REGULATION OF GROWTH AND THERMOPROTECTION OF THE BOVINE PREIMPLANTATION EMBRYO BY INSULIN-LIKE GROWTH FACTOR-1

By

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To my parents, my brothers, my nephews and my husband
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**LIST OF ABBREVIATIONS**

The following list describes abbreviations used in the dissertation. In addition, symbols for genes and proteins were used according to procedures outlined in the Guide for Authors to Biology of Reproduction ([http://www.biolreprod.org/site/misc/NomenBullets.xhtml](http://www.biolreprod.org/site/misc/NomenBullets.xhtml)). Gene symbols are used without definition and were obtained from the EntrezGene website of the National Center for Biotechnology Information ([http://www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene)).

<table>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>bST</td>
<td>Recombinant bovine somatotropin</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>COCs</td>
<td>Cumulus-oocyte complexes</td>
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<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ΔΔC&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Delta delta C&lt;sub&gt;T&lt;/sub&gt;</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate-treated</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EGA</td>
<td>Embryonic genome activation</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>IVP</td>
<td>In vitro production</td>
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<tr>
<td>ICM</td>
<td>Inner cell mass</td>
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<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>KSOM</td>
<td>Potassium simplex optimized medium</td>
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<td>KSOM-BE2</td>
<td>KSOM-bovine embryo 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
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<td>qPCR</td>
<td>Quantitative real-time RT-PCR</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
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<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
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<tr>
<td>SOF-BE1</td>
<td>Synthetic oviduct fluid –bovine embryo 1</td>
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<tr>
<td>TALP</td>
<td>Tyrodes albumin lactate pyruvate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBST</td>
<td>TBS + Tween 20</td>
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<tr>
<td>TCM</td>
<td>Tissue culture medium</td>
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<tr>
<td>TE</td>
<td>Trophectoderm</td>
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<tr>
<td>TL</td>
<td>Tyrodes lactate</td>
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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
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REGULATION OF GROWTH AND THERMOPROTECTION OF THE BOVINE
PREIMPANTATION EMBRYOS BY INSULIN-LIKE GROWTH FACTOR-1

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The function of the embryo depends upon regulation by maternally derived growth factors. One of these, IGF1, can affect function of the preimplantation bovine embryo by increasing the proportion of embryos that become blastocysts, reducing effects of heat shock on development and apoptosis, and enhancing survival rates of embryos transferred into heat-stressed recipients. It was hypothesized that pro-developmental actions of IGF1 are exerted after day 4 of development (when the embryonic genome is activated), and that the ability of IGF1 to protect embryos from heat shock is developmentally regulated and involves stimulation of genes promoting survival to stress.

In a series of experiments to determine the mechanism by which IGF1 increases competence to develop to the blastocyst stage, it was demonstrated that recombinant human IGF1 increased the proportion of oocytes becoming blastocysts when added from day 4-8 or day 0-8 but not from day 0-4 post-insemination. Furthermore, IGF1 promotes development to the blastocyst stage by regulating MAPK-dependent events because inhibition of MAPK signaling by the inhibitor PD 98059 reduced effects of IGF1. Moreover, actions of IGF1 involve increased expression of genes required for blastocoel formation as indicated by the observation that IGF1 increased expression of ATP1A1.
As expected, treatment of embryos with IGF1 at day 5 post-insemination reduced the block in development to the blastocyst stage caused by exposure of embryos to heat shock. In contrast, there was no thermoprotective action of IGF1 at the two-cell stage. Failure of IGF1 to protect two-cell embryos does not seem to be due to insufficient signaling molecules because IGF1R mRNA and protein was detected in two-cell and day 5 embryos, and the expression of mRNA encoding for other molecules involved in the IGF1 signaling pathway, such as PI3K, MAPK, RAF1, was higher in two-cell embryos. Thus, it is likely that IGF1 fails to be thermoprotective in two-cell embryos because of the increased sensitivity of these embryos to heat shock.

A final experiment evaluated gene expression in blastocysts treated with IGF1 using microarray technology to identify candidate genes responsible for the increased survival of IGF1-treated embryos transferred during heat stress. Culture with IGF1 caused altered expression of 102 genes (40 upregulated and 32 downregulated). Among these were genes involved in developmental processes, apoptosis and antioxidant defense.

Taken together, these investigations indicate that IGF1 can regulate embryonic development and resistance to heat stress but that these actions occur at or after day 4 of development, at a time after embryonic genome activation. Furthermore, the pro-developmental effects of IGF1 involve actions mediated by the MAPK pathway and include alteration of genes controlling formation of the blastocoelic cavity. Genes regulated by IGF1 at the blastocyst stage, such as those involved in development, apoptosis and protection from oxidative stress could be involved in the increase in embryonic survival after transfer to heat stressed recipients caused by IGF1.
CHAPTER 1
LITERATURE REVIEW

Introduction

Successful pregnancy is ensured when the zygote formed as a result of fertilization encounters a suitable environment that will nourish it and allow it to develop to term. One of the most critical periods of development occurs during the initial weeks of pregnancy when the embryo is dependent of growth factors, hormones, and cytokines derived from the oviduct and uterus [4-5]. Several factors can cause pregnancy loss during this period including fertilization of a compromised oocyte, chromosomal abnormalities, errors in embryonic development, infectious agents, and other inadequacies in the uterine environment [4, 6-7].

Embryonic mortality is a particular problem for lactating dairy cows. Only 30-40% of all inseminated cows become pregnant, and the pregnancy rate has declined in the last 4 decades [7-8]. There are many reasons for reduced fertility in dairy cows including altered follicular development [9-10], reduced steroid concentrations [11-12] and increased susceptibility to heat stress [13-14].

One possible strategy for improving fertility in compromised populations of animals is embryo transfer using in vitro production (IVP) of embryos. This technology was originally developed with the view to improve genetic selection by increasing the number of offspring from genetically-superior animals [15-16]. In cases where pregnancy rates to artificial insemination are low, however, such as during heat stress, transfer of IVP embryos can improve fertility in dairy cows [17-19].

Many advances in the techniques for in vitro maturation, fertilization and culture have been achieved. Nonetheless, in vitro embryo technologies pose several problems,
including reduced embryo survival after transfer, decreased survival to cryopreservation and increased neonatal calf loss [20-23]. Indeed, the embryo derived in vivo remains the predominant type of embryo used in embryo transfer. According to data from the International Embryo Transfer Society, only 34% of embryos transferred in 2008 were produced in vitro [24].

One reason for the altered development of the in vitro produced embryo is the difficulty in recreating in vitro the uterine environment with all the critical growth factors, cytokines, hormones and other substances present in that environment that regulate embryonic function. Accordingly, one approach to improve competence of the IVP embryo is to modify culture conditions to more closely mimic the reproductive tract. A molecule that holds promise for improving development of embryos in vitro is IGF1. Circulating IGF1 is synthesized and secreted primarily by the liver [25] and, in the cow, is also expressed in several reproductive tissues including ovary, oviduct, uterus and embryo [26-30]. IGF1 can affect function of the preimplantation bovine embryo by increasing the proportion of cultured embryos that become blastocysts [1, 3, 31-34], reducing effects of heat shock on development and apoptosis [1-2], and enhancing survival rates of embryos transferred into heat-stressed recipients [35].

Treatment of embryos cultured with IGF1 thus has the potential to increase blastocyst yield and to increase subsequent pregnancy rate after transfer into heat-stressed recipients. For IGF1 to be a practical treatment for enhancing embryonic resistance to stress in vivo, it must be active at the earliest stages of development when embryos are most susceptible to stress [36-37]. It is not known, however, how early in development IGF1 can affect embryo physiology. Moreover, little is known about the
molecular basis of how IGF1 mitigates the effects of heat stress on embryo development, increases embryonic survival following transfer during hot season and increases bovine embryonic development.

The objectives of this dissertation were to 1) determine the mechanisms by which IGF1 acts to increase the percent of oocytes becoming a blastocyst, 2) evaluate the molecular basis for the developmental acquisition of thermoprotective actions of IGF1 on preimplantation embryos, and 3) identify candidate genes induced by IGF1 in blastocysts that could mediate effects of IGF1 on embryonic survival during heat stress.

**Key Events During Preimplantation Development in the Bovine**

A series of key events takes place after fertilization to allow normal embryonic development. In the cow, the first week of pregnancy sees the embryo initiate cleavage divisions, activate its own genome and differentiate into trophectoderm (TE) and inner cell mass (ICM). Errors in these events, whether caused by environment or genetic or epigenetic factors, can lead to abnormal embryo development and pregnancy loss.

**Embryonic Genome Activation**

Embryonic genome activation (EGA) or the maternal-zygotic transition as it is sometimes called, is the process by which embryonic transcription is activated [38]. In the cow, EGA occurs between the 8-16 cell stage [39-40]. Until this time, maternal mRNA and protein support embryonic development. In the period before EGA, the embryo has a mRNA population similar to the one in the oocyte, whereas the 8-cell embryo exhibits an mRNA profile more comparable with the one found in blastocysts [41-42].

Activation of transcription is associated with increased translation of maternal mRNA for RNA polymerase IIA [43]. Inhibition of this polymerase has no effect on
development up to the 16-cell stage but blocks embryo development beyond the 16-cell stage [43], showing that transcription of embryonic genes is essential for normal development only after the 16-cell stage.

There is evidence for a limited amount of transcription before the 8-16 cell stage [42, 44]. Incubation of two-, four-, and eight-cell bovine embryos with $[^{35}\text{S}]\text{UTP}$ or $[^{3}\text{H}]\text{uridine}$ resulted in incorporation of label into the RNA, indicating transcriptional activity [43, 45]. Furthermore, two-cell embryos were capable of synthesizing HSPA1A in response to heat shock and this effect of heat shock was blocked by addition of transcriptional inhibitors such as $\alpha$-amanitin and actinomycin D [46-47].

The duration of the period of maternal control has been attributed to the stability of maternal mRNA [48]. Before EGA, there is a gradual degradation of maternal RNA and protein [38, 49] and a decrease in protein synthesis [50]. Some possible mechanisms for the degradation of maternal mRNA include binding of microRNAs to the 3’untranslated region of target RNA to repress their translation, regulatory RNAs that can bind to the 3’untranslated region and target the mRNA for degradation [38], and reduced availability of ribosome needed for translation [51].

**Epigenetic Modifications**

The newly formed embryo reprograms its new genome during early embryogenesis and preimplantation development [52]. Such reprogramming involves epigenetic modifications [53]. Inefficient reprogramming of DNA methylation may be in part responsible for low birth rates and development abnormalities. Abnormal DNA methylation has been found in bovine cloned embryos from the two-cell to the blastocyst stage compared to in vitro produced embryos [54-55]. Furthermore, Li et al. [56] found abnormal DNA methylation, histone acetylation and gene expression in
cloned calves that died during the perinatal period or at least 6 months after the prenatal period compared to in vivo produced animals.

Bovine embryos undergo DNA demethylation during early cleavage stages with demethylation reaching a nadir at the 8-cell stage (day 2-3 post-insemination) [57]. Demethylation is followed by de novo methylation beginning at the 8-cell to the 16-cell stage (day 4 post-insemination) [57-59]. However, the pattern of demethylation differs between paternally and maternally-derived DNA. Paternal DNA starts demethylation before the first cell division and demethylation is complete around the two-cell stage. Demethylation of maternal DNA does not begin until close to two-cell stage and is completed around the 8-16 cell stages when de novo methylation is initiated [57-58].

Methylation takes place on the 5′-cytosine residues at CpG dinucleotides [60-62] and is catalyzed by enzymes known as DNA methyltransferase (DNMT) [62-63]. The DNMT family members, which include DNMT1, DNMT2 and DNMT3 [63-64], are classified as de novo and maintenance methyltransferases [63]. DNMT1 is believed to function primarily as a maintenance methyltransferase although it also is involved in de novo methylation (methylation is re-established) [64]. There are three isotypes: DNMT1s (the somatic form), DNMT1o (oocyte-specific form) and DNMT1p (sperm specific form) [64-65]. DNMT2 has a weak methylation activity but may play a role in centromere function [63-64]. DNMT3a and DNMT3b and DNMT3L have been identified as de novo methyltransferases [63-64, 66-67].

In mouse, it was found that DNMT1 is expressed in oocytes and throughout preimplantation, DNMT2 has low abundance throughout preimplantation development and expression increases between 8-cell and morula/blastocyst stage, DNMT3a is high
in oocyte and early embryos and *DNMT3b* is low in oocytes and early embryos and increases in morulae and blastocysts [68]. In the bovine embryo, *DNMT1*, *DNMT2*, *DNMT3a* and *DNMT3b* are present from the 2-cell to the blastocyst stage [69].

DNA methylation plays an important role in genomic imprinting, both in silencing certain genes as well as activating others [62]. Imprinted genes are those where there is monoallelic expression that is parent-of-origin dependent [70]. The majority of paternally expressed genes enhance fetal growth while maternally expressed genes suppress fetal growth [62]. *IGF2* and *IGF2R* are two examples of imprinted genes (paternally and maternally expressed, respectively) [71]. Altered expression of *IGF2* was related to defects of organs in cloned calves experiencing neonatal death [72]. Imprinting is established in the germline [73], in female mammals, imprinting autosomal genes are established during folliculogenesis while in males, imprints are reset during fetal development [74]. Histone modification is another important epigenetic process whereby histones undergo acetylation, methylation, phosphorylation, ubiquitylation or sumoylation [60].

