Supplementing Bovine Embryo Culture Media to Improve the Production and Quality of In Vitro Produced Bovine Embryos

Lydia K. Wooldridge

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Animal and Poultry Science

Alan D. Ealy

Willard E. Eyestone

Kiho Lee

Sally E. Johnson

March 20, 2020

Blacksburg, VA

Keywords: Interleukin-6, STAT3, Leukemia Inhibitory Factor, Inner Cell Mass, Zinc

Improving Bovine Embryo Culture Media to Improve the Production and Quality of In Vitro Produced Bovine Embryos

Lydia K. Wooldridge

ABSTRACT

Initial studies in this work explored the role of interleukin-6 (IL6) and leukemia inhibitory factor (LIF) in preimplantation bovine embryos. Neither cytokine affected the total percentage of embryos which developed to the blastocyst stage in vitro. However, supplementation of IL6 increased blastocyst inner cell mass (ICM) cell number without affecting trophectoderm (TE) cell number. Additionally, we found that IL6 activated signal transducer and activator of transcription 3 (STAT3) specifically within ICM cells. LIF, however, did not affect ICM cell number or activate STAT3 in ICM cells, and was not pursued further. This increase in ICM cell number by IL6 was largely comprised of hypoblast ($GATA6^{+}$:$NANOG^{-}$) cells, and most IL6-responsive cells in day 9 blastocysts were hypoblast cells (as measured by STAT3 activation). However, some epiblast ($NANOG^{+}$) cells were also IL6-responsive, and IL6 appeared to initially slow epiblast differentiation. Finally, IL6-treated blastocysts also had increased transcripts of hypoblast/primitive endoderm (PE) markers. These results indicate that IL6 may improve pregnancy retention of IVP embryos by improving yolk sac development, but further work is needed to confirm this theory.

Activation of STAT3 by IL6 could be blocked with a chemical Janus kinase 2 (JAK2) inhibitor (AZD1480). JAK2 inhibition from day 5 to 8 resulted in blastocyst ICMs with fewer than 10% the normal cell number, regardless of IL6 supplementation. This indicates that STAT3 is critical for bovine ICM development. Further analysis revealed that inhibition of JAK2/STAT did not prevent ICM formation but disrupted its maintenance.

Additionally, we assessed the suitability of zinc sulfate and a bovine embryonic stem cell culture media (TeSR) for improving bovine embryo development in vitro. Zinc sulfate increased day 8 blastocyst total and ICM cell number. Therefore, zinc sulfate appears to improve blastocyst quality. The TeSR medium improved embryo development beyond day 8. In normal synthetic oviduct fluid, blastocysts degenerated after day 8, while blastocysts moved to TeSR had greatly increased cell numbers, and even exhibited PE migration out from the ICM, a phenomenon that has not been reported in vitro. This indicates that extended blastocyst culture is possible with TeSR media.

Improving Bovine Embryo Culture Media to Improve the Production and Quality of In Vitro Produced Bovine Embryos

Lydia K. Wooldridge

GENERAL AUDIENCE ABSTRACT

Bovine embryos have been produced in vitro for the purpose of being transferred to recipient cattle to produce a calf since the 1980s. This practice allows cattle breeders to increase the number of offspring from their best females each year, and also allows for more rapid progress in generational genetic improvement. However, only approximately 10% of bovine oocytes survive and produce a calf. This poor efficiency of bovine in vitro embryo production negatively impacts the procedure's widespread use. A significant portion of these embryo losses are likely a result of inadequate in vitro culture conditions, particularly of the embryo culture media, the fluid in which embryos are grown. This media is often called "synthetic oviduct fluid," or SOF, because it is designed to mimic the fluid present in the cow's oviduct, where the embryo would normally reside. However, SOF is much simpler in nature than actual cow oviduct fluid, and this leads to reduced embryonic survival of in vitro produced embryos.

Unfortunately, we know very little of what molecules control and promote bovine embryo development. Therefore, one major goal of bovine embryo research is to identify these factors and add them to SOF. The goal of this work was to examine the ability of three molecules, interleukin-6 (IL6), leukemia inhibitory factor (LIF), and zinc sulfate, to increase the number and quality of blastocysts produced through in vitro culture techniques. Additionally, I tested the replacement of SOF with a complex cell culture media, known as TeSR. This medium is more complex than SOF, and therefore should better promote embryo development.

This work revealed that IL6, but not LIF, improves in vitro produced (IVP) bovine blastocyst quality. Unfortunately, neither IL6 nor LIF affected the percentage of embryos which survived to the blastocyst stage. However, IL6, but not LIF, increased the number of cells in the inner cell mass (ICM) of the blastocysts. ICM cells are the portion of the embryo which will produce the future calf. IVP bovine embryos are known to have fewer cells than normal, in vivo derived, blastocysts, and this issue is believed to cause some embryonic death after embryo transfer. Therefore, treatment with IL6 may increase the percentage of embryos which will survive after transfer and produce a calf.

We also found the addition of zinc sulfate to SOF to benefit embryo quality. None of the concentrations of zinc significantly improved the percentage of embryos which survived to the blastocyst stage, but 2 μM zinc did increase ICM cell number. Like IL6, this may improve embryo survival after transfer.

The use of the TeSR media as a replacement for SOF had some benefits. Unfortunately, this media is unusable for producing embryos for transfer to recipients, as we discovered early embryos could not survive in the media. However, blastocyst-stage embryos thrived in it, and could be cultured in vitro for a longer period of time as a result. Therefore, this media will be a useful tool for studying bovine embryo development in vitro, however it is unlikely to benefit calf production.

In summary, this work provides evidence that zinc sulfate and IL6 are beneficial additions to SOF. However, future work is needed to determine if embryos produced with these factors are more able to produce a calf. Additionally, we discovered that TeSR is a superior extended blastocyst culture medium.

ACKNOWLEDGEMENTS

I would be a fool to think I got this far in life without help. Throughout my life, there are many people I've thanked in the back of my mind, but I haven't always been the best an expressing this appreciation. I hope to do that here.

First, I want to thank my family. Thank you to my parents, Tim and Debbie Michel for always supporting me and pushing me to do my best in school. It goes without saying, I've been influenced heavily by you two, and I hope you take some pride in knowing my achieving this degree is in large part thanks to you. Thank you to my P<0.05 other, my husband, Tim Wooldridge. Thank you for your endless love, encouragement and support of me and my career. Thank you for always doing your best to help me, so that I can be free to do study the science I love. And, of course, thank you for being the voice of reason sometimes and telling me to "stop" when I dive in over my head because I get excited about doing anything and everything. Last but not least, my grandparents, in-laws, my best friend Alex Rogers, and other family members, thank you for your words of encouragement and support over the years.

Thank you to those who have influenced my way of thinking and ability to think critically. In particular, thank you to Mr. Doug Smith, my high school English teacher. I will never forget the first thing you said to my sophomore English class as you walked into the room, swirling your lanyard around your fingers. "Feeble minds are easily manipulated." I took that as a challenge, along with that poster on your wall that read something like "Stupid people talk about other people. Average people talk of events. Smart people talk of ideas." I can easily say that, if it weren't for your teaching me to be a critical thinker and how to read between the lines, that I would not be the scientist that I am today. I'd like to think that, today, I am one of those "smart people," and a lot of that is thanks to you.

And then of course, since entering college, there have been a lot of people who have helped train me as a scientist. In particular, I'd like to thank Dr. Chenglin Chai, Dr. Jerry Taylor and Dr. Jonathan Green. Dr. Chai, thank you for giving me my first wet lab research opportunity. I will never forget the day you asked me, an undergraduate greenhouse worker who had never touched a pipette, to become your assistant technician. Thank you for taking the time to train me in the lab. I still remember a lot of things you told me, and I teach others the same things to this day. I can safely say that I don't think I would be the scientist I am today if you hadn't given me such a jumpstart on wet lab research. Dr. Taylor, thank you for giving me my next job opportunity to work as an assistant technician in Animal Science. Dr. Green, thank you for putting up with me asking you a million questions during my senior year at Mizzou. I learned a lot from you, both about biology and in finding the "holes" in my own thinking and reasoning. I've become a better scientist as a result. And thank you for giving me the opportunity to start a research project. Even though I didn't get very far because I graduated soon after, this was my first real experience which pushed me towards independent thinking in the lab.

I'd also like to thank everyone who has helped me since I've come to Virginia Tech. Dr. Saacke, thank you for coming up to me after my "WIP" presentation in the Reproductive Biology Club seminar meeting and giving me tips for working with embryos. Thanks to you, I learned how to make slide of blastocysts that actually look good. Which helped immensely with my dissertation and making figures for presentations and publications! Thank you to Dr. Akers and Cathy Parsons for letting me borrow your osmometer. Thank you to Brown Packing Company, and Desoto Biosciences for providing me with the bovine ovaries and oocytes I

needed to accomplish this work! And thank you Select Sires for donating the semen used to make these embryos.

Thank you to my committee members Dr. Sally Johnson, Dr. Kiho Lee and Dr. Will Eyestone for your advice and support over the years. And thank you to Dr. Johnson for helping me work out my pSTAT3^{Y705} immunofluorescence protocol!

Thank you to all the graduate students who have helped me over the years. Especially the Ealy lab members: Sarah McCoski, McCauley Vailes, Michelle Kott, Lauren Kimble, Zack Seekford and Savannah Speckhart. I never could have worked with so many COCs without your help getting them! Thank you to my office friends for providing comedic relief every now and again too! Also, I want to say thank you to the many undergraduates who have helped me as well. In particular, thank you to Chelsea Abbott Finn, Kayla Cook, Hannah Parker, Bailey McGill, Maddie Nardi and Katriona Lane Felkel. You guys rock!

And last but not least, thank you to my PhD adviser, Dr. Alan Ealy. Thank you for giving me this opportunity, and for putting up with me for the past five years. I know I can be a bit headstrong. Thank you for always listening to and supporting my ideas and giving me the power and freedom to work on my own. And thanks for putting up with me when I need to vent!

Table of Contents

List of Figures ..... **xiv**
List of Tables ..... **xvi**
List of Abbreviations ..... **xvii**
Chapter 1: Literature Review **1**
Introduction **1**
Proteins **3**
Insulin and Insulin-like Growth Factors **3**
Insulin 4
Insulin-like Growth Factor-I 5
Insulin-like Growth Factor-II 8
WNT Family and Inhibitors **8**
WNT Family Member 7A and 11 9
Dickkopf WNT Signaling Pathway Inhibitor 1 9
TGF-ß Superfamily & Antagonists **10**
Transforming Growth Factor-ß 10
Activin A 11
Bone Morphogenetic Proteins 12
Follistatin 13
Noggin 13
Cytokines **14**
Interleukin-1ß 14
Colony-stimulating factor 2 15
Leukemia inhibitory factor 16
Tumor Necrosis Factor-α 17
Stem Cell Factor 18
Growth Factors **18**
Fibroblast Growth Factors 18
Connective Tissue Growth Factor 20
Hepatoma-derived growth factor 20
Hepatocyte growth factor 21
Epidermal Growth Factor 22

Teratocarcinoma-derived growth factor 1 23
Transforming Growth Factor-α 23
Platelet-Derived Growth Factor 24
Nerve Growth Factor 24
Vascular Endothelial Growth Factor 25
Growth Hormone 26
Steroids 26
Cortisol 27
17ß-Estradiol 27
Progesterone 28
Other Molecules 28
Melatonin 28
Prostaglandin F2α 29
Lysophosphatidic Acid 29
Retinoids 30
Hyaluronic Acid 30
Thyroid Hormone 31
Cocktails 31
Conclusions & Moving Forward 32
Dissertation Hypothesis & Objectives 34
Chapter 2: Interleukin-6 increases inner cell mass numbers in bovine embryos 47
Introduction 47
Materials and Methods 48
In vitro embryo production 49
IL6 supplementation studies 50
Transcript profiling 52
Immunofluorescence and cell counting 53
Statistical analyses 55
Results 55
Transcript profiling 55
Study A: IL6 Treatment at Day 5 Post-Fertilization 56
Study B: IL6 Treatment at Day 3 or 5 Post-Fertilization 56

Study C: IL6 Treatment at Day 1 Post-Fertilization 57
Studies D and E: The Efficacy of Combined IL6 treatments at Day 1 and 5 Post-Fertilization 58
Composite Analysis of IL6 Effects on ICM cell numbers and the ICM:TE ratio 59
Study F: IL6 Supplementation During Individual Embryo Culture.....59
Discussion 60
Conclusions.....63
Chapter 3: Interleukin-6 requires JAK to stimulate inner cell mass expansion in bovine embryos 71
Introduction.....71
Materials and Methods 73
In vitro Embryo Production 73
IL6 Supplementation Study 74
Assessment of STAT3 Activity and Its Necessity for ICM Development.....74
qRT-PCR.....75
RNA Sequencing.....76
Immunofluorescence 77
Statistical Analyses.....79
Results.....79
IL6 Increases ICM and Total Cell Numbers in Day 7 Blastocysts but Not Morulae.....79
IL6 Activates the JAK/STAT3 Pathway in Bovine Embryos 80
IL6 has Minimal Effects on the Bovine Embryo Transcriptome 82
Discussion 83
Chapter 4: Interleukin-6 increases hypoblast proliferation and slows epiblast differentiation in the bovine blastocyst inner cell mass 96
Introduction.....96
Materials and Methods 97
In vitro Embryo Production 98
Study 1: IL6 Supplementation Prior To Blastocyst Formation and Day 9 Blastocysts 99
Study 2: IL6 Supplementation After Blastocyst Formation and Day 9 Blastocysts 99
Study 3: IL6 Supplementation Prior To Blastocyst Formation and Day 8 Blastocysts 99
Study 4: IL6 Washout at the Blastocyst Stage.....100
Study 5: Blastocyst STAT3 Response to IL6 Over Time.....100

Study 6: pSTAT3^{Y705} Colocalization with GATA6 or NANOG After IL6 Treatment in SOF-BEI 101
Study 7: Blastocyst Cell Numbers on Day 8, 9 and 10, and 24 Hour IL6 Treatments ...101
Study 8: Comparison of a TeSR Formulation to SOF-BEI 101
Study 9: pSTAT3^{Y705} Colocalization with GATA6 or NANOG After IL6 Treatment in TeSR 102
Immunofluorescence 102
Statistical Analyses....105
Results....106
IL6 Supplementation Prior to Blastocyst Formation Increases Epiblast, Hypoblast and Undifferentiated ICM Cell Number in Day 9 Blastocysts....106
IL6 Supplementation After Blastocyst Formation Does Not Affect Epiblast Cells 106
IL6 Supplementation Prior to Blastocyst Formation Decreases Epiblast Cell Number in Day 8 Blastocysts, but Does Not Affect the Number of NANOG^{+} Cells 107
IL6 Must be Present During Blastocyst Development to Affect ICM Cell Number 107
IL6's Ability to Activate STAT3 in the ICM Decreases with Time 108
IL6-Induced pSTAT3^{Y705+} ICM Cells Are Mainly Hypoblast in Day 9 Blastocysts Grown in SOF-BEI 109
ICM Cell Number Decreases After Day 8 in SOF-BEI 110
TeSR Bovine ESC Medium Supports Blastocyst Development Past Day 8 111
IL6 Induces STAT3 Activation in ICM Cells of Blastocysts Grown in TeSR....112
Discussion 113
Acknowledgements....118
Chapter 5: Leukemia inhibitory factor does not stimulate STAT3 activity in the bovine ICM, nor does it increase inner cell mass cell number 130
Introduction....130
Materials and Methods 131
In vitro Embryo Production 131
Treatment Preparation 132
LIF and STAT3 Activation....133
LIF's Effect on Day 8 Blastocyst Cell Numbers....133
Immunofluorescence 133
Cell Counting 134
qRT-PCR....135

Statistical Analyses....136
Results....136
LIF Activates STAT3 in Day 5 but Not Day 8 Embryos....136
LIF Increases Day 8 Advanced Blastocyst Formation but Not Cell Number....137
Transcripts for LIFR Decrease from Day 5 to Day 8....137
Discussion....138
Chapter 6: JAK2/STAT activity is not required for inner cell mass formation in bovine blastocysts but is required for hypoblast maintenance....144
Introduction....144
Materials and Methods....145
In vitro Embryo Production....145
ICM Formation Study....146
HYPO Maintenance Study....147
Whole ICM Maintenance Study....147
Immunofluorescence....148
Statistical Analyses....150
Results....150
JAK2/STAT activity is Not Required for ICM Formation....150
JAK2/STAT Activity is Necessary for Hypoblast Maintenance....151
JAK2/STAT Inhibition Predominantly Affects Hypoblast Cells....152
Blastocyst Age has Some Impact on Cell Numbers....152
Older Blastocyst HYPO Cells Exhibit Impaired Recovery After JAK Inhibition....153
Discussion....154
Chapter 7: Zinc Supplementation During In Vitro Embryo Culture Increases Inner Cell Mass and Total Cell Numbers in Bovine Blastocysts....167
Introduction....167
Materials and Methods....169
In vitro Embryo Production....169
Zinc Supplementation....169
Differential Cell Labeling in Blastocysts....170
Statistical Analysis....170
Results....171
Effects of Zinc Supplementation on Embryo Development....171

Zinc Supplementation Increases ICM and Total Cell Numbers in Blastocysts 171
Discussion 172
Chapter 8: Conclusions and Implications 177
References 185
Appendix A: Experimental Units and Analyzing Blastocyst Formation 210
Appendix B: Embryo Treatment by Culture Drop Injection 216
Procedure with calculation example 216
Appendix C: Removing the Zona Pellucida & Snap-freezing Bovine Embryos 217
Materials Needed: 217
Zona Pellucida Removal Instructions: 217
Snap-Freezing Instructions: 218
Appendix D: Immunolocalization Protocols for SOX2, Cleaved Caspase 3, Ki67, Tead4, GATA6, NANOG and CDX2 in Bovine Preimplantation Embryos 220
Solutions to Prep Prior to Staining 220
Universal Solutions 220
Permeabilizations Solutions 220
Antibodies 221
Plates Needed 221
Protocol 222
Fixation 222
Permeabilization 222
Blocking 222
Antibodies 222
DAPI 223
Slide Mounting 223
Appendix E: Immunolocalization Protocols for pSTAT3^{Y705} and its Combination with GATA6, NANOG or CDX2 in Bovine Preimplantation Embryos 224
Solutions to Prep Prior to Staining 224
Antibodies 225
Plates Needed 225
Protocol 226
Fixation 226
Permeabilization & Lipid Removal 226

Blocking 226
Antibodies 226
DAPI 227
Slide Mounting 227
Appendix F: Immunolocalization Protocols for GATA6, NANOG and CDX2 in Bovine Preimplantation Embryos 228
Solutions to Prep Prior to Staining 228
Antibodies 229
Plates Needed 229
Protocol 230
Fixation 230
Permeabilization 230
Blocking 230
Antibodies 230
DAPI 231
Slide Mounting 231

List of Figures

Figure 1-1. Summary of the receptor-binding bioactive factors which have been supplemented to in vitro produced bovine embryos. 46
Figure 2-1. Transcript abundances for IL6, IL6R and IL6ST from zygotes, 2-cell embryos, 8-cell embryos, morulae and blastocysts. 68
Figure 2-2. Representative images of differential cell staining in blastocysts collected at day 8 post-fertilization. 69
Figure 2-3. Pooled ICM cell counts and ICM to TE ratios from all studies. 70
Figure 3-1. Supplementation with IL6 stimulates ICM cell numbers in bovine blastocysts evaluated on day 7 post-fertilization. 91
Figure 3-2. Representative day 7 embryos treated with 0 or 100 ng/ml IL6 from day 1 to 7 post-fertilization.....92
Figure 3-3. IL6-dependent stimulation of STAT3 phospho-activation and nuclear localization in day 5 and 8 embryos. 93
Figure 3-4. The necessity for STAT3 activity in ICM development. 94
Figure 3-5. Expression analysis of IL6 family ligand and receptors, markers of embryonic lineages, and markers of pluripotency and differentiation after IL6 exposure.....95
Figure 4-1. Treatment with IL6 increases the number of HYPO and UN cells in the ICM and slows EPI differentiation. 119
Figure 4-2. IL6 must be present during blastocyst development to increase ICM cell number. 121
Figure 4-3. IL6's ability to stimulate STAT3 in the ICM wanes with time, and mostly stimulates HYPO cells. 122
Figure 4-4. The ICM shrinks with time in SOF-BEI, and IL6 can increase ICM cell number in day 7, 8 and 9 blastocysts. 123
Figure 4-5. Culturing day 7 blastocysts in TeSR medium increases blastocyst cell numbers, hatching, and PE migration. 124
Figure 4-6. Example images of ICM pSTAT3 ranks. 126
Figure 5-1. STAT3 activation profiles for LIF-treated bovine embryos.....140
Figure 5-2. Comparison of LIF versus IL6 supplementation on blastocyst formation and cell numbers. 141
Figure 5-3. Changes in *LIFR* and *IL6ST* transcript abundance between day 5 embryos and day 8 blastocysts.....142
Figure 6-1. Localization of SOX2 and CDX2 in the morula to expanded blastocyst stages in bovine blastocysts. 158
Figure 6-2. JAK2/STAT inhibition does not disrupt bovine ICM formation but does reduce ICM cell number. 159
Figure 6-3. JAK2/STAT inhibition after the ICM lineages are committed reduces HYPO but not EPI cell number.....160
Figure 6-4. JAK2/STAT inhibition disrupts bovine ICM maintenance. 161
Figure 6-5. Examples of blastocysts treated with DMSO or AZD1480. 163
Figure 6-6. ICM, HYPO, EPI and UN cell number decreases with blastocyst age. 164
Figure 6-7. Blastocyst ICMs do not recover after AZD1480 treatment.....165

Figure 6-8. 1 µM AZD1480 is sufficient to block IL6-induced STAT3 activation. 166
Figure 7-1. Effects of zinc supplementation during in vitro bovine embryo culture on cleavage rate and blastocyst development. 175
Figure 7-2. Effects of zinc supplementation during in vitro bovine embryo culture on total, ICM and TE cell numbers in day 8 blastocysts. 176
Figure 8-1. IL6 is among the factors which do not affect embryo survival to the blastocyst stage in vitro. 183
Figure 8-2. IL6 is the only factor currently known to increase blastocyst total cell numbers via the ICM.....184

List of Tables

Table 1-1: Table of Insulin and Insulin-Like Growth Factors.....36
Table 1-2. Table of WNT Family and Inhibitors 38
Table 1-3. Table of TGF-ß Superfamily & Antagonists 39
Table 1-4. Table of Cytokines.....40
Table 1-5. Table of Growth Factors 41
Table 1-6. Table of Steroids.....43
Table 1-7. Table of Other Molecules 44
Table 1-8. Table of Cocktails.....45
Table 2-1. Primers used for quantitative RT-PCR. 64
Table 2-2. Cleavage and blastocyst formation across each study. 65
Table 2-3. Embryonic ICM and TE cell counts in Day 8 blastocysts 67
Table 3-1. Primers used for quantitative RT-PCR. 89
Table 3-2. Antibody source and dilution information. 90
Table 4-1. Day 9 and 10 cell numbers from blastocysts grown in SOF-BEI or TeSR, with or without IL6. 127
Table 4-2. Formulation of the TeSR medium used 129
Table 5-1. Primers used for quantitative RT-PCR. 143
Table A-1. Experimental units for analyzing bovine blastocyst formation. 214

List of Abbreviations

ANOVA	Analysis of variance
ATRA	All-trans retinoic acid
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BMP5	Bone morphogenetic protein 5
BSA	Bovine serum albumin
CD44	Clusters of differentiation 44
CDX2	Caudal type homeobox 2
CTF1/CF1	Cardiotrophin-1
CFC	Cripto-1/FRL-1/Cryptic
CLCF1	Cardiotrophin-like cytokine factor 1
CNTF	Ciliary neurotrophic factor
COC	Cumulus-oocyte complex
CSF2	Colony stimulating factor 2
CSF2RA/B	Colony stimulating factor 2 receptors A or B
CTGF	Connective tissue growth factor
DAPI	4′,6-diamidino-2-phenylindole
DKK1	Dickkopf WNT signaling pathway inhibitor 1
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate-buffered saline
E2	Estradiol
ECM	Extracellular matrix
ED	Embryonic disk
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPI	Epiblast
ESC	Embryonic stem cell
ESR1/2	Estrogen receptors 1 or 2
FBS	Fetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FGF4	Fibroblast growth factor 4
FGF10	Fibroblast growth factor 10
FGF11	Fibroblast growth factor 11
FGFR	Fibroblast growth factor receptor
FGFR1/2/3	Fibroblast growth factor receptors 1, 2 or 3
FSH	Follicle stimulating hormone
GH	Growth hormone

GHR	Growth hormone receptor
GLM	General linear model
HA	Hyaluronic acid
HDGF	Hepatoma-derived growth factor
HGF	Hepatocyte growth factor
HNF4A	Hepatocyte nuclear factor 4 alpha
HS	Heparin sulfate
HSPA5	Heat shock protein family A member 5
HYPO	Hypoblast
ICM	Inner cell mass
IFNT	Interferon tau
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IL11	Interleukin-11
IL11RA	Interleukin-11 receptor subunit alpha
IL1B	Interleukin-1 beta
IL1R1/3	Interleukin-1 beta receptors 1 or 2
IL27	Interleukin-27
IL27RA	Interleukin-27 receptor subunit alpha
IL31	Interleukin-31
IL6	Interleukin-6
IL6R	Interleukin-6 receptor
IL6ST/gp130	Interleukin-6 signal transducer
IR	Insulin receptor
IU	International units
IVF	In vitro fertilization
IVP	In vitro produced
JAK	Janus kinase
JAK1/2	Janus kinases 1 or 2
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LOS	Large offspring syndrome
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MT1/2 or MTNR1A/B	Melatonin receptor 1 or 2
NCL	Nucleolin
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor

OPU	Ovum pick-up
OSM	Oncostatin M
P4	Progesterone
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factors
PDGFRA/B	Platelet-derived growth factors receptors A or B
PE	Primitive endoderm
PFA	Paraformaldehyde
PGF2α	Prostaglandin F2α
PGR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PTGFR	Prostaglandin F2α receptor
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitate real-time polymerase chain reaction
RARA/B/G	Retinoic acid receptors A, B or G
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPKM	Reads per kilobase of transcript, per million mapped reads
RTK	Receptor tyrosine kinase
RXRA/B/G	Retinoid X receptors A, B or G
SCF	Stem cell factor
SDHA	Succinate dehydrogenase flavoprotein subunit
SEM	Standard error of the mean
SFRP	Secreted frizzled-related protein
SMAD	Small mothers against decapentaplegic
SOCS3	Suppressor of cytokine signaling 3
SOF	Synthetic oviduct fluid
SOF-BEI	Synthetic oviduct fluid - bovine embryo 1
SOX2	Sex determining region Y-box 2
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
pSTAT3Y705	Phosphorylated STAT3 Tyrosine 705
STD	Standard deviation
T3	Triiodothyronine
T4	Thyroxine
TDGF1	Teratocarcinoma-derived growth factor 1
TE	Trophectoderm
TGFA	Transforming growth factor-α

TGFB1	Transforming growth factor beta
THRA/B	Thyroid hormone receptors A or B
TNF	Tumor Necrosis Factor
TNFR1/2	Tumor necrosis factor receptors 1 or 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UN	Undifferentiated inner cell mass cells
VEGF/VEGFA	Vascular endothelial growth factor
VEGFR1/2	Vascular endothelial growth factor receptors 1 or 2
WIF	WNT inhibitory factor
WNT	Wingless-related integration site
WNT11	Wingless-related integration site 11
WNT7A	Wingless-related integration site 7A

Chapter 1: Literature Review

Introduction

The first calf produced by in vitro fertilization (IVF) was born in 1981, almost 40 years ago [1], and now more than 700,000 in vitro produced (IVP) bovine embryos are transferred to recipients worldwide each year [2]. Utilization of ovum-pick up (OPU) and IVF techniques allows producers to increase the number of offspring produced by donor cows yearly, even to a greater extent than is possible with superovulation and subsequent embryo transfer [3]. Oocytes can be collected by OPU as frequently as once a week in cycling and pregnant cows, and even prepubertal heifers can be collected, allowing producers to shorten the generation interval and speed up genetic progress in cattle [3,4]. Calves have even been produced from post-mortem, abattoir-derived ovaries [2]. This practice can be economically advantageous for some producers, and can be utilized to impregnate dairy cattle whose offspring are not to be retained [5].

