipulators. During injections zygotes were held in place by a holding pipette which was made from Brosilicate Glass capillary tubes with outer diameter of (OD) 1.0mm, inner diameter (ID) of 0.75mm with 15 cm length, pulled by Sutter Micropipette puller P-97 (Sutter Instrument Co. Novato, California) with the following settings: P=50, 1/Heat=550, Pull=100, Vel=30, Del=10. The holding pipette was controlled by CellTram oil loaded manual piston pump which controlled suction pressure. siRNA was loaded into the sterile injection capillary (Femtotip II; Eppendorf) using a Eppendorf microloader. Injection details were controlled by a Femtojet injection system. The injector pressure was set to 150 hpa, the compensation
pressure was set to 10 hpa, and the injection time used was 0.2 sec which calculates to an injection volume of 15 pl, as suggested by manufacturer and Choi et al., 2009; Zhang, 2007. Visualization of the fluorescent siRNA in the cytoplasm of the zygote confirmed injection success. Zygotes were then cultured in groups of 30 in 30 μL drops of culture medium previously described (Ashkar et al., 2010).

![Image A](image1.png)

**Figure 2.** The microinjection of a single zygote. In A (100X magnification) the zygote has been localized and the injection needle has pierced the zona pellucida. A more magnified view in B (200X magnification) shows the injection needle piercing the zygote membrane.
Statistical Analysis

Statistics were analysed using the statistical software Stata\textsuperscript{(R)} Statistics/Data Analysis (StataCorp, College Drive, Texas, USA). A Chi squared test was performed on aggregated microinjection data to determine if there was statistical significance between the non injected, the scrambled injected, and the knockdown group for cleavage and blastocyst rates. A p value of 0.008 for blastocyst rates and a p value less than 0.001 for cleavage rates indicated that there was a significant difference within the three groups. A Chi squared test was then preformed comparing each group to determine which groups showed statistically significance differences which were determined as a p value less than 0.05.

The sex ratio of arrested embryos was analysed with a Test of Single Proportion for a statistical significance determined as a p value less than 0.05.
Results

Gene expression analysis

Female kidney tissue was used as a negative control for the gene expression analysis to ensure that amplification seen in bovine embryos was the result of Y-linked gene expression and not the expression of their similarly sequenced X-homologues. All X-homologues (DDX3X, EIF1AX, TSPYL2, USP9X, ZFX, and ZRSR2) showed expression in the female kidney, and none of the Y-linked genes (DDX3Y, EIF1AY, TSPY, USP9Y, ZRSR2Y, and ZFY) were expressed in female kidney (Fig. 2). All the Y-linked genes in this study showed expression in bovine testis tissue.

Figure 3. Expression of the X-homologues of the Y-linked genes analyzed in this study in female kidney tissue and male testis tissue. Transcripts for all X-homolog genes (DDX3X, EIF1AX, TSPYL2, USP9X, ZFX, and ZRSR2) were detected in both female and male samples using RT-PCR and gel electrophoresis. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); Female, female kidney tissue; Male, male testis tissue.
Blastocysts were pooled (10 and 20) and analysed for Y chromosome linked gene expression with RT-PCR. Pooled embryos were used to increase the concentration of transcripts. Previous investigation in our laboratory has concluded sex ratios to be at or close to 1:1 (King et al., 1991; Lechniak et al., 2003; Kochhar et al., 2003). We therefore expected each embryo pool to be approximately half male. Both pools of blastocysts expressed DDX3Y, EIF1AY, TSPY, USP9Y, ZFY, and ZRSR2Y (Fig. 3). None of the pools of blastocysts expressed HSFY or SRY (Fig. 3).

Figure 4. Expression of Y-linked genes in pooled bovine blastocysts. Transcripts were detected in both pools of 10 and 20 blastocysts for DDX3Y, EIF1AY, TSPY, USP9Y, ZFY, and ZRSR2Y for both primer sets.
using RT-PCR. Transcripts of *SRY* and *HSFY* were not detected in either pool of embryos using RT-PCR.

There was no Y-linked gene expression in the female kidney (negative control), whereas the male testis (positive control) expressed all of the genes. Transcripts of *GAPDH* were present in all samples.

L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); 10, pool of 10 blastocysts; 20, pool of 20 blastocysts; F, female kidney; M, male testis.