Histone acetyltransferases are enzymes involved in histone acetylation, and acetylated chromatin becomes more open and accessible for transcription [52, 75-76]. Acetylation can be removed by histone deacetylases leading to a more closed chromatin and repression of transcription [60, 75]. Histone modifications undergo dynamic changes during preimplantation development, for example, ICM and TE in the blastocyst have different histone modification profiles [60]. Expression of these enzymes was detected in all stages of bovine embryo development, indicating maternal and embryonic expression [77].
Polarization and Formation of the Blastocyst

After the first cleavage, the blastomeres of the two-cell embryo are at a right angle to each other. Thereafter, blastomeres divide asynchronously [51, 78]. The cells that divide early contribute more to the ICM than the cells that divide later [78-79]. This first division is thought to be involved in the establishment of the embryonic/abembryonic axis, which is involved in blastocyst polarity (ICM and polar TE at embryonic pole, and blastocoele and mural TE at the abembryonic pole) [79]. The polarization process is not well known in the cow but there are two models to explain the process in the mouse: the “inside-outside hypothesis” and the “cell polarity model”[79]. The first model proposes that cell position and cell-to-cell contact in the late morula determines cell fate [79]. The “cell polarity model” proposes that cell fate is established well before compaction, at the 8-cell stage in the mouse [80] and the differentiation pathway (ICM vs TE) depends upon whether cells undergo symmetric or asymmetric cell divisions lead to cell polarity along the radius of the early morula [79]. This process of polarization is an important determinant of cell fate because cells in contact with the external environment become TE while cells in the interior of the embryo are destined to form the ICM [78].

The first morphogenetic step of differentiation is the process of compaction, which occurs on day 4 to 5 post-insemination at the 16 to 32-cell stage in the cow [78] and is characterized by a change in appearance of the embryo so that individual cell borders are not discernable. Compaction is caused by establishment of adherens junctions between blastomeres [81-83]. In the absence of E-cadherin (a component of adherens junctions), for example, there is a decrease in the proportion of embryos becoming a blastocyst [84], and embryos fail to form trophectodermal epithelium [85]. The development of junctions between blastomeres results in the formation of different
compartments within the embryo. Some blastomeres remain in contact with the external environment whereas other cells are totally surrounded by other blastomeres.

Following compaction and establishment of cell polarity, the process of blastocyst formation begins involving cavitation and differentiation of blastomeres to TE and ICM. Cavitation is mediated by fluid transfer across the blastomeres and formation of a cavity filled with fluid, the blastocoel, at day 6-8 post-insemination [81]. Cavitation requires two cellular processes. First, water is moved into the interior extracellular space of the embryo by the combined actions of ATP1A1 (which uses ATP to pump Na\(^+\) out of and K\(^+\) into the cell) and aquaporins (which allow water movement directly across the cell) to form the fluid-filled blastocoelic cavity [81, 83]. The inhibition of ATP1A1 with ouabain caused a decrease in blastocyst diameter [86], and disruption in gene expression of ATP1A1 inhibited blastocyst formation [87].

Secondly, the intercellular junctions between cells (including tight junctions, adherens junctions and desmosome junctions) are required to maintain a impermeable seal between the inside and outside of the embryo and prevent blastocoelic fluid from diffusing out of the embryo [83, 88]. Tight junctions contribute to the maintenance of cell membrane polarity and intercellular signaling, and are composed of occludin and claudin proteins [83, 88].

Gap junctions allow communication between adjacent cells, and are composed of connexins [88-89]. The role of gap junctions for embryo development is controversial, since in some studies the use of inhibitors or knockout animals for connexin caused no interference in early embryo development [90-91], whereas in other studies using antibody inhibitors, embryo lethality was induced [83, 89]. Adherens junctions are
formed from E-cadherin and catenins and are involved in cell-to-cell adhesion, [88]. Desmosomes also play a role in cell-cell interaction, stabilizing the TE during blastocyst formation and expansion [88, 92].

**Challenges Associated with Production of In Vitro Produced Embryos**

Advances in in-vitro maturation, fertilization and culture are still required to optimize embryo competence for post-transfer development and eliminate inefficiencies and problems that limit use of IVP embryos [93]. As compared to embryos produced in vivo, IVP embryos have a reduced probability of developing to the blastocyst stage [94-95], decreased pregnancy rates and increased number of fetuses and calves with abnormalities [96-97], and lowered cryotolerance [17, 94, 98-100]. The reduced competence of the IVP embryo is associated with altered ultrastructural and physiological features such as decreased volume of mitochondria [101-102], higher rates of chromosomal abnormalities [103-105], and altered gene expression [102, 106-107]. There are several possible causes for reduced competence of IVP embryos that are described below.

**Oocyte Source**

In cattle, approximately 90% of immature oocytes undergo maturation in vitro and about 80% undergo fertilization, but only 20 to 40% reach the blastocyst stage [102]. Oocyte source is an important factor affecting in vitro embryo production. Many of the oocytes used in IVP are obtained from abattoir ovaries, and oocytes are collected from follicles of different sizes. Follicle diameter affects oocyte competence to develop to the blastocyst stage [108-112]. For example, 66% of oocytes from follicles > 6 mm became blastocysts after in vitro fertilization versus a value of 34% for oocytes from 2-6 mm follicles [108].
Differences in animal age also influence oocyte quality. Zygotes derived from prepuberal calf oocytes had ultrastructural abnormalities after maturation and cleaved and developed at a lower rate compared to adult cattle oocytes [113]. In another study, oocytes derived from cows had significantly higher blastocyst yield at day 8 than that from heifers [114-115].

**Conditions for Oocyte Maturation**

Oocytes matured in vivo are more competent than those matured in vitro. Rizos et al. [94] found a reduced rate of blastocyst development for oocytes matured in vitro as compared to those matured in vivo (39 and 58%, respectively). Katz-Jaffe et al. [116] reported that in vitro matured bovine oocytes had decreased amounts of mRNA for genes involved in volume regulation, osmoreception and cell cycle progression such as $AQP3$ and $SEPT7$, and increased expression of $SIAH2$, involved in stress-induced apoptosis. Also, mRNA for three imprinted genes, $IGF2R$, $PEG3$ and $SNRPN$, were present in higher amounts for oocytes matured in vitro [116]. Furthermore in vitro maturation can affect gene expression in the resulting blastocyst. The addition of bovine serum albumin in maturation medium increased expression of $IGF1R$, $IGF2$ and $IGF2R$ in day 9 bovine blastocysts and the use of fetal bovine serum in maturation medium increased mRNA for $HSPA1A$ [117].

Improvement of maturation conditions to increase oocyte maturation and blastocyst development can be achieved by addition of factors in maturation medium such as linolenic acid [118], sodium nitroprusside [119], leptin [120], polyvinyl alcohol-40 and follicle-stimulating hormone [121-122].
Fertilization

Fertilization conditions can affect developmental potential of the subsequent embryo. Using in vivo matured oocytes, Rizos et al.[94] reported that a greater proportion of inseminated oocytes became blastocysts when oocytes were fertilized in vivo (74%) than when oocytes were fertilized in vitro (58%).

Many aspects of in vitro fertilization have been studied to improve blastocyst development. Aging of sperm before in vitro fertilization reduced cleavage rates [123]. The use of heparin [124] and adjustment of sperm concentration for in vitro fertilization has improved fertilization and blastocyst yield [125]. Individual bulls require different concentration of heparin and sperm for in vitro fertilization [125]. Moreover, the sire used for fertilization can affect the proportion of embryos that develop to the blastocyst stage [126].

Different methods for sperm purification for in vitro fertilization have been used. Higher cleavage rates and embryo development were obtained when Percoll gradient was used for sperm purification than when a fertilization medium was used alone or with 20% bovine albumin serum [124]. Fertilization time is another factor that can influence blastocyst development. Recent data have shown that the proportion of cleaved embryos that developed to the blastocyst stage was higher for embryos produced by incubation of oocytes with sperm for 6 hr as compared to 9, 12 or 18 hr [127].

Culture Conditions for the Embryo

One important aspect of IVP affecting embryo competence is the period of embryo culture after fertilization. One model to demonstrate this concept has been to compare embryos produced and cultured in vitro with embryos produced by in vitro maturation and fertilization and then placed in the ewe oviduct for development to the blastocyst
stage. Embryos cultured in vivo had higher development to the blastocyst stage and higher survival rates to vitrification compared to embryos cultured in vitro [94, 98]. In another study [128], there was no difference in rate of blastocyst development between in vitro fertilized embryos cultured in vivo or in vitro but there were many transcripts that differed between the two types of embryos. For day 7 blastocysts, there was higher expression of IFNT, G6PD, BAX and SOX for embryos cultured in vitro and more expression of SOD2, IGF2, IGF1R and GJA1 for in vivo cultured embryos [128]. In another study, bovine blastocysts produced in vitro had higher amounts of mRNA for genes related to apoptosis (such as BAX and SOX) when compared to those produced in vivo [99, 129]. Lazzari et al. [130] found increased mRNA abundance for HSPA1A, SOD1, SLC2A3, SLC2A4, and IGF1R in bovine blastocysts produced in vitro compared with those produced either in the sheep oviduct or by superovulation.

More recently, a pairwise comparison identified 238 genes that were expressed with a twofold or more difference between embryos produced in vivo by artificial insemination and IVP or somatic cell nuclear transfer embryos [131]. Some of the genes that were upregulated in IVP embryos as compared to embryos produced by artificial insemination included HDAC3, DNMT1 and CDKN1C. Among the down-regulated genes were ESR1 and OXTR.

Although many treatments can affect blastocyst yield, few studies have examined embryonic survival after transfer. Addition of hyaluronan to culture increased blastocyst yield and improved embryo survival following vitrification [132] Another molecule that can affect competence of IVP embryos to survive transfer is CSF2. Addition of CSF2 to embryo culture from day 5 to 7 after insemination increased pregnancy rate and calving
rate after transfer and decrease pregnancy loss [133]. The gaseous environment may also affect embryo competence to establish pregnancy. To test this hypothesis, Merton et al. [134] cultured IVP embryos in a conventional incubator or in an incubator with carbon-activated air purification unit. While there was no difference in the proportion of embryos developing to the blastocyst stage, pregnancy rate was 12.9% - 14.6% higher for fresh and frozen/thawed embryos, respectively, produced in the filtered air incubator than in the standard incubator.

Taken together, results suggest that modification of embryo culture systems by providing an environment that more closely matches the uterine environment is a likely strategy to improve development and survival of IVP embryos. Important factors to consider are media composition, presence of growth factors and cytokines, and the gaseous environment inside the incubator.

**Use of IGF1 to Improve Embryonic Development in Vitro**

**Biology of IGF1**

The insulin-like growth factor family consists of three structurally related peptides: IGF1, IGF2 and insulin; three cell surface receptors (IGF1 receptor, IGF2 mannose-6-phosphate receptor and insulin receptor); and six IGF binding proteins (IGFBP-1 through 6; [135-136]). IGF1 consists of 70 amino acids and a molecular weight of 7.6 kDa [136]. The major secretagogue for IGF1 is growth hormone (GH) (or somatotropin) from the somatotroph cells of the anterior pituitary. GH acts in an endocrine fashion at the liver where it binds to its receptors and induces hepatic production of IGF1 [137-139].

Even though GH is the main regulator of circulating IGF1, insulin is a metabolic signal in the coupling of the GH/IGF1 axis. Insulin infusion in lactating cows increased
IGF1 plasma concentrations and mRNA expression for GHR and IGF1 in the liver [140].

The effect of insulin to increase hepatic IGF1 synthesis is mediated in part by the increase in GHR protein in the liver [141].

Nutritional status of the dairy cow can influence circulating concentrations of IGF1 [142-143]. Early postpartum, there is a period of negative energy balance which is associated with reduced expression of GHR-1A in the liver [144], reduced IGF1 synthesis [144-145] and increased concentrations of GH concentrations in plasma [146].

**Signaling by IGF1**

Actions of IGF1 are mediated by the IGF1 receptor, which is a transmembrane tyrosine kinase receptor that is activated with different potencies by at least three different ligands: IGF1, IGF2 and insulin. IGF1R is composed of two extracellular α-subunits and two transmembrane β-subunits, which are linked together by disulfide bonds to form an αβ IGF1 receptor [136, 147-148]. The α-subunits contain a cysteine-rich binding domain. IGF1 binds to the α-subunits of the IGF1R to cause a conformational change in the IGF1R β-subunits resulting in the tyrosine phosphorylation of the β-subunits. In addition, the β-subunits phosphorylation leads to the phosphorylation of tyrosine residues on several docking proteins, IRS-1 and Shc-homology protein [148]. The phosphorylation of IRS-1 triggers the activation of PI3K which increases the conversion of the PIP2 (a phospholipid component of cell membranes) to PIP3. PIP3 binds to domains of at least two proteins, PKB/AKT and PDK1. The PKB/AKT pathway has a role in regulation of apoptosis and cell survival, and PDK1 activation of PKC and PKA to regulate the cell cycle and growth (**Figure 1-1**) [3, 148-153].
Another signaling pathway activated by IGF1 includes the Ras/Raf/MAPK pathway. Binding of IGF1 to IGF1R causes phosphorylation of the docking protein Shc which causes formation of a complex between Shc and Grb2 (another adaptor protein). Grb2 binds to guanine nucleotide exchange factor SOS and this complex promotes the removal of GDP from Ras. Ras can then bind GTP, become activated and phosphorylate and activate Raf1. Raf1 activation, in turn, leads to a cascade of phosphorylation events to activate the MAPKK pathway. This pathway is important in cell differentiation, metabolism, mitogenic responses and cell cycle triggering (Figure 1-1) [136, 147-148, 154-155].
Figure 1-1. IGF1 signaling transduction mediated by IGF1R (modified from a drawing by Jousan [156]). Following the bind of IGF1 to IGF1R the Ras/Raf/MAPK and the PKB/AKT pathway are activated and can lead to increased activity of anti-apoptotic factors and inhibition of pro-apoptotic factors, thereby decreasing apoptosis, increasing cell differentiation, proliferation, cell cycle and growth. MAPK pathway can also be activated through Grb2/SOS signaling by activation of IRS-1.