Despite these obvious benefits of IVF technology, embryo and calf production from *in vitro* techniques still suffer from low efficiency. Approximately 20-40% of oocytes will develop to the blastocyst stage in vitro [6–10], and then only 30-40% of those blastocysts will produce a calf [11–13]. A number of factors affect the success of IVP embryos, some maternal (fertility of recipient), some embryonic (aneuploidies/mixoploidies, genetics of embryo, donor health history), and some are specifically derived from *in vitro* culture conditions (exposure to light, culture medium composition, fluctuating temperatures, etc.). Maternal and embryonic factors affecting fertility will always exist in reproduction, however the negative effects of *in vitro* culture conditions can and should be improved in order to better utilize IVF technology.

One focal point in bovine IVP embryo research has been to increase the complexity of embryo culture media to better mimic the uterine histotrophe (fluid in the uterine lumen containing various secretions). In general, most embryo-based media formations contain only salts, energy sources (lactate, pyruvate, phosphate, glucose, amino acids), and a macromolecule source (usually bovine serum albumin). Also, the media is often static (the embryos remain in the same droplet of media from zygote to blastocyst stage). True oviduct and uterine fluid contain a variety of enzymes, growth factors, extracellular vesicles, micronutrients, and many other components which ebb and flow with position in the tract, embryo-maternal crosstalk, and maternal stage in the estrous cycle. Most embryo culture media are completely devoid of such factors, except possibly culture media containing serum, which contains an unknown mix of proteins and other nutrients. However, many embryo culture media do not contain serum because it has been linked to an increased risk for producing calves with large offspring syndrome [14,15].

One proposed method to improve IVP blastocyst production and competency is to supplement *in vitro* culture media with biologically active growth factors or proteins secreted by the uterus, termed “embryokines” by Dr. Peter J. Hansen [16], or to add other biologically active molecules. This idea sparked the study of more than forty bioactive factors in *in vitro* bovine embryo culture systems over the last three decades, many of which have yielded positive results. However, the majority of these studies have not previously been reviewed, thus their information has not been compared collectively. This lack of connectivity has no doubt impeded the mass adoption of many of these bioactive factors in bovine embryo culture media.

I created this review to bring all these studies together in hopes that this will help advance the industry. More specifically, I collected information on receptor-binding proteins, hormones and

bioactive molecules which have been supplemented to bovine IVP embryos after fertilization (*i.e.* the culture period of in vitro embryo production). Concerning outcomes, I will focus primarily on blastocyst formation and pregnancy or calving outcomes, as these directly affect the efficiency and associated costs of calf production from IVP bovine embryos, and blastocyst cell number, as this is a commonly used indicator of blastocyst quality. I attempted to locate every manuscript meeting these criteria to the current date, but it is possible I missed some. However, some studies were purposefully excluded if they contained improper study design or analyses, or if they contained extremely low numbers (replicate or number of embryos or blastocysts).

I separated this review into three major bioactive factor groupings: proteins, steroids and other molecules. The protein group was split further into families, where possible. These families are Insulin and Insulin-like Growth Factors, WNT family members and inhibitors, Transforming Growth Factor-ß superfamily and antagonists, Cytokines, and Growth Factors. Additionally, I will briefly discuss manuscripts which used "cocktails" (*i.e*. 2+ bioactive factors) in bovine embryo culture.

Proteins

Insulin and Insulin-like Growth Factors

Insulin is a hormone secreted by pancreatic ß cells which regulates glucose uptake by cells and other processes [17]. The insulin-like growth factors, IGF1 and IGF2, on the other hand, are growth factors crucial for organismal growth and development and are secreted by a variety of cell types, although mainly the liver in the case of IGF1 [18]. Mice with null mutations of IGF1 or IGF2 are smaller than their wild type littermates, and exhibit organ hypoplasia, delays in bone ossification, and central nervous system abnormalities [19,20]. Conversely, IGF1 or IGF2

overexpression, which sometimes occurs from a loss of imprinting in IVP embryos, increases body weight and can result in Beckwith-Wiedmann syndrome in humans, or large offspring syndrome in cattle (both are overgrowth syndromes characterized by macrosomia) [21,22].

Both IGF1 and IGF2 predominantly signal through IGF1R, a member of the receptor tyrosine kinase (RTK) family, but can also signal via the insulin receptor (IR) [18]. The opposite is true for insulin, which has high affinity for IR, and some affinity for IGF1R. A third IGF receptor exists, named the IGF2R, but this receptor contains no intracellular signaling components and exists only to bind IGF1 or IGF2 to be internalized for degradation [18]. Transcripts for IGF1 and IGF2 are produced by the bovine oviduct and uterus [23–27]. Additionally, both IGF proteins are present in uterine luminal fluid [23]. Preimplantation bovine embryos also express transcripts for both IGFs, IGF1R, and IR, indicating a propensity for bovine embryos to respond to maternally-derived IGFs and insulin [28–31].

Insulin

Insulin has been supplemented to IVP bovine embryos at a large range of concentrations (from 1 pg/ml to 100 µg/ml). In most cases, insulin did not influence blastocyst formation or cell number (Table 1-1) [32–35]. However, a few studies detected an increase in blastocyst production after insulin treatment in the presence of glucose [36,37], although another group found that this combination did not increase blastocyst formation (Table 1-1) [38]. The requirement of glucose for an effect of insulin is plausible, as insulin's primary function is to stimulate glucose uptake by cells [17]. Additionally, glucose and insulin may both need to be present at the blastocyst stage, as blastocysts utilize more glucose than prior developmental stages [39,40]. However, one group showed that insulin in the presence of glucose did not

improve blastocyst cell number [32], while another group found this combination increases blastocyst cell number [36], and yet another group observed an increase without glucose [35]. A few groups have also found that insulin supplementation decreases apoptosis [32,34]. Ultimately, the benefits of insulin in embryo culture media, and whether glucose is co-required, are unclear at this time.

Insulin-like Growth Factor-I

The effects of IGF1 on bovine IVP embryo production has been studied extensively over the past three decades. Outcomes measured include blastocyst formation, blastocyst cell number, number and percent of cells showing signs of apoptosis, and post-vitrification survival. However, despite more the more than 20 papers published on IGF1's effects on bovine preimplantation embryos, the exact benefits of IGF1 supplementation are uncertain.

I found 23 published studies in which the effects of IGF1 on blastocyst development were assessed. Of the 23 publications examined, only 48% detected increases in blastocyst formation following IGF1 supplementation (see Table 1-1 for citations). Intriguingly, the majority of studies which utilized at least 250 embryos per treatment demonstrated an increase in blastocyst production, and the majority which used low numbers of embryos didn't see a difference (see Table 1-1 for citations), indicating a low n may be responsible for many of these discrepancies. Additionally, knockdown of the IGF1 receptor, IGF1R, resulted in a decrease in blastocyst formation and cell number in comparison to siRNA injected controls [41]. In the end, whether IGF1 may increase blastocyst formation, however it appears that a relatively large number of embryos are needed to detect this effect.

Supplementation of IGF1 does appear to benefit day 8 blastocyst cell number. Approximately two-thirds of the studies that examined inner cell mass (ICM) and trophectoderm (TE) numbers showed a benefit of IGF1 on total cell number (Table 1-1). Age of the blastocysts analyzed in these studies varied (day 6 to 8). When outcomes were separated by age of the blastocysts, 100% of the studies that examined day 8 blastocysts showed an increase in total cell number with 50-100 ng/ml IGF1 treatment [42–46], but inconsistent effects were detected in day 6 or 7 blastocysts [32,35,44,47–50]. This likely indicates that IGF1 does increase total cell number, but the increase is not observable or is less likely to be observed in younger blastocysts. Jousan and Hansen [49] also showed that the ability of IGF1 to increase blastocyst cell number is dependent on the Mitogen-activated protein kinase (MAPK) pathway, but not the Phosphoinositide 3-kinase (PI3K) pathway. However, it is unclear which cell type from the blastocyst is increased by IGF1 in day 8 blastocysts. Sirisathien and colleagues found it only increased ICM cell number [44], while Xie and colleagues [46] found it only increased TE cell number, and Sakagami and colleagues [45] found that it increased both ICM and TE cell numbers.

IGF1 was previously identified as an anti-apoptotic factor in mouse, rabbit and human embryos [51–53], which prompted several experiments analyzing its effects on apoptosis in bovine IVP embryos. A few groups reported that 100 ng/ml IGF1 decreased the number of apoptotic cells in day 6-7 blastocysts [32,48], but another group reported that the same concentration had no effect on apoptosis in day 7 blastocysts [54]. A fourth group utilized a lower concentration of IGF1, 50 ng/ml, and detected no effect on apoptotic cell number in day 7 blastocysts, but a decrease was observed in day 8 blastocysts [44]. Due to these conflicting reports, the effects of IGF1 on apoptosis in bovine blastocysts are unclear.

The ability of IGF1 to protect IVP embryos from a variety of stressors has also been tested. Treatment with IGF1 increased blastocyst formation and decreased the percentage of apoptotic cells in blastocysts when embryos were exposed to 1 and 2.5, but not 5 uM menadione (a source of reactive oxygen species (ROS)) [50]. However, IGF1 did not decrease ROS production by menadione, showing that it had some protective effect on the embryo. The ability of IGF1 to protect embryos against brief heat shock has also been analyzed. Day 5 embryos exposed to heat shock (41°C) without IGF1 had reduced total cell number and an increase in the percentage of cells that were TUNEL positive [55,56]. These effects were mitigated when IGF1 was supplemented during heat shock. Additionally, inhibition of the PI3K pathway blocked this protective effect of IGF1 on heat-shock induced apoptosis [49,56]. The ability of IGF1 to protect the embryo from heat shock appears to depend on stage of development though. IGF1 was unable to protect 2-cell embryos from the effects of heat shock (as measured by blastocyst formation), but was able to partially rescue blastocyst formation when day 5 embryos were heat shocked [57]. Finally, IGF1 does not appear to affect blastocyst re-expansion when supplemented prior to vitrification [58].

A few studies have examined the outcomes of transferred IGF1-treated embryos. In one study, supplementation of 100 ng/ml IGF1 from days 1 to 7 in vitro had no effect on conceptus recovery, length or Interferon tau (IFNT) secretion on day 14 when embryos were transferred in groups of 7-12 [59]. However, when single embryos were transferred to recipients, IGF1 treated embryos tended to be recovered at a higher rate, suggesting IGF1 may reduce pregnancy losses between day 7 and 14. In another study, 100 ng/ml IGF1 of IVP embryos prior to transfer was found to benefit pregnancy rate in heat-stressed, lactating Holsteins (Table 1-1) [60]. Treatment

with IGF1 increased day 53 pregnancy rate, but only tended to increase calving rate in this study. In conclusion, IGF1 appears to benefit calf production, at least during the hot months.

Insulin-like Growth Factor-II

Few studies have examined the effects of IGF2 supplementation on IVP bovine embryos, and, like IGF1, the effects of IGF2 on blastocyst development are unclear. Byrne and colleagues found that 1 and 100 ng/ml IGF2 increased day 7 blastocyst development, but strangely 10 ng/ml did not (Table 1-1) [32]. Another group corroborated that 10 ng/ml IGF2 did not increase blastocyst development [61], and another found that 50 ng/ml did not improve total blastocyst formation [62]. In addition, one study showed that 100 ng/ml IGF2 increased day 7 blastocyst total cell number, as well as decreased the percent of apoptotic cells (Table 1-1) [32]. More studies are needed to determine if 1 or 100 ng/ml IGF2 truly affects blastocyst development.

WNT Family and Inhibitors

The Wingless-related integration site (WNT) family is an evolutionary conserved group of glycolipoprotein ligands which are involved in many crucial and fundamental developmental processes, including stem cell proliferation and differentiation and body axis determination [63]. WNT signaling is complex, involving many different receptors and coreceptors and many regulatory steps with the potential for crosstalk. The most well-known receptor is the Frizzled receptor, but many others exist [64]. The WNT signaling pathways are categorized as canonical (ß-catenin dependent) or non-canonical (ß-catenin independent), but several sub-branches exist within these groupings, and some receptors can activate both types of pathways [64]. These

pathways are controlled both intracellularly by protein phosphorylation and extracellularly by antagonists such as DKK1, SFRP, WIF, Sclerostin, R-spondin and Norrin [64]. Certain WNTs utilize canonical or non-canonical pathways more frequently than the other [64].

Bovine embryos produce several WNTs and their receptors. One microarray analysis of bovine preimplantation embryos revealed the presence of transcripts for 16 different WNT ligands [65]. However, a RNA-seq analysis of morulae and blastocysts showed the expression of only 7 WNT ligands [66]. Various receptors, coreceptors, pathway regulators and signaling components have also been detected in bovine embryos [65,66]. Additionally, WNT7A is produced by the bovine endometrium, but not the preimplantation embryo [27,65,66]. However, despite being functional in preimplantation embryos, murine, porcine and bovine embryos do not need canonical WNT signaling to become blastocysts [65,67–70].

WNT Family Member 7A and 11

To date, a few WNT ligands, WNT7A and WNT11, have been supplemented to bovine embryos. Both WNT7A and WNT11 increased blastocyst formation on day 7, but neither had an effect on day 7 blastocyst cell number (Table 1-2) [66,71,72]. Future studies are needed to determine if WNT7A and/or WNT11 also affect the calving rate of IVP embryos.

Dickkopf WNT Signaling Pathway Inhibitor 1

One WNT canonical pathway inhibitor, Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1), has been tested on IVP bovine embryos. While DKK1 has not been shown to affect blastocyst formation [65,71,73,74], it decreased ICM, TE and total cell numbers of blastocysts in

one report [73], but not another [71] (Table 1-2). Interestingly, in one study DKK1 has been shown to skew the population of ICM cells toward hypoblast (GATA6+ NANOG-) [73]. As for its effects on transferred embryos, DKK1 treatment in vitro from day 5 to 7.5 produced longer day 15 conceptuses [74], but only transiently improved pregnancy rate on day 32 and not on days 64-76 or on calving (Table 1-2) [73].

TGF-ß Superfamily & Antagonists

Several members of the Transforming Growth Factor beta (TGFB1) family, as well as a few antagonists, have been supplemented to bovine IVP embryos. Members of the TGFB1 family utilize a hetero-tetrameric receptor, comprised of 2 type I and 2 type II receptors, both of which are Ser/Thr kinases [75–78]. The downstream effects of TGFB1 family members binding to their receptor complexes varies widely depending on the cell involved, however, they are perhaps most well-known for activating the Small Mothers Against Decapentaplegic (SMAD) family of proteins [75–79]. Both TGFB1 family receptors are expressed by bovine preimplantation embryos [80,81].

Transforming Growth Factor-ß

Transcripts for TGFB1 have been detected in the bovine endometrium [27,82]. To date, most studies have shown TGFB1 has no effect on blastocyst formation (Table 1-3) [61,80,83,84]. However, two groups did detect increases in blastocyst formation with 5 or 50 ng/ml TGFB1 [62,85]. Additionally, only two studies examined blastocyst cell number, and both found that

TGFB1 had no effect [83,85]. I could not find any studies which examined the effect of TGFB1 on pregnancy outcomes.

Activin A

Activins are also members of the TGFB1 family, and are involved in regulation of the hypothalamic-pituitary-gonadal axis, early embryonic development, and a number of other biological processes [77,78]. The name 'activin' comes from their ability to stimulate, or activate, the release of Follicle stimulating hormone (FSH) from the pituitary, which is opposite the function of an earlier identified group of FSH-release inhibitors, named inhibins. Transcripts for activin A were detected in preimplantation bovine embryos from the oocyte to blastocyst stages [86], and activin A is known to be produced by the bovine oviduct [87], suggesting a potential role for activin A in bovine preimplantation development.

The effects of activin A on blastocyst development, cell number, and apoptosis, have been tested by several groups. All groups found that activin A increased blastocyst formation (Table 1-3) [72,88–92]. However, one group found that only supplementation during early stages (beginning at day 1) increased blastocyst production [89], while others determined it didn't need to be supplemented until later stages [72,88,90,91]. Regardless, supplementation of activin A from day 1 to 8 was always found to be beneficial on blastocyst production [86,91]. No studies on the effects of activin A on IVP embryo pregnancy retention have been performed as of yet.

The effect of activin A on blastocyst cell number, however, is less certain. Most groups have found no effect [88,90,92], but Trigal and colleagues observed a decreased in TE cell number after activin A treatment, as well as an increase in apoptosis, despite an improvement in

blastocyst yield (Table 1-3) [91]. Because the majority of studies showed no effect of activin A on cell number, it seems unlikely that activin A truly decreases TE cell numbers. However, future studies involving activin A should be careful to confirm that activin A does not negatively impact blastocyst cell number.

Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are also members of the TGFB1 superfamily. More than a dozen BMPs have been identified, and various BMPs are involved in bone formation as well as the development of many other organs and tissues, including, but not limited to, heart, limb and gastrointestinal tract development [76,79]. Transcripts for several BMPs have been detected in preimplantation bovine embryos [28,93,94], as well as the oviduct [95].

The effects of BMPs 2, 4 and 5 have been tested on bovine preimplantation embryos to date. Lee and colleagues found BMP2 had no effect on blastocyst formation [93], however the protein was only supplemented from day 1 to 3 (Table 1-3). Rosa and colleagues found that BMP4 supplemented from day 1 to 9 decreased blastocyst formation but didn’t impact blastocyst cell number (Table 1-3) [96]. As for BMP5, Garcia and colleagues found that it increased blastocyst formation, and they only supplemented the protein from day 1 to 2 (Table 1-3) [97]. To summarize, BMP5 holds the most promise for benefiting IVP bovine embryos, but the effects of it being supplied throughout the culture period are currently unknown.

Follistatin

Follistatin is a secreted protein which antagonizes members of the TGFB1 superfamily, and is important for regulation of these proteins [98,99]. Follistatin null mice have defects of the skeleton and muscle tissue, and die soon after birth due to failure to breathe [100]. Follistatin transcripts have been detected in the bovine endometrium [101].

The laboratory of Dr. George W. Smith has found that supplementation of 1-10 ng/ml of follistatin to bovine embryos improves blastocyst formation, and increases blastocyst TE and total cell number (Table 1-3) [89,102]. This effect was apparent if follistatin was supplemented from day 1-3 or 4-7 post-fertilization; however the greatest improvement was observed when follistatin was present in the culture media from day 1 to 7 [102]. Future studies are needed to determine if these follistatin-treated embryos are more competent to maintain a pregnancy.

Noggin

Noggin is a secreted protein which binds to and sequesters members of the TGFB1 family, namely BMPs, to prevent them from binding their receptors [103,104]. Functionally, Noggin plays a role in many developmental processes, including somite patterning and neural tube closure [105], forebrain development [106], cartilage morphogenesis, and joint formation [107]. Mutations of and the absence of functional Noggin cause neural tube defects [105] and a number of joint abnormalities [107,108]. Noggin expression has not been detected in bovine endometrium [109] or preimplantation embryos [94] to the best of our knowledge.

To date, the effect of Noggin on IVP bovine embryos has only been tested once, and it was found to decrease blastocyst formation without affecting cell number (Table 1-3) [96]. However, only one concentration, 100 ng/ml, was tested.

Cytokines

Interleukin-1ß

Interleukin-1ß (IL1B) is a pro-inflammatory cytokine important for innate immunity [110], but is also known to have a role in ovulation [111]. To initiate signaling cascades, IL1B first binds its receptor, IL1R1, which then binds its coreceptor IL1R3/ILR1AP [110]. Transcripts for IL1B and IL1R1 are produced by the bovine endometrium and blastocyst [112].

The effects of IL1B on IVP bovine embryo development have been tested by Paula-Lopes and colleagues. The group supplemented 0.01 to 10 ng/ml IL1B under a variety of conditions, and found that IL1B does have some positive effects on blastocyst development, however only under very specific conditions (Table 1-4) [113]. Doses of 0.1 to 10 ng/ml increased blastocyst development, but only when treatment began at the start of the culture period versus 5 days after, and only if the embryos were cultured in larger groups (25-30 zygotes versus 10 in 50 µl drops of culture media). Because the same doses of IL1B did not benefit smaller group culture, the authors speculated that this indicates that IL1B likely indirectly increases blastocyst development by stimulating embryonic secretion of another factor. The authors determined this effect was not caused by persistent cumulus cells.

Colony-stimulating factor 2

Colony-stimulating factor 2 (CSF2), which is also known as granulocyte-macrophage colony-stimulating factor, was originally identified as a hematopoietic growth factor involved in myelopoiesis, but this function appears to be dispensable [114]. Instead, CSF2's more important role appears to be in inflammation [114]. CSF2 signals through a heterodimeric complex composed of CSF2RA and CSF2RB, which lack intrinsic kinase activity, but can stimulate the SRC family kinases, as well as the Janus-activated Kinase 2 and Signal Transducer and Activator of Transcription 5 (JAK2/STAT5), PI3K and MAPK pathways [114]. CSF2 is produced by the oviduct and uterus [27,114,115], but not the preimplantation bovine embryo [94]. Curiously, bovine preimplantation embryos lack transcripts for CSF2RB, but, as detailed below, several effects of CSF2 on bovine embryo development have been detected.

Initial studies with CSF2 (1997-2010) showed that supplementation of 1-50 ng/ml during embryo culture increased blastocyst development, and oxygen tension had no effect on this (Table 1-4) [62,115,116]. However, more recent studies show no detectable effect of 10 ng/ml CSF2 on blastocyst development (Table 1-4) [16,117–119]. Curiously, the latter studies utilized different sources of CSF2 than the initial studies, which utilized CSF2 from CIBA-GEIGY, a former Swiss company. Perhaps a difference in quality of the protein exists between the latter and former. The effects of CSF2 on blastocyst cell number have also been tested, but no difference was detected, other than a statistical tendency to increase ICM cell number (Table 1-4) [116,118]. Additionally, several studies have shown no effect of CSF2 on apoptosis in blastocysts, blastocyst grade, blastocyst diameter, blastocyst IFNT secretion, DNA methylation in the ICM or TE, or TE outgrowth formation [116–118,120]. However, CSF2 treatment does increase the survival of ICM-derived cell colonies [117].

After transfer to cattle, the effect of CSF2 is unclear. One study found CSF2 treatment increased calving rate in the cool season, and decreased pregnancy loss between day 30 and 35 in the hot season (Table 1-4) [116]. A second study found an increase in day 32 pregnancy rate and an improvement in survival curve, but no effect on calving rate (Table 1-4) [73]. Finally, a third study showed no difference in pregnancy rate or embryonic losses on day 30 or 60, although the number of pregnancies (6-10 per treatment) is low (Table 1-4) [119].

Leukemia inhibitory factor

Leukemia inhibitory factor (LIF) is a cytokine with known roles in a number of organs and diseases [121], but more relevant to embryo development and reproduction, LIF is utilized to maintain mouse embryonic stem cells (ESCs; derived from the blastocyst ICM) and is necessary for embryo implantation in mice [121–124]. LIF signals through a heterodimer complex of LIFR and gp130/IL6ST, and can activate JAK/STAT, MAPK, and PI3K signaling [121]. However, whether bovine preimplantation embryos express LIFR is unclear; Eckert and Niemann [125] reported only IVP embryos, and not *in vivo* produced, expressed transcripts for LIFR. Nevertheless, LIF has been a point of interest for bovine blastocysts because of its ability to activate STAT3 in mouse ESCs.

Numerous laboratories have tested the effect of LIF on blastocyst development, cell number and cryosurvival, but the results leave no clear answers. Several groups have reported no benefit of various doses of human LIF on blastocyst development (Table 1-4) [126–129]. In a few instances, LIF decreased day 8 blastocyst formation and quality [127,128], and decreased the percentage of hatched blastocysts in one case (Table 1-4) [129]. However, in some instances LIF increased blastocyst development and hatching (Table 1-4) [58,62,126,127,130,131]. As for its

effect on cryosurvival, some reported that LIF improves re-expansion and hatching post-thaw [58,127], but those same groups report no benefit in other instances [127,131]. The pattern is the same with blastocyst cell number, some groups have reported increases with LIF treatment [127,130,131], in other instances it had no effect [128,131], and in some cases it decreased blastocyst cell number (Table 1-4) [58,128,129]. Because of all these contrasting results, no conclusion on the effects of LIF can be made. Some of this disparity may be resultant of the wide range of doses utilized in these studies (500-6,000 IU or 2-100 ng/ml) or because of other protocol disparities.

Tumor Necrosis Factor-α

Tumor Necrosis Factor-α (TNF) is a cytokine that is mainly produced by macrophages and is known for its potent ability to induce tumor necrosis [132]. TNF is initially a transmembrane protein, but a metalloprotease, TNFα-converting enzyme, cleaves it to produce a soluble ligand. Both forms can bind and activate their receptors, TNFR1 (a.k.a. TNFRSF1A) or TNFR2 (a.k.a. TNFRSF1B) [132]. TNFRSF1A transcripts are present in bovine embryos at least to the morula stage [94]. TNF is largely known for its roles in inflammation and the immune system [132], and is present at elevated levels in the serum of cows with mastitis [133].

Unlike the other molecules I am reviewing, supplementation of TNF to bovine IVP embryos was expected to produce negative outcomes, as it may play a role in the reduced fertility of mastitic cows [134]. Indeed, Jackson and colleagues found that it decreased blastocyst development (Table 1-4) [134]. Therefore, I do not recommend supplementing this protein to embryo culture media, but thought it was pertinent to include.

Stem Cell Factor

Stem Cell Factor (SCF), or Kit ligand, is a cytokine that is produced by many cell types throughout the body. SCF's receptor is c-Kit (a.k.a. KIT), and SCF is involved in development of hematopoietic, germ and melanoblast cells, as well as other cell types [135]. Binding of SCF to c-Kit results in receptor dimerization and activates one or many pathways, including PI3K, Src family kinases, and Phospholipases C and D [135]. KIT transcripts are expressed by the preimplantation bovine embryo [94] and SCF is produced by bovine endometrial cells *in vitro* [136].

To date, SCF has been tested by Dhali and colleagues. Supplementation of 50 ng/ml SCF had no effect on blastocyst formation (Table 1-4) [137]. Further work is needed to determine if SCF affects blastocyst cell number and/or pregnancy retention.