Individual blastocysts (N=18) were analyzed for expression of all the Y-linked genes using RT-qPCR. As seen with the pooled blastocysts in figure 3, transcripts were found for *DDX3Y, EIF1AY, TSPY, USP9Y, ZFY*, and *ZR2SRY*, but not for *HSFY*, or *SRY* (Table 2). A total of 10 of the 18 blastocysts expressed some or all of the Y-linked genes making it likely that these were all male and those that did not express the Y-linked genes were likely female. Table 2 lists these blastocysts as “presumptive males” and “presumptive females”. To confirm this, a group of ten single blastocysts were sexed and analysed for gene expression. It was seen that only the male blastocysts expressed the Y-linked genes, three of which (*DDX3Y, USP9Y, ZR2SRY*) were expressed in 100% of the males, however this was dependent on the primer set used (Table 2).

Another 22 blastocysts were analysed the same way but only using the primer sets which amplified the genes in 100% of the males (*DDX3Y-1, USP9Y-1, ZR2SRY-1*) and validated our results (Table 2). None of the female blastocysts expressed the Y-linked genes (Table 2).
Table 2. Expression of Y-linked genes in sexed and unsexed individual bovine blastocysts
Each gene shows the proportion of positively expressing blastocysts over total number of blastocysts analysed. In parentheses is the percentage of blastocysts showing expression.

<table>
<thead>
<tr>
<th>Primer used</th>
<th>Unsexed (presumptive males)</th>
<th>Unsexed (presumptive females)</th>
<th>Sexed males</th>
<th>Sexed females</th>
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</thead>
<tbody>
<tr>
<td>DDX3Y-1</td>
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<td>17/17 (100)</td>
<td>0/15 (0)</td>
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<td>1/5 (20)</td>
<td>0/5 (0)</td>
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<td>3/5 (60)</td>
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<td>4/5 (80)</td>
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<td>0/5 (0)</td>
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<td>GAPDH</td>
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<td>8/8 (100)</td>
<td>17/17 (100)</td>
<td>15/15 (100)</td>
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</table>
Blastocysts were sexed using genomic TSPY for RT-PCR and SRY RT-qPCR, with ZAR1 as an autosomal positive control gene for both methods. For the RT-PCR sexing, genomic TSPY was present in the male, but not the female blastocysts, whereas the autosomal gene ZAR1 was present in both (Fig. 4). Out of the 32 blastocysts that were sexed, 17 were male and 15 were female (53.1% male and 46.9% female); the sex ratio did not differ from the expected 1:1 ratio.

Figure 5. Results of DNA-based sexing and expression analysis in eight individual blastocysts on RT-PCR. Embryos were sexed using genomic TSPY as a male-specific marker and genomic ZAR1 as an autosomal control. The Y-linked genes (DDX3Y, USP9Y, and ZRSR2Y) were expressed in all male but not female blastocysts. GAPDH was used as a reference gene and was expressed in all embryos, regardless of sex. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); 1-4, female blastocysts; 5-8, male blastocysts; F, female kidney; M, male testis.

To further this investigation of Y-linked genes in pre-implantation embryos, two pools of 30 embryos for each developmental stage (2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst) were also analyzed for transcripts of USP9Y (USP9Y-1 primer set) and ZFY (ZFY-1 primer set) using RT-PCR. USP9Y expression was seen in one of the 4-cell embryo pools and both of the embryo pools from 8-cell to blastocyst, but was not seen in the 2-cell embryo pools (Fig. 5). ZFY
expression was seen in both embryo pools from 8-cell to blastocyst, but was not expressed in the 2-cell or 4-cell embryo pools (Fig. 6). To ensure the RT-PCR conditions were sensitive enough to detect both USP9Y and ZFY transcripts at levels as low as the 2-cell pools, blastocysts which already tested positive for each gene were diluted to levels similar to those in the 2-cell pools. It was seen that primers for USP9Y and ZFY were able to detect transcripts in these diluted pools under these RT-PCR conditions which mimicked the estimated amount of male cells in each 2-cell embryo pool (Fig. 7).

**Figure 6.** USP9Y gene expression in pooled bovine embryos (product size 285bp). Transcripts were detected in one of the two 4-cell pools and both embryo pools from 8-cell through to blastocyst using (RT-PCR). USP9Y did not show expression in either 2-cell embryo pool or in the female kidney sample (negative control), whereas the male testis sample (positive control) did show USP9Y expression. GAPDH was present in all samples. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); #1, first embryo trial; #2, second embryo trial; F, female kidney; M, male testis.
Figure 7. ZFY gene expression in pooled bovine embryos (product size 170 bp). Transcripts were detected in both embryo pools from 8-cell through to blastocyst using (RT-PCR). USP9Y did not show expression in either embryo pools for 2-cell or 4-cell, or in the female kidney sample (negative control), whereas the male testis sample (positive control) did show ZFY expression. GAPDH was present in all samples. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); #1, first embryo trial; #2, second embryo trial; F, female kidney; M, male testis.