IGF2R is a mannose 6-phosphate receptor that has high affinity for IGF2 and low affinity for IGF1 and is not believed to have a major role in IGF signal transduction. It is thought to regulate IGF2 by targeting it for clearance and degradation, and hence inhibiting IGF2 actions [136, 157-158]. IGF1 and IGF2 actions are modified by IGFBP which bind to IGF1 and IGF2 with high-affinity. At least 99% of the IGFs in circulation are bound to the IGFBP, which increase IGF half-life and deliver the growth factors to tissues. Different actions of IGFPB are summarized on Table 1-1.
Table 1-1. Known actions of insulin like growth factor binding proteins

<table>
<thead>
<tr>
<th></th>
<th>Affinity to IGF</th>
<th>Modulation of IGF action</th>
</tr>
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<tbody>
<tr>
<td>IGFBP-1</td>
<td>1 and 2</td>
<td>inhibit and/or potentiate</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>2 more than 1</td>
<td>Inhibit</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>1 and 2</td>
<td>inhibit and/or potentiate</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>1 and 2</td>
<td>Inhibit</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>2 more than 1</td>
<td>Potentiate</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>2 more than 1</td>
<td>Inhibit</td>
</tr>
</tbody>
</table>

Modified from Rajaram et al. [159]

The IGFBP can either inhibit or potentiate IGF actions (Table 1-1) by sequestering IGF from the IGF1R, or releasing IGFs to bind the IGF1R. The release of IGF from the IGFBP can be induced by actions of endoproteases [160]. In addition, however, IGFBP can bind to cell surfaces or cell matrices and thereby experience a decrease in affinity for IGF1, and release the growth factor to act on cells [135, 157, 160]. Furthermore, IGFBP may have bioactivity independent of IGF or without triggering IGF1R signaling through interactions of specific domains in the IGFBP with specific domains on the cell surface [136, 157, 160-161].

**IGF1 in the Reproductive Tract**

While the primary source of circulating IGF1 is the liver, it is also expressed locally in several reproductive tissues. In cattle, this includes the ovary [29], oviduct [28], uterus [26, 29] and embryo from the two-cell to the blastocyst stage [27, 30, 162].

Transcripts for *IGF1* were detected in cumulus oocyte complexes derived from follicles that were 3 to 6 mm and 8 to 16 mm in diameter [163]. *IGF1R* mRNA was detected in oocyte and granulosa cells of preantral and antral follicles [164-165]. *IGF1* mRNA in the corpus luteum was highly expressed during the early luteal phase, with a
decrease from day 5 to 7 and then an increase from day 8 to 18 [166]. Amounts of IGF1 mRNA decreased after experimentally-induced luteolysis [167]. The role of IGF1 in the corpus luteum is not clear but IGF1 stimulates luteal progesterone secretion [168].

Transcripts for IGF1 were localized in the mucosa and muscle layers of the ampulla and isthmus in the bovine oviduct [28]. Highest expression of IGF1 mRNA in the oviduct was on day 3 post-insemination for non-lactating beef heifers and on day 0-1 for lactating dairy cows [28].

IGF1 mRNA expression in the uterus was higher at estrus and lowest during the early and late luteal phases, and mainly localized in the sub-epithelial stroma underlying the uterine luminal epithelium [169]. IGFBP1 and IGFBP3 mRNA have also been found in the uterus of ewes and heifers with expression greater in pregnant than in cyclic animals [170]. Amounts of IGFBP1 mRNA expression in the ewe uterus are upregulated by progesterone and IFNT [170].

Expression of IGF1, IGF2 and IGFBP were studied in the uterus of postpartum dairy cows at day 14 postpartum. IGF1 mRNA was localized in the sub-epithelial stroma of inter-caruncular and caruncular endometrium while IGF2 and IGF1R mRNA were localized in the deep endometrial stroma, the caruncular stroma and myometrium [171]. Expression of IGFBP3 was found in the luminal epithelium, IGFBP2, IGFBP4, IGFBP5 and IGFBP6 in the stroma and IGFBP4 and IGFBP5 in the myometrium [171].

**Actions of IGF1 on Embryonic Development and Survival**

IGF1 has an important effect on preimplantation embryonic development. In mice, addition of IGF1 added to culture medium beginning at the two-cell stage increased the proportion of embryos becoming a blastocyst and the number of cells in the ICM of the blastocyst [172-173].
In other species too, there are data supporting a relationship between IGF1 and embryo development and survival in vitro and in vivo. Addition of IGF1 to culture medium increased the proportion of embryos becoming blastocysts in the bovine [31-34], ovine [174], buffalo [175], pig [152], mouse [173] and human [176]. Additionally, IGF1 is mitogenic and can increase total cell number of bovine blastocysts [177-180] and decrease the number of apoptotic blastomeres [1, 3, 153, 178]. Matsui et al. [181] used a monoclonal antibody specific for the α subunit of IGF1 receptor to block IGF1 actions on embryo development to the morula stage, showing that actions of IGF1 are mediated by IGF1R.

Culture of bovine embryos with IGF1 increased steady-state amounts of mRNA for IGFBP2, IGFBP5 and decreased mRNA for IGF1R in blastocysts [182]. In another study, IGF1 altered amounts of several transcripts in blastocysts [183]. In particular, IGF1 increased mRNA for IGFBP3 and DSC2 and tended to increase amount of ATP1A1 and BAX mRNA. Also, there was a decrease in transcripts for HSPA1A and IGFI R [183].

**IGF1 and Fertility During Heat Stress**

**Effect of Heat Stress on Fertility**

Heat stress is a major cause of poor reproductive function in lactating dairy cattle. It has been shown that a 0.5 1°C increase in uterine temperature on the day of insemination reduce conception rates by 12.8% [184]. Heat stress decreases fertility by several actions including a decrease in blood flow to the uterus [185], reduced duration of estrus, impaired follicular development and oogenesis, and altered follicular steroid production [14, 186-187]. Oocytes that develop under elevated temperatures have altered membrane composition, which was associated with decreased oocyte viability.
and developmental competence [187-191]. Also in vitro studies showed that heat-shocked oocytes had increased apoptosis and decreased cleavage rates [192].

Elevated temperatures (i.e., heat shock) such as experienced by heat stressed females can also have deleterious effects on preimplantation embryos. Early embryonic development was compromised in cows exposed to heat stress in the first seven days of pregnancy [193-194]. In vitro exposure of embryos to elevated temperatures reduced development to the blastocyst stage [1, 3, 195].

The magnitude of the effect of heat shock on development of preimplantation bovine embryos is developmentally regulated. Heat stress reduced blastocyst yield in superovulated cows when applied on day 1 of pregnancy but not at days 3, 5, or 7 of pregnancy [194]. Heat stress also had a greater effect on embryonic mortality when applied early in gestation in pigs [196]. In vitro, embryos were more affected by heat shock when exposed early in development (two to four-cell stage) than when given later in development (day 4-5 post-insemination) [36, 197-198]. Edwards et al. [36] showed that heat shock at 41°C for 12 hr decreased blastocyst development for two-cell and four-cell embryos (0% vs 26% and 10% vs 25% for heat shocked and control, respectively), but did not affect morula (42% vs 37% heat shocked and control, respectively).

There are many physiological effects of elevated temperature on the embryo that could be responsible for disrupted development. Heat shock of the two-cell embryo caused swelling of mitochondria and disruption of microfilaments and microtubules to cause movement of organelles towards the center of the blastomere [199-200]. Heat shock can increase intracellular levels of reactive oxygen species (ROS) [198], which is
correlated with DNA fragmentation [201], and with an increase in embryonic mortality [202]. Exposure to elevated temperature can also induce apoptosis. This effect occurs in maturing oocytes [187, 192] and embryos after the 8-16 cell stage [1, 3, 153, 203]. Overall protein synthesis in oocytes and embryos can also be reduced by heat shock, although heat shock protein 70 increases during heat shock [10, 47], presumably to block apoptosis and stabilize proteins denatured by heat shock [204].

Furthermore, Rivera et al. [205] have shown that two-cell embryos submitted to heat shock were arrested and did not pass the eight-cell stage. One possible cause for this embryonic arrest could be the increase in oxidative stress leading to higher levels of p66shc mRNA. P66Shc is a stress adaptor protein associated with early embryonic arrest [206-208] and it regulates mitochondrial metabolism by modulating the amount of ROS released into the cytosol [209].

**Heat Shock and IGF1**

IGF1 can reduce effects of elevated temperature on the bovine preimplantation embryo. Culture of embryos in the presence of IGF1 diminished the negative effects of heat shock administered at day 5 post-insemination on the percent of oocytes becoming blastocysts and number of apoptotic blastomeres [1, 3, 153]. The anti-apoptotic actions of IGF1 are mediated through activation of the PI3K/AKT pathway because either a PI3K inhibitor or an AKT inhibitor blocked the anti-apoptotic actions of IGF1 in heat-shocked embryos [3, 153]. In addition to protecting embryos from elevated temperature, IGF1 can act as a survival factor to reduce effects of hydrogen peroxide on mouse preimplantation embryos [210], induction of apoptosis by camptothecin and actinomycin D in mouse embryos [211] by menadione in bovine embryos [212] and by ultraviolet radiation in rabbit embryos [213].
IGF1 can also improve competence of an embryo to establish and maintain pregnancy following transfer to recipients provided the recipients are exposed to heat stress. Lactating cows exposed to heat stress were more likely to become pregnant following transfer of an in vitro produced embryo if the embryo was cultured in the presence of IGF1 [33]. In another experiment, the effect of IGF1 on post-transfer embryo survival in lactating cows was evaluated for warm and cool seasons [35, 133]. During the hot season, pregnancy rate was higher for cows receiving an embryo treated with IGF1 (18% vs 33% for control and IGF1, respectively). During the cold season, however, there was no difference in pregnancy rate between recipients receiving control or IGF1 treated embryos (27.6% vs 23%, control and IGF1, respectively) [35]. Similar results were seen in another experiment with lactating cows [133] except that the IGF1 effect was not significant.

The mechanism by which IGF1 improves embryo survival after heat shock and after transfer into heat-stressed recipients is not known. The inhibition of apoptosis caused by IGF1 is probably not responsible for increased survival of embryos to elevated temperature because a similar protective effect was not caused by administration of a caspase-3 inhibitor [3]. In fact, development is sometimes more likely to be blocked by heat shock when caspase-3 activity is inhibited [3, 214]. Among the actions of IGF1 that could improve survival after heat shock are mitogenesis, to increase blastomere proliferation [178-180], and increased expression of SLC2A genes, to increase uptake of energy substrates [215].

To determine possible factors responsible for increased survival of embryos treated with IGF1 after transfer into heat-stressed recipients, Block et al. [183]
measured characteristics of blastocysts produced in the presence of IGF1. There was no effect of IGF1 on blastocyst total cell number, the proportion of apoptotic blastomeres or the ratio of TE: ICM. However, IGF1 increased transcript abundance for \textit{ATP1A1} and \textit{DSC2} that are involved in blastocyst formation.

One possible approach to reduce the effects of heat stress on fertility is to administer recombinant bovine somatotropin (bST). This hormone increases plasma concentrations of IGF1 [216-218] and, in some studies, increases pregnancy rates following timed artificial insemination [218-221]. There is little evidence that bST can improve pregnancy rate in heat-stressed cows. However, in one study bST increased plasma concentration of IGF1 in lactating cows exposed to heat stress but did not have a significant effect on pregnancy rates (14.8\% vs 17.2\% for control and bST) [2]. Bell et al. [222] found similar results in another study (22.4\% pregnancy rate for control vs 24.8\% pregnancy rate for bST). One possible reason for the ineffectiveness of bST is that IGF1 induced by bST may not be thermoprotective in embryos at the earliest stages of development, when thermosensitivity is highest. It is not known whether IGF1 has thermoprotective actions on bovine embryos at stages earlier than day 5 [1, 3].

\textbf{Hypothesis and Objectives}

This dissertation focuses on two of the main actions of IGF1 on development of the preimplantation bovine embryo - the increase in proportion of embryos that develop to the blastocyst stage and the thermoprotective effects of IGF1 during culture and after transfer into recipients.

Development from the one-cell stage to the blastocyst stage is accompanied by an increase in cell number. The rate of increase in cell number, in turn, depends upon the ratio between cell proliferation and apoptosis (Figure 1-2). In addition, the embryo must
overcome cell arrest, an event in which the embryo enters a senescence-like stage where cells stop dividing [207]. Furthermore, development to the blastocyst stage requires a sequential series of events beginning with degradation of maternal RNA, embryonic genome activation, compaction, blastocoele formation and differentiation of cells into the TE and ICM (Figure 1-2).
Figure 1-2. Potential actions of IGF1 on embryonic development. Development from the 1-cell stage to the blastocyst stage depends upon an increase in cell number and the rate of increase in cell number depends upon the ratio between cell proliferation and apoptosis. In addition, embryo development can be blocked by embryo arrest. Furthermore, development to the blastocyst stage requires a sequential series of events beginning with degradation of maternal RNA, EGA, compaction, blastocoele formation and differentiation of cells into the TE and ICM.
Given this scenario, one action of IGF1 that could result in an increase in the proportion of embryos becoming blastocysts would be an increase in embryo cell number. This action could involve stimulation of cell proliferation and/or a decrease in the number of blastomeres undergoing apoptosis. In addition IGF1 could block cell arrest, stimulate embryonic genome activation, or activate genes involved in compaction or blastocyst formation. Experiments to determine whether IGF1 affects competence to develop to the blastocyst stage before or after day 4 of development can help distinguish between these possible mechanisms, because effects on maternal mRNA degradation and embryonic genome activation would occur before day 4 while effects on compaction and blastocyst formation would occur after day 4.