Growth Factors

Fibroblast Growth Factors

The Fibroblast Growth Factor (FGF) family is comprised of 18 different secreted signaling proteins and 4 intracellular signaling proteins [138]. These FGFs are involved in a number of developmental processes, perhaps most notable for this review is FGF control of hypoblast development in mice and cattle [138–143].

The secreted FGFs bind to members of the FGFR family with the help of either heparin (FGFs 1-10, 16-18, 20, 22) or members of the Klotho family (FGFs 15/19, 21 or 23) as a cofactor [138]. Four members of the FGFR family exist (FGFRs 1-4), and two (FGFR1 and

FGFR3) also exist in two major splice variants [138]. Activated FGF-HS/Klotho-FGFR complexes control multiple intracellular signaling pathways, including the MAPK, PI3K, Phosphoinositide phospholipase C-γ (PLCγ), and STAT pathways [138]. Additionally, FGFs and their ligands have been localized to cell nuclei, where they can carry out other functions through currently unknown mechanisms [138]. On top of that, four members of the FGF family are not secreted (FGF11-14), and do not interact with RTKs [138]. Instead, these intracellular FGFs interact with voltage gated sodium channels and microtubules, but little is known about their exact functions [138].

The FGF ligands are expressed by nearly all adult tissues, and several are produced by bovine embryos [28,138]. Additionally, bovine preimplantation embryos produce transcripts for all members of the FGFR family [144,145]. Intriguingly, in bovine blastocysts, FGFR2 and 4 were localized exclusively to the TE, but FGFR1 and 3 were located in both the ICM and TE [144].

Various FGFs (1, 2, 4 and 10) have been supplemented to IVP bovine embryos. A few studies have shown that doses of 0.05-50 ng/ml FGF2 have no effect on blastocyst development [46,61,62,84], but a high dose (500 ng on day 0 and 500 again on day 4 for a total of 1 ug/ml) increased day 7 blastocyst formation (Table 1-5) [146]. In direct contrast though, one study found positive effects with a low dose (50 pg/ml) [147]. FGF2 also does not appear to protect day 5 embryos from the negative effects of heat shock [146]. Most studies have shown that FGF2 does not affect blastocyst cell number [46,145,146], but one study showed a positive effect (Table 1-5) [84]. Additionally, FGFs 1 and 10 had no effect on blastocyst cell number (Table 1-5) [145]. However, FGFs 1, 2 and 10 have been shown to stimulate trophoblast proliferation in a bovine trophoblast cell line [46]. Also, FGF4 can skew the ICM toward the hypoblast fate [142], and FGF2 promotes bovine primitive endoderm outgrowth culture [143]. No studies have yet

been conducted to examine the effect of *in vitro* FGF supplementation on pregnancy retention and calving rate.

Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) is a member of the Cellular Communication Network family of extracellular matrix proteins (ECM). CTGF is mostly known for being a regulatory protein, capable of mediating some ligand-receptor binding, including that of integrins, heparin sulfate proteoglycans, lipoprotein receptor related proteins, and RTKs [148]. Additionally, CTGF is also involved in cell adhesion, migration, proliferation, and ECM remodeling [148]. Concerning bovine reproduction, CTGF is one of the most prominently expressed ligands by the bovine endometrium [27].

A single study examining CTGF supplementation has been conducted to date. Kannampuzha-Francis and colleagues found that supplementation of CTGF had no effect on IVP bovine blastocyst formation, but doses of 0.1 and 1 nM increased ICM cell number in day 7 blastocysts (Table 1-5) [88]. To date, no studies have examined the effect of CTGF on IVP embryo pregnancy retention.

Hepatoma-derived growth factor

Hepatoma-derived growth factor (HDGF) is involved in ribosome biogenesis, RNA processing, DNA damage repair, transcriptional regulation, cell proliferation and migration, and organ development [149–153]. The signaling mechanisms of HDGF are largely unknown, but nucleolin (NCL) was recently identified as a receptor [154]. HDGF transcripts and protein are

produced by the bovine endometrium [155,156], and transcripts for HDGF and NCL have been detected in the bovine preimplantation embryo [28].

The effects of HDGF on bovine IVP embryos appear to be limited. Gómez and colleagues reported that 100 ng/ml HDGF increased day 7 blastocyst formation in some but not all instances, and this effect was lost on day 8, or if BSA was present in the culture medium (Table 1-5) [155,157]. Increasing day 7 but not day 8 blastocyst formation may indicate that HDGF increases the speed of embryo development to the blastocyst stage, rather than embryo survivability. Paradoxically though, the same manuscript also showed that 0.1 and 1 ng/ml HDGF decreased day 7 blastocyst formation (Table 1-5). Additionally, the authors found that 100 ng/ml HDGF increased TE and total cell number in group-cultured blastocysts, but when the embryos were cultured individually, HDGF also increased ICM in addition to TE and total cell numbers (Table 1-5) [155]. In another study by the same authors, HDGF had no effect on pregnancy rate, calving rate, or birth weight of calves produced from IVP embryos (Table 1-5) [157].

Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a pleiotropic cytokine known to have epithelial regenerative effects in a number of organs, as well as other functions [158,159]. The receptor for HGF is a RTK called Met/c-Met, and HGF-Met binding can induce a number of cell signaling pathways, including MAPK, PI3K and STAT3 [158–160]. The ligand HGF is produced by the uterus [27], and Met transcripts are expressed by bovine morulae and blastocysts [28].

Kannampuzha-Francis and colleagues are the only group to have tested HGF thus far, and they found that 0.01 nM HGF decreased blastocyst development, but 0.1 and 1 nM HGF had no effect (Table 1-5) [88]. No dose affected day 7 blastocyst cell number (Table 1-5).

Epidermal Growth Factor

Epidermal growth factor (EGF) belongs to the family of EGFR ligands, which includes EGF, transforming growth factor α, amphiregulin, and others [161]. EGF is involved in a number of different cancers, as well as the development of the skin, lungs, mammary glands, prostate, pancreas, gastrointestinal tract, and central nervous system [162]. To initiate intracellular signaling, EGF binds to its receptor, EGFR, an RTK, and has been shown to stimulate multiple signaling pathways, including the MAPK and PI3K pathways [161,163]. Transcripts for EGF are produced by the oviduct and uterus in cattle [27], and by oocytes and up to the 4 cell stage in bovine embryos [94]. Unlike EGF, however, EGFR is expressed by bovine oocytes and embryos to the blastocyst stage, suggesting the potential for maternal-embryonic cross talk [94].

Several groups have examined the effects of EGF on bovine blastocyst formation. Approximately half have shown that 5-10 ng/ml EGF increases blastocyst formation (Table 1-5) [43,44,46,85,164], but several groups have found no effect of EGF on blastocyst development (Table 1-5) [61,83,84,165,166]. Additionally, most studies have shown that 25-100 ng/ml of EGF does not affect blastocyst development (Table 1-5) [43,164]. However, Yang and colleagues [85] observed an increase in blastocyst development with 100 ng/ml (Table 1-5). In most studies, EGF did not affect blastocyst cell number (Table 1-5) [43,44,46,83,85,166,167], but Sirisathien and colleagues [44] reported that it decreased the percentage of cells which were apoptotic. In contrast though, two studies showed that EGF increased blastocyst cell number

(Table 1-5) [45,84]. No studies looking at the effect of EGF individually on pregnancy retention of IVP embryos have been conducted thus far.

Teratocarcinoma-derived growth factor 1

Teratocarcinoma-derived growth factor 1 (TDGF1), also known as Cripto, is a member of the EGF-CFC family, and can be either secreted or membrane bound [168–170]. TDGF1 can function as both a coreceptor and a ligand, where it binds to the GRP78 receptor (a.k.a HSPA5) [169,171]. TDGF1 over-expression has been associated with several cancers, but TDGF1 also plays roles in development and embryonic stem cell differentiation [168]. In the mouse blastocyst, TDGF1 is expressed in all cells of the blastocyst, but later becomes restricted to the epiblast [172,173]. Additionally, TDGF1's receptor, HSPA5, is expressed by bovine preimplantation embryos [94].

Thus far, only one study examining the effects of TDGF1 on bovine IVP embryos exists. The authors found that TDGF1 had no effect on blastocyst development when supplemented from day 5 to 7, however the authors did find that 0.01 nM increased blastocyst total cell number [88].

Transforming Growth Factor-α

Transforming growth factor-α (TGFA) is a member of the EGFR ligand family, and, while it binds EGFR just like EGF, it is known to produce functionally distinct cellular responses [174]. TGFA is involved in many biological processes, including development of several organs and a number of diseases [175,176].

The effect of TGFA on bovine blastocyst development is unclear. In one study, TGFA had no effect on blastocyst development [61], another study found that it increased blastocyst development [147], but, in one last study, a decrease in blastocyst development was observed (Table 1-5) [166]. I am not aware of any studies which examined blastocyst cell number after TGFA treatment.

Platelet-Derived Growth Factor

Platelet-derived Growth Factors (PDGF) are a family of growth factors that are comprised of two subunits (PDGF-A, B, C and/or D) and are involved in the growth of connective tissue and the formation of embryonic blood vessels and various organs [177,178]. The PDGFs utilize two receptors, PDGFRA and PDGFRB, which are class III RTKs [177,178]. Transcripts for these receptors have been detected in bovine preimplantation embryos [28,94]. PDGF-A transcripts have been detected in the bovine preimplantation embryo [94], and PDGF-B transcripts have been detected in the oviduct [179].

To date, three studies have examined the effect of PDGF on bovine blastocyst development. Two studies found that PDGF-AB and BB had no effect on blastocyst formation [61,180], but another showed PDGF-BB decreased blastocyst formation (Table 1-5) [147]. No studies have examined PDGF's effects on blastocyst cell number.

Nerve Growth Factor

Nerve Growth Factor (NGF) is largely known as a neurotrophic factor involved in the survival and differentiation of peripheral nerve cells, but it is also involved in the development of

a variety of other structures and organs [181–184]. NGF can utilize two different receptors, $p75^{NTR}$ (a.k.a. NGFR) and the Trk receptors, TrkA, TrkB and TrkC (a.k.a. the NTRKs) [185]. The ligand NGF is produced by the female bovine reproductive tract [27] and NGFR transcripts appear to be produced at the blastocyst stage in bovine embryos [94]. Both NGF transcripts and protein have been detected in the bovine oviduct [186].

To date, only one study has examined the effects of NGF treatment on bovine blastocyst development. Flood and colleagues found that supplementation of 10 ng/ml NGF to bovine embryos had no effect on blastocyst formation (Table 1-5) [61]. No studies have examined NGF's effects on blastocyst cell number or IVP embryo pregnancy retention.

Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF/VEGFA) is a key angiogenic and pro-survival factor for vascular endothelial cells, but is also known to affect other cell types [187]. Additionally, VEGF is necessary for embryonic angiogenesis, and even heterozygous null VEGF embryos are embryonic lethal [187]. VEGF can utilize two different RTKs, VEGFR-1 (a.k.a FLT-1) or VEGFR-2 (a.k.a KDR or FLK1), and is perhaps most well-known for activating the PLCγ-PKC-MAPK pathway [188]. VEGFR-1 transcripts are expressed by the bovine preimplantation embryo, and VEGFR-2 transcripts appear to be present in early (1-16 cell) embryos [94]. VEGF is also one of the most highly expressed transcripts in the oviduct at day 5 post-ovulation [27], suggesting a potential role for it in early embryo development.

I located one manuscript which examined VEGF supplementation to IVP embryos. Lou and colleagues found that 5 ng/ml did not affect blastocyst formation (Table 1-5) [189]. Moving

forward, it might be interesting to see if in vitro treatment with VEGF has any effects on the trophoblast/placenta and yolk sac, since it is known to play an important role in angiogenesis of these structures [187]

Growth Hormone

Growth hormone (GH) is largely known for its ability to stimulate body growth both directly and indirectly through IGF1 stimulation, but it is also involved in metabolism, reproduction, and other physiological functions [190]. Growth hormone utilizes one receptor, known as Growth Hormone Receptor (GHR), a cytokine-type receptor, which it uses to activate many pathways, including the JAK-STAT ad PI3K pathways [190]. GHR is highly expressed in the liver, but also is expressed by other cells, including the bovine preimplantation embryo [191,192]. I could not find evidence that GH is produced by the uterus, or present in the uterine lumen fluid, however this does not mean it is not present in the histotrophe.

Several groups have tested the effects of GH supplementation on bovine blastocyst production and cell number. In all instances, 100 ng/ml GH increased blastocyst formation and cell number (Table 1-5) [42,191,193,194]. However, Iwata and colleagues did not find a benefit on day 60 pregnancy rate (Table 1-5) [194].

Steroids

Unlike proteins, relatively little research has been performed on the effects of steroid hormones added to post-fertilization bovine embryo culture media. Steroid hormones are all derivatives of cholesterol and can be divided into two classes: corticosteroids (cortisol and

others) and sex steroids (progesterone, estradiol and others). These classes can then be divided further based on the receptor the steroid binds. Steroid hormones perform a variety of functions, depending on the specific steroid they are, and the tissue they are acting on. I located only two manuscripts which examined the effects of various steroids on bovine IVP embryo production. These steroid hormones are cortisol (a.k.a. hydrocortisone), 17ß-estradiol (E2) and progesterone (P4).

Cortisol

Cortisol is a glucocorticoid, which binds the glucocorticoid receptor, and is involved in many bodily functions, including the stress response and metabolism [195,196]. Glucocorticoid receptor transcripts have been detected in bovine preimplantation embryos [197], as well as cortisol in oviductal fluid [198]. However, Banliat and colleagues did not find any effects of cortisol on blastocyst formation or cell number, but they did find that it decreased blastocyst survival after freezing (Table 1-6) [199].

17ß-Estradiol

Estradiol, or E2, is a sex steroid largely known for its role in female reproduction, but it also has functions in metabolism and male reproduction [200]. Estradiol utilizes two nuclear receptors, ERα (ESR1) and ERß (ESR2), but membrane-bound receptors for E2 have also been found [200,201]. Transcripts for ESR2 have been detected in early stage bovine embryos (oocyte to 8-cell) [94], and E2 can be found in oviductal fluid [198]. However, Banliat and colleagues found that E2 supplementation had no effect on blastocyst production or cell number (Table 1-6)

[199]. Further work is likely needed to determine if blastocyst-stage embryos possess ESR1 or ESR2 protein, and thus the ability to respond to E2.

Progesterone

Progesterone, or P4, is another sex steroid like E2, and is crucial for pregnancy retention and proper embryo development in cattle [202,203]. Progesterone utilizes the progesterone receptor (PGR), of which transcripts are present in bovine preimplantation embryos [204]. Additionally, P4 can be detected in oviductal fluid [198]. It does not appear that P4 affects IVP blastocyst development though (Table 1-6) [199,205]. However, Larson and colleagues found that 1 or 100 ng/ml decreased blastocyst cell number [205], but Banliat and colleagues found that 55 ng/ml did not affect cell number, and improved cryo-survival (Table 1-6) [199].

Other Molecules

Melatonin

Melatonin is a hormone secreted mainly by the pineal gland and gastrointestinal tract that regulates circadian rhythm but also has other functions [206,207]. Melatonin can utilize either MT_1 (MTNR1A) or MT_2 (MTNR1B) receptors, both of which are G-protein coupled receptors [206,207]. Both receptors have been detected on bovine preimplantation embryos [208,209], but because Melatonin can act as a free-radical scavenger, it does not necessarily require the presence of its receptors to benefit bovine embryo production.

All reports thus far indicate that melatonin supplementation increases IVP bovine blastocyst production (Table 1-7) [209–212], but Papis and colleagues noted this effect only occurred in

high oxygen culture, whereas at low oxygen melatonin decreased blastocyst formation [212]. I could not determine the oxygen concentrations used in the other manuscripts. Additionally, melatonin appears to boost blastocyst cell number (Table 1-7) [209,211], can increase hatching after thawing [210], and improves blastocyst production in the presence of paraquat, a pesticide known to adversely affect reproduction [211]. Future studies are needed to determine if melatonin supplementation also benefits pregnancy retention of IVP embryos.

Prostaglandin F2α

Prostaglandin F2α ($PGF_{2\alpha}$) is a prostaglandin produced by the uterus which causes luteolysis [213]. To do this, $PGF_{2\alpha}$ binds to its receptor, PTGFR, a G-protein coupled receptor [214], which has been detected in bovine preimplantation embryos [215]. However, Scenna and colleagues found that $PGF_{2\alpha}$ supplementation to IVP embryos decreased blastocyst formation (Table 1-7) and this effect was not common to other prostaglandins, as Prostaglandin E2 had no effect [216]. Thus, it seems unlikely that $PGF_{2\alpha}$ would benefit embryo production.

Lysophosphatidic Acid

Lysophosphatidic Acid (LPA) is a phospholipid which also functions as a signaling molecule. LPA can utilize different G protein-coupled receptors, LPARs 1-6, to alter cell proliferation, cytoskeletal properties and more [217]. Transcripts for LPARs 1-4 have been detected in bovine preimplantation embryos [94,218], and LPA is produced by the bovine uterus [219]. However, Torres and colleagues showed that LPA supplementation did not affect IVP bovine blastocyst

production (Table 1-7) [218]. To date, no studies have examined LPA's effect on blastocyst cell number or IVP embryo pregnancy retention.

Retinoids

Retinoids are analogs of vitamin A which are vital for proper embryo development [220,221]. Retinoids bind to nuclear receptors, RARs (RARA, RARB, RARG) or RXRs (RXRA, RXRB, RXRG) [220,221], which are expressed in bovine preimplantation embryos [222–224]. I found three manuscripts which tested the effects of retinoids and their receptor agonists on bovine IVP embryos. In one case, all-trans retinoic acid (ATRA) increased blastocyst formation [225], but in two others it did not (Table 1-7) [226,227]. Similarly, in two cases ATRA increased blastocyst cell number [225,226], but in another it had no effect (Table 1-7) [227]. Additionally, in one manuscript 0.1 µM LG100268 (a RXR agonist) increased blastocyst formation [227], but in another it did not (Table 1-7) [225]. The receptor agonist LG100268 also appears to be detrimental for blastocyst cell number (Table 1-7) [227], and it has been observed to increase apoptosis [225]. In the end, it is unclear if retinoid or RXR agonist supplementation benefits blastocyst production.

Hyaluronic Acid

Hyaluronic acid (HA) is glycosaminoglycan and a major component of extracellular matrix, although it can also act as a signaling molecule by binding to CD44 [228]. Through signaling, HA is involved in inflammation and other processes [228]. The receptor, CD44, is expressed by bovine preimplantation embryos [229]. Furnus and colleagues found that supplementing 1 mg/ml

increased blastocyst formation [230], but Saeed-Zidane and colleagues found no benefit (Table 1-7) [167]. Both groups found no effect on blastocyst cell number (Table 1-7) [167,230]. However, Furnus and colleagues found that HA did not affect post-thaw survival, but Saeed-Zidane and colleagues found it increased post-thaw re-expansion [167,230]. Thus, whether HA supplementation benefits blastocyst production or post-thaw development is unclear.

Thyroid Hormone

The thyroid hormones, T_3 and T_4, are involved in various aspects of development, growth and metabolism [231]. These thyroid hormones travel through the body to bind one of their receptors, THRA or THRB, to elicit a response [231]. Thyroid hormones have been detected in bovine uterine homogenate and preimplantation embryos express both receptors [232]. I located one manuscript examining the effects of their supplementation on bovine IVP embryos. Ashkar and colleagues found that a mix of T_3 and T_4 increased blastocyst formation (Table 1-7) [233]. No studies have examined cell number or pregnancy retention after their supplementation.

Cocktails

We also collected manuscripts which examined the effects of 'cocktails' (*i.e.* 2+ factors present in the culture media) on IVP bovine embryo development post-fertilization. The idea behind using multiple factors is that this even further increases the complexity of the embryo culture media and should hopefully better mimic the uterine environment and produce positive effects. Additionally, some factors may have synergistic effects. Indeed, many of the papers showed positive effects on either blastocyst formation or cell number (See Table 1-8 for more

detail) [43,45,46,58,62,84,85,234,235]. The combination of EGF and FGF2 appears promising for benefiting blastocyst production, for example. Several groups have found improvements to both the percentage of blastocysts formed and their cell number with this combination, with or without IGF1 [46,84,234]. Unfortunately though, Vailes and colleagues found this combination plus IGF1 did not improve pregnancy rate up to day 56, but no studies have examined calving as of yet [234].

Conclusions & Moving Forward

In summary, I collected information of the effects of 40 bioactive factors on bovine IVP blastocyst and calf production. I summarized the results in Figure 1-1. In many cases, a beneficial effect was found on either bovine blastocyst production, or on blastocyst cell number. Both outcomes are important, as an increased number of blastocysts could mean more calves can be produced, and increased cell number could indicate embryo quality was improved [236,237]. However, scarcely few studies have examined calving outcomes after supplementation of bioactive factors to culture media (Fig. 1-1). This represents a large gap in our knowledge of whether these factors are truly beneficial to vitro embryo production efficiency, and this is likely why the use of these factors has not been widely adopted.

Of course, calving studies are costly and attaining the number of pregnancies needed to detect differences can be difficult or impossible for some research groups. Thus, understandably, the embryokine to be studied for calving effects needs to be very promising, and, as I have shown here, a considerable number of bioactive factors (11/40) are in a “gray area” (i.e., <75% of studies agree on the effect of said bioactive factor on blastocyst formation; See Fig. 1-1). I tried to highlight a few possible reasons for these differences in the “Notes” section of Tables 1-1-7.

Even further, 18 of the 40 factors only had one study behind them. Repeatability would certainly make these factors more appealing for calving studies.

I chose to highlight two things in the "notes" sections of the tables. First, I noted if the blastocyst formation study had a "low n" (*i.e.* <250 embryos cultured per treatment). This is important, because IVP embryos exhibit a large amount of variation (see Appendix A), and this easily can lead to Type I and Type II errors. Of the 115 observations herein (115 is the number of rows in Tables 1-1-7 where blastocyst formation was a measured outcome), 76, or 66% had a low n. In the case of IGF1's effect on blastocyst formation, this problem may explain why different studies came to different conclusions. Secondly, I also noted the use of undefined culture media (i.e. serum-containing or co-culture), which could confound results in comparison to more defined media.

Moving forward, it is clear there is great need for more calving studies if the use of bioactive factors is to be widely adopted, but improvements can be made elsewhere as well. The use of sufficient experimental units and statistics to analyze blastocyst production is a must. Bovine embryos exhibit a large amount of variation, week to week and droplet to droplet (see Appendix A). It is important to account for this natural variation and hopefully this might reduce discrepancies between studies in the future. Additionally, the inclusion of blastocyst cell counting in all manuscripts would be useful, as these techniques are relatively easy to do.

In the end, the most promising factors for improving blastocyst production are WNT7A, WNT11, activin A, follistatin, BMP5, GH, Melatonin and Thyroid Hormone, as 75+% of studies conducted on them thus far have shown a benefit to blastocyst production. For improving quality by increasing blastocyst cell number, IGF1, IGF2, Follistatin, CTGF, HDGF, TDGF1, GH,

Melatonin and ATRA are likely the best choices. As for improving calving, IGF1 and CSF2 appear hopeful.

Dissertation Hypothesis & Objectives

For my dissertation, the overarching objective was to improve IVP bovine blastocyst quality by supplementing molecules that are absent in traditional synthetic oviduct fluid (SOF). The first and largest objective was to determine the function of Interleukin-6 (IL6) in preimplantation development and to determine if its supplementation was beneficial to IVP embryos. The second objective was to determine if zinc addition to SOF improved blastocyst production and cell number.

The cytokine IL6 is most commonly known for its functions in the innate immune response, however bovine preimplantation embryos express not only IL6, but also both its receptor subunits, IL6R and IL6ST, indicating that preimplantation bovine embryos have the propensity to respond to IL6 [94,238–242]. IL6 is the flagship member of the IL6 family of cytokines which are well known for their ability to activate STAT3, a transcription factor crucial for the maintenance of the ICM and embryonic stem cells in mice [122–124,243]. This is interesting because bovine IVP embryos are known to have poor quality ICMs [237,244–246], and STAT3 might be an important factor in the bovine ICM. At the start of this work, IL6 had not been previously supplemented to bovine IVP embryos, but LIF, another family member, had been. LIF is the cytokine most commonly used to maintain STAT3 signaling in murine ESCs. This prompted several studies on LIF between 1994 and 2017, but, unfortunately, the effects of LIF on blastocyst production and cell number were discordant (See Table 1-4) [58,126–129,131]. However, work in the pig found that IL6 increased blastocyst formation and ICM cell number

[247], and in the mouse, IL6, and not LIF, controls blastocyst STAT3 activity in the ICM [248]. This led us to the hypothesis that IL6 is the predominant STAT3 controller in bovine embryos, and its supplementation would promote blastocyst formation and ICM development in bovine IVP embryos.

Under this hypothesis, our objectives were five-fold. First, I sought to determine if IL6 affected blastocyst formation or ICM cell number. Second, I investigated whether IL6 could activate STAT3 in bovine IVP embryos, especially within ICM cells. Third, I examined whether IL6 had any effect on the different populations of the ICM, the epiblast or hypoblast. Fourth, I investigated whether a recombinant bovine LIF could mimic IL6's effects. And fifth, I studied whether STAT3 is required for bovine ICM formation or maintenance.

Concerning zinc, zinc is an essential trace element that is absent in most embryo culture media which do not include serum. Zinc is a component of many proteins and transcription factors, including those important for cell proliferation and DNA repair [249–251], and zinc supplementation had previously been shown to benefit bovine in vitro maturation [252–254]. Therefore, I hypothesized that the addition of zinc to serum-free SOF would improve blastocyst production and cell number.

Table 1-1: Table of Insulin and Insulin-Like Growth Factors

Table 1						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
Insulin	Increased; 75 ng/ml	N/A	N/A	x	x	[37]
	Increased; 1.7 µM	Increased; 1.7 µM	N/A			[36]
	No effect; 10 µg/ml	Increased; 10 µg/ml	N/A	x		[35]
	No effect; 170 µM	No effect; 170 µM	N/A	x		[33]
	No effect; 1.8 pM, 1.8 nM	No effect; 1.8 pM, 1.8 nM	N/A	x		[32]
	No effect; 5 µg/ml	No effect; 5 µg/ml	N/A	x		[34]
IGF1	Increased; 50 ng/ml	N/A	N/A		x	[255]
	Increased; 100 ng/ml	No effect; 100 ng/ml	N/A			[47]
	Increased; 100 ng/ml	Increased; 100 ng/ml	N/A	x		[42]
	Increased; 13 nM	Increased; 13 nM	N/A	?		[32]
	Increased; 50 ng/ml	Increased; 50 ng/ml	N/A	x		[256]
	Increased; 50 ng/ml	Increased; 50 ng/ml	N/A		x	[43]
	Increased; 100 ng/ml	N/A	N/A			[55]
	Increased; 10, 100, 200 ng/ml	N/A	N/A			[257]
	Increased; 50, 100 ng/ml	Increased; 50, 100 ng/ml	N/A			[45]
	Increased; 50 ng/ml	Increased; 50 ng/ml	N/A	x		[46]
	Increased; 100 ng/ml	N/A	N/A	?		[72]
	Increased; 50 ng/ml	N/A	N/A	x		[62]
	No effect; 50 ng/ml	N/A	N/A	x	x	[258]
	No effect; 20 ng/ml	N/A	N/A	x		[147]
	No effect; 10 ng/ml	N/A	N/A	x		[61]
	No effect; 100 ng/ml	N/A	N/A	x		[84]
	No effect; 20 ng/ml	Increased; 20 ng/ml	N/A	x		[35]
	No effect; 100 ng/ml	No effect; 100 ng/ml	N/A	x	x	[48]
	No effect; 100 ng/ml	Increased; 100 ng/ml	N/A	x		[49]
	No effect; 100 ng/ml	No effect; 100 ng/ml	N/A	?		[54]
	No effect; 100 ng/ml	N/A	N/A	x		[137]
	No effect; 100 ng/ml	No effect; 100 ng/ml	N/A	x		[50]
	No effect; 10 ng/ml	N/A	N/A	x		[166]
	No effect; 100 ng/ml	N/A	N/A			[58]
	N/A	N/A	Increased; 100 ng/ml			[60]
IGF2	Increased; 0.013 nM	Increased; 0.13 nM, 13 nM	N/A	?		[32]
	No effect; 50 ng/ml	N/A	N/A	x		[62]
	No effect; 10 ng/ml	N/A	N/A	x		[61]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured.

Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An “x” in a cell indicates the study meets this criterion. A “?” indicates we could not determine this information from the manuscript. The “Low n” column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the “Cell Number” or “Pregnancy/Calving” columns. The “Undefined Medium” column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-2. Table of WNT Family and Inhibitors

Table 2						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
WNT7A	Increased; 66 ng/ml	No effect; 66 ng/ml	N/A			[71]
	Increased; 66 ng/ml	N/A	N/A	?		[72]
WNT11	Increased; 2.5 µg/ml	No effect; 2.5 µg/ml	N/A	x		[66]
DKK1	No effect; 50, 100, 200, 400 ng/ml	N/A	N/A			[65]
	No effect; 100 ng/ml	Decreased; 100 ng/ml	Day 32 Increased; Calving no effect			[73]
	No effect; 100 ng/ml	No effect; 100 ng/ml	N/A			[71]
	No effect; 100 ng/ml	N/A	N/A	?		[74]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-3. Table of TGF-ß Superfamily & Antagonists

Table 3						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
TGFB1	Increased; 5 ng/ml	No effect; 5, 10 ng/ml	N/A	x		[85]
	Increased; 50 ng/ml	N/A	N/A	x		[62]
	No effect; 10 ng/ml	N/A	N/A	x		[61]
	No effect; 2 ng/ml	No effect; 2 ng/ml	N/A	x		[83]
	No effect; 1 ng/ml	N/A	N/A	x		[84]
	No effect; 50, 100 ng/ml	N/A	N/A	x		[80]
Activin A	Increased; 1, 10, 100 ng/ml	No effect; 10 ng/ml	N/A	x		[92]
	Increased; 10 ng/ml	N/A	N/A	x	x	[89]
	Increased; 10, 100 ng/ml	No effect; 10, 100 ng/ml	N/A	x		[90]
	Increased; 10 ng/ml	Decreased; 10 ng/ml	N/A	x		[91]
	Increased; 1 nM	No effect; 0,01, 0.1, 1 nM	N/A			[88]
	Increased; 1 nM	N/A	N/A	?		[72]
BMP2	No effect; 1, 10, 100 ng/ml	N/A	N/A	x	x	[93]
BMP4	Decreased; 100 ng/ml	No effect; 100 ng/ml	N/A	x		[96]
BMP5	Increased; 100 ng/ml	N/A	N/A	x	x	[97]
Follistatin	Increased; 1, 10 ng/ml	Increased; 10 ng/ml	N/A	x		[89]
	Increased; 10 ng/ml	Increased; 10 ng/ml	N/A		x	[102]
Noggin	Decreased; 100 ng/ml	No effect; 100 ng/ml	N/A	x		[96]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-4. Table of Cytokines

Table 4						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
IL1B	Mixed; Various doses	N/A	N/A	x		[113]
CSF2	Increased; 1, 10 ng/ml	N/A	N/A	x		[115]
	Increased; 10 ng/ml	No effect; 10 ng/ml	Increased; 10 ng/ml			[116]
	Increased; 50 ng/ml	N/A	N/A	x		[62]
	No effect; 2, 5, 10, 50 ng/ml	No effect; 2, 5, 10, 50 ng/ml	N/A	x		[118]
	No effect; 10 ng/ml	N/A	N/A			[117]
	No effect; 10 ng/ml	N/A	No effect; 10 ng/ml	?		[119]
	N/A	N/A	Increased day 32; No effect on calving			[73]
LIF	Increased; 100 ng/ml	Increased; 100 ng/ml	N/A	x		[131]
	Increased; 100 ng/ml	Decreased; 100 ng/ml	N/A			[58]
	Increased; 50 ng/ml	N/A	N/A	x		[62]
	No effect; 0.5, 1, 2, 4, 5, 6k IU	N/A	N/A	x	x	[126]
	No effect; 1000 IU	Decreased; 1000 IU	N/A	x	x	[129]
	Decreased; 100 ng/ml	Increased; 100 ng/ml	N/A	x		[127]
	Decreased; 100 ng/ml	Decreased; 100 ng/ml	N/A	x		[128]
TNF	Decreased; 25 ng/ml	N/A	N/A	x	x	[134]
SCF	No effect; 50 ng/ml	N/A	N/A	x		[137]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-5. Table of Growth Factors

Table 5						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
FGF2	Increased; 50 pg/ml	N/A	N/A	x		[147]
	Increased; 1 µg/ml	No effect; 5, 100, 500 ng/ml or 1 µg/ml	N/A	x		[146]
	No effect; 10 ng/ml	N/A	N/A	x		[61]
	No effect; 0.05, 1, 10 ng/ml	Increased; 1 ng/ml	N/A	x		[84]
	No effect; 10 ng/ml	No effect; 10 ng/ml	N/A	x		[46]
	No effect; 50 ng/ml	N/A	N/A	x		[62]
	N/A	No effect; 50 ng/ml	N/A			[145]
FGF1	N/A	No effect; 50 ng/ml	N/A			[145]
FGF10	N/A	No effect; 50, 500 ng/ml	N/A			[145]
CTGF	No effect; 0.01, 0.1, 1 nM	Increased; 0.1, 1 nM	N/A			[88]
HDGF	Increased; 100 ng/ml	N/A	No effect; 100 ng/ml	x		[157]
	Mixed; various doses	Increased; 100 ng/ml	N/A	x		[155]
HGF	Decreased; 0.01 nM	No effect; 0.01, 0.1, 1 nM	N/A			[88]
EGF	Increased; 10, 100 ng/ml	No effect; 10 ng/ml	N/A	x	x	[85]
	Increased; 5 ng/ml	No effect; 5 ng/ml	N/A	x	x	[43]
	Increased; 5 ng/ml	No effect; 5 ng/ml	N/A		x	[256]
	Increased; 10 ng/ml	N/A	N/A	x		[164]
	Increased; 100, 200 ng/ml	Increased; 100, 200 ng/ml	N/A			[45]
	Increased; 10 ng/ml	No effect; 10 ng/ml	N/A			[46]
	No effect; 10 ng/ml	N/A	N/A	x		[61]
	No effect; 10 ng/ml	No effect; 10 ng/ml	N/A	x		[83]
	No effect; 10 ng/ml	Increased; 10 ng/ml	N/A	x		[84]
	No effect; 10 ng/ml	N/A	N/A	x		[165]
	No effect; 10 ng/ml	No effect; 10 ng/ml	N/A			[166]
	No effect; 10 ng/ml	No effect; 10 ng/ml	N/A			[167]
TDGF1	No effect; 0.01, 0.1, 1 nM	Increased; 0.01 nM	N/A			[88]
TGFA	Increased; 1 ng/ml	N/A	N/A	x		[147]
	No effect; 10 ng/ml	N/A	N/A	x		[61]
	Decreased; 10 ng/ml	N/A	N/A	x		[166]
PDGF	No effect; 10 ng/ml	N/A	N/A	x		[61]
	No effect; 1, 10, 100 ng/ml	N/A	N/A	x		[180]
	Decreased; 1 ng/ml	N/A	N/A	x		[147]
NGF	No effect; 10 ng/ml	N/A	N/A	x		[61]
VEGF	No effect; 5 ng/ml	N/A	N/A	x	x	[189]
GH	Increased; 100 ng/ml	Increased; 100 ng/ml	N/A	x		[42]
	Increased; 100 ng/ml	N/A	N/A		?	[191]
	Increased; 100 ng/ml	Increased; 100 ng/ml	No effect; 100 ng/ml	x	x	[194]
	N/A	Increased; 100 ng/ml	N/A			[193]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured.

Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An “x” in a cell indicates the study meets this criterion. A “?” indicates we could not determine this information from the manuscript. The “Low n” column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the “Cell Number” or “Pregnancy/Calving” columns. The “Undefined Medium” column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-6. Table of Steroids

Table 6						
Steroid	**Outcomes**			**Notes**		
	Blastocyst Formation	**Cell Number**	**Pregnancy/ Calving**	**Low n**	**Undefined Medium**	**Ref**
Cortisol	No effect; 40 ng/ml	No effect; 40 ng/ml	N/A		x	[199]
E2	No effect; 120 pg/ml	No effect; 120 pg/ml	N/A		x	[199]
P4	No effect; 1, 100 ng/ml	Decreased; 1, 100 ng/ml	N/A	x		[205]
	No effect; 55 ng/ml	No effect; 55 ng/ml	N/A		x	[199]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

. An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-7. Table of Other Molecules

Table 7						
	Outcomes			Notes		
	Blastocyst Formation	Cell Number	Pregnancy/ Calving	Low n	Undefined Medium	Ref
Melatonin	Increased; 1 µM	N/A	N/A	x		[210]
	Increased; 1 µM, 10 nM	Increased; 1 µM	N/A	x	x	[209]
	Increased; 1 µM	Increased; 1 µM	N/A	?	x	[211]
	Increased; 1 µM	No effect; 1 µM	N/A	x		[212]
PGF2α	Decreased; 0.1, 1, 10, 100 ng/ml	N/A	N/A	x		[216]
LPA	No effect; 1 mM	N/A	N/A		x	[218]
Retinoids & RXR Agonists	LG Increased; 0.1 µM	LG Decreased; 10 µM	N/A		x	[227]
	ATRA Increased; 0.7 µM	ATRA Increased; 0.7 µM	N/A			[225]
	ATRA No effect; 0.7 µM	ATRA Increased; 0.7 µM	N/A		x	[226]
Thyroid Hormones	Increased;50 ng/ml	N/A	N/A	x	x	[233]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

Table 1-8. Table of Cocktails

Table 8				
Molecules	**Outcomes**			**Ref**
	Blastocyst Formation	**Cell Number**	**Pregnancy/ Calving**	
EGF, TGFB1	Increased	No effect	N/A	[85]
	No effect	No effect	N/A	[83]
EGF, FGF2	Increased	Increased	N/A	[84]
EGF, IGF1	Increased	N/A	N/A	[43]
	Increased	Increased	No effect	[45]
	No effect	No effect	N/A	[165]
IGF1, IGF2, FGF2, LIF, CSF2, TGFB1, PDGF, Hyaluranon	Increased	No effect	N/A	[235]
IGF1, IGF2, FGF2, TGFB1, CSF2, LIF	Increased	Increased	N/A	[62]
LIF, IGF1	Increased	No effect	N/A	[58]
EGF, FGF2, IGF1	Increased	Increased	N/A	[46]
	Increased	N/A	No effect	[234]
EGF, HA	No effect	No effect	N/A	[167]
Cortisol, E2, P4	No effect	No effect	N/A	[199]

White cells with black text indicate this outcome was examined, but the embryokine was found to have no effect. White cells with grey text and "N/A" indicate this outcome was not measured. Green cells indicate a positive effect was detected. A blue cell indicates that a positive effect was detected but that this effect was inconsistent in the same manuscript. Red cells indicate a negative effect was detected. Pink cells indicate a negative effect was detected, but inconsistently in the same manuscript. Yellow cells indicate that both positive and negative results were reported. In each cell is the dose, or doses, of the embryokine which produced this effect.

An "x" in a cell indicates the study meets this criterion. A "?" indicates we could not determine this information from the manuscript. The "Low n" column highlights studies which had <250 zygotes/embryos per treatment for blastocyst formation analyses only. This column does not apply to the "Cell Number" or "Pregnancy/Calving" columns. The "Undefined Medium" column indicates that the culture media either contained serum and/or co-culture with other cells.

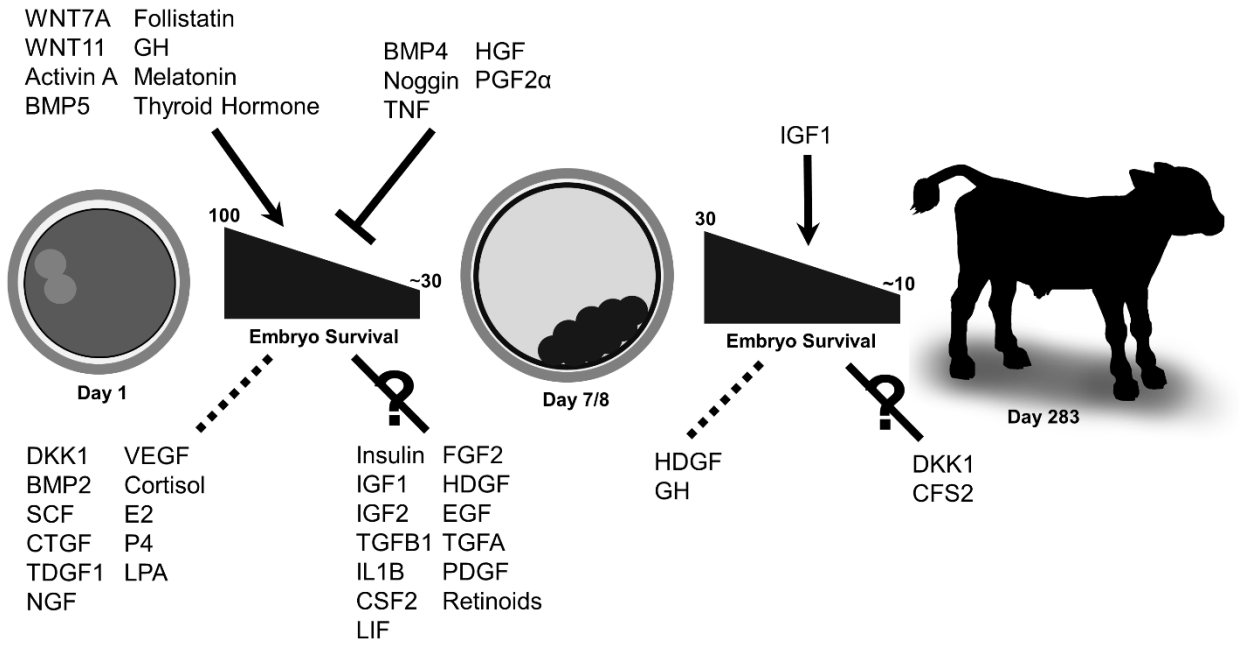

Figure 1-1. Summary of the receptor-binding bioactive factors which have been supplemented to in vitro produced bovine embryos. The arrow indicates those factors improve emrbyo survival to the indicated day of development. The barred line indicates those factors decrease embryo survival to the indicated day of development. The dashed line indicates those factors have no effect on embryo survival to the indicated day of development. The line with a "?" indicates less then 75% of studies report the same effect for those factors.

Chapter 2: Interleukin-6 increases inner cell mass numbers in bovine embryos

Introduction

The first lineage specification event during mammalian embryogenesis is the differentiation of the trophectoderm (TE) and the inner cell mass (ICM). The TE will from the outermost layer of the fetal portion of the placenta and the ICM develops into hypoblast and epiblast lineages. The hypoblast will contribute to the extraembryonic endoderm, which forms the yolk sac, while the epiblast gives rise to the three embryonic germ layers and additional extraembryonic lineages. Proper ICM specification and development is crucial to embryo survival. Loss of either ICM lineage is embryonic lethal in mice [259,260].

In humans, a prominent ICM, as assessed microscopically, is associated with reduced early embryonic loss and increased implantation and live birth rates [261–263]. A similar scenario also exists in cattle, where the current consensus is that bovine embryo culture conditions fail to adequately promote proper ICM development, and this contributes to at least some of the pregnancy losses that occur after transfer of *in vitro*-produced (IVP) bovine embryos [244,264]. Bovine IVP blastocysts have fewer ICM cells, elevated apoptosis in the ICM, and produce smaller embryonic disks than their *in vivo*-produced counterparts [237,265]. In some cases, embryonic disks could not be detected in IVP conceptuses [244–246,266]. Also, fewer pregnancies are maintained by IVP conceptuses that lack visible embryonic disks when compared with IVP conceptuses containing prominent embryonic disks [244]. Unfortunately, the embryonic and uterine-derived factors controlling ICM specification and development remain largely unknown in cattle, humans and other mammals.

Two members of the interleukin-6 (IL6) family of cytokines, IL6 and leukemia inhibitory factor (LIF), have been identified as important mediators of embryonic cell development and maintenance. Outside of embryonic development, these two cytokines are best known for their roles in inflammation, cancer, metabolism, and placental development and implantation [238,267]. Both IL6 and LIF are also known for their abilities to maintain murine embryonic stem cells, which are derived from the ICM of murine blastocysts, by initiating the signal transducer and activator of transcription 3 (STAT3) signaling cascade [122–124]. Both IL6 and LIF can activate STAT3 signaling in various cell types, however, each ligand utilizes a ligand-specific receptor subunit (IL6R or LIFR) and a common subunit that contains the signal transducing regions (IL6ST, GP130). Specifically, LIF requires a heterodimer of IL6ST and LIFR while IL6 uses a heterotrimer composed of two IL6ST subunits and one IL6R subunit [238,268].

Bovine IVP embryo responses to LIF supplementation are varied, and the effects of IL6 on IVP bovine embryo production have not been explored [58,127–129]. An embryotrophic role for IL6 is suggested in other species. In the pig, IL6 supplementation during culture increased parthenogenetic blastocyst formation and ICM cell numbers [247]. In the mouse, IL6, and not LIF, appears to be responsible for blastocyst-stage nuclear STAT3 activity in the ICM [248]. Six studies were completed to test the hypothesis that IL6 supplementation promotes blastocyst formation and ICM development in bovine preimplantation embryos.

Materials and Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials. Unless specified otherwise, reagents were purchased from ThermoFisher Chemical Company (Waltham, MA).

In vitro embryo production

Bovine embryos were produced by *in vitro* maturation, fertilization and culture procedures described previously with some modifications [46,269]. Cumulus-oocyte complexes (COCs) were harvested from ovaries purchased from Brown Packing Company (Gaffney, SC, USA) or COCs were purchased from DeSoto Biosciences (Seymour, TN) and incubated overnight for 21 to 24 h at 38.5°C in 5% CO_2 in groups of 20-35 in 500 µl TCM-199 containing Earle's salts and supplemented with 10% [v/v] fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia, USA), 25 µg/ml bovine follicle stimulating hormone (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 2 µg/ml estradiol (Sigma-Aldrich; St. Louis, MO), 22 µg/ml sodium pyruvate, 1mM L-alanyl-L-glutamine (Glutamax) and 25 µg/ml gentamicin sulfate. No differences in embryo responses to treatments were observed between COCs harvested in these two manners. For fertilization, COCs were washed in HEPES-SOF and placed in groups of 150-200 in 3 ml SOF-FERT covered by paraffin oil (Ovoil; Vitrolife, Göteborg, Sweden) [46,73,270]. Frozen semen from four Holstein bulls (donation from Select Sires, Plain City, OH, USA) was thawed, and spermatozoa were isolated through a biphasic (40% and 80%, [v/v]) Bovipure™ density gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA) before addition to the fertilization media at a concentration of 1 million sperm/ml fertilization media. Day of fertilization was designated as day 0. After incubation for 14 to 18 hours at 38.5°C in 5% CO_2 in humidified air, presumptive zygote-cumulus complexes were denuded, washed in HEPES-SOF and, unless otherwise stated, placed in groups of 20-30 in droplets of 50 µl of SOF-BE1 covered by paraffin oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 in humidified air [146].

In one study (Study B), embryos visually appraised as healthy (little to no evidence of blastomere degeneration) were harvested at day 3 post-fertilization and transferred to medium containing treatments. For all other studies involving treatments after day 1 post-fertilization, a new treatment method was developed to reduce the amount of handling each embryo experienced and ultimately reduce culture stresses and improve overall development. For this new method, treatments were administered directly to existing drops of embryos via the addition of 2 µl of treatment-concentrated SOF-BE1. This method did not affect treatment outcomes. In studies that began at day 1, presumptive zygotes were transferred directly to medium containing treatment.

IL6 supplementation studies

For all studies, a concentrated IL6 stock (10 µg/ml; recombinant bovine, Kingfisher Biotech, St. Paul, MN, USA) was prepared in SOF containing 1% [w/v] bovine serum albumin (BSA; Sigma-Aldrich) and stored in aliquots at -80°C. Control treatments consisted of carrier only (1% BSA). Stocks were only thawed once and were used immediately after thawing. Cleavage was assessed at day 3 post-fertilization. Blastocyst formation was recorded at day 7 and 8 post-fertilization.

In the first study (Study A), treatments of 0, 1, 10 or 100 ng/ml IL6 were administered at day 5 post-fertilization (n=20-28 embryos/50 µl drop; 3-4 drops/treatment; 4 replicates). Representative day 8 blastocysts were collected from each treatment group (0, 1, 10 and 100 ng/ml IL6) and processed for cell counting (n=34-39 blastocysts/treatment over 4 replicates).

In the second study (Study B), day 3 embryos (n=80 embryos/treatment; 10 embryos/drop; 6 replicates) were selected and moved to new drops containing either the control

treatment or 100 ng/ml IL6. At day 5, half of the drops from each treatment group received 100 ng/ml IL6 via a treatment-concentrated injection to the drops, while the other half received carrier. This created four total treatments: no IL6 (carrier-only), 100 ng/ml IL6 beginning at day 3, 100 ng/ml IL6 beginning at day 5 and 200 ng/ml IL6 administered in 100 ng/ml doses at days 3 and 5. Representative day 8 blastocysts were collected from each treatment group (n=11-16 blastocysts/treatment over 3 replicates) and processed for cell counting.

In the third study (Study C), day 1 embryos were placed in medium containing 0, 1, 10 or 100 ng/ml IL6 (23-26 embryos/drop; 2-4 drops/treatment; 4 replicates). At day 8, representative blastocysts were collected from each treatment group (n=27-41 blastocysts/treatment over 4 replicates) and processed for cell counting.

In the fourth study (Study D), 100 ng/ml IL6 was administered at day 1, day 5, or both day 1 and 5 (total 200 ng/ml IL6), or 200 ng/ml IL6 was administered at day 1. Controls received carrier only at each time point (19-27 embryos/drop; 2-4 drops/treatment/replicate; 6 replicates). At day 8 post-fertilization, representative blastocysts from each treatment group were collected (n= 27-28 blastocysts/treatment over 5 replicates) and processed for cell counting.

In the fifth study (Study E), 0, 100 or 200 ng/ml IL6 was administered at day 5 post-fertilization, or 100 ng/ml was administered on both days 1 and 5 (for a total of 200 ng/ml IL6). Controls received carrier only at both time points (19-30 embryos/drop; 2-6 drops/treatment/replicate; 4 replicates). At day 8 post-fertilization, representative blastocysts from each treatment group were collected (n= 38-44 blastocysts/treatment over 4 replicates) and processed for cell counting.

In the sixth study (Study F), embryos were cultured individually (1 embryo/5 µl drop; 25 embryos/treatment; 4 replicates) in medium containing 0, 100 or 200 ng/ml IL6 beginning at day

1. At day 4, each individual culture drop received an additional 1 µl of SOF-BE1 containing either carrier only (for 0 and 200 ng/ml groups) or concentrated IL6 to deliver an additional 100 ng/ml IL6 (for a total of 200 ng/ml IL6 over day 1 and 4). A group-culture control treatment (25 embryos/50 µl drop; 1-2 drops/replicate) lacking IL6 supplementation was also included. Development was assessed at day 4, 7 and 8. No cell counting was completed.

Transcript profiling

Random samplings of zygotes, 2-cell embryos, 8-16 cell embryos, morulae and blastocysts from the control group (0 ng/ml IL6) were collected at day 1, 2, 4, 6 and 8 post-fertilization, respectively. At each stage, 10 embryos were pooled before RNA extraction. Between 3 to 5 pools of embryos were collected at each stage of development. After washing in Dulbecco's phosphate-buffered saline (DPBS) containing 0.2% [w/v] polyvinylpyrrolidone (PBS-PVP), embryos were collected into <10 µl PBS-PVP in microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted by using the PicoPure RNA Isolation Kit (Applied Biosystems, Inc., Foster City, CA, USA). The entire RNA sample was incubated with RNase-free DNAse I (20 µl reaction volume) for 30 minutes at 37°C followed by 10 minutes at 75°C. The RNA (15 µl) was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.) in a total reaction volume of 30 µl. Negative control samples did not receive reverse transcriptase. For PCR, SybrGreen PCR Master Mix (Applied Biosystems, Inc.) was mixed with RT product and 500 nM concentration of forward and reverse primers for a total reaction volume of 10 µl. After activation/denaturation (95 °C for 10 minutes), a two-step amplification sequence was set for 50 cycles (95°C for 15 s,

57°C for 1 min) on an Eppendorf RealPlex 4 MasterCycler. Each sample and primer combination were run in triplicate.

Each primer pair (Table 2-1) was identified using the Primer-BLAST Program from the National Center for Biotechnology Information (U.S. National Library of Medicine, Bethesda, MD) and synthesized by Integrated DNA Technologies (IDT; San Diego, CA). Primer efficiency standard curve analysis was completed to verify adequate primer efficiency (76-103% efficiency). Dissociation curve analysis (57 to 95°C) was completed after each PCR amplification to confirm the presence of one amplicon. Succinate dehydrogenase flavoprotein subunit (SDHA) was used as a housekeeping gene based on previously verified stability across early embryonic stages [271]. The abundance of *SDHA* was not influenced by embryo stage in this work. The relative abundance of each target transcript was expressed as fold change from the embryo stage containing the lowest abundance for the specified transcript by using the $2^{[-ddCt]}$ approach.

Immunofluorescence and cell counting

At day 8 post-fertilization, ICM and TE cell numbers were determined in a subset of blastocysts [272]. In some replicates which yielded low numbers of blastocysts, all blastocysts were examined. In other replicates with large blastocyst numbers, up to 15 blastocysts per treatment were sampled. The selected blastocysts were representative of the types (non-expanded, expanded or hatched) of blastocysts present in each treatment group. On rare occasions (~3% of total blastocysts sampled), abnormal blastocysts were identified after staining (<60 total cells). These were excluded despite appearing of good quality upon visual appraisal. The cutoff of 60 cells was chosen because bovine blastocysts should begin blastulation after the

64-cell stage [240]. The incidence of these blastocysts was not restricted to or affected by any one treatment. In studies A, B, C, D and E, a total of 3, 5, 9, 3 and 2 blastocysts were excluded for this reason, respectively. By treatment, a total of 5, 3, 5, 5 and 4 blastocysts were excluded for the 0 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml and 200 ng/ml treatment groups, respectively. These numbers are combined from all studies which included each specific treatment, regardless of the time when the treatment began. The numbers of blastocysts examined in Table 2-2 do not include these abnormal blastocysts.