Figure 8. Expression of ZFY (product size 170 bp) and USP9Y (product size 285 bp) in diluted blastocyst samples. Expression of ZFY and USP9Y was seen in both diluted samples, and in the male testis sample (positive control). L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water), 150, amount of cells equivalent to a single blastocyst (Thouas et al., 2001); 30, number of male cells in each pool of 30 2-cell embryos following a 1:1 sex ratio; M, male testis.

In addition to analyzing USP9Y and ZFY expression in healthy embryos, expression was also analyzed in embryos that had arrested development at the 2-, 4-, and 8-cell stage. Two pools of 30 arrested embryos were analyzed for transcripts of USP9Y and ZFY using RT-PCR.
GAPDH was expressed in each embryo pool; however neither *USP9Y* (Fig. 8) nor *ZFY* (Fig. 9) were expressed by these arrested embryos.

**Figure 9.** *USP9Y* gene expression in pooled bovine arrested embryos (product size 285 bp). Transcripts were not detected in either arrested embryo pool for 2-cell, 4-cell and 8-cell using RT-PCR. *USP9Y* did not show expression in the female kidney sample (negative control), whereas the male testis sample (positive control) did show *USP9Y* expression. GAPDH was present in all samples. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); #1, first embryo trial; #2, second embryo trial; F, female kidney; M, male testis.

**Figure 10.** *ZFY* gene expression in pooled bovine arrested embryos (product size 170 bp). Transcripts were not detected in either arrested embryo pool for 2-cell, 4-cell and 8-cell using RT-PCR. *ZFY* did not show expression in the female kidney sample (negative control), whereas the male testis sample (positive control) did show *ZFY* expression. GAPDH was present in all samples. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); #1, first embryo trial; #2, second embryo trial; F, female kidney; M, male testis.
Arrested embryos and oocytes were also analysed for X homologues of USP9Y. USP9X was expressed by pooled oocytes and by at least one pool of arrested embryos of the 2-, 4-, and 8-cell stage (Fig. 10).

![Figure 11. USP9X gene expression in pooled bovine arrested embryos and matured oocytes.](image)

Transcripts were detected in one pool of embryos arrested at the 2-cell, 4-cell, and 8-cell stage and both oocyte pools using RT-PCR. USP9X showed expression in both the female kidney sample and male testis sample. L, 100 base pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); #1, first embryo trial; #2, second embryo trial; F, female kidney; M, male testis.

The sex ratio of male arrested embryos produced during normal IVF was found to be 0.59:1.00. Embryo sexing of 8 arrested embryos using RT-PCR is shown in Fig. 11. Total number of arrested embryos sexed is shown in Fig. 12.

![Figure 12. DNA-based sexing of 8 embryos arrested at the 2-8 cell stage using RT-PCR.](image)

Embryos were sexed using TSPY as a male-specific marker and genomic GAPDH as an autosomal control. L, 100 base
pair ladder (Life Technologies, Burlington, ON, Canada); N, negative control (water); 1-2 and 4, male embryos; 3 and 5-8, female embryos; M, male testis; F, female kidney.

Sexing of embryos arrested at the 2-8 cell stage

Figure 13. Results of sexing 100 arrested 2-cell, 4-cell, and 8-cell embryos using RT-PCR. 37 arrested embryos were found to be male and 63 arrested embryos were found to be female. The male to female sex ratio is 0.59:1.00. * indicates significant differences at the level of p<0.05.

Microinjection

Zygotes were individually injected with siRNA to knockdown USP9Y transcripts or were injected with a control of scrambled siRNA and compared to development of non injected embryos. There were significant differences between the cleavage rates of all three groups but no significant differences between the blastocyst rates (Table 3, Fig. 13 and 14). Previous
Investigation in our laboratory has concluded sex ratios of blastocysts produced during normal IVF conditions to be at or close to 1:1 (King et al., 1991; Lechniak et al., 2003; Kochhar et al., 2003). The sex ratio of blastocysts from the injection groups did not differ from the expected 1:1 ratio. Of the scrambled injection group there were three males and three female embryos sexed and of the knockdown injection group there was one male and one female embryo sexed.