A second goal of this dissertation is to evaluate whether the ability of IGF1 to protect embryos from heat shock [1, 3] is developmentally regulated, i.e., whether IGF1 can protect embryos from heat shock at early stages of development when the embryo is most susceptible to elevated temperature [36, 198]. It is possible that the early embryo may lack signaling molecules for IGF1 in sufficient quantity for IGF1 to affect embryo function or that lack of activation of the embryonic genome may limit cellular responses to IGF1 early in development. In addition, because the early embryo is so susceptible to heat shock, the thermoprotective actions of IGF1 may not be sufficient to overcome damage caused by heat shock. The question of developmental regulation of IGF1 thermoprotection is an important one practically because it relates to the likelihood of identifying treatments to improve fertility in heat-stressed cows. bST, which stimulates secretion of IGF1 [216], increased fertility of lactating cows not exposed to heat stress [219-220] but did not increase pregnancy rates of heat stressed cows [2, 222].
A third goal of the study was to identify genes whose expression is regulated by IGF1 at day 7 of development to help understand the mechanism by which embryos treated with IGF1 are better able to establish pregnancy in heat stressed cows than control embryos. In particular, it is hypothesized that IGF1 causes differential expression of genes related to survival from stress (*HSPA1A, SOD2, GPX*), embryonic growth (*IGF1, IGF1R, SLC2A, BMP15, PED*) or apoptosis.
CHAPTER 2
ACTIONS OF INSULIN-LIKE GROWTH FACTOR-1 TO INCREASE DEVELOPMENT OF BOVINE EMBRYOS TO THE BLASTOCYST STAGE

Introduction

Proper development of the embryo is dependent upon maternal signals. While embryos can grow in simple defined media, the pattern of development can be disrupted. In the cow, for example, in vitro produced embryos suffer from a variety of morphological and molecular abnormalities and competence of the resultant embryo to survive freezing or transfer into recipients is reduced compared to embryos produced in vivo [223-224]. Lack of maternal signals controlling development is responsible for at least some of the problems inherent in the embryo produced in vitro since potential for development to the blastocyst stage, cryotolerance and gene expression can be made more similar to that of embryos derived in vivo if in vitro produced embryos are returned to the oviduct after fertilization [224].

Growth factors and cytokines that can affect embryonic development have been identified in a variety of species. In the cow, these include vascular endothelial growth factor [225], epidermal growth factor [226], colony stimulating factor 2 [133, 227], leukemia inhibitory factor [228] and interleukin-1β [229]. The mechanisms by which embryonic development is improved by these factors are not known. Effects on the proportion of embryos that develop to the blastocyst stage could be caused by stimulation of cell proliferation, inhibition of apoptosis and embryo arrest, or promotion of key events such as maternal RNA degradation, embryonic genome activation, compaction and blastocoel formation.

Here we evaluated how one growth factor capable of regulating embryonic development, IGF1, increases the proportion of embryos that develop to the blastocyst
stage. IGF1 is mainly produced in the liver upon stimulation by growth hormone [137, 230] although some local synthesis in the ovary, uterus and embryo has been reported [27, 29, 231]. Treatment with IGF1 can increase the proportion of embryos becoming blastocysts in the bovine [31-32, 34] ovine [174], buffalo [175], pig [152], mouse [172] and human [176]. In the cow, the model species examined for the present study, IGF1 also improves resistance of preimplantation embryos to heat shock [1, 3] and oxidative stress [212], alters expression of several genes at the blastocyst stage [183] and improves embryo survival after transfer into heat-stressed recipients [35, 133].

Specific objectives of the current study were to determine whether the pro-developmental actions of IGF1 are exerted before or after day 4 of development (i.e., on events occurring through the period of genomic activation versus events coincident with compaction and blastocoele formation), whether MAPK or PI3K signaling pathways mediate effects of IGF1, and whether IGF1 alters expression of genes controlling blastocoel formation.

Materials and Methods

Materials

Unless otherwise mentioned, reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA). HEPES-Tyrodes Lactate (TL) and IVF-TL, solutions were purchased from Caisson (Sugar City, ID) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (HEPES-TALP), and IVF-TALP as previously described [232]. Oocyte collection medium was tissue culture medium-199 (TCM-199) with Hanks salts without phenol red (HyClone, Logan, Utah) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Gibco,
Grand Island, NY) with Earle’s salts supplemented with 10% (v/v) bovine steer serum, 2 
µg/ml estradiol 17-β, 20 µg/ml bovine follicle stimulating hormone (Folltropin-V; 
Bioniche, London, ON, Canada), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin 
sulfate, and 1 mM glutamine. Percoll was from GE Healthcare (Uppsala, Sweden).

Frozen semen from various bulls was donated by Southeastern Semen Services 
(Wellborn, FL). The embryo culture medium was Synthetic Oviduct Fluid-Bovine 
Embryo 1(SOF-BE1). The formulation was as described by Fischer-Brown et al. [233] 
except that bovine serum albumin was omitted, the concentration of sodium lactate was 
5 mM and additional components were added as follows: polyvinyl alcohol (1 mg/ml), 
alanyl-glutamine (1 mM), sodium citrate (0.5 mM) and myo-inositol (2.77 mM).

Recombinant human IGF1 was purchased from Sigma. A vial containing 50 µg of 
lyophilized IGF1 was rehydrated with 200 µl of water, and this stock solution was then 
stored at -20°C in 5 µl aliquots until dilution to the requisite concentration with SOF-BE1 on the day of use.

Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-
bin/primer3plus/primer3plus.cgi) or were based on Sakurai et al. [234] (CDX2), and 
were synthesized by Integrated DNA Technologies 

**In Vitro Production of Embryos**

Ovaries were obtained from Central Beef Packing Co. (Center Hill, FL), and 
transported in 0.9% (w/v) NaCl solution at room temperature. Cumulus-oocyte 
complexes (COCs) were obtained by slicing 2 to 8 mm follicles on the surface of 
ovaries. Those COCs containing at least one layer of compact cumulus cells and even granulation were washed in oocyte collection medium. COCs were matured for 20-22 hr
in groups of 10 in 50 µl drops of oocyte maturation medium overlaid with mineral oil at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. Matured COCs were then washed in HEPES-TALP and transferred in groups of 200 to a 35 mm petri dish containing 1700 µl of IVF-TALP supplemented with 80 µl PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl), and fertilized with 120 µl Percoll-purified spermatozoa (~ 1x10⁶ sperm cells). Sperm were prepared from a pool of frozen-thawed semen from three different bulls; a different set of bulls was generally used for each replicate). After 6 to 10 hr of fertilization in an atmosphere of 5% CO₂ in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortexing for 4 min in HEPES-TALP and hyaluronidase (10,000 U/ml in 600 µl HEPES-TALP medium) and washed in HEPES-TALP. Embryos were then placed in groups of 30 in 50 µl drops of SOF-BE1 overlaid with mineral oil. Embryos were cultured at 38.5°C in an atmosphere of 5% CO₂ in humidified air.

**Concentration-Dependent Actions of IGF1 to Increase Blastocyst Development**

Following fertilization, embryos were washed and cultured in 50 µl drops of SOF-BE1 (control), or SOF-BE1 containing 10, 100, or 200 ng/ml IGF1. Concentrations were chosen so that the second concentration was within the range of values for IGF1 in blood of lactating cows [235-236]. The percentage of oocytes that cleaved was observed at day 3 post-insemination and the percentage of embryos that became blastocyst was observed at day 7 and day 8 post-insemination. The experiment was replicated 4 times using 231 to 284 embryos per group.
Determination of the Stage of Development at Which IGF1 Acts to Increase Blastocyst Development

This experiment tested whether IGF1 improves developmental competence by acting from day 0-4 post-insemination (i.e., on events occurring through the period of genomic activation at the 8-16 cell stage) or from day 4 to day 8 post-insemination (i.e., coincident with compaction and blastocoel formation). Following fertilization, putative zygotes were washed and assigned to one of four treatments: control, IGF1 from day 0-8 post-insemination, IGF1 from day 0-4 post-insemination or IGF1 from day 4-8 post-insemination. Embryos were placed in groups of 30 in 50 µl drops of SOF-BE1 ± 100 ng/ml IGF1 at day 0. For all treatments, embryos were washed at day 4 and transferred to fresh medium containing SOF-BE1 ± 100 ng/ml IGF1. The percent of oocytes that cleaved was assessed at day 4 post-insemination and the percent that became blastocysts was determined at day 7 and 8 post-insemination. The experiment was replicated 5 times using 332 to 356 embryos per group.

Role of MAPK and PI3K Signaling Pathway in IGF1 Actions

Two experiments were performed to test whether the increase in blastocyst development caused by IGF1 is mediated by the MAPK or PI3K pathways using PD98059 and LY294002 as inhibitors. Embryos were produced as described above and cultured in SOF-BE1 from day 0-4 post-insemination. At day 4, embryos were placed in groups of 30 in 50 µl drops of SOF-BE1 containing treatments. For the MAPK experiment, treatments were SOF-BE1 containing 0.1% dimethyl sulfoxide (DMSO vehicle), SOF-BE1 containing 0.1% DMSO and 100 ng/ml IGF1, SOF-BE1 containing 0.1% DMSO, 100 µM PD 98059 and SOF-BE1 containing 0.1% DMSO (vehicle), 100 ng/ml IGF1 and 100 µM PD 98059. For the PI3K experiments, treatments were similar
except that PD 98059 was replaced with 100 µM LY294002. Embryo development was assessed at day 7 and 8 post-insemination. The inhibitor experiment was replicated 5 times using 308 to 378 embryos per group, and the PI3K inhibitor was replicated 7 times using 399 to 515 embryos per group.

**Action of IGF1 on Expression of Genes Controlling Compaction and Blastocyst Formation**

Following fertilization, putative zygotes were washed and cultured in 50 µl drops of SOF-BE1. At day 4, embryos were transferred to fresh medium of either SOF-BE1 or SOF-BE1 containing 100 ng/ml IGF1. Morula and early blastocysts were selected at day 6 and frozen at -80°C until RNA extraction. Total cellular RNA was extracted from groups of 20 embryos using the Arcturus PicoPure RNA Isolation kit (MDS, Analytical Technologies) following the manufacturer's instructions. After RNA extraction, all samples were treated with DNase (DNAse I Kit RNase-free; New England Biolabs) and then cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City-CA). Reverse-transcribed cDNA was subjected to real-time PCR amplification using a 25 µl reaction consisting of 2.5 µl of cDNA sample, 12.5 µl of SYBR Green (Applied Biosystems), 2.5 µl (1 µM) of primers (Table 2-1) and diethylpyrocarbonate-treated (DEPC) water. Quantitative real-time RT-PCR (qPCR) was performed using an ABI 7300 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The thermal cycles was performed as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C of 15 sec and 60°C for 1 min. To obtain the fold difference, data were analyzed using the delta-delta cycle threshold ($\Delta\Delta C_{\text{t}}$) method described previously [237]. cDNA concentration-
dependent amplification was validated by making standard curves for all genes using serial 5-fold dilutions. This experiment was replicated 6 times.

**Statistical Analysis**

Data on the percent of oocytes that cleaved and became a blastocyst were analyzed by least-squares analysis of variance (ANOVA) using the Proc GLM procedure of the Statistical Analysis System (SAS for Windows, Version 9.2 Cary, NC). Percent data were transformed by arcsin-transformation before analysis. The mathematical model included main effects of replicate, treatment or treatments and all interactions. Replicate was considered random and other main effects were considered fixed. For the qPCR experiment, Cycle threshold (C<T>) and ΔC<T> were analyzed statistically but data are presented as fold differences. All values reported are least-squares means ± SEM. Probability values were based on analysis of arcsin-transformed data while least-squares means were from analysis of untransformed data.

The following orthogonal contrasts were used to determine differences between individual concentrations of IGF: 0 vs others, 100 vs 10 and 200 and 10 vs 200. For other analyses, identification of means that differed significantly was determined using the pdiff procedure of SAS.

**Results**

**Concentration-Dependent Actions of IGF1 to Increase Blastocyst Development**

Treatment with IGF1 did not affect the percent of oocytes that cleaved by day 3 post-insemination (Figure 2-1A) but increased the percent of embryos that became a blastocyst at day 7 (p<0.05; Figure 2-1B) and 8 (p=0.05; Figure 2-1C). At day 7, there was no statistical difference between 10, 100 and 200 ng/ml. At day 8, the percent of
oocytes that became a blastocyst was higher (P<0.05) for 100 ng/ml than for 10 or 200 ng/ml.

**Determination of the Stage of Development at Which IGF1 Acts to Increase Blastocyst Development**

As expected, treatment did not affect cleavage (Figure 2-2A) and addition of IGF1 from day 0-8 post-insemination increased (p<0.05) the percent of oocytes that became blastocysts at day 7 (Figure 2-2B) and 8 post-insemination (Figure 2-2C). A similar increase in percent of oocytes developing to the blastocyst stage was observed when embryos were cultured with IGF1 from day 4-8 (P<0.05 vs controls) but not when IGF1 was added from day 0-4 (Figure 2-2B and 2-2C). Representative image of day 8 blastocysts are shown in Figure 2-3.

**Effect of Inhibition of MAPK and PI3K Signaling on Actions of IGF1 to Promote Development**

Results from the experiment with the MAPK inhibitor PD 98059 are shown in Figure 2-4. Development at both day 7 and 8 was affected by inhibitor x IGF1 interactions (P<0.05). These interactions reflected the fact that IGF1 increased development in the absence of the inhibitor but not in the PD 98059-treated group.

Results from the experiment with the PI3K inhibitor LY-294,002 are shown in Figure 2-4. The inhibitor reduced the percent of oocytes that were blastocysts at day 7 and 8 post-insemination (P<0.05) and IGF1 tended to increase blastocyst development in the absence and presence of LY-294,002 at day 7 (P=0.09) and day 8 (P=0.08). There were no inhibitor x IGF1 interactions because IGF1 increased development in the presence and absence of the inhibitor.
**Action of IGF1 on Expression of Genes Controlling Compaction and Blastocyst Formation**

There was no effect of IGF1 on expression of *CDX2* or *OCLN* at day 6 post-insemination. IGF1 decreased the steady state expression of *CDH1* \( (P<0.05) \) and there was a tendency \( (P=0.07) \) for *ATP1A1* expression to be higher in IGF1 treated embryos (Figure 2-5).