Embryos were fixed in 4% [w/v] paraformaldehyde for 15 minutes at room temperature, permeabilized using 0.25% [v/v] Triton-X for 20 minutes and blocked with 10% [v/v] Horse Serum for 1 hour at room temperature. Embryos were then incubated overnight at 4°C with anti-CDX2 primary antibody (Biogenex, AM392-5M, sold ready-to-use), washed, and incubated for 1 hour at room temperature with either donkey anti-mouse FITC or Alexa Fluor 647 (Invitrogen, A16018 or A31571, 1:200 dilution for either). Embryo DNA was then stained with DAPI (1µg/ml) for 5 minutes at room temperature. Embryos were then placed in 10% [v/v] ProLong™ Gold Antifade diluted with PBS-PVP and imaged by flattening on a glass slide lined with a thin layer of petroleum jelly. Immunoreactive complexes and DNA staining were visualized by using an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epifluorescence illumination system. Images were captured with a DS-L3 digital camera and assembled with NIS-Elements Software (Nikon Instruments, Melville, NY). The program, FIJI (ImageJ) was used to label and record individual nuclei by utilizing the cell counter plugin to count nuclei staining for CDX2 ($CDX2^+$, indicating TE) and only DAPI ($CDX2^-/DAPI^+$, indicating ICM) [273].

Statistical analyses

All analyses except for the individual culture study were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). Relative mRNA abundance data were log-transformed before analysis. For each embryo development study, the IVP replicate was used as the experimental unit. Replicate was considered a random independent variable for all cleavage and blastocyst formation analyses. Percentage data (*e.g.* blastocyst formation rates) were arcsine-transformed before analysis but are presented as non-transformed means and SEM. The Tukey honestly significant difference test was used for all cleavage and blastocyst formation data. Blastomere numbers and differential staining analysis used individual embryos as the experimental unit. Individual comparisons of blastomere numbers were partitioned further by using the Probability of Difference (PDIFF) test of SAS. Chi-square analysis was used to analyze the individual embryo culture study. Statistical significance was determined at $P \leq 0.05$.

Results

Transcript profiling

Transcripts for *IL6* and *IL6R* were detected in each RNA preparation examined at the zygote, 2-cell, 8-16 cell, compact morula and blastocyst stages. Transcripts for *IL6ST* were detected in each of the zygote and blastocyst samples, in 4 of 5 8-16 cell pools, and in 3 of 5 compact morula stage pools. Neither *IL6* nor *IL6ST* transcript abundance were altered across each of the stages (Figure 2-1). However, the abundance of *IL6R* transcripts was greater ($P < 0.05$) at the 8-cell stage than at the 2-cell, morula or blastocyst stages (Figure 2-1).

Study A: IL6 Treatment at Day 5 Post-Fertilization

In this first study, recombinant bovine IL6 was supplemented from day 5 to 8 post-fertilization. The day 5 time point was chosen because it corresponds with the initiation of blastomere compaction and ICM and TE specification in bovine IVP embryos [274,275]. A dose-response study was completed using IL6 concentrations based on work completed in porcine embryos [247]. None of these concentrations affected the percentage of embryos which formed blastocysts at day 7 or 8 (Table 2-2). Exposure to 1 or 10 ng/ml IL6 did not affect ICM, TE, or total cell numbers or the ICM:TE ratio. However, 100 ng/ml IL6 increased both ICM and total cell numbers ($P<0.05$) (Table 2-3). The number of TE cells remained unchanged, but the ICM:TE ratio was increased ($P<0.05$) in this treatment group. Figure 2-2 provides images of representative blastocysts from the 0 and 100 ng/ml IL6 treatment groups.

Study B: IL6 Treatment at Day 3 or 5 Post-Fertilization

This follow-up study examined whether the 100 ng/ml IL6 concentration could influence blastocyst formation and/or ICM and TE cell numbers when provided at day 3, as the embryonic genome is being activated (8 to 16-cell stages in cattle), and to determine whether this response at day 3 is comparable to providing IL6 at day 5 [276]. Also, IL6 administration at both day 3 and 5 (100 ng/ml from day 3 to 5, 200 ng/ml from day 5 to 8) was tested to determine whether this supplementation scheme further improved any outcomes.

Supplementation with 100 ng/ml IL6 either at day 3 or 5 did not affect blastocyst formation at day 7 or 8, but the combined, day 3 and 5 IL6 treatment tended to increase ($P=0.06$) blastocyst development at day 8 but not day 7 (Table 2-2). Supplementing IL6 in a single dose at either day 3 or 5 increased ICM cell numbers ($P<0.05$; Table 2-3). The combined day 3 and 5

IL6 treatment did not further improve ICM cell numbers. TE cell numbers were not affected by any IL6 treatment. There was a tendency (P=0.09) for IL6 treatment at day 3 to improve total blastomere numbers, but total numbers were not altered in the other treatment groups. Supplementation with IL6 at day 3 or day 5 increased (P<005) the ICM:TE ratio. This ratio was increased further (P<0.05) by supplementing IL6 at both day 3 and 5.

Study C: IL6 Treatment at Day 1 Post-Fertilization

This study began IL6 supplementation at day 1 post-fertilization (*i.e.* beginning of embryo culture) to test the limits in the duration of IL6 supplementation required to produce ICM responses at day 8. This study design also permitted assessment of how IL6 supplementation affected cleavage rates. A dose-response study was completed to verify previous findings that 100 ng/ml IL6 was required to achieve greater ICM cell numbers.

Cleavage rates tended to be greater (P=0.09) for embryos treated with 1 ng/ml IL6 when compared with controls, but cleavage rates were unaffected by exposure to 10 or 100 ng/ml IL6 (Table 2-2). Blastocyst formation was unaffected by treatment with 1, 10 or 100 ng/ml IL6. The 100 ng/ml IL6 treatment increased (P<0.05) ICM and total cell numbers in day 8 blastocysts (Table 2-3). Neither 1 nor 10 ng/ml IL6 affected ICM or total cell numbers. No changes in TE cell numbers were observed with any of the IL6 treatments. Oddly, the ICM:TE ratio differed between the 1 and 10 ng/ml IL6 treatments, but both treatments as well as the controls remained lower than the 100 ng/ml IL6 treatment group (P<0.05).

Studies D and E: The Efficacy of Combined IL6 treatments at Day 1 and 5 Post-Fertilization

A subsequent study (Study D) was designed to evaluate whether ICM responses to IL6 could be enhanced further by increasing the dose of IL6 at day 1 (200 ng/ml) or by sequential IL6 supplementation at both days 1 and 5. Media was not exchanged in this treatment group, so these embryos were exposed to 100 ng/ml beginning at day 1 and 200 ng/ml from day 5 to 8. Cleavage and blastocyst rates were not affected by the various IL6 supplementation schemes (Table 2-2). When compared with the control, ICM and total cell numbers were increased ($P<0.05$) by supplementing 100 ng/ml IL6 at day 1 or 5 or 200 ng/ml IL6 at day 1 (Table 2-3). When IL6 was administered on both days 1 and 5, ICM cell numbers but not total cell numbers were greater ($P<0.05$) than when embryos received IL6 only on day 1 or 5. The number of TE cells were not affected by any treatment. The ICM:TE ratios followed the same response as the ICM cell numbers, where adding 100 ng/ml IL6 at either day 1 or 5 increased ($P<0.05$) the ICM:TE ratio when compared with the control. Providing 100 ng/ml IL6 at both day 1 and 5 further increased ($P<0.05$) the ICM:TE ratio. Supplementation with 200 ng/ml IL6 at day 1 provided similar results to when 100 ng/ml IL6 was provided only once during embryo culture. The 200 ng/ml IL6 treatment beginning at day 1 did not produce the same responses as the dual day 1 and 5 IL6 treatment strategy.

Study E was designed to confirm whether the sequential addition of IL6 at day 1 and 5 could be replicated, and to test whether exposure to 200 ng/ml IL6 at day 5 but not at earlier times could produce the same beneficial treatment effect as the dual IL6 treatment strategy. Neither cleavage nor blastocyst development were influenced by the IL6 supplementation schemes examined (Table 2-2). As before, supplementing 100 ng/ml IL6 at day 5 increased ($P<0.05$) ICM and total cell numbers when compared with the control (Table 2-3). However, no

further increases in ICM or total cell numbers were detected when the embryos were supplemented with 200 ng/ml IL6 at day 5 or 100 ng/ml IL6 on both days 1 and 5. Again, TE cell numbers were unaffected by IL6 exposure. The ICM:TE ratio was greater ($P<0.05$) in all IL6-treatments than the control but did not differ from one another.

Composite Analysis of IL6 Effects on ICM cell numbers and the ICM:TE ratio

A final examination of the effects of 100 ng/ml IL6 supplementation on ICM cell numbers and ICM:TE ratio were explored by graphing each data point from each experiment, regardless of the time when IL6 supplementation was initiated (Fig. 2-3). An increase in ICM cell numbers and ICM:TE ratio was detected when all data were combined ($P<0.0001$).

Study F: IL6 Supplementation During Individual Embryo Culture

A final study examined whether IL6 supplementation could overcome the developmental block that occurs when bovine embryos are cultured individually in relatively large drops of culture medium. The IL6 dosages chosen for this study were selected based on a pilot study (data not shown). Individually-cultured zygotes that lacked IL6 supplementation underwent cleavage, albeit at a reduced level ($P<0.05$) when compared with group-cultured control zygotes (Table 2-2). None of these individually-cultured control embryos reached the blastocyst stage. By contrast, cleavage rate was not different between group-cultured controls and individually-cultured zygotes supplemented with 100 ng/ml IL4 at both day 1 and 4 or with zygotes supplemented with 200 ng/ml IL6 at day 1. Blastocysts were detected at day 8 but not day 7 in IL6-supplemented, individually-cultured embryos, although the percentage of blastocysts were less than the group-cultured controls ($P<0.05$). The low number of blastocysts recovered from

this study did not permit us to examine how IL6 supplementation affected ICM, TE and total cell numbers and the ICM:TE ratio.

Discussion

Bovine IVP embryos typically are of lower competency after transfer than *in vivo*-produced embryos. This is attributed in part to *in vitro* culture conditions lacking critical embryokines and selective nutrients that the oviduct and uterus produce in early pregnancy. This makes IVP embryos a nice model for investigating ways to improve embryo development and competency in cattle and potentially other species. Work in the mouse and pig has indicated a potential role for IL6 in ICM maintenance [247,248]. Also, a recent report found that *IL6* transcripts were among the most prominently expressed embryokines in the bovine oviduct and endometrium at day 3 and 5 post-estrus [27,277,278]. This previous work provided the impetus for us to explore IL6 as an embryokine.

The bovine embryo also produces *IL6* transcripts in both the ICM and TE in blastocysts [94,239–242]. Our transcript profiling work confirmed the presence of *IL6* transcripts in bovine embryos between the 1-cell and blastocyst stages. We also confirmed the presence of transcripts for both IL6 receptor subunits (*IL6R* and *IL6ST*) throughout early embryo development. *IL6R* was expressed constitutively and was greater in abundance at the 8-cell stage than other stages (excluding the zygote stage). This suggests that *IL6R* transcription ensues as embryonic genome activation begins. No apparent changes in *IL6ST* transcript abundance were detected across the stages examined. However, *IL6ST* mRNA could not be detected in a few of the 2-cell and 8-16 cell embryo samples. We did not pursue if *IL6ST* was truly absent in these samples or if this outcome was caused by using too little RNA. The absence of transcripts also does not guarantee

the absence of the mature protein, especially when transcripts were detected at earlier stages of development.

Supplementation with IL6 had no definitive effects on cleavage rates and blastocyst formation when embryos were cultured in groups. This finding contradicts a report in pigs, where improvements in blastocyst development were observed [247]. However, IL6 supplementation was beneficial to embryo development when provided to individually-cultured embryos. A low-density culture environment was employed (1 embryo/ 5 µl medium). This culture scheme usually prevents normal embryo development, presumably because of the lack of conditioning factors that embryos produce in group culture. These positive effects on cleavage and blastocyst rates implicates IL6 as a potential embryokine for mediating embryo development in stressful environments but not when culture conditions are adequate for normal development.

The most notable outcome of this work was observing changes in the composition of blastocysts exposed to IL6 during *in vitro* embryo development. Improvements in ICM cell numbers were consistently observed after IL6 supplementation, and IL6 promoted ICM development regardless of when it was first administered. In most studies, blastomere numbers within the ICM were nearly doubled in embryos receiving 100 or 200 ng/ml IL6 but not lower IL6 concentrations. Sequential IL6 administration at days 1 and 5 further increased ICM cell numbers in one study (Study D) but failed to do so in another (Study E). This contrast in outcomes may be due solely to chance, although we cannot discount that some unknown factor, such as the genetics of the embryos (ovaries were of unknown origin in every study), may have produced these different responses to sequential IL6 treatment. Regardless, in both studies, this double-treatment scheme still increased ICM cell numbers when compared to the controls.

Individual embryo responsiveness to IL6 varied. However, overwhelmingly positive increases in ICM cell numbers were observed in every study (see Fig. 2-3). This positive effect of IL6 on ICM cell numbers also was detected in porcine embryos (1.7-fold increase versus controls), suggesting that this phenomenon is not restricted solely to cattle [247].

Another interesting finding from this work was the lack of IL6 effect on TE cell numbers. This explains why substantial increases in the ICM:TE ratio were seen. It also implicates the improvements in ICM cell numbers as the sole reason for the improvements in total embryo cell numbers. The mechanism(s) of action for IL6 are only beginning to be explored, but this work indicates that IL6 solely targets the ICM during early embryogenesis. This observation is consistent with other work that implicates IL6 as a pluripotency factor for mouse embryonic stem cells and for its role in controlling STAT3 activity in early stage mouse embryos [122,248]. STAT3 is a primary mediator of ICM lineage maintenance in mice [243,248].

It was also interesting that supplementation with IL6 promoted ICM development regardless of when it was first administered during embryo culture. It was surprising to observe a beneficial effect of IL6 supplementation at day 1. Sufficient amounts of biologically active IL6 may have survived from day 1 to later dates in culture when IL6 could influence embryonic gene expression. The functional lifespan of IL6 was not examined. Alternatively, IL6 provided at day 1 could function post-transcriptionally. More work is needed to clarify this activity.

To enable ICM and TE cell counting, we utilized an immunofluorescence protocol to mark the CDX2-positive TE cells. This is a TE specific marker in bovine blastocysts [7,272,279–282]. Binding specificity of the antibody used herein has been verified recently by the absence of staining in CDX2 knock-down bovine blastocysts [283]. We did not utilize an ICM-specific marker. An ICM-specific marker exists for bovine blastocysts, SOX2, however, we chose not to

utilize it as this staining is not exclusively nuclear and makes the individual nuclei obscure [284]. Instead, we assumed that CDX2-negative, DAPI-positive nuclei were ICM cells.

We realize the number of blastocysts utilized for cell counting in some treatments and studies appears low (*e.g*. n=11-16 in study B). In these studies, the effect of 100 or 200 ng/ml IL6 on ICM cell numbers and the ICM:TE ratio is so profound that we do not need to utilize many embryos to detect a difference. Moreover, one can see that this effect can be consistently produced in Fig. 2-3 (different symbols indicate different studies).

Conclusions

This work provides evidence that IL6 functions as an embryokine in bovine preimplantation embryos. The beneficial effects of IL6 include increasing ICM blastomere numbers and supporting embryonic development in individual embryo culture systems. The implications of enhancing ICM development in IVP embryos has yet to be explored, but all indications are that IL6 may improve IVP bovine embryo competency, since small ICMs in IVP bovine embryos likely contribute to many of the early pregnancy failures observed in cattle receiving these embryos [244–246,266].

Table 2-1. Primers used for quantitative RT-PCR.

Gene	Reference or Genbank accession no.	Primer sequence (5′ - 3′)	Product size (bp)
IL6	NM_173923.2	**Forward:** CCAGCCACAAACACTGACCT **Reverse:** TAGCTCTCAGGCTGAACTGC	121
IL6R	NM_001110785.3	**Forward:** AAGTGCACACCCGTCGTATT **Reverse:** TCAGATTCAAGGCTGCTGGG	114
IL6ST	XM_010816769.3	**Forward:** GTCTCATGCTCACGGCACTA **Reverse:** CGCGTCTGATTTGCCAACAA	220
SDHA	(Goossens *et al.* 2005	**Forward:** GCAGAACCTGATGCTTTGTG **Reverse:** CGTAGGAGAGCGTGTGCTT	185

Table 2-2. Cleavage and blastocyst formation across each study.

Study	Treatment	Treatment start	IVF replicates	Total embryos	Cleavage %±SEM *	Day 7 blastocyst %±SEM *	Day 8 blastocyst %±SEM *
A	0 ng/ml IL6	Day 5	4	317	N/A	12.8 ± 1.3 [a]	21.8 ± 3 [a]
	1 ng/ml IL6	Day 5		316		10.8 ± 1.4 [a]	21.8 ± 3.7 [a]
	10 ng/ml IL6	Day 5		323		16.8 ± 3.3 [a]	23.7 ± 4.6 [a]
	100 ng/ml IL6	Day 5		320		12.7 ± 4.2 [a]	23.7 ± 6.3 [a]
B	0 ng/ml IL6	Day 3	6	240	N/A	10.8 ± 4 [a]	24.2 ± 6.3 [a]
	100 ng/ml IL6	Day 3		240		14.6 ± 3.4 [a]	30.4 ± 6.8 [a]
	100 ng/ml IL6	Day 5		240		13.8 ± 3.3 [a]	25.8 ± 6.9 [a]
	100 + 100 ng/ml IL6	Day 3 + Day 5		240		12.5 ± 4.8 [a]	32.9 ± 6.4 [a†]
C	0 ng/ml IL6	Day 1	4	249	73.4 ± 3.7 [a]	12.3 ± 4.2 [a]	20.1 ± 3.5 [a]
	1 ng/ml IL6	Day 1		297	80.1 ± 5.9 [a†]	17.9 ± 2.5 [a]	27.1 ± 2.4 [a]
	10 ng/ml IL6	Day 1		301	76.4 ± 5.6 [a]	18.1 ± 3.7 [a]	27.7 ± 4.7 [a]
	100 ng/ml IL6	Day 1		248	76.4 ± 3.6 [a]	19.4 ± 4.1 [a]	29.6 ± 4.2 [a]
D	0 ng/ml IL6	Day 1	6	395	74.8 ± 4.5 [a]	11.5 ± 1 [a]	20.3 ± 2.1 [a]
	100 ng/ml IL6	Day 1		437	76.8 ± 2.2 [a]	10.8 ± 1.7 [a]	21.5 ± 2.5 [a]
	100 ng/ml IL6	Day 5		392			21.6 ± 2.4 [a]

	100 + 100 ng/ml IL6	Day 1 +		440	[a]	10.9 ± 1 [a]	19.4 ± 3.7 [a]
	200 ng/ml IL6	Day 5		418	77.8 ± 3.8 [a]	10.2 ± 1.8 [a]	18.9 ± 1.7 [a]
		Day 1			75.2 ± 5.2 [a]	9.8 ± 1.6 [a]	
					76.7 ± 3.7 [a]		
E	0 ng/ml IL6	Day 5	4	347	87.7 ± 0.02 [a]	15.8 ± 0.05 [a]	27.9 ± 0.04 [a]
	100 ng/ml IL6	Day 5		337	85.3 ± 0.01 [a]	17.4 ± 0.01 [a]	26.1 ± 0.02 [a]
	200 ng/ml IL6	Day 5		392	83.9 ± 0.03 [a]	20.5 ± 0.04 [a]	30.4 ± 0.03 [a]
	100 + 100 ng/ml IL6	Day 1 + 5		357	81.1 ± 0.04 [a]	18.9 ± 0.04 [a]	29.1 ± 0.02 [a]
F	Group Culture 0 ng/ml IL6	Day 1	4	175	138/175 [a]	17/138 [a]	29/138 [a]
	Individual Culture 0 ng/ml IL6	Day 1		100	65/100 [b]	0/65 [b]	0/65 [b]
	Individual Culture 100 + 100 ng/ml IL6	Day 1 + Day 4		100	79/100 [a]	0/79 [b]	7/79 [c]
	Individual Culture 200 ng/ml IL6	Day 1		100	73/100 [ab]	0/73 [b]	7/73 [c]

Different superscripts within each study denote differences. Significance established at P<0.05.

† indicates a trend (P =0.06 in study B and P=0.09 in Study D) when compared with the control).

* Data for studies A-E are presented as the Mean% ± SEM. Data from study F are presented as proportions.

Table 2-3. Embryonic ICM and TE cell counts in Day 8 blastocysts

Study	Treatment	Treatment start date	IVF replicates	Number of blastocysts	ICM	TE	Total	Ratio
A	0 ng/ml IL6	Day 5	4	39	53.9 ± 4.2 [a]	113.5 ± 5.1 [a]	167.4 ± 8.3 [a]	0.47 ± 0.03 [a]
	1 ng/ml IL6	Day 5		35	52.2 ± 3.3 [a]	108.6 ± 6.3 [a]	160.8 ± 8.0 [a]	0.51 ± 0.04 [a]
	10 ng/ml IL6	Day 5		34	60 ± 3.6 [a]	116.2 ± 6.5 [a]	176.2 ± 8.2 [a]	0.56 ± 0.05 [a]
	100 ng/ml IL6	Day 5		34	98 ± 6.5 [b]	113.2 ± 6.3 [a]	211.2 ± 10.3 [b]	0.92 ± 0.07 [b]
B	0 ng/ml IL6	Day 3	3	16	36.8 ± 4.3 [a]	95.6 ± 8.1 [a]	132.5 ± 11.7 [a]	0.38 ± 0.03 [a]
	100 ng/ml IL6	Day 3		11	61.8 ± 8.1 [b]	106.3 ± 10.6 [a]	168.1 ± 14.0 [a†]	0.65 ± 0.11 [b]
	100 ng/ml IL6	Day 5		11	58.9 ± 7.6 [b]	97 ± 16.5 [a]	155.9 ± 22.5 [a]	0.7 ± 0.09 [b]
	100 + 100 ng/ml IL6	Day 3 + Day 5		12	74.3 ± 8.7 [b]	87.3 ± 7.2 [a]	161.5 ± 13.4 [a]	0.87 ± 0.10 [c]
C	0 ng/ml IL6	Day 1	4	27	56.4 ± 4.1 [a]	111 ± 7.9 [a]	167.3 ± 9.8 [a]	0.56 ± 0.05 [ab]
	1 ng/ml IL6	Day 1		39	52.5 ± 3.0 [a]	111.7 ± 5.4 [a]	164.2 ± 7.3 [a]	0.49 ± 0.03 [a]
	10 ng/ml IL6	Day 1		39	59.3 ± 3.9 [a]	102.3 ± 4.7 [a]	161.6 ± 6.8 [a]	0.62 ± 0.05 [b]
	100 ng/ml IL6	Day 1		41	88.3 ± 4.6 [b]	112.7 ± 5.0 [a]	200.9 ± 7.6 [b]	0.82 ± 0.05 [c]
D	0 ng/ml IL6	Day 1	5	28	46.4 ± 3.6 [a]	99.6 ± 7.2 [a]	146 ± 8.6 [a]	0.52 ± 0.05 [a]
	100 ng/ml IL6	Day 1		27	79.2 ± 4.7 [b]	112.1 ± 7.0 [a]	191.3 ± 10.2 [b]	0.75 ± 0.05 [b]
	100 ng/ml IL6	Day 5		28	79.4 ± 6.0 [b]	108.1 ± 6.6 [a]	187.5 ± 10.6 [b]	0.77 ± 0.05 [b]
	100 + 100 ng/ml IL6	Day 1 + Day 5		28	100 ± 8.4 [c]	106.2 ± 4.8 [a]	206.2 ± 10.1 [b]	0.98 ± 0.09 [c]
	200 ng/ml IL6	Day 1		27	77.4 ± 6.9 [b]	104.5 ± 5.9 [a]	181.9 ± 11.3 [b]	0.75 ± 0.06 [b]
E	0 ng/ml IL6	Day 5	4	41	56.4 ± 4.1 [a]	112.3 ± 7.5 [a]	168 ± 10.3 [a]	0.53 ± 0.03 [a]
	100 ng/ml IL6	Day 5		38	88.7 ± 6.2 [b]	111.4 ± 6.9 [a]	200.1 ± 8.4 [b]	0.83 ± 0.05 [b]
	200 ng/ml IL6	Day 5		44	93.7 ± 5.2 [b]	116.6 ± 5 [a]	210.3 ± 8.4 [b]	0.83 ± 0.05 [b]
	100 + 100 ng/ml IL6	Day 1 + Day 5		44	89.8 ± 5.7 [b]	115 ± 6.5 [a]	204.8 ± 10.5 [b]	0.81 ± 0.05 [b]

Different superscripts in each study denote differences. Significance established at $P<0.05$.

† indicates a trend ($P=0.09$).

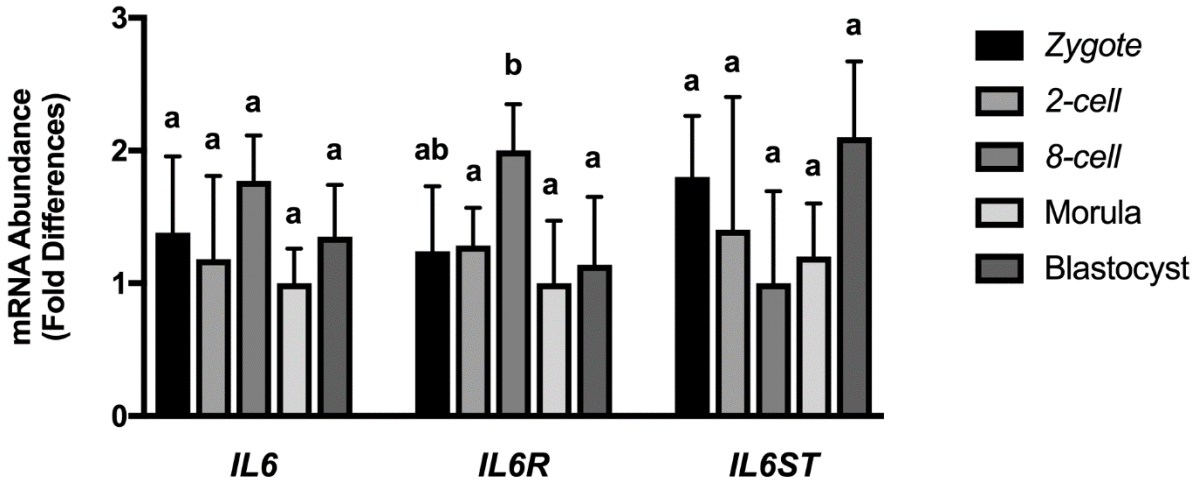

Figure 2-1. Transcript abundances for IL6, IL6R and IL6ST from zygotes, 2-cell embryos, 8-cell embryos, morulae and blastocysts. Total RNA was isolated from 3 to 5 pools of 10 embryos from each developmental stage before reverse transcription. The relative abundance of each target transcript is expressed as fold change from the embryo stage containing the lowest abundance for the specified transcript by using the $2^{[-ddCt]}$ approach. Corresponding means and SEMs are indicated by the bars. Different superscripts within each transcript indicates differences ($P<0.05$).