Table 3. Total cleavage and blastocyst rates from siRNA injections calculated from total number of embryos put into culture. There were significant differences in cleavage rate at the level of p<0.05 between all three groups as indicated by different letters (a,b,c). There was no significant difference in blastocyst rate between the three groups.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Cleavage Rate (%)</th>
<th>Blastocyst Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non Injected</strong></td>
<td>N=222</td>
<td>62.6&lt;sup&gt;a&lt;/sup&gt; (N=139)</td>
<td>23.0 (N=32)</td>
</tr>
<tr>
<td><strong>Scrambled Injected</strong></td>
<td>N=190</td>
<td>48.9&lt;sup&gt;b&lt;/sup&gt; (N=93)</td>
<td>17.2 (N=16)</td>
</tr>
<tr>
<td><strong>Knockdown Injected</strong></td>
<td>N=185</td>
<td>37.3&lt;sup&gt;c&lt;/sup&gt; (N=69)</td>
<td>14.2 (N=10)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>N=597</td>
<td>50.4 (N=301)</td>
<td>19.3 (N=58)</td>
</tr>
</tbody>
</table>
Figure 14. Total cleavage rates from siRNA injections calculated from total number of embryos put into culture. There were significant differences in cleavage rate between groups as indicated by a line and p value less than 0.05.

Figure 15. Total blastocyst rates from siRNA injections calculated from total number of embryos showing cleavage. There was a significant difference in blastocyst rate between non injected and knockdown groups as indicated by a line and p value less than 0.05.
Table 4. Total number of embryos, cleavage numbers, and blastocyst numbers, of non injected group per injection run.

<table>
<thead>
<tr>
<th>Run</th>
<th>Total</th>
<th>Cleavage</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>62</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>#2</td>
<td>47</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>#3</td>
<td>64</td>
<td>41</td>
<td>12</td>
</tr>
<tr>
<td>#4</td>
<td>49</td>
<td>34</td>
<td>5</td>
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</tbody>
</table>

Table 5. Total number of embryos, cleavage numbers, and blastocyst numbers, of scrambled-injected group per injection run

<table>
<thead>
<tr>
<th>Run</th>
<th>Total</th>
<th>Cleavage</th>
<th>Blastocyst</th>
</tr>
</thead>
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<tr>
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<td>26</td>
<td>10</td>
</tr>
<tr>
<td>#2</td>
<td>52</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>#3</td>
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<tr>
<td>#4</td>
<td>44</td>
<td>21</td>
<td>1</td>
</tr>
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</table>

Table 6. Total number of embryos, cleavage numbers, and blastocyst numbers, of knockdown-injected group per injection run.

<table>
<thead>
<tr>
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<th>Total</th>
<th>Cleavage</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
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<td>0</td>
</tr>
<tr>
<td>#2</td>
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<td>1</td>
</tr>
<tr>
<td>#3</td>
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<td>26</td>
<td>4</td>
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<tr>
<td>#4</td>
<td>41</td>
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<td>5</td>
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Discussion

For this thesis eight Y linked genes (DDX3Y, EIF1AY, HSFY, SRY, TSPY, USP9Y, ZFY, and ZRSR2Y) were examined in pooled pre-implantation bovine blastocysts to determine the presence of their transcripts and to determine the consistency of expression in individual blastocysts. Two of these genes (USP9Y and ZFY) were analyzed further in earlier pre-implantation embryo stages (2-, 4-, 8-, 16-cell, and morula) to determine the timing of expression and to compare their expression in normal embryos to their expression in embryos that had spontaneously arrested development at the 2-, 4-, and 8-cell stages. The transcripts of USP9Y were also targeted and knocked down in zygotes using RNA interference to observe its impact on pre-implantation male embryo development.

Transcripts from six of the eight Y linked genes that were analysed were found to be present in pooled and individual bovine blastocysts (DDX3Y, EIF1AY, TSPY, USP9Y, ZFY, and ZRSR2Y) whereas two genes were not detected in this study (HSFY and SRY). DDX3Y, USP9Y, and ZRSR2Y also showed expression in 100% of male blastocysts. Transcripts of USP9Y and ZFY were present in pooled embryos of the blastocyst, morula, 16-cell, and 8-cell stages and transcripts of USP9Y were also present at the 4-cell stage. Transcripts of these two genes however were not present in arrested embryos of the 2-, 4-, or 8-cell stage. The introduction of siRNA designed to knockdown transcripts of USP9Y resulted in a significant decrease in the number of embryos that cleaved but did not show a significant difference in survival to the blastocyst stage or in the sex ratio in embryos that survived to the blastocyst stage.
This is the first study to identify the expression of *EIF1AY*, *TSPY*, and *USP9Y* in bovine embryos, the first study to examine Y chromosome gene expression in arrested embryos, and the first study to examine the effects of *USP9Y* transcript knockdown on embryo development.