**Discussion**

Insulin-like growth factor is an important maternal determinant of embryonic survival that can promote development to the blastocyst stage [31-32, 34], protect the embryo from several stresses [1, 3, 212] and increase competence for development to term, at least in heat-stressed females [35, 133]. As shown here, IGF1 exerts its pro-developmental effects at concentrations that are within the range of those found in the blood of lactating and non-lactating cows [235-236]. Since addition of IGF1 to culture medium at day 4 post-insemination increased blastocyst development while IGF1 from day 0-4 had no statistical difference from the control group on development, IGF1 exerts actions on development at a time after embryonic genome activation [49, 58] and when the embryo is undergoing compaction [238], DNA methylation [58], proliferation and blastocoel formation [238]. Furthermore, the pro-developmental effects of IGF1 involve actions mediated by the MAPK pathway and include alteration of genes controlling formation of the blastocoelic cavity.

Activation of IGF1 receptors leads to signaling through at least two main pathways, MAPK and PI3K that engage transcriptional and non-transcriptional events leading to a stimulation of cell proliferation and differentiation, inhibition of apoptosis and cytokine signaling [239]. The MAPK pathway is one of the pathways for the proliferative
actions of IGF1 [240-241] and it is possible, therefore, that the main action of IGF1 that increases blastocyst development is an increase in cell number. In this way, more embryos could reach a critical cell number necessary for differentiation into the blastocyst. Other molecules that stimulate proliferation also can increase the proportion of embryos that develop to the blastocyst stage [225-226]. Another possible way to control cell number, a reduction in apoptosis, does not seem to be major mechanism for the pro-developmental effects of IGF1 because inhibition of the PI3K pathway, which mediates the effects of IGF1 on apoptosis [3], did not prevent the ability of IGF1 to increase blastocyst development. There was, however, a reduction in the proportion of embryos that became blastocysts caused by addition of LY294002, indicating the importance of this signaling pathway for embryonic development.

There was evidence in the present study that IGF1 increases development to the blastocyst stage, at least in part, by regulating expression of genes involved in compaction and blastocoel formation. Compaction occurs on day 5 post-insemination at the 32-cell [238, 242] and blastocoel formation occurs beginning at day 6-7 post-insemination [243-244]. The process of compaction, which is necessary for subsequent development of the blastocyst, involves formation of junctional complexes involving CDH1 and OCLN [83]. There was no effect of IGF1 on steady state mRNA content of OCLN at day 6 post-insemination, but IGF1 decreased expression of CDH1. The decrease in CDH1 expression could represent a transient decrease in this adhesion molecule in preparation for blastocoel formation. By the blastocyst stage, Block et al. [183] found that IGF1 did not affect expression of CDH1. There was a tendency for IGF1 to increase expression of ATP1A1 at day 6 post-insemination, a time before most
embryos in our culture system have a visible blastocoele. Block et al. [183] also observed a tendency for IGF1 to increase ATP1A1 transcript abundance in day 7 blastocysts. Na+/K+ ATPase activity is involved in active transport of ions across the TE to form the fluid-filled blastocoelic cavity [81, 83]. It is not known whether effects of IGF1 on molecular events leading to blastocoel formation occur because of increased proliferation mediated by the MAPK pathway or whether regulation of these genes is independent of changes in the rate of cell proliferation.

There was no effect of IGF1 on the abundance of transcripts for the trophoblast marker, CDX2, [245]. This result suggests that IGF1 is not involved in differentiation of the TE and is consistent with the observation that IGF1 did not alter the TE:ICM ratio [183].

It is also possible that IGF1 promotes development to the blastocyst stage by regulating energy metabolism. There is an increase in oxygen consumption and glucose uptake at compaction and a larger increase at the blastocyst stage [246]. One action of IGF1 is increased transport of glucose [215]. In another study, however, IGF1 did not affect expression of the glucose transporters SLC2A1, SLC2A3 or SLC2A8 in bovine blastocysts [183].

In conclusion, IGF1 promotes development to the blastocyst stage by regulating MAPK-dependent events at day 4 or later. Among the actions likely to be important for the pro-developmental actions of IGF1 at this time are an increase in proliferation and promotion of blastocoel formation through regulation of expression of ATP1A1.
<table>
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Figure 2-1. Concentration-dependent effects of IGF1 on the percent of oocytes that cleaved (Panel A) and that became blastocysts at day 7 (Panel B) and day 8 (Panel C) post-insemination. Concentration of IGF1 did not affect cleavage rate (p>0.05). IGF1 increased the percent of oocytes becoming a blastocyst at day 7 and day 8 compared to control (p<0.05 and p=0.05 respectively). At day 7 there was no statistical difference between 10, 100 and 200 ng/ml, and at day 8 the percent of oocytes that became a blastocyst was higher for 100 ng/ml (P<0.05) than for 10 or 200 ng/ml. Data are least-squares means ± SEM of results from 4 replicates involving 231 to 284 oocytes per group.
Figure 2-2. Improvement in blastocyst development when IGF1 is added from day 4-8 of culture but not statistical different from controls when added from day 0-4. Data are least-squares means ± SEM and represent the percent of oocytes that cleaved (Panel A) and that became blastocysts at day 7 (Panel B) and day 8 (Panel C) post-insemination. Embryos were either cultured without IGF1, IGF1 from day 0-8 post-insemination, day 0-4 post-insemination or day 4-8 post-insemination. The main effect of treatment was significant for results at day 7 (p<0.05) and 8 (p<0.01) and differences between individual means (p<0.05) are indicated by different superscripts above each bar. Data are least-squares means ± SEM of results from 5 replicates involving 332 to 356 oocytes per group.
Figure 2-3. Representative images of day 8 embryos when IGF1 was used in different days of culture. Panel A, control group without IGF1; Panel B, IGF1 was added from day 0-4 of culture; Panel C IGF1 was added from day 4-8 of culture and Panel D, IGF1 was added from day 0-8 of culture.
Figure 2-4. Effect of the MAPK inhibitor PD 98059 and the PI3K inhibitor LY294002 on actions of IGF1 to increase the percent of blastocysts at day 7 and day 8 post-insemination. Black circles represent absence of inhibitor, and open circles represent the presence of inhibitor. For MAPK inhibitor PD 98059, development at both day 7 and 8 was affected by inhibitor x IGF1 interactions (P<0.05). For the PI3K inhibitor LY-294,002 experiment, the inhibitor reduced the percent of oocytes that were blastocysts at day 7 and 8 post-insemination (P<0.05) with or without IGF1 and IGF1 tended to increase blastocyst development in the absence and presence of LY-294,002 at day 7 (P=0.09) and day 8 (P=0.08).
Figure 2-5. Effects of IGF1 on expression of genes involved in compaction and blastocoel formation. Control group is represented by the black bars and the IGF1 treated group is represented by the white bars. IGF1 decreased the steady state expression of CDH1 (p<0.05) and there as a tendency of IGF1 to increase steady state expression of ATP1A1 on day 6 embryos (P=0.07).
CHAPTER 3
DEVELOPMENTAL CHANGES IN THERMOPROTECTIVE ACTIONS OF INSULIN-LIKE GROWTH FACTOR-1 ON PREIMPLANTATION BOVINE EMBRYOS

Introduction

Adverse effects of elevated temperature (i.e., heat shock) on development of the preimplantation embryo are one of the causes of reduced fertility during heat stress [14, 204]. Resistance of the preimplantation bovine embryo to heat shock can be modified by genetic, developmental and microenvironmental inputs. For example *Bos indicus* embryos are more resistant to heat shock than *B. taurus* embryos [247-250]. The embryo also acquires resistance to elevated temperature during development. Thus, exposure of cows to heat stress on day 1 after estrus reduced embryonic development to the blastocyst stage but heat stress at days 3, 5 and 7 had no effect [194]. Similarly, exposure to elevated temperature in vitro caused a greater reduction in development for two-cell embryos than for ≥ 16 cell embryos at day 5 of development [36, 197, 204]. Among the microenvironmental inputs affecting embryonic resistance to heat stress is the growth factor IGF1. Treatment of embryos with IGF1 reduces the magnitude of effects of heat shock on development and apoptosis at day 5 of development [1, 3]. Moreover, treatment with IGF1 in vitro enhances survival rates of blastocysts transferred into heat-stressed recipients [35].

Circulating IGF1 is synthesized and secreted primarily by the liver [251] although it is also expressed in several reproductive tissues including, in the cow, ovary, oviduct, uterus and embryo [26, 28-29]. Secretion by liver is increased by growth hormone [251]. Interestingly, however, injection of recombinant growth hormone into lactating cows exposed to heat stress did not increase fertility even though circulating concentrations of IGF1 were increased [2, 222]. One possible explanation is that, although IGF1 can
protect more advanced embryos from effects of heat stress [11-13], it cannot block effects of heat stress on the oocyte or early embryo. Not only are early cleavage-stage embryos maximally sensitive to elevated temperature [36, 197-198, 204], but lack of activation of the embryonic genome [49] may limit cellular responses to IGF1.

The present study had two objectives. The first was to determine whether the thermoprotective actions of IGF1 on the preimplantation bovine embryo were developmentally regulated so that the two-cell embryo was refractory to IGF1. The second was to determine the molecular basis for the improved competence of embryos treated with IGF1 to establish pregnancy after transfer to heat-stressed recipients [35]. Since this beneficial effect of IGF1 has not been observed when embryos were transferred to recipients not exposed to heat stress [35], it was hypothesized that IGF1 would enhance genes involved in cytoprotection and inhibit genes that would exacerbate effects of heat shock on the embryo.

**Materials and Methods**

**Embryo Culture Media and Additives**

Unless otherwise mentioned, reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA). HEPES-Tyrodes Lactate (TL) and IVF-TL solutions were purchased from Caisson Laboratories (Sugar City, ID). These media were used to prepare HEPES-Tyrodes Albumin Lactate Pyruvate (HEPES-TALP), and IVF-TALP as previously described [232]. Oocyte collection medium was TCM-199 with Hank’s salts without phenol red (HyClone, Logan, Utah) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 (Gibco, Grand Island, NY) with Earle’s salts supplemented with
10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-β, 20 µg/ml bovine follicle stimulating hormone (Folltropin-V; Bioniche, Bellevue, Ontario, Canada, 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL). The embryo culture medium was Potassium Simplex Optimized Medium (KSOM) that contained 1 mg/ml bovine serum albumin and was obtained from Caisson. On the day of use, KSOM was modified to produce KSOM-BE2 (KSOM-bovine embryo 2) as described previously [252]. Recombinant human IGF1 was purchased from Sigma-Aldrich. A vial containing 50 µg of lyophilized IGF1 was rehydrated with 200 ml of water. This stock solution was then stored at -20°C in 5 µl aliquots until use, when a single aliquot of IGF1 was diluted with KSOM-BE2 to a final concentration of 100 ng/ml.

**In vitro Production of Embryos**

Embryo production was performed as previously described [1, 3] using in vitro maturation of oocytes and in vitro fertilization. Immature COCs were collected from ovaries obtained from Central Packing Co. (Center Hill, FL, USA). Harvested COCs were washed in oocyte collection medium and allowed to mature for 20-22 hr in groups of 10 in 50 µl drops of oocyte maturation medium overlaid with mineral oil and at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. Matured COCs were then washed in HEPES-TALP and transferred to a 35 mm petri dish containing 1700 µl of IVF-TALP supplemented with 80 µl PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl), and fertilized with 120 µl Percoll-purified spermatozoa (~ 1x10⁶ sperm cells). To eliminate bull effects, a pool of frozen-thawed semen from three different bulls were used for each replicate; a separate pool was used for each
replicate. After 6 to 10 hr of fertilization, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortexing in HEPES-TALP and hyaluronidase (10,000 U/ml in 600 µl HEPES-TALP medium) for 4 min, washed in HEPES-TALP, and placed in groups of 30 in 50 µl drops of KSOM-BE2 overlaid with mineral oil. For most of the culture period, embryos were cultured at 38.5°C in a humidified atmosphere of 5% O₂ and 5% CO₂ (v/v) with the balance nitrogen. During the heat shock period, both control and heat-shocked embryos were placed in an atmosphere of 5% (v/v) CO₂ in humidified air. This atmosphere, which contains a higher oxygen content (21%) was used during heat shock because high oxygen tension exacerbates the deleterious effect of temperature on embryonic development to the blastocyst stage [253].

**Protective Effect of IGF1 on Heat Shocked Embryos at 41°C**

Two-cell embryos were selected at 28-30 hr post-insemination. Embryos with ≥ 16 cells were selected at day 5. Embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml IGF1 (treated group) or KSOM-BE2 only (control group) and then assigned randomly to temperature treatment. After 1 hr of preincubation with or without IGF1, embryos received one of two thermal treatments in an atmosphere of 5% CO₂ in air as follows: 38.5°C for 24 hr or 41°C for 15 hr and 38.5°C for 9 hr. Embryos were then washed 3 times in KSOM-BE2 drops to remove IGF1, placed in fresh drops of KSOM-BE2 and cultured in an atmosphere of 5% O₂ until day 8 post-insemination at 38.5°C when development to the blastocyst stage was assessed. The experiment with two-cell embryos was replicated 11 times using 169 to 174 embryos per group and the experiment for day 5 embryos was replicated 15 times using 193 to 201 embryos per group.
Protective Effect of IGF1 on Day 5 Embryos Exposed to Heat Shock at 42°C

Another experiment was performed where day 5 embryos were exposed to a more severe heat shock of 42°C. Day 5 embryos with at least 16 cells were selected and randomly transferred to a fresh drop of KSOM-BE2 + 100 ng/ml IGF1 (treated group) or KSOM-BE2 only (control group), and then assigned randomly to treatment. After 1 hr of pre-incubation with or without IGF1, embryos received thermal treatments in 5% CO₂ in humidified air as follows: 38.5°C for 24 hr or 42°C for 15 hr and 38.5°C for 9 hr. Embryos were then washed 3 times in KSOM-BE2 drops to remove IGF1, placed in fresh drops of KSOM-BE2 and cultured in 5% O₂ at 38.5°C until day 8 post-insemination when development to the blastocyst stage was assessed. The experiment was repeated 4 times using 59-60 embryos per group.