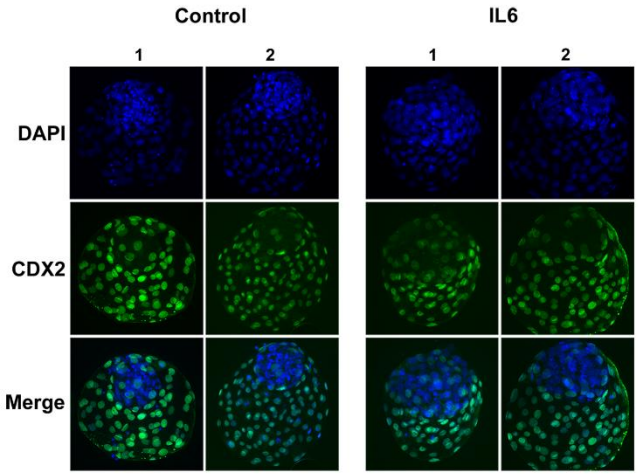

Figure 2-2. Representative images of differential cell staining in blastocysts collected at day 8 post-fertilization. Embryos either received 0 or 100 ng/ml IL6 beginning at day 5 post-fertilization. *Panel A:* Blastocysts were harvested at day 8, fixed, immunostained, and physically flattened between a slide and coverslip. Photographs represent a single plane of focus. Nuclei representing TE are indicated by $CDX2^{+}/DAPI^{+}$ staining (green) and the ICM nuclei are $CDX2^{-}/DAPI^{+}$ (blue). Control embryo number 1 had 42 ICM cells and 94 TE cells, while control embryo number 2 had 53 ICM cells and 120 TE cells. IL6-treated embryo number 1 had 86 ICM cells and 99 TE cells, while IL6-treated embryo number 2 had 76 ICM cells and 143 TE cells.

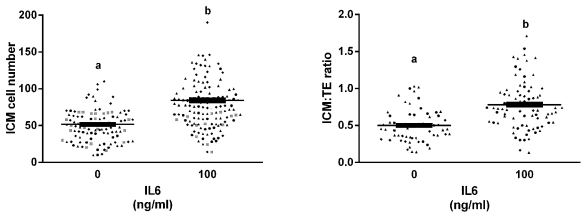

Figure 2-3. Pooled ICM cell counts and ICM to TE ratios from all studies. All 100 ng/ml treatments of IL6 and associated controls were utilized, regardless of time point of treatment. No other doses of IL6 (1, 10 or 200 ng/ml) are included in this figure. Data from different studies are indicated by different symbols. *Panel A*: Individual ICM counts for embryos receiving either no treatment or 100 ng/ml IL6. *Panel B*: Individual ICM:TE ratios for embryos receiving either no treatment or 100 ng/ml IL6. Corresponding means and SEMs are indicated by the bars. Different superscripts within each panel indicates differences ($P<0.05$).

Chapter 3: Interleukin-6 requires JAK to stimulate inner cell mass expansion in bovine embryos

Introduction

The mammalian blastocyst inner cell mass (ICM) contains the ability to form every cell type within the adult body. It is of no surprise, then, that defects of the ICM can result in lethality [243,259,260]. Understanding the development and needs of the ICM is crucial for embryos grown in vitro. Preimplantation embryos of several species, including humans, mice, rabbits and cattle, have been cultured in vitro to the blastocyst stage for decades [285,286]. However, in vitro culture systems lack the complexity of the oviductal environment, and this can hinder ICM development. This is particularly true for the bovine preimplantation embryo, which, when cultured in vitro, produces a smaller ICM during culture and after uterine transfer [59,237,244,265,266]. One way we may be able to improve *in vitro* produced (IVP) bovine embryo competency is by supplementing IVP embryos with growth factors and cytokines produced by the oviduct and uterus. These bioactive factors are often referred to as embryokines, and their supplementation can improve IVP blastocyst development and pregnancy retention after embryo transfer [116,234,287]. We recently discovered that interleukin-6 (IL6) increases the number of cells in the ICM of bovine blastocysts on day 8 of IVP development [8].

IL6 is perhaps the most well-known member of the IL6 cytokine family. Other members of this family include cardiotrophin-1 (CTF1 or CF1), cardiotrophin-like cytokine factor 1 (CLCF1), leukemia inhibitor factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and interleukins 11, 27 and 31 (IL11, IL27, IL31). All IL6 cytokine family members act through a receptor complex comprising a ligand-specific receptor subunit that permits high affinity ligand binding (*e.g.* IL6R for IL6) and a common subunit (IL6ST, also known as gp130)

that contains signal transducing regions [238,288]. All family members utilize the IL6ST subunit to control several signal transduction pathways, the most prominent of which is the Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) system. The JAK/STAT3 pathway is essential for the ICM lineage in mice and cattle, but the specific ligands that regulate JAK/STAT3 activity in bovine embryos is unclear [243,289]. Several indications exist that members of the IL6 cytokine family serve in this capacity. Some family members serve as mouse embryonic stem cell pluripotency factors [124,290,291]. Also, IL6 supplementation stimulates STAT3 phosphorylation and increases ICM cell numbers in porcine blastocysts [247].

The following work was completed to answer several questions about IL6's effects on bovine preimplantation embryos. First, we investigated the developmental stage at which IL6 first increases embryo cell number. We also used this information to determine if IL6 increases ICM cell number by skewing lineage specification toward ICM cells, or by increasing expansion of the ICM lineage. Second, we investigated IL6's ability to activate the JAK/STAT3 pathway and the necessity of this pathway for IL6's effects. Finally, we utilized RNA sequencing to dissect the outcomes of IL6 treatment on blastocyst transcripts and to profile IL6 family member transcript abundance. Most of the studies began at day 5 of embryonic development because embryonic genome activation had occurred by this stage of development (8- to 16-cell stage), and thus, embryos could respond to external signals [276], and because these embryos were preparing to undergo compaction and embryonic lineage specification.

Materials and Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials. Unless specified otherwise, reagents were purchased from ThermoFisher Chemical Company (Waltham, MA).

In vitro Embryo Production

Bovine embryos were produced by *in vitro* maturation, fertilization and culture procedures described previously [8,269]. In brief, cumulus-oocyte complexes (COCs) were collected by slashing follicles and placed in groups of 20-35 in 500 µl TCM-199 containing Earle's salts and supplemented with 10% [v/v] fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia, USA), 25 µg/ml bovine follicle stimulating hormone (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 2 µg/ml estradiol (Sigma-Aldrich; St. Louis, MO), 22 µg/ml sodium pyruvate, 1mM L-alanyl-L-glutamine (Glutamax) and 25 µg/ml gentamicin sulfate. The COCs were matured for 21 to 24 hours at 38.5°C in 5% CO_2 in humidified air. The COCs were washed in HEPES-SOF and placed in groups of 150-200 in 3 ml SOF-FERT covered in paraffin oil (Ovoil; Vitrolife, Göteborg, Sweden) [73,270]. Frozen semen from four Holstein bulls (donation from Select Sires, Plain City, OH, USA) was thawed, and spermatozoa were isolated through a biphasic (40% and 80%, [v/v]) Bovipure™ density gradient (Nidacon; Spectrum Technologies, Healdsburg, CA, USA) before addition to the fertilization media at a concentration of 1 million sperm/ml fertilization media. After incubation for 14 to 18 hours at 38.5°C in 5% CO_2 in humidified air, presumptive zygote-cumulus complexes were denuded by gentle pipetting. Denuded presumptive zygotes were then washed in HEPES-SOF

and placed in groups of approximately 20-30 in droplets of 50 µl of SOF-BE1 covered by paraffin oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 in humidified air [146].

IL6 Supplementation Study

A concentrated IL6 (recombinant bovine; Kingfisher Biotech, St. Paul, MN, USA) stock (10 µg/ml) was prepared in SOF containing 1% [w/v] bovine serum albumin (BSA) and stored in aliquots at -80°C. Stocks were only thawed once and were used immediately after thawing.

Either 0 or 100 ng/ml IL6 was provided on day 1 post-fertilization (n=23-29 embryos/drop; 2-7 drops/treatment, 6 replicates; total 578-657 embryos/treatment). On day 7, embryos were separated by stage and fixed for immunofluorescent differential cell counting utilizing caudal type homeobox 2 (CDX2) a TE-specific transcription factor (see below for antibody specifications and procedures) [283]. Stages included morulae (compacted blastomere mass but no signs of a blastocoel cavity; n=15-23/treatment), early blastocysts (blastocoel cavity <50% of interior space; n=7-17/treatment), regular blastocysts (blastocoel ≥50% of interior space, not expanded; n=37-43/treatment), and expanded blastocysts (increase in diameter with or without zona pellucida hatching; n=27-31/treatment).

Assessment of STAT3 Activity and Its Necessity for ICM Development

Day 5 embryos were transferred to drops of SOF-BE1 containing 5 µM AZD1480 (JAK1/2 inhibitor; Selleckchem, Houston, TX, USA) or carrier only (0.005% [v/v] DMSO) for 4 h and then were treated with 0 or 100 ng/ml IL6 for 30 minutes before fixation in 4% [w/v] paraformaldehyde. This JAK1/2 inhibitor was chosen based on a previous observation of its ability to block STAT3 activation in bovine embryos [289]. Embryos (n=3-5 embryos/replicate;

3 replicates; total 10-12 embryos/treatment) were processed for immunofluorescence analysis of STAT3 phosphorylation (pSTAT3^{Y705}) and nuclear localization (see below for antibody specifications and procedures). Another experiment utilized blastocysts harvested on day 8 post-fertilization (n=3-5 blastocysts/replicate; 2 replicates; total 7-8 blastocysts/treatment) and incubated with 0 or 100 ng/ml IL6 for 30 minutes before fixation and pSTAT3^{Y705} analysis.

In a follow-up experiment, embryos harvested on day 5 post-fertilization were placed into drops of SOF-BE1 (n=10 embryos/drop; 10-30 embryos/treatment/replicate; 6 replicates; total 90-130 day 5 embryos/treatment) containing either 0 or 5 µM AZD1480. After 4 h, each drop of embryos received an injection of 1 µl SOF-BE1 containing concentrated IL6 or carrier, to produce a final in-drop concentration of 0 or 100 ng/ml IL6. Blastocyst development was assessed on day 7 and 8 post-fertilization. On day 8, representative blastocysts (n=6-17 blastocysts/treatment over 4 replicates) were collected, fixed and processed for cell counting. In one replicate of this study, the presence of SOX2, an ICM marker, was examined to confirm that the non-CDX2-stained cells (CDX2^{-} cells) that reside within the inner clump of cells in blastocysts were indeed ICM cells.

qRT-PCR

On day 7, embryos were treated with 100 ng/ml IL6 or carrier only and incubated for 6 hours at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 in humidified air. After 6 hours, pools of blastocysts (n=11-19/treatment/replicate; 3 replicates) were collected, zona pellucidae were removed by brief exposure to Acidic Tyrode's Solution (Sigma-Aldrich), washed 4 times in sterile Dulbecco's phosphate-buffered saline (DPBS) containing 0.2% [w/v]

polyvinylpyrrolidone (PBS-PVP), collected into <10 µl PBS-PVP in microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C.

Total RNA was extracted by using the Qiagen RNeasy Micro Kit (Qiagen, Germantown, MD). The entire RNA sample was incubated with RNase-free DNAse I (Applied Biosystems, Inc., Foster City, CA, USA) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.). Negative controls did not receive reverse transcriptase. SybrGreen PCR Master Mix (Applied Biosystems, Inc.) was mixed with reverse transcription product and 250 nM forward and reverse primers. After activation/denaturation (95°C for 10 minutes), a two-step amplification sequence was set for 50 cycles (95°C for 15 s, 57°C for 1 min). Each sample and primer combination were run in triplicate. Each primer pair (see Table 3-1 for primer list) was identified using the Primer-BLAST Program from the National Center for Biotechnology Information (U.S. National Library of Medicine, Bethesda, MD, USA) and synthesized by Integrated DNA Technologies (IDT; San Diego, CA, USA). Primer efficiency standard curve analysis was completed to verify adequate primer efficiency (≥ 90% efficiency). Dissociation curve analysis (60 to 95°C) was completed after each PCR amplification to confirm the presence of one amplicon. *SDHA* was used as a housekeeping gene based on previously verified stability across early embryonic stages [271]. The $2^{[-ddCt]}$ approach was used to generate mRNA abundances relative to the non-IL6-treated embryos, depending on the study.

RNA Sequencing

Day 5 embryos (n=25-31 embryos/drop; 5-6 drops/treatment; 3 replicates), were treated with 100 ng/ml IL6 or carrier only via the addition of 2 µl of treatment concentrated SOF-BEI to

their existing culture drop. On day 8, blastocysts (n = 10/treatment/replicate) were collected from each treatment group and their zona pellucidae were removed by brief exposure to Acidic Tyrode's Solution. Blastocysts were then washed 4 times in sterile PBS-PVP, collected into <10 µl PBS-PVP in microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted by using the PicoPure RNA Isolation Kit (Applied Biosystems, Inc.). Genomic DNA was removed on-column by using the RNase-Free DNase 50 kit (Qiagen). Samples were shipped in dry-ice to Cofactor Genomics (St. Louis, MO, USA) for picoRNA library construction and sequencing using an Illumina-based platform with single end 75 base reads. Remaining blastocysts in each replicate were fixed for cell counting to confirm IL6 batch bioactivity (n=17-24 blastocysts/treatment).

Sequence analysis was performed using CLC Genomics Workbench 11.0.1 (Qiagen). Reads were imported and adapters and low-quality reads were removed. The sequences were then aligned to the *Bos taurus* reference genome (Ensembl;UMB3.1). Expression values were expressed as reads per kilobase of transcript per million (RPKM). Differential gene expression was determined by empirical analysis using the Robinson and Smyth Exact Test [292]. False discovery rate (FDR) was controlled at a rate of 5% using the Benjamini-Hochberg method [293]. The Panther Classification system (version 14.0) was used to classify genes based on gene families, molecular function, biological processes and interacting pathways. Heat maps were generated through GraphPad Prism (Version 8.0.2).

Immunofluorescence

Embryos were removed from culture and washed twice in PBS-PVP. For SOX2 immunostaining, zona pellucidae were removed by brief exposure to Acidic Tyrode's solution.

Regardless of the target, embryos were fixed in 4% [w/v] paraformaldehyde for 15-20 minutes at room temperature and washed three times in PBS-PVP. See table 3-2 for antibody information. For CDX2 and SOX2 studies, embryos were permeabilized using 0.25% [v/v] Triton-X for 20 minutes and then washed twice in wash buffer (DPBS containing 0.1% [w/v] BSA and 0.1% [v/v] Tween20) before blocking for 1 hour at room temperature in 10% horse serum. For pSTAT3^{Y705} studies, embryos were permeabilized with 70% [v/v] ethanol for 5 minutes and washed once in blocking buffer (10% [v/v] Horse Serum with 0.1% [v/v] Triton-X100) followed by two washes in wash buffer. Blocking for this antibody was completed with 10% [v/v] Horse Serum containing 0.1% [v/v] Triton-X. All embryos were incubated overnight at 4°C in primary antibodies for CDX2 (Biogenex, San Ramon, CA; AM392-5M, sold ready-to-use), pSTAT3^{Y705} (Cell Signaling Technologies, Danvers, MA; 9145T; 1:100) or SOX2 (R&D Systems, Minneapolis, MN; AF2018-SP; 1:300). Embryos were then washed three times and incubated at room temperature for 1 hour with donkey anti-mouse FITC, 488 or 647 (for CDX2), donkey anti-rabbit 555 (for pSTAT3^{Y705}), or donkey anti-goat 647 (for SOX2). Embryos were washed three times and incubated for 5 minutes at room temperature with DAPI (1µg/ml). After washing once in PBS-PVP, the embryos were placed in 10% [v/v] ProLong™ Gold Antifade diluted with PBS-PVP and either imaged by flattening on a glass slide or not flattening (for z-series acquisition), depending on the study. Immunoreactive complexes and DNA staining were visualized by using an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epifluorescence illumination system. Images were captured with DS-L3 digital camera and assembled with NIS-Elements Software (Nikon Instruments, Melville, NY). Cell counting was completed by using the cell counter plugin in the program FIJI (ImageJ) to label and record

individual nuclei stained for CDX2 ($CDX2^+$, indicating TE) or DAPI only ($CDX2^-/DAPI^+$, indicating ICM) [273].

Statistical Analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). Individual comparisons were partitioned further, when necessary, by using the probability of difference (PDIFF) test of SAS. Relative mRNA abundance data were log-transformed before analysis, but data are presented as fold change with standard error. For each embryo development study, the replicate was used as the experimental unit. This replicate refers to the mean developmental percentages observed on each in vitro embryo production occasion (*i.e.* each IVP run). Percentage data (*e.g.* blastocyst formation rates) were arcsine-transformed before analysis but are presented as non-transformed means and SEM. The Tukey honestly significant difference test was used for all blastocyst formation data. Blastomere numbers and differential staining analysis used individual embryos as the experimental unit. Statistical significance was determined at $P \leq 0.05$.

Results

IL6 Increases ICM and Total Cell Numbers in Day 7 Blastocysts but Not Morulae

Previous work determined that IL6 supplementation beginning at day 5 post fertilization increases ICM cell numbers at day 8 post-fertilization [8]. A follow-up study examined whether beginning IL6 treatment on day 1 post-fertilization affected ICM and TE cell numbers in embryos harvested on day 7 post-fertilization (Fig. 3-1). No effect of IL6 was detected on the

incidence of morulae and early and expanded blastocyst formation on day 7 (data not shown). However, IL6 treatment tended (P=0.06) to reduce regular (7.5±2 versus 13.1±2% for controls) and total blastocyst formation (15.4±3 versus 20.4±4% for controls). Treatment with IL6 did not affect total cell number in morulae (Fig. 3-1A). Supplementation with IL6 increased (P<0.05) ICM cell numbers in early, regular and expanded blastocysts (Fig. 3-1B, D, F). Treatment also increased (P<0.05) total cell numbers in regular and expanded blastocysts but not early blastocysts. Numbers of TE cells were reduced (P <0.05) by IL6 treatment in early blastocysts but were unchanged in regular and expanded blastocysts. The ICM:TE ratio was increased (P<0.05) by IL6 at each stage of blastocyst development (Fig. 3-1C, E, G). Example images of immunostained embryos within each stage are presented in Figure 3-2.

IL6 Activates the JAK/STAT3 Pathway in Bovine Embryos

The mode of IL6 action on embryonic cells was examined by determining whether IL6 stimulates STAT3 phosphorylation and nuclear localization. On day 5 post-fertilization, control embryos showed no nuclear pSTAT3^{Y705} staining, but embryos treated with IL6 for 30 minutes contained nuclear-localized staining in most cells (Fig. 3-3A). When the study was completed on day 8 of development, control blastocysts showed slight nuclear pSTAT3^{Y705} staining in all ICM cells and no staining in the TE (Fig. 3-3B). Exposure to IL6 for 30 minutes intensified nuclear pSTAT3^{Y705} staining specifically in the ICM (Fig. 3-3B).

A second study examined the ability of IL6 to stimulate STAT3 activity by examining *SOCS3* expression, a STAT3-inducible signal silencer [294]. This study began at day 7 to capture blastocyst responses to IL6 treatment. Exposure of day 7 embryos to 100 ng/ml IL6 for 6 h increased *SOCS3* transcript abundance (Fig. 3-3C).

The next series of studies examined the consequences of blocking IL6-induced STAT3 activation beginning at day 5 post-fertilization. Pre-treatment with 5 µM AZD1480, a pharmacological inhibitor of JAK1/2, effectively blocked IL6-induced pSTAT3^{Y705} staining and nuclear localization on day 5 post-fertilization (Fig. 3-3A). Examples of cell staining in day 8 blastocyst with or without AZD1480 exposure is provided in Figure 3-4 (panels A-L). Supplementation with IL6 beginning on day 5 did not affect total blastocyst or expanded blastocysts development on day 7 or 8 (Fig. 3-4M). Treatment with AZD1480 alone reduced ($P<0.05$) the percentage of total blastocysts on day 7 and 8 and reduced ($P <0.05$) the percentage of expanded blastocysts on day 8 (Fig. 3-4M). Co-supplementation of AZD1480 and IL6 partially restored blastocyst development on day 7 and 8 (Fig. 3-4M).

As expected, ICM cell numbers were increased ($P<0.05$) after IL6 exposure beginning on day 5, but ICM cell numbers were reduced ($P<0.05$) following exposure to AZD1480 beginning on day 5, regardless of whether IL6 treatment was administered (Fig. 3-4N). Neither AZD1480 treatment, IL6 supplementation nor the combination of treatments affected TE cell numbers. Total blastomere numbers were reduced with AZD1480 treatment in both IL6 treated and non-treated embryos ($P<0.05$; data not shown).

To confirm that the DAPI^{+}/CDX2^{-} nuclei in AZD1480 treated blastocysts were ICM cells, a subset of embryos was stained for SOX2, a pluripotency marker commonly used to mark the ICM (Fig. 3-4B, E, H, K) [284]. Numerous SOX2^{+} nuclear staining was detected in the BSA controls and IL6-treated groups (Fig. 3-4B, E). However, only a few SOX2^{+} nuclei were detected in embryos exposed to AZD1480 (Fig. 3-4 H, K). Curiously, this JAK inhibition resulted in some cells dual-staining for CDX2 and SOX2 (indicated by arrows in Fig. 3-4H-I and K-L).

IL6 has Minimal Effects on the Bovine Embryo Transcriptome

Blastocysts produced from embryos treated with IL6 or carrier only from day 5 to day 8 were harvested, pooled, and processed for RNA sequencing. Blastocysts not used for the RNA analysis were processed for cell counting. Treatment with IL6 increased ($P<0.05$) ICM (74.71±5.5 versus 47.2±6.6 cells in controls), TE (131.8±8.2 versus 105.3±8.7 cells in controls), and total cell numbers (206.5±12.3 versus 152.5±14.4 cells in controls). Treatment with IL6 also increased ($P<0.05$) the ICM:TE ratio (0.6±0.05 versus 0.4±0.04 in controls).

The analysis contained an average of 47,021,778 reads/sample and identified 22,104 transcripts and 19,981 genes (see Supplementary File 1). Treatment with IL6 affected the expression of 91 genes (≥1.5-fold; ≥1.0 RPKM; FDR<0.05), with 60 up-regulated and 31 down-regulated (Supplementary File 1). Panther analysis identified genes associated with signaling molecules (PC00207; *FAM43B, A2M, FZD10, GDF15*), receptors (PC00197; *GPR68, NPSR1, SORL1, LAMA1, FZD10*), and transcription factors (PC00218: *IRF1, BHLHE40, ZNF423*). Panther Pathway Analysis identified genes involved with chemokine and cytokine signaling (P00031; *VWF, ITGA2, NFKB*) and integrin signaling (P00034; *LAMA1, ITGA2, CAV1*). Panther-GO Slim Analysis for molecular function identified several genes associated with protein binding (GO:0005515; *EXOC3L4, FZD10, KIFC1, CSTB, TRIB3, SERPINA3, IGFBP4, TMEM88, SEPT4, CAV1, GDF15, FAM43B, A2M, CIITA, BHLHE40*).

The dataset was used to describe the abundance of IL6 family member ligands and receptors in bovine blastocysts (Fig. 3-5A). For IL6 family receptors, *IL6R*, *IL6ST* and *IL11RA* expression was detected (RPKM > 1.0). *CNTFR*, *LIFR* and *IL27RA* were present in low abundance (RPKM 0.2-1.0) and other receptors were not detected (RPKM < 0.2). Treatment with IL6 increased (FDR < 0.05) *IL6R* abundance but not the other receptors. The most abundant

IL6 family ligand was *IL6*. Both *IL27* and *CTF1* were also detected at moderate abundance (RPKM > 1.0) and *CLCF1* was detected at low abundance (RPKM 0.2-1). Other ligands were not detected (RPKM < 0.2).

The expression profiling dataset also was used to examine the expression of genes associated with ICM, TE and primitive endoderm (PE) lineages and with pluripotency and differentiation (Fig. 3-5 B-C) [295]. No changes in gene expression were detected in ICM and TE lineage markers, but two PE lineage markers were expressed in greater amounts (FDR < 0.05) after IL6 treatment (*PDGFRA, HNF4A*) (Fig. 3-5B). Also, only 3 of the 19 genes identified in the dataset associated with pluripotency and differentiation were increased (FDR < 0.05) after IL6 treatment (*STAT1, STAT3, OTX2*) (Fig. 3-5C).

Discussion

Interleukin-6 is best known as a pro-inflammatory cytokine that regulates the acute phase response and other inflammatory responses [238,296]. This group recently described that IL6 also serves as an embryotrophic factor in cattle, and it contains the unique ability to stimulate ICM cell numbers in bovine blastocysts [8]. This work built upon previous findings and described the apparent signaling mechanism responsible for this IL6 action.

The ontogeny of IL6's effect on ICM cell numbers was assessed by examining changes in ICM cell numbers as blastocysts began forming on day 7 post-fertilization. We were unable to discriminate between future ICM and TE cells at the morula stage with CDX2 differential staining. However, no changes in blastomere numbers were detected at the morula stage after IL6 treatment. Therefore, it seems probable that IL6's proliferative or anti-apoptotic actions are not occurring until the blastocyst begins to form. It is difficult to speculate whether IL6 promotes

blastomere allocation to the ICM as the blastocyst is being formed or if IL6 promotes ICM cell lineage expansion after the ICM is formed. We found that early blastocysts on day 7 contain greater ICM cell numbers and fewer TE cell numbers after IL6 treatment, suggesting a lineage reallocation. However, regular and expanded blastocysts contained greater ICM but similar TE cell numbers, suggesting ICM cell proliferation after IL6 treatment. Moreover, there also may be proliferation in TE cells between the early and later stages of development.

In our previous work, we did not detect an effect of IL6 on TE cell numbers [8]. However, in this work, we detected two occasions when TE cell numbers changed after IL6 treatment. The first was in early blastocysts and the second effect was found in the day 8 blastocysts used as controls for our RNA sequencing study. The reasons for these occurrences were not pursued and it is not clear if this effect can be replicated or if it occurred on these occasions merely due to chance. The noteworthy effect of IL6 on ICM numbers was pursued instead of these minor changes in TE numbers that occurred in some but not all studies.

Previously, no known activator of STAT3 in bovine preimplantation embryos and blastocysts had been described. This work discovered that IL6 activates STAT3 in bovine embryos. Nuclear staining of $STAT3^{Y705}$ was detected in IL6-treated embryos in nearly all blastomeres on day 5 and solely within the ICM on day 8. Additionally, IL6 increased expression of *SOCS3*, a STAT3-responsive gene, in day 7 blastocysts after 6 hours of treatment. Similar findings have been observed in other species. Blocking embryo-derived IL6 activity (by antibody-based neutralization) in mouse blastocysts reduced STAT3 phosphorylation and nuclear localization [248]. IL6 also stimulated STAT3 phosphorylation in pig blastocysts, although this activation could be detected both in the ICM and TE [247]. The *SOCS3* increase was not observed in our RNA sequencing data. This is not surprising given that embryos were

exposed to IL6 for 72 hours in the RNA sequencing study, and SOCS3 expression is known to spike initially but decrease after long-term IL6 treatment [294].