*SRY* transcripts were not able to be detected in pooled or individual blastocysts even though it has been detected before as early as the 4- to 8-cell stage (Gutierrez-Adan et al., 1997). Using the same primer set (*SRY*-3) and similar methods still had a negative outcome. The main difference between the two methods was the use of *SRY* specific primers in the reverse transcription step which may have allowed for more specific detection. *HSFY* transcripts were also not detected in the pooled or individual embryos and it was concluded that *SRY* and *HSFY* may be expressed in bovine embryos but at levels much lower than the rest of the Y linked genes analysed.

*DDX3Y, EIF1AY, TSPY, USP9Y, ZFY*, and *ZRSR2Y* all showed expression in the pooled blastocysts; however, consistency varied in individual blastocysts. Of the 18 individual blastocysts analyzed 10 showed at least some expression of *EIF1AY, TSPY*, and *ZFY*, and all 10 showed expression of *DDX3Y, USP9Y, ZRSR2Y*, as well as the reference gene *GAPDH*. These 10 blastocysts were considered males since only male embryos express Y linked genes. The other eight blastocysts tested positive for the reference gene *GAPDH* and were considered female embryos because they did not express any Y linked genes. Since Y linked gene expression is male specific these findings represent the first case of embryo sexing using gene expression instead of genomic genes. Only *DDX3Y, USP9Y*, and *ZRSR2Y* showed consistent expression making them good candidates for embryo sexing using RNA; however, primer selection is
important as only one primer set for each gene (DDX3Y-1, USP9Y-1, ZRSR2Y-1) amplified transcripts in all male blastocysts. To confirm these findings the same gene expression analysis was performed on half of an individual blastocyst while the other half underwent traditional genomic sexing. All 17 sexed male embryos showed expression of DDX3Y, USP9Y, ZRSR2Y, and the reference gene GAPDH. The 15 sexed female embryos showed no expression of any Y linked genes but consistent expression of the reference gene GAPDH. Therefore this method of RNA sexing can be incorporated into most studies which use at least half of the blastocyst for RNA isolation. Since this is the first study to attempt RNA based sexing the procedure is limited to blastocysts and further analysis in different embryonic stages would be useful.

These findings show that several Y linked genes are expressed during pre-implantation development and furthermore three Y linked genes seem to be expressed by all male blastocysts. This is a strong endorsement of the argument that the Y chromosome is active in pre-implantation development (Burgouyne, 1993) and these results also narrow down which Y linked genes may be most important during pre-implantation development. Two primer sets were used to examine the expression of these Y linked genes in blastocysts and the product was sequenced to confirm primer specificity and decrease the possibility of a false positive. To further the analysis of Y linked gene expression in bovine pre-implantation embryos, two genes (USP9Y and ZFY) were examined in earlier stages using the same primer sets used for analysis in blastocysts. These genes were chosen because they represent different areas of the Y chromosome. ZFY is found on the short arm of the Y chromosome where genes are involved with testis development while USP9Y is a long arm gene involved with sperm production (Jobling & Tyler-Smith, 2003; Sun et al., 1999; Brown et al., 1998). ZFY has previously been
shown to be expressed in the bovine pre-implantation embryo as early as the 8-cell stage (Peippo et al., 2002) but USP9Y has not been previously examined. Neither of these genes have been examined in spontaneously arrested embryos, and given their different locations on the Y chromosome and different gene actions they were chosen to be examined further.

Both pools of embryos showed expression of USP9Y and ZFY as early as the 8-cell stage, with one pool of 4-cell embryos showing expression of USP9Y. One explanation for finding transcripts of USP9Y in only one of the two pools of 4-cell embryos could be that USP9Y begins expression during the 4- to 8-cell stage. The embryos were harvested at the same time and while they were all 4-cell embryos they may be developing at different rates and the pool that was negative for USP9Y transcripts could be comprised of slower developing male embryos which had not enough time to transcribe detectable levels RNA. Both pools of embryos at every later stage of development (8-cell, 16-cell, morula, and blastocysts) showed expression of USP9Y and ZFY.