Developmental Changes in Expression of Genes Involved in IGF1 Signaling

In one experiment, embryos were cultured in 50 µl drops of KSOM-BE2 in 5% O₂. Two-cell embryos were selected at 28-30 hr post-insemination and embryos ≥ 16 cells were selected from separate wells at day 5 post-insemination. Selected embryos were washed in Ultraspec™ RNase free water (Biotecx – Houston, TX, USA) and stored at -80°C until RNA extraction. For each stage of development, total RNA was extracted from five groups of 20 embryos each using Arcturus PicoPure RNA Isolation Kit (MDS, Analytical Technologies, Sunnyvale, CA, USA) following the manufacturer's instruction. RNA was frozen at -80°C. Quantitative PCR was performed by Mogene, LLC (Saint Louis, MO, USA). RNA was quantified using the Ribogreen RNA quantification Kit (Invitrogen, Carlsbad, CA, USA) and then subjected to quantitative real-time RT-PCR (qPCR) using the TaqMan® RNA-to-CT™ 2-Step Kit (Applied Biosystems, Foster City, CA, USA) in 25 µl reactions. In the first step, cDNA is generated and in the second the
cDNA is quantitated using the TaqMan Gene Expression Master Mix. The thermal cycles for qPCR was 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C of 15 sec and 60°C for 1 min. The qPCR reactions were performed and fluorescence quantified with the ABI 7900HT system (Applied Biosystems, Foster City, CA, USA). The genes included *IGF1R, RAF1, MAPK* and *GAPDH*, with the last gene serving as an endogenous control (housekeeping gene) (Table 3-1). Each qPCR was run in triplicate.

For a second experiment, genes of interest included *IGF1R, PI3K, HK2* and *Hist2h2aa2* a housekeeping gene. Embryos were produced as described above and 7 groups of 25 two-cell embryos and 7 groups of 25 day 5 embryos were selected. RNA was extracted using Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA) following the manufacturer’s instruction. RNA was frozen at -80°C and sent to the University of Missouri for qPCR. Primer sets for genes were designed by the public domain primer design software Primer3 http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were chosen based on the published sequences of the bovine genome and the primers used had a product size of 150 to 300 bp in length (Table 3-2). cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse-transcribed cDNA was subject to real-time PCR amplification using 25 µl master mix reactions consisting of 12.5 µl of SYBRGreen (Applied Biosystems, Foster City, CA, USA), 5 µl (1 µM) of the primers (2.5 µl forward and 2.5 µl reverse), 5 µl of diethylpyrocarbonate-treated (DEPC) water and 2.5 µL of cDNA sample (1 ng/ µl). The PCR reactions were performed and fluorescence quantified with the ABI 7300 Applied Biosystems system (Applied Biosystems, Foster City, CA, USA). The thermal cycles for real-time PCR was
50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C of 15 sec and 60°C for 1 min. Each PCR was run in duplicate. For both experiments, responses were quantified using the C_T method, and the ΔΔC_T method was used to determine fold change.

**Immunofluorescent Analysis of Insulin-like Growth Factor 1 Receptor (IGF1R)**

This experiment was designed to evaluate the presence of IGF1R in two-cell and day 5 embryos. Two-cell embryos were selected at 28-32 hr post-insemination and embryos ≥ 16-cells were selected on day 5 post-insemination. Embryos were fixed in 4% (v/v) paraformaldehyde in 10 mM KPO_4_, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml polyvinylpyrrolidone (PVP) for 15 min at room temperature. After fixation, embryos were washed in PBS-PVP and stored in PBS-PVP at 4°C until immunofluorescent analysis within 3 days of fixation.

Fixed embryos were permeabilized with 0.25% (v/v) Triton-X in PBS for 10 min at room temperature and then washed three times in Tris buffered saline [TBS (10 mM Tris, pH 7.2, 0.9% (w/v) NaCl)] containing 0.1% (v/v) of Tween-20 (TBST). Embryos were blocked for 1 hr in PBS containing 20% (v/v) normal goat serum (Pel-Freez Biologicals, Roger, AR, USA) at room temperature and then washed two times in TBST. Afterwards, embryos were incubated overnight with the primary protein G-purified rabbit polyclonal antibody to IGF1R (Abcam, Cambridge, MA, USA) at a concentration of 2.5 µg/ml in TBST-BSA [TBST containing 0.1% (w/v) bovine serum albumin]. The negative control group was incubated with rabbit IgG (2 µg/ml, Sigma-Aldrich). After incubation, embryos were washed in TBST 3 times for 5 min each and incubated for 1 hr in the dark with 2 mg/ml anti-rabbit IgG F(ab')_2 fragment labeled with Alexa Fluor 555 (Cell Signaling, Danvers, MA, USA) diluted 1:1000 in TBST-BSA. Embryos were washed 3
times in TBST for 5 min each, and nuclear labeling was performed with 1 μg/ml of 4',6-
diamidino-2-phenylindole (DAPI) in TBST-BSA for 15 min. Embryos were rinsed in PBS-
PVP and placed in approximately 100 μl drops of PBS-PVP on a FluoroDish (World 
Precision Instruments, Inc, Sarasota, FL, USA) for analysis. Embryos images were 
extracted and examined with a laser confocal scanning microscope (Leica TCS SP5, 
Bannockburn, IL, USA).

Seven consecutive section were merged and the merged image was subjected to 
image analysis using ImageJ 1.43t software (National Institute of Health, Bethesda, MA, 
USA). Pixel intensity of membrane-assocaited immunofluorescence was analyzed and a 
threshold was chosen to designate the area of the picture to be analyzed (plasma 
membrane) while excluding lower-intensity staining in the cytoplasm. Pixel intensity in 
the selected areas was measured and a mean gray value calculated. A total of 5 two-
cell and 5 day 5 embryos were analyzed.

**Microarray Hybridization**

Embryos were cultured in KSOM-BE2 ± 100 ng/ml IGF1 in a 5% O₂ atmosphere. 
At day 7, grade 1 blastocysts [254] were selected. A total of four pools of 30 control 
blastocysts and four pools of 30 IGF1-treated blastocysts were produced. RNA was 
extracted using RNeasy Micro kit (Qiagen Inc, Valencia, CA, USA). Samples were 
frozen at -80°C and sent to Mogene LLC (St. Louis, MO, USA), an Agilent Certified 
Service Provider, for microarray analysis using the Agilent bovine gene expression 
microarray 4x44k (AMIDID 023647, Agilent Technologies, Santa Clara CA, USA). The 
array contains 43,803 bovine probes represented and the probes were developed by 
clustering more than 450,000 mRNA and EST sequences of the bovine genome (btau 
4.0).
Concentration of total RNA was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and integrity determined by Agilent 2100 Bioanalyzer RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara CA, USA). A representative analysis is shown in figure 3-1. The remainder of the RNA was amplified using Agilent Quick-Amp. Labeling Kit. Only those amplification reactions yielding amplified RNA of consistent size range and quantity across samples were utilized in subsequent microarray experiments. A total of 1.2 µg of the amplified material was labeled using the ULS aRNA fluorescent Labeling Kit (Kreatech Biotechnology, LG, Amsterdam). The hybridizations were setup so that, for two pairs of samples, RNA from the control embryos were labeled with Cy3 and RNA from IGF1 treated embryos were labeled with Cy5, whereas for the other two pairs, RNA from control embryos were labeled with Cy5 and RNA from the IGF1 group were labeled with Cy3. Arrays were hybridized for 17 hr at 65°C and 10 rpm. Arrays were washed following procedures described in the Agilent Gene Expression manual and were scanned at 5 µm on an Agilent C Scanner (Agilent Technologies, Santa Clara CA, USA).

Hybridizations were prepared using 1.65 µg of sample (825 ng per dye assignment) per array. Prior to hybridization, sample combinations (47.8 µl including 10x Blocking Agent) were fragmented with 2.2 µl of Agilent 25x Fragmentation Buffer (Agilent Technologies, Santa Clara CA, USA) at 60°C for 30 minutes. After fragmentation, 5 µl of Kreablock was added to each tube followed by 55 µl of Agilent 2x Hi-rpm Hybridization Buffer. This mixture was applied to an Agilent bovine gene
expression microarray 4x44k (AMIDID 015354, Agilent Technologies, Santa Clara CA, USA).

**Microarray Data Analysis**

The microarray image extraction and data pre-processing were performed using Agilent’s Feature Extraction software v 9.5 (Agilent Technologies, Santa Clara CA, USA). The intensity of each spot was summarized as the median pixel intensity, and then the generated values were transformed to log. The lowess method was used to normalize intensity within each array. Microarray data were analyzed using the JMP® Genomics 4 for SAS® 9.1.3 software (SAS Inst., Inc., Cary, NC). The quantile normalization method was performed for the data global normalization and least-squares analysis of variance conducted using the PROC ANOVA procedure of JMP® Genomics 4 for SAS® 9.1.3 to identify differentially regulated genes. The model included replicate and treatment. Replicate (array) was considered random and treatment was considered fixed. Correction for false discovery rate was performed by the Benjamini and Hochberg method [255] with a maximum false discovery rate of 0.01. P values were adjusted to a false discovery rate of 0.01 and genes with a fold change of at least 1.5 and a probability of P ≤ 0.05 were considered differentially expressed.

**qPCR**

Validation of the microarray data was conducted by performing qPCR on three genes that were upregulated by IGF1 (NFATC3, PPIP5K2, TGFB2), three genes downregulated by IGF1 (RAD23A, H1FOO, FADS6) and on GAPDH, which was used as a housekeeping gene. Primer sets for genes were designed by the public domain primer design software Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 3-3). To confirm primer specificity, amplicon
size was determined by agarose gel electrophoresis and amplicons were sequenced at
the Genetic Analysis Core Laboratory of the Interdisciplinary Center for Biotechnology
Research, University of Florida.

cDNA was generated using High Capacity cDNA Reverse Transcription Kit
(Applied Biosystems, Foster City, CA) and cDNA concentration-dependent amplification
was validated by making standard curves for all genes using serial 5-fold dilutions using
CT1 cells (trophectoderm cells). Reverse-transcribed cDNA was subjected to qPCR
amplification using SyberGreen a 10 µl reaction consisting of 1 µl of cDNA sample (20 ng/ µl), 5 µl of SYBR Green (Applied Biosystems), 1 µl of 1 µM primers (forward and
reverse) and 2 µl of DEPC water. qPCR was performed using a Bio-Rad C100 thermal
cycler -CFX96-Real-Time system (Bio-Rad, Hercules, CA, USA). Due to differences in
primer annealing temperature, the thermal cycle for four of the genes tested (RAD23A,
PPIP5K2, NFATC3, TGFB2) was performed as follows: 50°C for 2 min and 95°C for 10
min followed by 50 cycles of 95°C of 15 sec and 60°C for 1 min. The thermal cycle for
the other two genes (FADS6 and H1FOO) was 50°C for 2 min and 95°C for 10 min
followed by 50 cycles of 95°C of 15 sec, 55°C for 30 sec and 74°C for 30 sec.

Statistical Analysis

Data on development and immunocytochemistry (pixel intensity) were analyzed
by least-squares analysis of variance (ANOVA) using the Proc GLM procedure of the
percent of oocyte that cleaved and became blastocyst were transformed by arcsin
transformation before analysis. The mathematical model included main effects of
replicate, temperature, and IGF1 treatment and all interactions. Replicate was
considered random and other main effects were considered fixed. Probability values
were based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data. Pixel intensity data were analyzed with embryo as the experimental unit and stage of development at the dependent variable.

For data from qPCR experiments, all C_T responses from genes of interest were normalized to the housekeeping GAPDH gene using the ΔC_T method. The ΔΔC_T for each sample was calculated by subtracting the ΔC_T of IGF1 from the control in the same replicate. Fold change was determined by solving for 2^-ΔΔC_T relative to the controls. Treatment effects were analyzed by the median scores procedure of SAS (SAS for Windows, Version 9.0, Cary, NC, USA.

Results

Thermoprotective Actions of IGF1 on Two-cell and Day 5 Embryos

The first experiment to evaluate whether the thermoprotective effect of IGF1 on bovine embryos was developmentally regulated used a heat shock of 41°C for 15 hr. For two-cell embryos, heat shock reduced the percent of embryos that became blastocysts at day 8 (P<0.005). IGF1 did not protect two-cell embryos from heat shock as indicated by a lack of effect of IGF1 or the IGF1 x heat shock interaction (Figure 3-2A). For day 5 embryos, which are known to be more resistant to heat shock than two-cell embryos, culture at 41°C heat shock did not reduce the percent of embryos that became a blastocyst and there was no effect of IGF1 or IGF1 x heat shock (Figure 3-2B).

Given the resistance of day 5 embryos to a heat shock of 41°C, another experiment was performed where day 5 embryos were exposed to a more severe heat shock of 42°C for 15 hr. The percent of embryos that became blastocysts was reduced by heat shock (P<0.001) and increased by IGF1 (P=0.05) (Figure 3-3). Even though
there was no interaction, the increase in development caused by IGF1 was greater for embryos at 42°C than for embryos at 38.5°C. Thus, IGF1 reduced the effects of 42°C on development.

**Gene Expression of Molecules Involved in IGF1 Signaling**

In the first experiment, expression of *IGF1R, RAF1* and *MAPK* were higher in two-cell embryos compared to day 5 embryos (P<0.001; Figure 3-4A). In the second experiment (Figure 3-4B), there was again a trend for *IGF1R* mRNA to be higher at the two-cell stage but the difference was not significant. Another gene, *PI3K*, had higher expression at the two-cell stage (P<0.001), while a third gene, *HK2*, was expressed more highly for day 5 embryos (P<0.001).

**Presence of IGF1R in Two-cell and Day 5 Embryos**

Immunofluorescent labeling was performed to detect the presence of IGF1R in two-cell and day 5 embryos. Immunoreactive IGF1R was localized to the plasma membrane at both stages of development (Figure 3-5A). Intensity of staining was quantified and there was no difference (P>0.05) in the amount of immunoreactive IGF1R between the two stages of development (Figure 3-5B).