The inhibition of the JAK/STAT3 pathway prevented the IL6-induced expansion of ICM cell numbers in bovine blastocysts. Additionally, a few of the embryos exposed to the JAK1/2 inhibitor began degenerating approximately 24 to 48 h after blastocyst formation (2 of 36 JAK1/2-treated blastocysts versus none of the non-treated blastocysts). We also experienced some difficulty obtaining acceptable quality blastocysts for cell counting in JAK1/2-inhibited blastocysts (5 of 11 blastocysts were not used because of poor quality). These observations resemble the rapid, post-blastocyst degeneration observed in STAT3-null mouse embryos [243,248]. Therefore, present findings point to IL6 acting through STAT3 to mediate ICM development in cattle, pigs and mice. However, we cannot confirm that the JAK/STAT3 is the only pathway IL6 employs to impact ICM development because inhibition of the JAK/STAT3 pathway always results in a loss of ICM cells, regardless of IL6 supplementation.

Most of the cell counting studies herein did not utilize an ICM-specific marker. We concluded that blastomeres lacking CDX2 represent ICM. In one study, we confirmed these $CDX2^-$ cells were ICM cells by SOX2 immunostaining. Other studies did not utilize SOX2 for cell counting because it can be detected both in the nucleus and cytoplasm of the bovine ICM, and this complicates cell counting [284]. Curiously, the JAK1/2-inhibited blastocysts contained a subset of $SOX2^+$ and $CDX2^+$ dual-positive nuclei (arrows in Figure 3-4 H-I and K-L). The reason for this phenomenon was not explored. This could suggest a possible disruption of lineage specification or it may simply reflect an artifact of cell immunostaining.

It is interesting that IL6 supplementation may partially alleviate the negative effect of JAK1/2 inhibition on blastocyst formation. Inhibition of JAK1/2 reduced blastocyst formation on

day 7 and 8, but blastocyst rates were partially restored with IL6 and AZD1480 co-supplementation. This suggests that IL6 may act on embryos through non-STAT3-dependent systems to control blastocoel formation and/or TE development. However, IL6 supplementation did not affect blastocyst formation in a group culture setting in this and previous work, but this group recently showed that supplementing IL6 during a high-stress situation (individual embryo culture), facilitated blastocyst formation [8]. This may indicate that IL6's ability to promote blastocyst development in bovine embryos is observed primarily during sub-optimal culture conditions.

The RNA profiling work provided minimal insights into the mechanisms that mediate IL6's effects on the ICM. Time-response studies and studies that specifically examine the ICM likely are needed to decipher gene expression changes associated with IL6 treatment. However, this dataset was useful for describing IL6 as a major controller of bovine embryogenesis within the IL6 family. *IL6R* was the most abundantly expressed ligand-specific receptor. Moreover, IL6 supplementation increased *IL6R* expression. Likewise, *IL6* was the most abundantly expressed ligand. The sequencing also suggests that many of the other IL6 family members are unlikely to affect embryonic STAT3 expression in bovine blastocysts. Either the ligand-specific receptor is absent, or endometrial and/or embryonic ligand expression is lacking during the time of blastocyst development. One family member of note was IL11. Blastocyst expression of *IL11RA* is interesting because IL11 is a putative uterine-produced embryokine. It exhibits a temporal expression pattern in bovine endometrium, with greatest abundance on day 7 post-estrus [297].

The RNA-sequencing dataset also provided evidence that IL6 supplementation had minimal effects on embryo lineage formation and early pluripotency and differentiation potential. IL6 supplementation did not alter the expression of lineage markers associated with

ICM and TE development, but alterations in primitive endoderm (PE) lineage markers were detected. The PE will produce the yolk sac, and the yolk sac of IVP bovine embryos is poorly vascularized in comparison to embryos produced *in vivo* [298,299]. We detected an increase in *PDGFRA2* and *HNF4A* transcripts with IL6 treatment, both of which are vasculogenesis and angiogenesis factors associated with the PE. Therefore, these changes in gene expression may reflect downstream IL6 effects that may improve yolk sac development and function. Only 3 of the 19 pluripotency and differentiation markers found in the RNA sequencing dataset were increased by IL6 treatment (*STAT1, STAT3, OTX2*). Previous reports found that expression for each of these transcriptional regulators is controlled by IL6 family members in non-embryonic tissues, indicating that it not unexpected to detect these responses to IL6 treatment in bovine embryos [300,301]. Future studies will be necessary to confirm if these differences in transcript abundance also yield protein and functional differences.

In summary, this work provides additional evidence that IL6 functions as an embryokine in bovine preimplantation embryos. IL6 activates STAT3 phosphorylation and nuclear localization specifically within the ICM. Also, blocking the JAK/STAT3 pathway prevents the beneficial effects of IL6 on ICM development. However, due to the apparent necessity of STAT3 for ICM survival, we cannot completely confirm that IL6 acts solely through the JAK/STAT3 pathway to elicit its ICM effects. The physiological relevance of IL6 remains speculative. It is expressed in the oviduct, endometrium and embryo during early pregnancy in cattle and a recent report found that *IL6* transcripts were among the most prominently expressed cytokines in the oviduct on day 3 and 5 and in the endometrium on day 5 [8,247,294]. However, it remains unknown if IL6 is required for normal bovine embryo development or if it functions primarily as a facilitator of ICM development only during *in vitro* embryo production. Lastly, evidence

provided herein suggests that IL6 is the predominant mediator of IVP embryo ICM development among IL6 family members. The implications of enhancing ICM development in IVP embryos has yet to be explored, but all current indications suggest that IL6 treatment could improve IVP embryo survival after transfer in cattle.

Table 3-1. Primers used for quantitative RT-PCR.

Gene	Reference or Genbank accession no.	Primer sequence (5′ - 3′)	Product size (bp)
SOCS3	NM_174466.2	**Forward:** CGAGAAGATCCCTCTGGTGT **Reverse:** CGGTCTTCCGACAGAGATGT	77
SDHA	Goossens *et al.* 2005	**Forward:** GCAGAACCTGATGCTTTGTG **Reverse:** CGTAGGAGAGCGTGTGCTT	185

Table 3-2. Antibody source and dilution information.

Epitope	Source	Catalog Number	Working Concentration or Dilution*	Host Species
CDX2	Biogenex	AM392-5M	Ready-to-use	Mouse
$pSTAT3^{Y705}$	Cell Signaling Technologies	9145T	1:100**	Rabbit
SOX2	R&D Systems	AF2018-SP	1:300**	Goat
Donkey anti-mouse FITC secondary	Invitrogen	A16018	1:200**	Donkey
Donkey anti-mouse 647 secondary	Invitrogen	A31571	10 µg/ml	Donkey
Donkey anti-mouse 488 secondary	Invitrogen	A21202	10 µg/ml	Donkey
Donkey anti-rabbit 555 secondary	Invitrogen	A31572	10 µg/ml	Donkey
Donkey anti-goat 647 secondary	Invitrogen	A21447	10 µg/ml	Donkey

*All antibodies (except the CDX2 antibody) were diluted in 1% [v/v] Horse Serum in PBS-PVP.

** The protein concentration of this antibody preparation is not provided. It is supplied as a liquid.

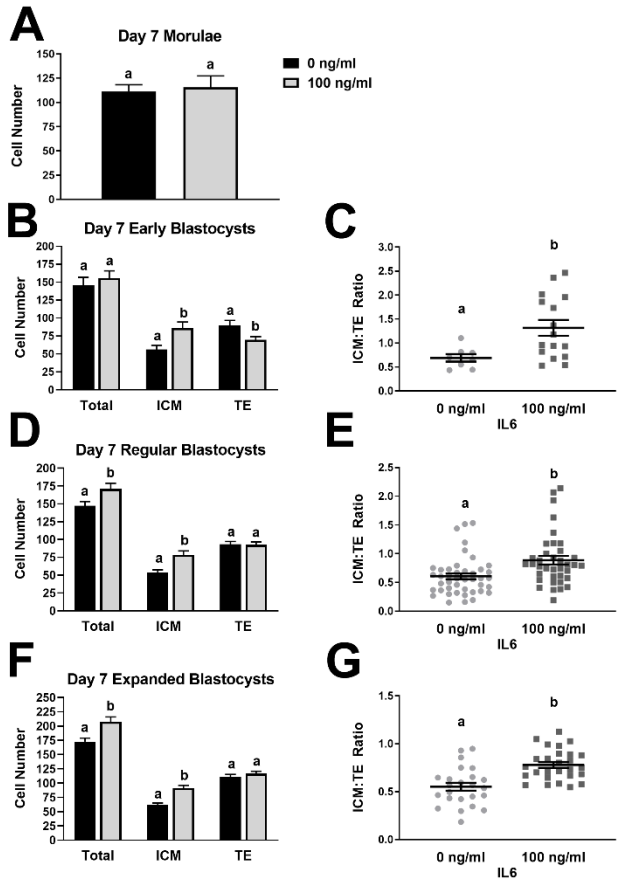

Figure 3-1. Supplementation with IL6 stimulates ICM cell numbers in bovine blastocysts evaluated on day 7 post-fertilization. Embryos received either 100 ng/ml IL6 or carrier only (0 ng/ml). Morula and blastocysts were separated by stage on day 7, fixed and immunostained. *Panel A:* Average total cell counts for morulae. *Panels B, D, F:* Total cell numbers and numbers of ICM and TE cells in early (B), regular (D) and expanded (F) blastocysts. *Panels C, E, G*: The ICM:TE ratios for individual day 7 embryos categorized as early (C), regular (E) and expanded (G) blastocysts. Corresponding means and SEMs are indicated by the bars. Different superscripts within each panel indicate differences (P<0.05).

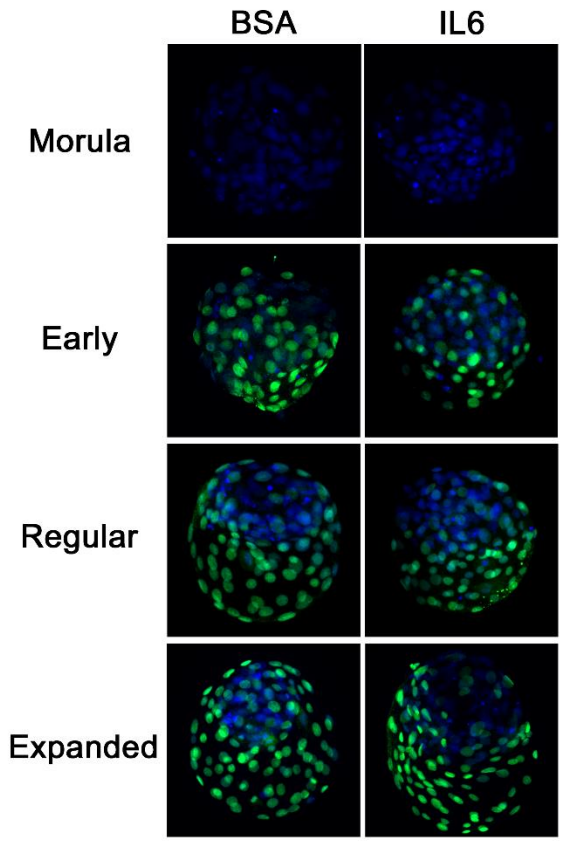

Figure 3-2. Representative day 7 embryos treated with 0 or 100 ng/ml IL6 from day 1 to 7 post-fertilization. On day 7, embryos were harvested, grouped based on stage of development and treatment, fixed and immunostained. DAPI staining was used at the morula stage to determine total cell numbers. Differential cell staining was completed at the blastocyst stages. Nuclei representing TE are indicated by $CDX2^{+}/DAPI^{+}$ staining (green) and the ICM nuclei are $CDX2^{-}/DAPI^{+}$ (blue).

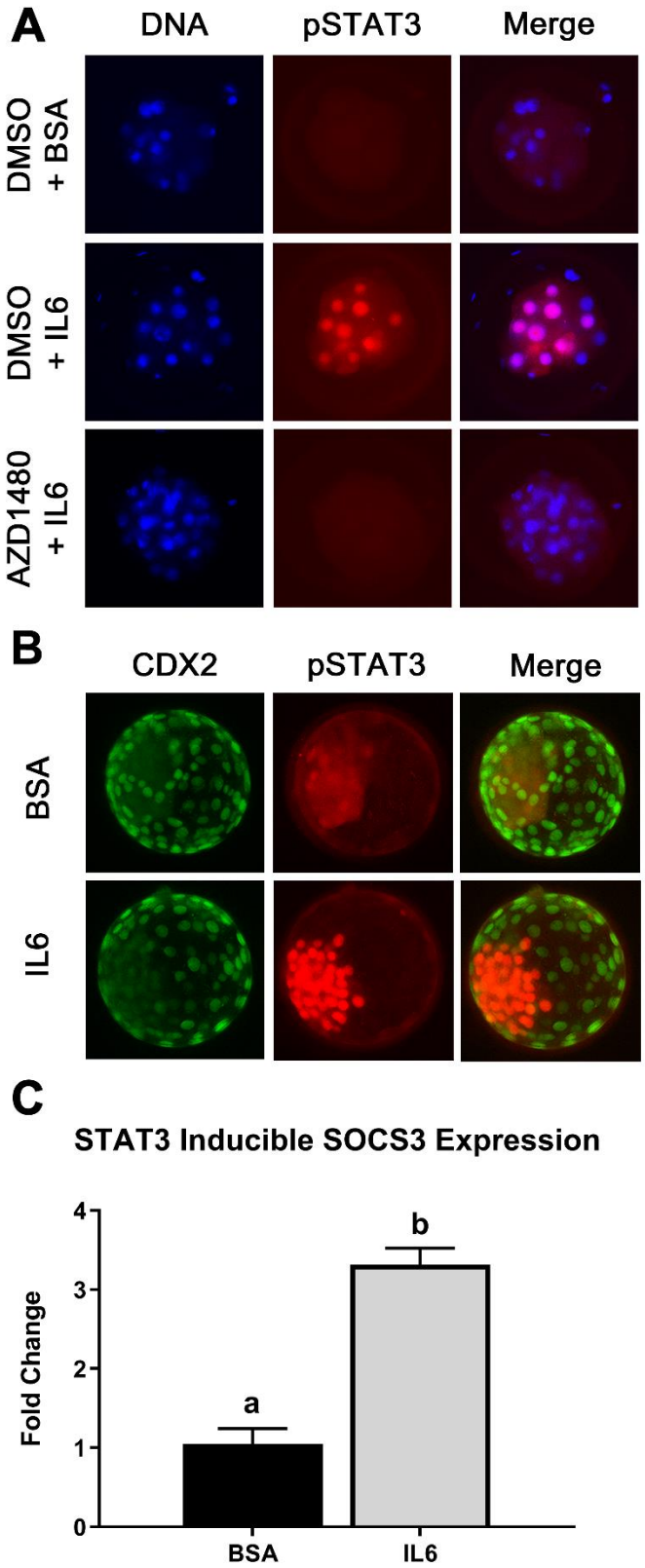

Figure 3-3. IL6-dependent stimulation of STAT3 phospho-activation and nuclear localization in day 5 and 8 embryos. *Panel A*: A representative sampling of embryos (>8-cells) that were pre-treated with 0 (DMSO only) or 5 µM AZD1480 for 4 hours before treatment with 0 (BSA only) or 100 ng/ml IL6 for 30 minutes. Embryos were immunostained to detect pSTAT3^{+} (red) and counterstained with DAPI (blue) to detect all nuclei. *Panel B*: A representative sampling of day 8 blastocysts treated with 0 or 100 ng/ml IL6 for 30 minutes before immunostaining to detect pSTAT3^{+} nuclei (red) and CDX2^{+} (TE marker, green). All images were obtained by z-series acquisition and subsequent focusing of the image via an Extended Depth of Field module (Nikon). *Panel C:* Influence of 100 ng/ml IL6 on *SOCS3* expression after 6 hours of treatment in day 7 blastocysts. Different superscripts within each panel indicate differences ($P<0.05$).

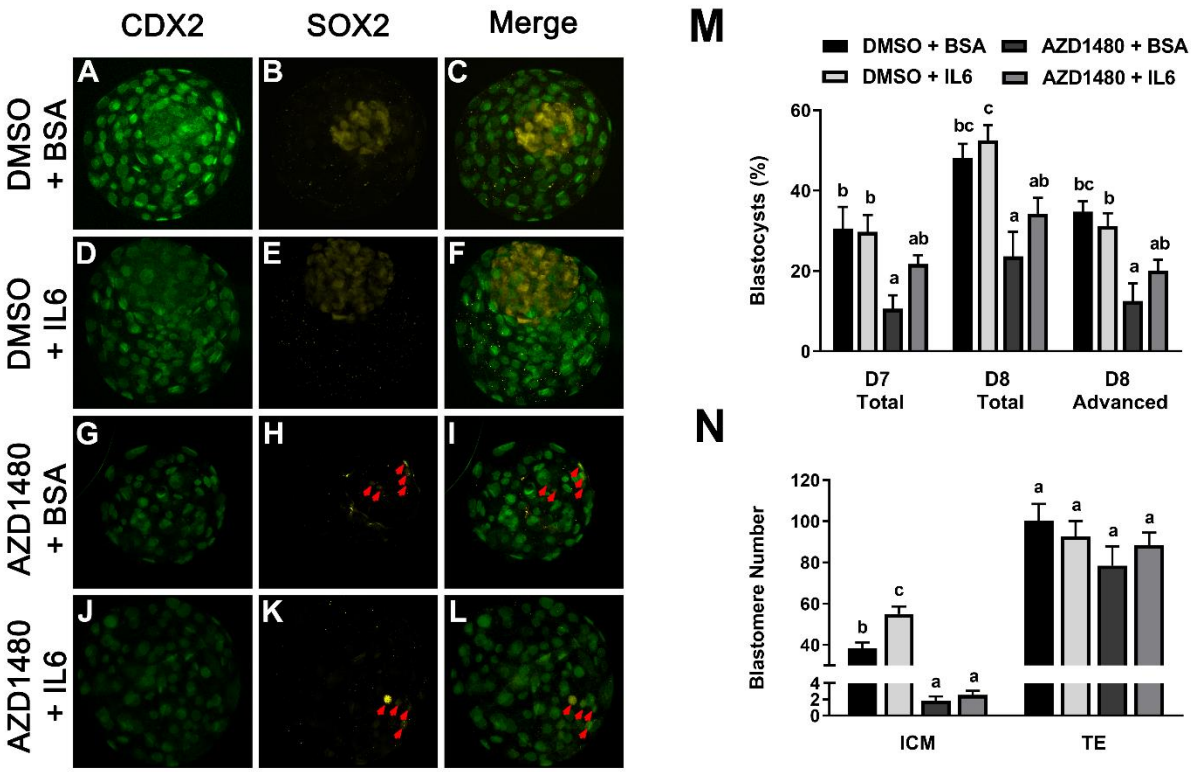

Figure 3-4. The necessity for STAT3 activity in ICM development. On day 5 post-fertilization, embryos (>8-cell) were exposed to either 0 (DMSO only) or 5 µM AZD1480 and either 0 (BSA only) or 100 ng/ml IL6 until day 8 post-fertilization. *Panels A-L*: Representative images of blastocysts exposed to the JAK inhibitor and/or IL6. Embryos were immunostained to detect TE ($CDX2^+$ nuclei; green) or ICM ($SOX2^+$ nuclei and cytoplasmic; yellow). Images were obtained after flattening blastocysts between a slide and coverslip. *Panel M*: The effect of JAK inhibition on blastocyst formation and cell number. *Panel N:* Effect of supplementation with or without the JAK inhibitor and IL6 on TE and ICM cell numbers. Different superscripts within each panel indicate differences ($P<0.05$).

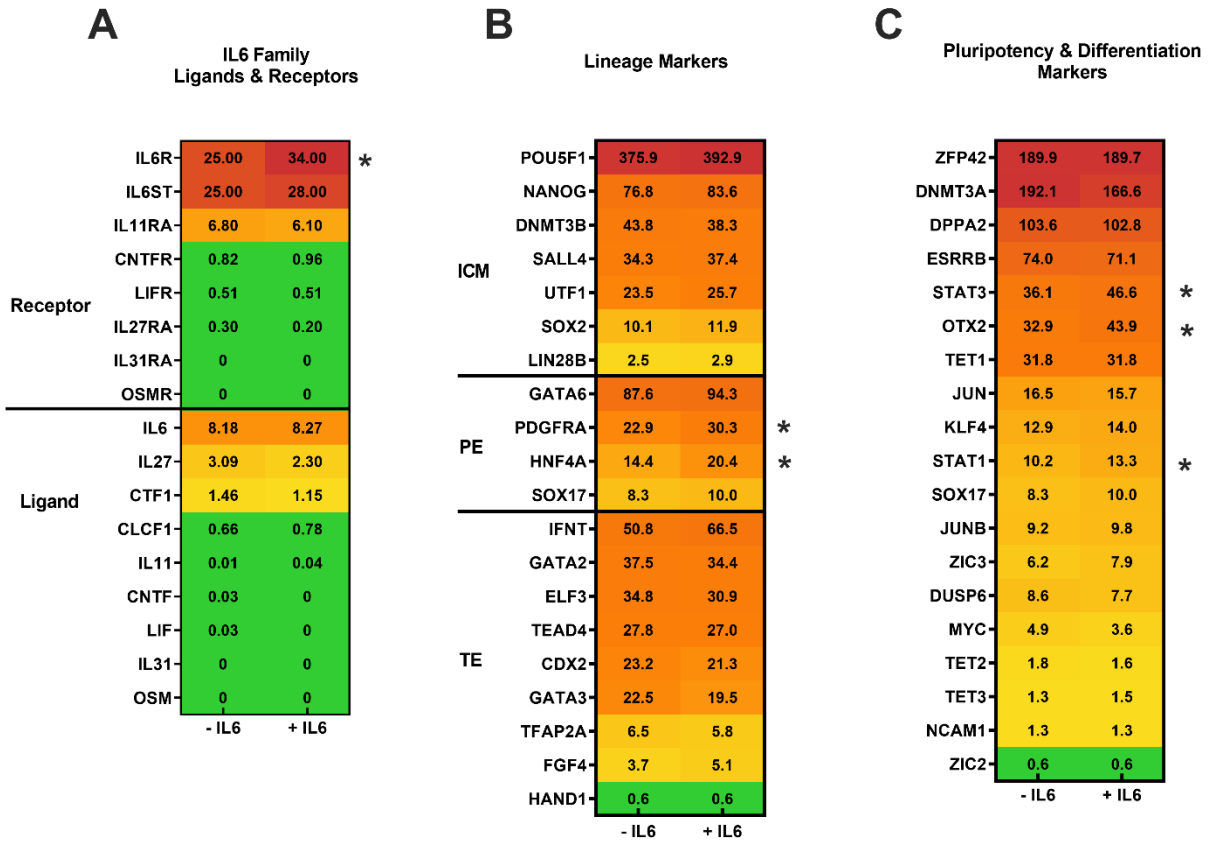

Figure 3-5. Expression analysis of IL6 family ligand and receptors, markers of embryonic lineages, and markers of pluripotency and differentiation after IL6 exposure. Supplementation with 0 or 100 ng/ml IL6 was completed from day 5 to 8 post-fertilization. Blastocysts recovered on day 8 were processed for RNA extraction and RNA-sequencing was completed using an Illumina-based platform and the CLC Genomics Workbench 11.0.1 to align sequences to the *Bos taurus* reference genome (Ensembl;UMB3.1). *Panel A*: Abundances of IL6 family member ligands and receptors with and without IL6 supplementation. *Panel B*: Transcript abundance for genes associated with ICM, PE and TE lineages. *Panel C*: Transcript abundance of pluripotency and differentiation markers identified in the dataset. Expression values in each rectangle are expressed as reads per kilobase of transcript per million (RPKM). Asterisks indicate transcripts with differential expression based on IL6 treatment (FDR < 0.05).

Chapter 4: Interleukin-6 increases hypoblast proliferation and slows epiblast differentiation in the bovine blastocyst inner cell mass

Introduction

Interleukin-6 (IL6) is a cytokine, commonly known for its role in stimulating the acute-phase response in the innate immune response to illness [302]. Intriguingly, bovine preimplantation embryos produce transcripts for IL6 and both of its receptor subunits needed to transduce signals [8,240,241]. IL6 signals through a heterotrimer complex composed of one ligand-specific alpha subunit, IL6R, and two beta subunits, both IL6ST (a.k.a. gp130), which are not ligand specific, but contain intracellular signal-transducing regions [302]. Ligand binding of IL6 to IL6R results in recruitment of two IL6ST units, and downstream activation of intracellular signaling pathways, including the PI3K, MAPK, and JAK/STAT cascades [302]. However, IL6 is perhaps best known for activating STAT3 signaling [303]. The transcription factor STAT3 is involved in numerous cancers, inflammation, and even murine embryonic stem cell (ESC) maintenance [122–124,238,303]. ESCs are derived from the inner cell mass (ICM) of blastocysts, making STAT3 an important focal point in blastocyst development as well.

The ICM is a primitive structure, present in a very early embryonic stage, the blastocyst, and gives rise to the embryo proper as well as the extraembryonic endoderm and mesoderm. Despite our long-time ability to culture *in vitro*-produced (IVP) bovine embryos to the blastocyst stage, several indications exist that the IVP bovine ICM is inferior to its *in vivo* produced counterpart. These IVP ICMs contain fewer cells [237], have elevated apoptosis [304–306], and commonly produce small and defective embryonic disks after transfer [244,246]. These abnormalities may be responsible for the low calving rates of IVP embryos [116,307,308]. This lab recently

reported that IL6 supplementation during *in vitro* development of bovine embryos increases ICM cell number in IVP bovine blastocysts and stimulates STAT3 activity in ICM cells [8,309]. This discovery invites the idea that IL6 may be useful as a tool to improve blastocyst quality and thereafter pregnancy retention of bovine IVP embryos. However, the ICM is not comprised of a single cell type; soon after its own specification, the ICM differentiates into two lineages, the epiblast (EPI), which will produce the embryo proper and extraembryonic mesoderm, and the hypoblast (HYPO), which produces the extraembryonic endoderm, otherwise known as the yolk sac. The effect of IL6 treatment on these two lineages in the bovine blastocyst is currently unknown.

The following work was performed to address the question of how IL6 affects the EPI and HYPO lineages after treatment *in vitro*. We hypothesized that IL6 stimulates proliferation of one or both lineages. Additionally, through these studies we discovered the inadequacy of our culture medium to support development beyond day 8 post-fertilization. To remedy this, we tested the suitability of a bovine ESC medium for blastocyst culture until day 10, with or without IL6 supplementation.

Materials and Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials from a commercial slaughterhouse that followed humane slaughter practices according to USDA guidelines. Reagents were purchased from ThermoFisher Chemical Company (Waltham, MA), unless otherwise specified.