Transcripts of USP9Y were not present in the 2-cell embryo pools, and transcripts of ZFY were not present in the 2- or 4-cell embryo pools. There are several possibilities for a false negative outcome. The most likely explanation is that the RT-PCR conditions were not sensitive enough to detect transcripts at such low cell numbers. However given an approximate 1:1 sex ratio and pools of 30 embryos only about 15 embryos per pool would be male and therefore able to express Y chromosome linked genes. In the pool of 30 2-cell embryos this would equal a total of 30 male cells, compared to a single blastocyst which has around 150 cells (Thouas et al., 2001). To test the RT-PCR conditions a pool of blastocysts which already tested positive for
transcripts of *USP9Y* and *ZFY* was diluted to equal the same number of male cells expected to be present in the 2-cell embryo pool. As seen in Figure 7, both primers for *USP9Y* and *ZFY* were able to detect transcripts in the diluted pools, indicating that these RT-PCR conditions are sensitive enough to detect transcripts at low levels. Therefore either expression is extremely low or *USP9Y* is not expressed at the 2-cell stage and *ZFY* is not expressed at the 2- or 4-cell stage. This is expected as these stages of embryo development occur prior to the major genome activation in cattle, which happens at the 4- to 8-cell stages (Telford et al., 1990; Nothias et al., 1995).

*USP9Y* and *ZFY* transcripts were also not detected in the 2-, 4-, or 8-cell arrested embryo pools. It was determined that normal 8-cell embryos express both these genes, however arrested 8-cell embryos do not. Since these embryos were harvested on day eight (to ensure that they had truly arrested development) it was unknown if their expression pattern would be more similar to the stage of development that was reached (2-, 4-, or 8-cell) or to the age of the embryo (day eight). It was seen that normal 8-cell embryos as well as day eight blastocysts express both *USP9Y* and *ZFY*, but the lack of expression of these genes in arrested embryos indicates that they behave differently than normal embryos. It is possible that these embryos did express *USP9Y* and *ZFY* while at the 8-cell stage on day four, before an unknown reason caused them to arrest development. These transcripts may have degraded by day eight when they were harvested resulting in a false negative. However, it is not uncommon for RNA in an embryo or oocyte to remain for multiple days; maternal transcripts must be able to survive from before ovulation to the embryonic genome activation stage without being degraded (reviewed by Schoenwolf et al., 2009; Senger, 2003). In the mouse oocyte poly(A)-RNA
degrades slowly with a half life of 8-12 days (Brower et al, 1981). However, given the possibility that these arrested embryos may have expressed *USP9Y* and *ZFY* at some point in their development they still stopped this expression before day eight which is unlike normal embryos of the same stage or age. Furthermore, transcripts of *USP9X* (the X homolog of *USP9Y*) were found to be present in at least one pool of 2-, 4-, and 8-cell arrested embryos. These transcripts were either maternal which showed that transcripts lasted for several days without degradation, or they were embryonic which showed that these arrested embryos were not fully transcriptionally silent.

Embryos for *USP9Y* knockdown analysis were assigned to three groups. The non injected group was used as the IVF control and represented normal *in vitro* embryo production and survival rates. The scrambled siRNA injection group was used to account for the stress and damage to the embryo from the act of injection and the presence of siRNA in the cytoplasm. The *USP9Y* knockdown siRNA injection group was used to analyse the effect of zygote growth and survival without or with decreased levels of *USP9Y* transcripts. There was a significant decrease seen in cleavage rates between all three groups but no significant difference in survival rates to the blastocyst stage, however a trend of decreased survival was observed in the knockdown group.

*USP9Y* and *ZFY* are both active at the 8-cell stage which is the earliest point of development where the embryonic genome first becomes active (Telford et al., 1990; Nothias et al., 1995). These genes are also expressed at every later pre-implantation stage indicating that they may be involved in some developmental pathways during this time. This may explain
the observation that male embryos show increased proliferation resulting in faster development, higher cell numbers, and higher mitotic indices (Xu et al., 1992). Furthermore at the blastocyst stage *DDX3Y, USP9Y,* and *ZRSR2Y* are expressed in 100% of males indicating that Y chromosomal genes may be involved in development and certain genes may be more important than other Y chromosomal genes. If this is true then lacking these transcripts might have a negative impact on development. When this was investigated in embryos that had spontaneously arrested at the 2-, 4-, and 8-cell stage it was discovered that these embryos do not show expression of *USP9Y* or *ZFY*. This lack of expression may be a contributing factor to why these embryos have arrested development. Furthermore when individual arrested embryos were sexed it was found that significantly more female embryos had arrested development. Previous work has demonstrated that early cleaving embryos which are more likely to be male are also more likely to develop to the 8-cell stage or beyond (Yadav et al., 1993). Perhaps embryos naturally lacking these transcripts were not able to survive as well in vitro as those that did as seen by the higher number of female embryos that arrested development.