**Effect of IGF1 on Gene Expression in Blastocysts**

Using the criteria of a minimum 1.5-fold difference and P≤0.05, a total of 102 genes were differentially expressed between IGF1 and control embryos. A total of 72 genes were annotated, with 40 genes upregulated by IGF1 (Table 3-4) and 32 genes downregulated by IGF1 (Table 3-5).

The differentially expressed genes were used to query the DAVID bioinformatics database (http://david.abcc.ncifcrf.gov) to identify biological process ontologies in which genes are represented. When ontologies containing only one or two differentially
expressed genes or organ-specific ontologies were removed, there were a total of 10 ontology terms in which differentially expressed genes were represented (Table 3-6). Six of these terms were related to developmental processes: embryonic development, embryonic morphogenesis, anatomical structure morphogenesis, anatomical structure development, cell development, and cellular component morphogenesis. The set of developmental genes in the ontologies included 7 upregulated by IGF1 (in order of fold-change: ODZ4, SLC40A1, ANXA2, NFATC3, TGFB, BMP7 and DYRK3) and 9 downregulated by IGF1 (CNTNAP, NRG2, DPYSL4, ALDH1A2, FBN2, TNFRSF11A, NODAL, MMP13, and NEURL). In addition to the genes involved in developmental processes identified by DAVID, other genes involved in development were differentially regulated. Genes upregulated by IGF1 were CAB39 and SRPX2 while genes downregulated by IGF1 were GFAP, PARD3B, NT5E, CBX1, KREMEN, IFITM3, and ARHGEF10L.

Other biological process ontologies in DAVID containing more than two differentially regulated genes were as follows: the transmembrane receptor protein serine/threonine kinase signaling pathway, for which three genes were upregulated by IGF1 (FNTA, BMP7, and TGFB2); the positive regulation of cell proliferation ontology, with three upregulated genes (IL6ST, FNTA, and TGFB2) and three downregulated genes (ALDH1A2, TNFRSF11A, and NODAL); cellular responses to cell signaling: response to steroid hormone stimulus; and response to external stimulus. Included in these last two ontologies were four upregulated genes (IL6ST, NFATC3, TGFB2, and BMP7) and 5 downregulated genes (ALDH1A2, MMP15, MST1 MMP13, and NEURL).
Further analysis of the set of differentially-regulated genes was performed to test the hypothesis that IGF1 increases genes involved in cytoprotection. To this end, the list of differentially expressed genes was evaluated for the presence of heat shock protein genes as well as genes involved in DNA repair, protection from reactive oxygen species and apoptosis. No members of the heat shock protein family were differentially regulated and only one DNA repair gene (RAD23A) was affected by IGF1. There were, however, two genes involved in protection from oxidative stress that were upregulated by IGF1 (COQ9 and GSTM2) and one such gene downregulated (MST1). A total of 5 anti-apoptotic genes were upregulated by IGF1 (IL6ST, EIF3A, NFATC3, DYRK3, and ANP32B) and 5 pro-apoptotic genes were downregulated by IGF1 (DPYSL4, MST1, TNFRSF11A, NODAL and ARHGEF10L). In addition, IGF1 also upregulated two pro-apoptotic genes (IER3IP1, and RNASEL), and downregulated one anti-apoptotic gene (NT5E).

There were a group of 5 antiviral genes that were differentially regulated including three genes increased by IGF1 (MAN2A2, CPSF3, and RNASEL) and two genes inhibited by IGF1 (MON1B and IFITM3).

The data base was also queried to determine whether genes reported to be regulated by IGF1 in bovine blastocysts using PCR [183] were regulated by IGF1 in the present study. However, none of the genes, which were ATP1A1, BAX, DSC2, HSPA1A, IGFBP3, and IGF1R, were differentially regulated.

**Validation of Microarray Data by qPCR**

Results of microarray analysis were confirmed for four of six genes analyzed by qPCR (Figure 3-6). In particular, NFATC3, PPIP5K2, and TGFβ2, which were upregulated by IGF1 as determined by microarray analysis, were also higher in the
IGF1 group by qPCR (P<0.05). Also, \textit{FADS6}, which was downregulated by IGF1 in the microarray analysis, was lower in the IGF1 group by qPCR (P<0.05). Another two genes that were downregulated by IGF1 as determined by microarray analysis were not downregulated as determined by qPCR. One gene (\textit{RAD23A}) was upregulated as determined by qPCR (P<0.05) while the other (\textit{H1FOO}), was not different between control and IGF1 although the magnitude of difference between IGF1 and control was in the opposite direction than for the microarray results.

\textbf{Discussion}

In addition to increasing competence to develop to the blastocyst stage [31, 34], IGF1 acts as a survival factor in the preimplantation embryo to protect against elevated temperature [1, 3], oxidative stress [210, 212], tumor necrosis factor α [256], camptothecin and actinomycin D [211]. Results from the present study demonstrate that thermoprotective actions of IGF1 are developmentally regulated because IGF1 diminished the effects of heat shock on development for day 5 embryos ≥ 16 cells but had no thermoprotective effect for two-cell embryos exposed to heat shock. Moreover, the failure of IGF1 to protect two-cell embryos is probably not because signaling molecules required for IGF actions are depleted. Indeed, \textit{IGF1R} mRNA was higher for two-cell embryos than for day 5 embryos and the amount of immunoreactive IGF1R was similar for two-cell and day 5 embryos. Similarly, mRNA for three key molecules in the IGF1 signaling cascade, \textit{RAF1}, \textit{MAPK}, and \textit{PI3K}, [150] were higher for two-cell embryos. Reduction in transcript abundance from the two-cell stage to morula stage is a very common pattern in the bovine embryo [107, 257], probably because of degradation of maternally-derived mRNA and because transcription is inhibited until the 8-16 cell stage [49]. The one gene whose transcript abundance increased from the two-cell stage
to day 5 was *HK2*. A similar change in hexokinase mRNA from the two-cell to morula stage was seen earlier using non quantitative RT-PCR [258]. This increase probably reflects the increased utilization of glucose associated with compaction [259].

There are two other possible reasons why IGF1 failed to increase resistance of two-cell embryos to heat shock. One possibility is that the block to transcription in the two-cell embryo prevents changes in gene expression required for thermotolerance. A second possibility is that the damage to the two-cell embryo caused by heat shock is too great for IGF1 to counter. As shown in this study and others [36, 197-198], the two-cell embryo is more susceptible to heat shock than day 5 embryos. In the present study, for example, exposure to 41˚C decreased development of two-cell embryos while having no effect on development of day 5 embryos. The reason for increased susceptibility of the two-cell embryo to heat shock is not known but could involve transcriptional silencing [49], increased production of free radicals in response to heat shock [198], and decreased amounts of the intracellular antioxidant glutathione [260].

One of the characteristics of the bovine embryo produced in vitro in the presence of IGF1 is increased potential for survival after transfer into recipients, but only when those recipients are exposed to heat stress [35, 261]. Thus, IGF1 changes some aspect of embryo function (gene expression, epigenetic regulation, etc.) that, through interactions with heat-stress induced changes in maternal function, enhances embryo survival. Microarray analysis was performed to identify genes or gene clusters that might be involved in this effect of IGF1. In general, the pattern of gene expression was largely similar between control and IGF1-treated blastocysts. Only a small number of differentially-expressed genes were identified and the change in transcript abundance
caused by IGF1 was most typically between 1.5 and 2.0 fold. None of a set of 6 genes whose expression was increased in IGF1-treated blastocysts [183] was identified as being regulated by IGF1 in the present experiment. It is possible that the microarray analysis underestimated the genes regulated by IGF1 or that only a few changes are involved in the post-transfer consequences of treatment with IGF1.

Among the genes whose expression changed in response to IGF1 were several involved in apoptosis and protection from reactive oxygen species. Regulation of these genes could conceivably increase post-transfer survival in heat-stressed recipients by protecting the embryo from effects of maternal hyperthermia. Of the 5 anti-apoptotic genes upregulated by IGF1, three are involved in cell signaling. In particular, IL6ST is part of the IL6 receptor complex that inhibits apoptosis through phosphorylation of STAT [262], Dyrk3 is a kinase that phosphorylates and activates the anti-apoptotic protein SIRT1 [263], and NFATC3 is a transcription factor that increases production of BCL2 [264]. ANP32 is a substrate of caspase 3 that limits apoptosis [265], presumably by competing with other substrates for the enzyme. EIF3A is a translation initiation factor whose overexpression can inhibit apoptosis in cancer cells [266]. Five pro-apoptotic genes were also downregulated by IGF1. DPYSL4 is one of the mediators of p53-induced apoptosis [267], MST1 is a Sterile20-like kinase that promotes apoptosis through several pathways [268], TNFRSF11A, also called RANK, is a ligand for the pro-apoptotic TNF family member RANK [269], NODAL is a pro-apoptotic member of the TGFB family [270], and ARHGEF10L is a member of the RhoGEF family of guanine nucleotide exchange factors (GEFs) that activate Rho GTPases which in turn can activate apoptosis [271]. It is true that IGF1 also increased expression of two pro-
apoptotic genes (IER3IP1, and RNASEL) and downregulated one anti-apoptotic gene (NT5E). However, the anti-apoptotic effect of IGF1 on the preimplantation bovine embryo has been demonstrated directly through studies evaluating induction of apoptosis by heat shock [1, 3] and menadione [212].

Effects of heat shock on development involve reactive oxygen species; heat shock increases production of reactive oxygen species and addition of certain antioxidants can reduce the effects of heat shock [198, 202]. Thus, the increase in expression of two genes involved in protection from reactive oxygen species could facilitate survival after heat shock. One of the antioxidant genes was GSTM2, which utilizes glutathione to reduce electrophilic molecules [272]. In addition, GSTM2 can serve as a prostaglandin E synthase [273]. The other gene upregulated by IGF1 was COQ9, an endogenous lipophilic antioxidant [274-275]. Treatment with IGF1 also decreased expression of MST1. While this kinase is involved in blocking free radical generation caused by FOXO regulation of superoxide dismutase and catalase [276], it is also pro-apoptotic [277-278] so inhibition of its expression could contribute to embryo survival.

It is probably unlikely that the actions of IGF1 to improve embryonic survival during heat stress are simply the result of increasing embryonic resistance to maternal heat stress. Indeed, the embryo is substantially resistant to maternal hyperthermia by the blastocyst stage [36, 194, 197-198] and embryo transfer can minimize the seasonal variation in fertility in lactating dairy cows [14]. Moreover, treatment of embryos with IGF1 increases pregnancy rate in heat-stressed embryo transfer recipients to a value higher than that seen in embryo transfer recipients not exposed to heat stress [35]. One interpretation of this observation is that increased survival after transfer is to do a
combination of changes in embryonic function caused by IGF1 treatment and the maternal microenvironment established by heat stress. A large number of genes involved in developmental processes were affected by IGF1 (9 upregulated genes and 16 downregulated genes) and some of these genes could be important for embryonic survival in association with other changes in embryonic function caused by maternal hyperthermia.

One process that IGF1 may be regulating is neurulation. The default fate of embryonic ectoderm is neural tissue and this process is inhibited early in development by BMP4 [279]. Several genes involved in neural function or differentiation were inhibited by IGF1 including CNTNAP2, a member of the neurexin family of receptors and cell adhesion molecules involved in synapse formation [280], GFAP, a glial intermediate filament protein [281], DPYSL4, a member of a family of cytosolic phosphoproteins involved in brain development [282], ALDH1A2, which catalyzes formation of retinoic acid [283], which in turn promotes neural crest cell formation from embryonic stem cells [284], KREMEN, a receptor for Dickkopf 1 that promotes embryonic stem cell differentiation towards neuroectoderm [285], ARHGEF10L, an activator of Rho GTPases that participate in neural tube closure [286] and NRG2, a member of the neuroregulin family of receptor ligands that are involved in development of the nervous system [287].