RNA interference was used to examine the effect of male embryo development with a knockdown of *USP9Y* transcripts. *USP9Y* was chosen because it exhibited expression as early as the 4-cell stage and also in all later stages of pre-implantation development, as well as consistent expression in all male blastocysts making it the best candidate for having an effect on embryo development. Knockdown of *USP9Y* transcripts showed a significant decrease in the cleavage rate of developing embryos and a non-significant decrease in the blastocyst rate. This knockdown also did not eliminate all male blastocysts indicating that while *USP9Y* may have
had an effect on early embryo development it was not essential for survival. Since this knockdown targeted transcripts of USP9Y and since cleavage rate was affected there must be something involving USP9Y transcripts at the 2-cell stage, however, transcripts at this stage were not detected. It is possible that they are present but in lower, undetectable amounts, or other factors may be at play. A study examining the duration of gamete interaction saw that a shorter interaction time between sperm and oocytes resulted in a sex ratio skew toward males (Kochhar et al., 2003). Yadav et al., 1993 suggested that sexual differences may begin before the embryonic genome is thought to be activated and is due to either differential processing of X- and Y-bearing sperm within the zygote or to very early differential gene expression derived from X- and Y-bearing sperm. Further investigation into Y chromosome transcripts around the time of fertilization may reveal that USP9Y transcripts are being delivered to the oocyte along with the male genome as has been demonstrated before with other spermatozoa transcripts (protamine-2 and clusterin) (Ostermeier et al, 2004).

Regardless, if the mRNA knockdown was complete, the knockdown of USP9Y transcripts did not eliminate all male embryos developing to the blastocyst stage indicating that it is not essential for male survival. It could be that Y chromosome transcripts are in place to help “at risk” male embryos cleave and survive while healthier male embryos can survive without it. If so that may help explain why there were still male embryos surviving to the blastocyst stage even with USP9Y transcript knock down as they were healthier embryos overall and the unhealthy embryos that did not naturally express USP9Y showed arrested development at the 2-, 4-, and 8-cell stage. Further analyses into the sex ratios of the arrested embryos from this
knockdown group may reveal a change in the sex ratio towards more males who are “at risk” and need transcripts of *USP9Y* to survive.

The Y chromosome has two main known functions: testis formation and sperm production. While it is obvious that Y linked genes are not directly involved in these pathways during pre-implantation embryo development, it does not mean that they are not involved at some level. The similarities between sperm development and early embryo development are quite striking as both processes involve rapid cellular divisions without accompanied cytoplasmic growth. Since some of the genes involved with spermatogenesis were expressed in the early embryo it was possible that they were facilitating the same type of cellular division. For example, *USP9Y* is a gene involved with spermatogenesis which codes for a deubiquitylating enzyme (Lahn & Page, 1997; Brown et al., 1998). Ubiquitin is a small regulatory protein found in almost all tissues of eukaryotic organisms (Wilkinson, 2005) and deubiquitylating enzymes (DUBs) have a role in post transcriptional modification of ubiquitin giving them the potential to regulate any ubiquitin mediated cellular process. A DUB named ubiquitin-specific protease 6 (UBP6) was the first DUB to be identified as an oncogene (a factor that promotes cancer usually by affecting the cell cycle to promote proliferation) (Onno et al., 1993; Papa et al., 1993). Further research into DUBs and disease uncovered that another DUB by the name of ubiquitin-specific protease 7 (UBP7) forms a stabilizing complex with mouse double minute 2 homolog (MDM2), a negative regulator of p53 resulting in increased cell proliferation (Li et al., 2002; Li et al., 2004; Cummins et al., 2004; Hu et al., 2006). Furthermore two more DUBs, ubiquitin carboxyl-terminal hydrolase 33 (UBP33) and ubiquitin specific peptidase 20 (UBP20) are involved with Von Hippel-Lindau disease; an autosomal dominant disease causing a variety of
tumors. These two deubiquitylating enzymes act to stabilize the a-subunit of the transcription factor hypoxia-inducible factor-1 (HIF-1) (Li et al., 2005) which regulates the genes involved in glucose metabolism and cell proliferation (Semenza, 2003). Deubiquitylating enzymes show a pattern of being involved with increased cellular proliferation. If USP9Y transcripts follow this pattern it is obvious why it is important for spermatogenesis which involves rapid cellular divisions, and if this same effect is also seen during pre-implantation embryo development it would result in an increased rate of cellular division and faster progression through the stages of cleavage for any embryo expressing this gene. Since only male embryos express USP9Y it may be the reason for the advanced developmental rate seen by male pre-implantation embryos. Since USP9Y is a gene from the long arm of the Y chromosome it follows with Burgoyne’s 1993 work which showed male embryos that had deletions from the long arm of the Y chromosome developed at a slower rate more similar to female embryos.