In conclusion, thermoprotective actions of IGF1 are developmentally regulated with the two-cell embryo being refractory to IGF1. Failure of IGF1 to protect two-cell embryos is probably not because signaling molecules required for IGF1 actions are depleted but rather either because the block to transcription in the two-cell embryo
prevents changes in gene expression required for thermotolerance or that the damage to the two-cell embryo caused by heat shock is too great for IGF1 to counter. In any case, refractoriness of the early preimplantation embryo to protective actions of IGF1 can be used to explain why a treatment like bovine somatotropin that regulates IGF1 secretion was not effective for increasing fertility of females exposed to heat stress [2, 222]. Results also indicate that the improved competence for post-transfer survival of bovine embryos caused by treatment with IGF1 is associated with changes in expression of genes involved in developmental processes, apoptosis, and protection from reactive oxygen species.
### Table 3-1. Primer sets for quantitative real-time RT-PCR (Exp.1)

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<th>Primer/Probe</th>
<th>Sequence</th>
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<tr>
<td>IGF1R</td>
<td>XM_606794.1</td>
<td>Forward</td>
<td>AGTTATCTCCGGTCTCTGAGG</td>
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<td></td>
<td>Reverse Probe</td>
<td>CTTATTGGCGTTGAGGTATGC</td>
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<td>/56-FAM/TTTTGCTTAGGCTGGAGGTGCT/3IABlk_FQ/</td>
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<td>AAGCTATACAAAGAAGCTGCC</td>
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<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>/56-FAM/TGGTAGCTGACTGCGCTGAGAAAGTG/3IABlk_FQ/</td>
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<td>NM_175793</td>
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### Table 3-2. Primer sets for quantitative real-time RT-PCR (Exp.2)

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<td>Reverse</td>
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<td>PI3K</td>
<td>NM_174576.1</td>
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<tr>
<td>Bos taurus tumor necrosis factor receptor superfamily, member 11a, NFkB activator (<em>TNFRSF11A</em>)</td>
<td>XM_609364</td>
<td>15</td>
<td>10</td>
<td>-1.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Bos taurus similar to Homo sapiens nodal homolog (mouse) (<em>NODAL</em>)</td>
<td>XM_609225</td>
<td>8</td>
<td>5</td>
<td>-1.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Predicted: Bos taurus similar to kringle-containing transmembrane protein 1 (<em>KREMEN1</em>)</td>
<td>XM_602679</td>
<td>11</td>
<td>7</td>
<td>-1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Predicted: Bos taurus similar to neuralized-like protein 1 (h-neuralized 1) (h-neu) (RING finger protein 67), transcript variant 1 (<em>NEURL</em>)</td>
<td>XM_587462</td>
<td>59</td>
<td>39</td>
<td>-1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Salmo salar UV excision repair protein RAD23 homolog A (rd23a)</td>
<td>NM_001141812</td>
<td>405</td>
<td>269</td>
<td>-1.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Bos taurus MON1 homolog B (yeast) (<em>MON1B</em>)</td>
<td>NM_001037454</td>
<td>24</td>
<td>16</td>
<td>-1.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Bos taurus membrane-spanning 4-domains, subfamily A, member 5 (<em>MS4A5</em>)</td>
<td>NM_001078146</td>
<td>15</td>
<td>10</td>
<td>-1.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Bos taurus matrix metalloproteinase 13 (collagenase 3) (<em>MMP13</em>)</td>
<td>NM_174389</td>
<td>11</td>
<td>8</td>
<td>-1.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Bos taurus Rho guanine nucleotide exchange factor (GEF) 10-like (<em>ARHGEF10L</em>)</td>
<td>NM_001046297</td>
<td>9</td>
<td>6</td>
<td>-1.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Bos taurus interferon induced transmembrane protein 3 (1-8U) (<em>IFITM3</em>)</td>
<td>NM_181867</td>
<td>217</td>
<td>146</td>
<td>-1.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Table 3-6. Significant biological process gene ontology terms for differentially expressed genes in blastocysts\(^a\)

<table>
<thead>
<tr>
<th>Gene ontology (GO)</th>
<th>Action of IGF1</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0009790: embryonic development</td>
<td>UP: BMP7, TGFB2, ODZ4</td>
<td>DWN: ALDH1A2, NODAL, FBN2, MMP13, NRG2</td>
</tr>
<tr>
<td>GO:0048598: embryonic morphogenesis</td>
<td>UP: BMP7, ODZ4</td>
<td>DWN: ALDH1A2, NODAL, FBN2, MMP13</td>
</tr>
<tr>
<td>GO:0009653: anatomical structure morphogenesis</td>
<td>UP: BMP7, SLC40A1, TGFB2, ANXA2, ODZ4</td>
<td>DWN: ALDH1A2, NEURL, NODAL, FBN2, MMP13</td>
</tr>
<tr>
<td>GO:0008284: positive regulation of cell proliferation</td>
<td>UP: FNTA, IL6ST, TGFB2</td>
<td>DWN: ALDH1A2, TNFRSF11A, NODAL</td>
</tr>
<tr>
<td>GO:0048545: response to steroid hormone stimulus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0048856: anatomical structure development</td>
<td>UP: ANXA2, TGFB2, DYRK3, BMP7, NFATC3, SLC40A1, ODZ4</td>
<td>DWN: NEURL, NODAL, DPYSL4, MMP13, ALDH1A2, TNFRSF11A, CNTNAP2, FBN2</td>
</tr>
<tr>
<td>GO:0048468: cell development</td>
<td>UP: BMP7, TGFB2</td>
<td>DWN: ALDH1A2, NEURL, NODAL, CNTNAP2</td>
</tr>
<tr>
<td>GO:0007178: transmembrane receptor protein serine/threonine kinase signaling pathway</td>
<td>UP: FNTA, BMP7, TGFB2</td>
<td></td>
</tr>
<tr>
<td>GO:0009605: response to external stimulus</td>
<td>UP: IL6ST, BMP7, NFATC3, TGFB2</td>
<td>DWN: ALDH1A2, NEURL, MST1, MMP15</td>
</tr>
<tr>
<td>GO:0032989: cellular component morphogenesis</td>
<td>UP: BMP7, TGFB2</td>
<td>DWN: NEURL, NODAL</td>
</tr>
</tbody>
</table>

\(^a\) The analysis was conducted using David software (http://david.abcc.ncifcrf.gov/). The only ontologies shown are those with more than two differentially expressed genes in an ontology and where the ontology was not an organ-specific term.
Figure 3-1. Representative results of analysis of RNA from KSOM (control) and IGF1 treated embryos used for microarray, determined by Agilent 2100 Bioanalyzer RNA 6000 Pico Labchip Kit.
Figure 3-2. Effect of IGF1 on the reduction in development caused by a heat shock of 41°C at the two-cell stage and day 5 of development (embryos ≥ 16 cells). Data in Panel A are from two-cell embryos and data in Panel B are from day 5 embryos at 41°C. In two-cell embryos, there was a decrease in the percent of embryos becoming a blastocyst caused by heat shock (P<0.005) but no effect of IGF1 or IGF1 x temperature. For day 5 embryos, there was no effect of temperature, IGF1 or the interaction.
Figure 3-3. Effect of IGF1 on the reduction of development caused by exposure of day 5 embryos (≥ 16 cells) to a heat shock at 42°C. There was a decrease in the percent of embryos becoming blastocyst at day 8 caused by heat shock (P<0.001) and an increase in percent blastocyst caused by IGF1 (P<0.05).
Figure 3-4. Changes in expression of genes involved in IGF1 signaling at the two-cell and day 5 (≥16 cells) stage as determined by qPCR. In the first experiment (panel A), steady state amounts of mRNA for IGF1R, RAF1 and MAPK were higher for two-cell embryos (P<0.001). In the second experiment (panel B), there was a nonsignificant tendency for expression of IGF1R to be higher at the two-cell stage. Amounts of mRNA for PI3K were higher for two-cell embryos (P<0.001) while amounts of HK2 mRNA were higher for day 5 embryos (P<0.001).
Figure 3-5. Expression of IGF1R protein in two-cell and day 5 (≥ 16 cells) embryos. Panel A represents immunocytochemistry staining for two-cell embryos and day 5 embryo. Red fluorescence denotes positive staining of IGF1R. As shown by quantitative analysis in panel B, there was no difference in pixel intensity between two-cell and day 5 embryos.
Figure 3-6. Fold-change in gene expression using qPCR (y axis) and microarray hybridization (x-axis) for a selected group of six differentially expressed genes. Fold change values are calculated as IGF1/control. N.S = non-significant.
CHAPTER 4
GENERAL DISCUSSION

Insulin-like growth factor is an important maternal determinant of embryonic survival that can promote development to the blastocyst stage [31-32, 34], protect the embryo from several stresses [1, 3, 212] and increase competence for development to term, at least in heat-stressed females [35, 133]. The overall goal of this dissertation was to understand the molecular basis for the developmental acquisition of thermoprotective actions of IGF1 on preimplantation embryos and the thermoprotective effects of IGF1 during culture and after transfer into recipients. A schematic diagram illustrating the conclusions of the dissertation is shown in Figure 4-1.

For both effects on development and on thermotolerance, the embryo appears resistant to IGF1 until sometimes between the two-cell stage and day 4 after fertilization. Thus, addition of IGF1 from day 0-4 had no effect on the proportion of embryos becoming blastocysts while addition from day 4-8 increased the percent of embryos becoming blastocysts in a manner involving MAPK-regulated events (Chapter 2). Similarly, IGF1 protected embryos from heat shock at day 5 but not at the two-cell stage. Developmental changes in actions of IGF1 appear not to be due to a lack of IGF1 signaling molecules because IGF1R and mRNA for selected genes involved in IGF1 signaling were present at the two-cell stage (Chapter 2). It seems most likely that the reason for unresponsiveness to IGF1 relates to the lack of transcriptional capacity for the early embryo until embryonic genome activation at the 8-16 cell stage [39]. Indeed, one effect of IGF1 was increased expression of ATPA1A (Chapter 2) and this action may contribute to embryo competence to become a blastocyst.
The MAPK pathway is one of the signaling pathways for the proliferative actions of IGF1 [240-241]. Inhibition of the MAPK pathway decreased the effect of IGF1 on embryo development (Chapter 2), and it is possible, therefore, that the main action of IGF1 for increasing blastocyst development is an increase in cell number. It is controversial whether IGF1 increases cell number in the bovine embryo; some studies did not show an increase in blastocyst cell number [183] while other studies did [177, 179]. Since the inhibition of MAPK pathway did not block overall embryo development, future studies to evaluate different pathways by which IGF1 improves embryo development, such as PDK1 or JAK-STAT [148, 288-289], could be important. In addition, it would be of interest to determine whether IGF1 increases other genes involved in embryo compaction and blastocyst formation such as zonula occludens, and the aquaporins [81, 87]
Figure 4-1. Developmental actions of IGF1 to promote blastocyst formation and protect from heat shock. Note that effects of IGF1 to increase competence of an embryo to become a blastocyst between day 4 and 8 post-insemination. Similarly, IGF1 can protect embryos from heat shock at day 5 but not at the two-cell stage. Actions of IGF1 to increase development involve MAPK-dependent events and include increased expression of ATPA1A. Failure of the embryo to respond to IGF1 before day 4 appears not to be due to a lack of IGF1 signaling molecules because IGF1R and mRNA for selected genes involved in IGF1 signaling were present at the two-cell stage. Note also that the blastocyst produced in the presence of IGF1 has increased potential for survival when transferred into heat stressed recipients [35]. This effect of IGF1 is associated with changes in expression of genes involved in development, apoptosis and protection from free radicals.
While lack of transcriptional regulation may be one reason why IGF1 cannot protect two-cell embryos from heat shock, it is also possible that the increased sensitivity of two-cell embryos to elevated temperatures due to higher production of ROS [198] or other reasons makes the damage caused by heat shock too severe to be reversed by IGF1. Some of the deleterious effects of ROS include DNA strand breaks, mitochondrial damage [290], and embryonic arrest. Rivera et al. [205] have shown that two-cell embryos submitted to heat shock were arrested and did not pass the eight-cell stage. One possible cause for embryonic arrest could be an increase in oxidative stress leading to higher levels of p66shc mRNA. P66Shc is a stress adaptor protein associated with early embryonic arrest [206-208] and it regulates mitochondrial metabolism by modulating the amount of ROS released into the cytosol [209]. Another possibility to explain increased sensitivity of the two-cell embryo to heat shock is that maternal mRNAs and proteins may be more sensitive to elevated temperatures. Embryonic development during the early cleavage stages is supported by maternal mRNAs and proteins synthesized and stored during oogenesis [39], and these stores are important during the interval of fertilization and embryonic genome activation.

Heat stress reduces the duration of estrus, impairs follicular development and oogenesis, decreases follicular steroid production [14, 186-187] and decreases IGF1 concentration in the blood and follicular fluid [291]. Addition of IGF1 to maturation medium was shown to stimulate oocyte maturation, cumulus expansion and cleavage rate after fertilization [292]. Furthermore, the use of bST has been shown to increases plasma concentrations of IGF1 [216, 218], which could be used as an in vivo treatment to prevent effects of heat stress. In vivo studies showed that bST treatment increased
IGF1 content in the follicular fluid, improved follicular development prior to ovulation [293], and increased fertilization rate and embryonic development [294]. However, the lack of thermoprotective effects of IGF1 on two-cell embryos would make treatment of cows with bST at early stages of pregnancy ineffective for preventing effect of heat stress on fertility. Future experiments could be conducted to evaluate whether oocytes can be protected from heat shock by IGF1 and to determine whether this beneficial effect would carry over into the period of embryonic development. Furthermore, it is not known whether an increase of IGF1 in reproductive tract can increase fertility.

Moreira et al. [294] found that treatment of recipient cows with bST increased pregnancy rates after transfer of embryos flushed from donor cows without bST. Perhaps bST and IGF1 can also improve the uterine environment. Futures studies could be done to evaluate effects of bST on gene expression in the uterus of cows under heat stress.

In our study, microarray analysis showed that IGF1 changed expression of genes involved in apoptosis and protection from reactive oxygen species in day 7 blastocysts, which could conceivably increase post-transfer survival in heat-stressed recipients by protecting the embryo from effects of maternal hyperthermia. Furthermore, a large number of genes involved in developmental processes were affected by IGF1 and some of these genes could be important for embryonic survival in association with other changes in embryonic function caused by maternal hyperthermia. The fact that IGF1 increased potential for survival after transfer into recipients, but only when those recipients are exposed to heat stress [35, 261], suggests that beneficial effects of IGF1
on embryo function only affect embryonic survival in conjunction with other changes in embryo function caused by heat-stress induced changes in maternal function.

Taken together, these investigations indicate that IGF1 can regulate embryonic development and resistance to heat stress but that these actions occur at or after day 4 of development, at a time after embryonic genome activation. Furthermore, the pro-developmental effects of IGF1 involve actions mediated by the MAPK pathway and include alteration of genes controlling formation of the blastocoelic cavity. Genes regulated by IGF1 at the blastocyst stage, such as those involved in development, apoptosis and protection from oxidative stress could be involved in the increase in embryonic survival after transfer to heat-stressed recipients caused by IGF1.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Aline Quadros Santos Bonilla was born in Itabuna and raised in Ilhéus, Bahia, Brazil. In 2000 she received her degree in veterinary medicine from the Universidade Federal de Viçosa, and in 2003, she finished her master’s program at the same University. Her M.S. thesis, concerning in nutrition and reproduction in Nelore bulls, was completed under the direction of Dr José Domingos Guimarães. Following graduation, Dr Bonilla worked in veterinary service and embryo transfer in Barrado Garças – MT and then Campo Grande MS, Brazil. In 2006, Dr. Bonilla and her husband Luciano moved to Gainesville and she started her Doctor of Philosophy degree in the animal molecular and cellular biology graduate program, in the laboratory of Dr Peter J. Hansen. In the fall of 2010, Dr. Bonilla will start a post-doctoral program.