Why is the Y chromosome active so early in development? Two theories are discussed here; (a) male embryos need to grow at an advanced rate to reach a healthy male birth weight facilitated by the Y chromosome, (b) under the stress of an in vitro environment male embryos respond by expressing Y linked genes that aid in development and survival.

(a) Given that human and cattle birth weight is higher in male than female offspring (Karn & Penrose, 1951; Martin & Blunn, 1952), and given a similar gestation time for males and females it is required that male fetuses have an advanced growth rate to reach their birth weight in time. Under this stress of reaching a healthy birth weight in time male embryos must develop at an advanced rate as soon as possible, and given that the Y chromosome is the only
difference between early male and female embryos it must be actively involved in
development. This theory coincides with a hypothesis by Mittwoch (1989) who proposed that
embryonic sexual dimorphism might begin as early as the time of fertilization.

(b) The stress of an in vitro environment on embryo development is known, and has
been demonstrated that oxygen concentration during embryo culture in vitro has an impact on
blastocyst gene expression (Harvey et al., 2004). It may be that there is a response to this stress
to allow the embryo to cope and survive better. If the Y chromosome does aid in development
it may be an evolutionary response for male embryos to express Y linked genes that allow them
to survive better in vitro. While this does go against the Trivers-Willard hypothesis (TWH) which
states that females would be favoured during stress (Trivers & Willard, 1973) it does coincide
with the sex ratio skewed towards males seen during in vitro embryo production but not seen
during artificial insemination or natural breeding (Bousquet et al, 1999; Camargo et al., 2010).

One of the major benefits of this study is to further the understanding of the Y
chromosome during pre-implantation development. It is essential to discover in entirety the Y
linked genes that are expressed during this time. The number of genes analysed in blastocysts
in this thesis represents approximately 30% to 44% of the gene families located on the Y
chromosome based on human and chimpanzee Y chromosome content. Once the remaining
genes are examined in blastocysts and earlier stages their expression can be compared to
spontaneously arrested embryos. As this is the first study to examine Y linked gene expression
in spontaneously arrested embryos only transcripts of two genes (USP9Y and ZFY) have been
analysed. Further analysis will reveal whether other Y linked genes are/are not expressed by
arrested embryos. Some Y linked genes expressed by arrested embryos may not be essential for
development but may aid “at risk” embryo development to the blastocyst stage. This can be
examined by continuing the RNA interference study on additional Y linked genes once their
expression pattern in pre-implantation embryos is complete.

The new method of embryo sexing via RNA has potential for use in research and the
dairy industry. Currently the only method of embryo sexing uses DNA and each sample can only
be used for DNA based research. With RNA based sexing it is now possible to couple
transcription studies and embryo sex. If these studies are successful in identifying other Y linked
genes active in the pre-implantation embryo and which of those are important for development
they could be used as a health marker for early male embryos. If the conditions are improved
and sensitive enough for a single blastomere then this health check and sexing step could be
accomplished as early as the 8-cell stage when these genes begin expression.
Summary & Conclusion

Objective one demonstrated that the Y chromosome is transcriptionally active during pre-implantation development as seen by the expression of *DDX3Y, EIF1AY, TSPY, USP9Y, ZFY,* and *ZRSR2Y*. This expression may be important because *DDX3Y, USP9Y,* and *ZRSR2Y* were expressed in 100% of male blastocysts examined, and both *USP9Y* and *ZFY* began expression at the same time as the rest of the embryonic genome. Objective two demonstrated that the Y chromosome may be important for development. Embryos that had arrested development at the 2-, 4-, and 8-cell stage did not express *USP9Y* and *ZFY*, and more female than males embryos showed arrested development indicating that development might have been compromised without the expression of Y linked genes. Objective three demonstrated that the Y chromosome, specifically *USP9Y*, is not essential for male pre-implantation embryo development *in vitro* but may have an effect at the time of cleavage. Taken together this thesis provides evidence for the importance of the Y chromosome during male pre-implantation bovine embryo development.
References


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## Appendix I

Primer sequences used for PCR and qPCR experiments.

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<th>Size (bp)</th>
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<th>Gene name and symbol</th>
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<td>238</td>
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<td>(Hamilton et al., 2011)</td>
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