In vitro Embryo Production

Bovine blastocysts were produced by *in vitro* maturation, fertilization and culture procedures described previously [309,310]. In brief, cumulus-oocytes complexes (COCs) were extracted from ovaries by slashing follicles with a scalpel and washed in BoviPlus Oocyte Washing Medium (Minitube USA, Inc. Verona, WI). After washing, COCs were cultured in groups of 20-30 in 500 µl of maturation medium [309] covered in paraffin oil (Ovoil; Vitrolife, Göteborg, Sweden). Maturation lasted 21-24 hours, in 5% CO2 in humidified air at 38.5°C. The COCs were then washed in HEPES-SOF [73], and placed in groups of 150-200 in 3 ml SOF-FERT [270] under paraffin oil. Pooled semen from four Holstein bulls (donation from Select Sires, Plain City, OH, USA) was thawed, and spermatazoa were isolated using a biphasic gradient (40 and 80% [v/v] Bovipure™; Nidacon; Spectrum Technologies, Healdsburg, CA, USA) before washing once in SOF-FERT. Sperm were added to the fertilization dish at 1 million sperm/ml medium, and the COCs and sperm were co-incubated overnight (14-18 hours) in humidified air at 5% CO2 at 38.5°C. The following morning, the presumptive zygote-cumulus complexes were denuded by gentle pipetting in HEPES-SOF and placed in groups of approximately 25 in drops of 50 µl SOF-BEI [146] under paraffin oil. The embryos were incubated in 5% CO2, 5% O2 and 90% N2 in humidified air at 38.5°C for the rest of their culture period, as specified in each experiment below. Day of fertilization was designated as day 0. Unless specified otherwise, treatments were administered to existing drops by addition of 2 µl of treatment-concentrated SOF-BEI, to achieve a final in-drop concentration of 0 or 100 ng/ml IL6. For blastocysts sampled on day 8, regular, expanded and hatched blastocysts were selected for fixation. For blastocysts sampled on day 9, non-expanded blastocysts appeared degenerate (darkened cytoplasm, severe evidence of blebbing and fragmentation), and only expanded and hatched

blastocysts were considered viable and fixed. For blastocysts sampled on day 10, non-hatched blastocysts appeared degenerate, and only hatched blastocysts were considered viable and fixed.

Study 1: IL6 Supplementation Prior To Blastocyst Formation and Day 9 Blastocysts

Treatments of 0 or 100 ng/ml IL6 were administered on day 5. Four days later, on day 9, blastocysts were collected from each treatment and fixed for CDX2, GATA6 and NANOG immunostaining, as described below. This study was replicated four times, with a total of 46-52 blastocysts analyzed per treatment.

Study 2: IL6 Supplementation After Blastocyst Formation and Day 9 Blastocysts

On day 7, blastocysts were collected from their existing drops, and sorted individually into new 50 µl drops of SOF-BEI, containing either 0 or 100 ng/ml IL6. The blastocysts were cultured in their new treatment drops for 48 hours, and then were collected and fixed for CDX2, GATA6 and NANOG immunostaining, as described below. This study was replicated three times, using a total of 39-42 blastocysts per treatment.

Study 3: IL6 Supplementation Prior To Blastocyst Formation and Day 8 Blastocysts

Treatments of 0 or 100 ng/ml IL6 were administered on day 5. On day 8, blastocysts were sampled from each treatment and fixed for CDX2, GATA6 and NANOG immunostaining, as described below. This study was replicated three times, with a total of 28-29 blastocysts analyzed per treatment.

Study 4: IL6 Washout at the Blastocyst Stage

Treatments of 0 or 100 ng/ml IL6 were administered on day 5. Two days later (day 7), blastocysts and morulae were removed from treatments, separated by stage of development, and washed twice in 50 µl drops of SOF-BEI. After washing, the embryos were placed in a final 50 µl drop of fresh SOF-BEI in groups of 2-7. On day 9, all blastocysts were fixed and kept separate by treatment and day 7 stage of development, before being processed for NANOG, GATA6 and CDX2 staining as described below. This study was replicated twice. A total of 5-12 day 7 morulae and 24-28 day 7 blastocysts were analyzed per treatment.

Study 5: Blastocyst STAT3 Response to IL6 Over Time

On day 6.5, 7, 8 or 9, blastocysts were treated with 100 ng/ml IL6 for 30 minutes. On day 8 and 9, some blastocysts received 0 ng/ml IL6 in some replicates for reference. After incubation, the blastocysts were fixed and processed for $pSTAT3^{Y705}$ immunostaining, as described below. Samples were collected from each stage of development in at least two replicates. A total of 27-73 blastocysts were analyzed per time point and per treatment.

For analysis, the degree of STAT3 staining in each ICM was scored categorically. A rank of 1 indicated no ICM cells were positive for $pSTAT3^{Y705}$. A rank of 2 indicated <5 cells were positive. A rank of 3 indicated >5, but not all, ICM cells were positive. A rank of 4 indicated all ICM cells were positive. See figure 4-6 for examples.

Study 6: pSTAT3^{Y705} Colocalization with GATA6 or NANOG After IL6 Treatment in SOF-BEI

Treatments of 0 or 100 ng/ml IL6 were administered on day 9, and the blastocysts were incubated for 30 minutes before being fixed and immunostained for either NANOG and pSTAT3^{Y705}, or GATA6 and a different pSTAT3^{Y705} antibody, as described below. A total of 63 blastocysts from 4 replicates were stained for NANOG, and 24 were pSTAT3^{Y705} positive and used for analysis. For GATA6 staining, 26 of 58 blastocysts from 3 separate replicates were pSTAT3^{Y705} positive and used for analysis.

Study 7: Blastocyst Cell Numbers on Day 8, 9 and 10, and 24 Hour IL6 Treatments

On day 7, 8 or 9, blastocysts were treated with 0 or 100 ng/ml and, after 24 hours, the blastocysts were collected and fixed for CDX2 staining, as described below. A total of 11-19 blastocysts/treatment/time point were analyzed from 3 replicates.

Study 8: Comparison of a TeSR Formulation to SOF-BEI

We also tested the efficacy of a bovine ESC medium, a TeSR formulation, on blastocyst development past day 8. The exact formulation for this medium can be found in table 4-2 [295,311].

On day 7, existing drops of embryos in SOF-BEI were randomly allocated to BEI or TeSR treatment. For TeSR, blastocysts were removed from their drops of SOF-BEI and cultured in groups of 6 or 7 in 50 µl of TeSR medium covered in paraffin oil. For SOF-BEI, blastocysts remained in their original drops. We previously determined that this method resulted in higher blastocyst cell number on day 10 in comparison to blastocysts which moved to new droplets of

SOF-BEI on day 7, presumably because of conditioning factors in the original drop. In both SOF-BEI and TeSR, the blastocysts were cultured to day 9, and in two replicates, some drops were also cultured to day 10. In addition to being cultured in TeSR or SOF-BEI, the embryos also received 0 or 100 ng/ml IL6 beginning on day 7. On day 9 and 10, blastocysts were collected and fixed for NANOG, GATA6 and CDX2 immunostaining, as described below. A total of 28 to 41 day 9 blastocysts were analyzed per media/treatment from 4 replicates. For day 10, a total of 4 to 11 hatched blastocysts were fixed per media/treatment from 2 replicates.

Study 9: pSTAT3^{Y705} Colocalization with GATA6 or NANOG After IL6 Treatment in TeSR

Embryos were cultured until day 7 in SOF-BEI. On day 7, all blastocysts were removed from their drops and moved to new 50 µl droplets of TeSR medium. The blastocysts were cultured in groups of 5-8 until day 9. Treatments of 0 or 100 ng/ml IL6 were administered on day 9. The blastocysts were incubated with their treatments for 30 minutes, before half were fixed for pSTAT3^{Y705} and GATA6 immunostaining, and the remaining half were stained for NANOG and pSTAT3^{Y705} (different antibody than the one used with GATA6), as described below. A total of 12 blastocysts were treated with 0 ng/ml, and 37 were treated with 100 ng/ml from 2 replicates.

Immunofluorescence

Blastocysts were collected and washed twice in Dulbecco's Phosphate Buffered Saline (DPBS), with 0.2% [w/v] polyvinylpyrrolidone (PBS-PVP), before fixation in 4% [w/v] paraformaldehyde for 15-20 minutes at room temperature. The blastocysts were then washed three more times in PBS-PVP and were either stored in PBS-PVP at 4°C for up to one week

before processing or were processed for immunostaining immediately. One of four different protocols, described below, were used, depending on the study. All permeabilization and blocking steps were performed at room temperature. All secondary antibody incubations were performed at room temperature for 1 hour. All blastocysts were washed three times in wash buffer (DPBS containing 0.1% [w/v] BSA and 0.1% [v/v] Tween20) following primary and secondary antibody incubations. The antibody buffer used was 1% horse serum.

For CDX2 standalone staining, the blastocysts were permeabilized for 20 minutes with 0.25% Triton-X in DPBS and blocked for one hour with 10% [v/v] horse serum. The blastocysts were then incubated with mouse monoclonal IgG1 anti-CDX2 antibody (Biogenex, San Ramon, CA, AM392-5M, sold ready-to-use) for 1 hour at room temperature or overnight at 4°C, before a final incubation with a donkey anti-mouse IgG secondary (Alexafluor 488 or 647; 1:200).

For CDX2, NANOG and GATA6 co-staining, the blastocysts were permeabilized for 30 minutes with 0.5% Triton-X in DPBS and blocked for one hour with 10% [v/v] horse serum. Due to antibody overlap, two rounds of primary and secondary antibody incubations were completed. First, the blastocysts were incubated with a mix of rabbit polyclonal IgG anti-GATA6 (Cell Signaling Technology, Danvers, MA; 5851T; 1:500) and mouse monoclonal IgG1 anti-NANOG (eBioscience; 14-5768-82; 1:200) for 1 hour at room temperature, before incubation with a mix of donkey anti-rabbit IgG and donkey anti-mouse IgG secondary antibodies (Alexafluor 647 for NANOG, and 555 for GATA6; both 1:200). For the next round, the blastocysts were incubated with mouse monoclonal IgG1 anti-CDX2 antibody (same as above) for 1 hour at room temperature, before a final incubation with a donkey anti-mouse IgG secondary (Alexafluor 488; 1:500).

For pSTAT3^{Y705} and NANOG co-staining, the blastocysts were incubated for 5 minutes in room temperature 70% ethanol, before additional permeabilization and blocking in 10% [v/v] horse serum containing 0.5% Triton-X for 1 hour at room temperature. The blastocysts were then incubated with a mix of two primary antibodies, rabbit polyclonal IgG anti-pSTAT3^{Y705} (Cell Signaling Technologies; 9145T; 1:100) and mouse monoclonal IgG1 anti-NANOG (same as above; 1:200) for 1 hour at room temperature or overnight at 4°C. The secondary antibodies utilized were donkey anti-mouse IgG and anti-rabbit IgG Alexafluors (555 for STAT3, and 647 or 488 for NANOG; both 1:200).

For pSTAT3^{Y705} and GATA6 co-staining, the blastocysts were incubated for 5 minutes in room temperature 70% ethanol, before additional permeabilization and blocking in 10% [v/v] horse serum containing 0.5% Triton-X for 1 hour at room temperature. The blastocysts were then incubated with a mix of two primary antibodies, mouse monoclonal IgG2b anti-pSTAT3^{Y705} (Santa Cruz Biotechnology, Dallas, TX; sc-8059; 1:200) and rabbit polyclonal IgG anti-GATA6 (same as above; 1:500) for either 1 hour at room temperature or overnight at 4°C. The secondary antibodies utilized were donkey anti-mouse IgG and anti-rabbit IgG Alexafluors (555 for GATA6, and 488 for pSTAT3; both 1:200).

After incubation with the appropriate secondary antibodies, the blastocysts were incubated with 1 µg/ml DAPI for 5 minutes at room temperature, before washing once in PBS-PVP, and flattening on a glass slide lined with petroleum jelly. Staining was visualized with an Eclipse Ti-E inverted microscope equipped with an X-cite 120 epifluorescence illumination system and DS-L3 digital camera. Images were captured with NIS-Elements Software (Nikon Instruments, Melville, NY), and cell counting was completed with the cell counter plugin in the program FIJI (ImageJ) [273]. Cells dual positive for GATA6 and NANOG were considered undifferentiated

ICM (UN) cells. In studies utilizing the CDX2, NANOG and GATA6 staining protocol, some secondary mis-binding occurred between the CDX2 secondary and the NANOG primary, due to the CDX2 and NANOG antibodies both being mouse IgG1 antibodies. We were aware of this issue, and to ensure our cell counts were accurate, cells which were $CDX2^{+}$:$NANOG^{+}$ were identified and labeled as EPI or UN, and not TE cells.

Statistical Analyses

All analyses were completed using the Statistical Analysis System (SAS for Windows; SAS Institute Inc., Cary, NC, USA). For all data, the individual blastocyst was considered the experimental unit. Differences in cell number were analyzed by least-squares ANOVA, using the general linear model (Proc GLM). Replicate was considered a random independent variable for all ANOVAs. On a few occasions, the data was determined to be right-tailed, and was cube-root transformed before analysis because some data points were zeros. All data presented here are non-transformed means and SEMs. Individual comparisons were partitioned further by using the Probability of Difference (PDIFF) test in SAS. For analysis of differences in score distribution, Fisher's Exact Test was used when observations were low in number, or a Chi-squared Test was used if the n was sufficient (Proc Freq in SAS). Statistical significance was determined at $P \leq 0.05$.

Results

IL6 Supplementation Prior to Blastocyst Formation Increases Epiblast, Hypoblast and Undifferentiated ICM Cell Number in Day 9 Blastocysts

Because the ICM produces two cell types, we analyzed the effect of IL6 on the number of cells in the EPI and HYPO in day 9 blastocysts. Day 9 was selected as the end point because all or most ICM cells should be committed at this time point [73]. An example of a blastocyst stained for GATA6, NANOG and CDX2 can be seen in Figure 4-1A.

IL6 supplementation increased ($P<0.01$) the number of ICM cells in day 9 blastocysts (Fig. 4-1B) and tended ($P=0.08$) to increase the number of cells in the TE. Treatment with IL6 also increased ($P<0.01$) in total cell number (202.7 ± 10.9 versus 150.8 ± 6.8 cells for IL6 treated compared to the controls, respectively). Dissecting the ICM revealed that IL6 increased ($P<0.05$) the number of EPI, HYPO, and UN cells (Fig. 4-1B).

IL6 Supplementation After Blastocyst Formation Does Not Affect Epiblast Cells

We also administered IL6 treatment to day 7 blastocysts to examine the effects on the ICM lineages in day 9 blastocysts. Again, IL6 increased ($P<0.01$) the number of ICM, TE and total cells in the blastocysts (Fig. 4-1C). The number of HYPO and UN cells were also increased ($P<0.05$) with IL6 treatment (Fig. 4-1C). No effect on EPI cell number was observed (Fig. 4-1C).

IL6 Supplementation Prior to Blastocyst Formation Decreases Epiblast Cell Number in Day 8 Blastocysts, but Does Not Affect the Number of NANOG$^+$ Cells

Because of our previous data indicated that IL6 increases ICM cell number on day 8, and because of the interesting day 9 data above, we examined the ICM lineages after IL6 treatment on day 8. Again, IL6 increased ($P<0.05$) the number of cells in the ICM, however, no increase in TE or total cell number was observed in this study (Fig. 4-1D). Just as we observed in day 9 blastocysts, IL6 increased (<0.01) the number of HYPO and UN cells (Fig. 4-1D). However, at this time point, IL6 decreased ($P<0.05$) the number of EPI cells (Fig. 4-1D) but had no effect ($P=0.5$) on the number of NANOG$^+$ cells (includes EPI and UN; Fig. 4-1E).

IL6 Must be Present During Blastocyst Development to Affect ICM Cell Number

In this study, we sought to answer whether IL6 must be present at the blastocyst stage to increase cell number, or if the previously observed increases in ICM cell number were an effect of IL6 programming prior to the blastocyst stage. Embryos were treated with 0 or 100 ng/ml IL6 from day 5 to 7, washed in new SOF-BEI, and cultured for two more days. IL6 treatment from day 5 to 7 had no effect ($P>0.05$) on ICM, TE, total (Fig. 4-2), EPI, HYPO, or UN cell number in day 9 blastocysts (EPI, HYPO and UN cell number not shown). The results were the same whether only the embryos which were day 7 morulae, day 7 blastocysts, or both combined were analyzed.

Because no differences in treatments were observed, we next examined any differences in cell number between the day 9 blastocysts which were either a morula or blastocyst on day 7. No difference ($P>0.05$) in ICM or HYPO cell numbers were detected between the day 7 morula and blastocyst groups (data not shown). However, the day 9 embryos which were morulae on day 7

had fewer (P<0.05) TE, EPI and total cells, but had more (P<0.05) UN ICM cells (data not shown).

IL6's Ability to Activate STAT3 in the ICM Decreases with Time

We previously reported that IL6 treatment stimulates STAT3 activation and nuclear localization in the ICM cells of blastocysts [309]. Here, we dissected the effect of IL6 on STAT3 in the ICM by ranking the degree of $pSTAT3^{Y705}$ staining in the ICM.

First, day 8 and 9 blastocysts were given 0 or 100 ng/ml IL6 to confirm that IL6 did increase STAT3 staining. The distribution of STAT3 scores as well as the averages were different (P<0.05) between treatments at day 8 as well as day 9 (data not shown).

Next, we examined the averages of scores after only IL6 treatment across all time points. The scores tended (P=0.06) to decrease between days 6.5 and 7 and decreased (P<0.05) between days 7 and 8 (Fig. 4-3A). No differences (P>0.05) in distribution of scores was detected between days 8 and 9 (Fig. 4-3A).

Finally, we examined changes in the proportion of scores 4, 2 and 3 combined, or 1 between consecutive time points with IL6 treatment. The proportion of rank 4 blastocysts did not differ between day 6.5 and 7, or day 8 and 9 (Fig. 4-3B). However, a reduced proportion (P<0.05) of blastocysts ranked 4 on day 8 in comparison to day 7 (Fig. 4-3B). The proportion of ranks 2 and 3 combined did not differ across any time points (Fig. 4-3B). Ranks 2 and 3 were combined for this analysis as these ranks both represent blastocysts which only had a partial ICM response to IL6. No difference in the proportion of rank 1 blastocysts was observed from day 6.5 to 7, or day 8 to 9, but the proportion increased (P<0.01) from day 7 to 8 (Fig. 4-3B).

IL6-Induced pSTAT3^{Y705+} ICM Cells Are Mainly Hypoblast in Day 9 Blastocysts Grown in SOF-BEI

To examine STAT3 colocalization with ICM lineage markers, we first utilized a pSTAT3^{Y705} antibody which we and others have previously used in bovine embryos [289,309] and examined NANOG colocalization. An example of a blastocyst with pSTAT3^{Y705} and NANOG staining can be seen in Figure 4-3C. A summary of the number of pSTAT3^{Y705+} cells in the NANOG-stained blastocysts can be seen in Figure 4-3D, including a breakdown of the number and percentage which were also NANOG^{+}. The majority of pSTAT3^{Y705+} cells did not also have NANOG staining. A breakdown of the percentage of pSTAT3^{Y705+} blastocysts which exhibited either 100% pSTAT3^{Y705} colocalization with NANOG, partial colocalization, or no colocalization can be seen in Figure 4-3E. No blastocysts had 100% colocalization of pSTAT3^{Y705} and NANOG and the majority had no colocalization at all.

Next, we identified another pSTAT3^{Y705} antibody which was compatible with our GATA6 antibody. An example of a blastocyst stained using this method can be seen in Figure 4-3F. A summary of the number of pSTAT3^{Y705+} cells in the GATA6-stained blastocysts can be seen in Figure 4-3G, including a breakdown of the number and percentage which were also GATA6^{+}. The majority of pSTAT3^{Y705+} cells were also GATA6^{+}. A breakdown of the percentage of pSTAT3^{Y705+} blastocysts which exhibited either 100% pSTAT3^{Y705} colocalization with GATA6, partial colocalization, or no colocalization can be seen in Figure 4-3H. No blastocysts had 0% colocalization of pSTAT3^{Y705} and GATA6, and the majority had 100% colocalization.

ICM Cell Number Decreases After Day 8 in SOF-BEI

Because we observed day 9 blastocysts with fewer ICM cells than day 8 blastocysts in separate studies, we designed a study to examine ICM cell number over time cultured *in vitro* to determine if cell death is occurring. We also included 0 or 100 ng/ml IL6 treatments because we previously observed a decrease in IL6-STAT3 response over time and hypothesized that IL6's ability to increase ICM cell number would be lost with time.

First, we compared only the 0 ng/ml IL6 treated blastocysts to observe changes in blastocyst cell number over time without treatment. The number of cells in the ICM did not differ in day 9 blastocysts compared to day 8 and in day 10 blastocysts compared to day 9 (Fig. 4-4A). However, when day 10 blastocysts were compared to day 8, ICM cell number decreased ($P<0.05$; Fig. 4-4A). Trophectoderm cell number did not change from day 8 to 9 but increased ($P<0.05$) from day 9 to 10 and 8 to 10 (Fig. 4-4A). In the end though, because ICM cells were lost and TE cells were gained, total cell number did not differ between any time points (Fig. 4-4A).

Next, we examined the effect of IL6 treatment when administered for 24 hours either at day 7, 8 or 9. Overall, IL6 increased ($P<0.01$) ICM cell number, had no effect on TE cell number, and increased total cell number ($P<0.05$). When we separated treatment effects by time, IL6 tended ($P=0.08$) to increase ICM cell number from day 7 to 8 and ($P=0.06$) 9 to 10, but increased ($P<0.05$) ICM cell number from day 8 to 9 (Fig. 4-4B). IL6 had no effect on TE cell number at any time point (Fig. 4-4B). Total cell number did not change after IL6 treatment from day 7 to 8, or day 9 to 10, but increased ($P<0.05$) when IL6 was supplemented from day 8 to 9 (Fig. 4-4B).

TeSR Bovine ESC Medium Supports Blastocyst Development Past Day 8

We next tested a bovine ESC medium to determine if blastocyst development could be improved with a more complex medium formulation. Blastocysts were either cultured in SOF-BEI until day 9, or blastocysts were collected on day 7 and moved to new drops of TeSR medium and cultured until day 9. Half of the drops in each media contained 0 or 100 ng/ml IL6 as well.

Examples of blastocysts grown in SOF-BEI and TeSR can be seen in Figure 4-5A. On day 9, blastocysts grown in TeSR medium had more ($P<0.01$) ICM, TE, EPI, HYPO, and total cells than the blastocysts grown in SOF-BEI (Table 4-1, Comparison 1). The number of UN ICM cells did not differ between media (Table 4-1, Comparison 1). Additionally, more ($P<0.01$) blastocysts hatched by day 9 in TeSR medium in comparison to SOF-BEI (Fig. 4-5B), producing more viable day 10 blastocysts. On day 10, blastocysts grown in TeSR had increased ($P<0.05$) numbers of ICM, TE, HYPO and total cells (Table 4-1, Comparison 2). No differences were observed between EPI and UN ICM cell numbers at this time point (Table 4-1, Comparison 2).

Comparing IL6 treatments on day 9, in SOF-BEI, IL6 increased ($P<0.05$) ICM, HYPO, and UN cell number (Table 4-1, Comparison 3). In SOF-BEI, IL6 had no effect on TE, EPI, or total cell number (Table 4-1, Comparison 3). In TeSR, IL6 had no effect on ICM, EPI or HYPO cell number, but tended to increase TE ($P=0.06$) and total ($P=0.08$) cell number and increased ($P<0.01$) the number of UN ICM cells (Table 4-1, Comparison 4).

Because of the low number of day 10 blastocysts analyzed, we combined 0 and 100 ng/ml IL6 treatments to analyze the effects of day on blastocyst cell number. In SOF-BEI, ICM, HYPO, EPI and total cell number did not change between day 9 and 10 ($P>0.05$), but the number of UN ICM cells decreased ($P<0.05$) while the number of TE cells tended to increase ($P=0.06$)

(Table 4-1, Comparison 5). In TeSR, the number of cells in the ICM and EPI did not change ($P>0.05$) from day 9 to 10, but the number of TE and total cells increased ($P<0.05$) (Table 4-1, Comparison 6). Additionally, the number of HYPO cells tended to increase ($P=0.07$), while the number of UN ICM cells decreased ($P<0.05$) (Table 4-1, Comparison 6).

Surprisingly, we observed evidence of primitive endoderm (PE) migration (HYPO moving out from ICM to line the TE) in day 9 and 10 blastocysts grown in TeSR, but we have never observed this behavior in blastocysts grown in SOF-BEI. An example of a TeSR blastocyst with PE migration can be seen in Figure 4-5A, row 3. To quantify this difference, we analyzed the data as a binomial using chi-square. Obviously, TeSR medium had more ($P<0.01$) PE migration than blastocysts grown in SOF-BEI (0/76 blastocysts in SOF-BEI versus 29/98 in TeSR), and a greater proportion ($P<0.01$) of day 10 TeSR blastocysts had PE migration in comparison to day 9 TeSR blastocysts (Fig. 4-5C). However, IL6 treatment decreased ($P<0.01$) the proportion of day 9 blastocysts which had PE migration in TeSR (20/46 blastocysts versus 9/52 in 0 vs 100 ng/ml IL6, respectively).

IL6 Induces STAT3 Activation in ICM Cells of Blastocysts Grown in TeSR

To confirm whether or not IL6 was able to stimulate STAT3 activation and nuclear localization in blastocysts grown in TeSR medium, we analyzed $pSTAT3^{Y705}$ and GATA6 or NANOG colocalization in day 9 blastocysts. Treatment with 100 ng/ml IL6 increased ($P<0.01$) the proportion of blastocysts which had $pSTAT3^{Y705+}$ cells in their ICM cells (1 of 12 blastocysts versus 22 of 37 in 0 versus 100 ng/ml IL6, respectively). In 13 $pSTAT3^{+}$ blastocysts, a total of 279 ICM cells were $pSTAT3^{+}$, but only 37 cells, or 13.3% were also $NANOG^{+}$. In a separate

group of 9 blastocysts, 119 cells were pSTAT3^{+}, and 118, or 99.2%, were also positive for GATA6.

Discussion

We recently reported on IL6's ability to activate STAT3 in bovine preimplantation embryos and increase ICM cell number in blastocysts [8,309]. In this work, we dissected IL6's effect on the two ICM lineages, the EPI and HYPO, and explored the use of a bovine ESC medium as an extended blastocyst culture medium.

Treatment with IL6 mainly affected the HYPO population and produced an approximate doubling in HYPO cell number. This suggests IL6 stimulates proliferation of this lineage, which may benefit transferred embryos. The HYPO lineage eventually forms the yolk sac, a transient embryonic structure important for nutrient exchange. Some evidence suggests that IVP bovine embryos have defective yolk sac development [298,299]. In future studies, it will be interesting to see if IL6 improves yolk sac development after IVP embryo transfer.

We also showed that IL6 increases EPI cell number and appears to slow down EPI differentiation. We speculate that IL6 acts to temporarily hold EPI-precursors in a stem-like state, similar to what has been observed in mouse ESCs, albeit with LIF [312]. Additionally, if IL6 supplementation began after the blastocyst had already formed, no effect on the number of EPI cells was observed, indicating that IL6 is also only capable affecting EPI cell number when supplemented during blastocyst formation.

In complement to our cell count data, our STAT3 studies also suggest that the epiblast is slightly responsive to IL6, but the hypoblast is much more responsive. With time, the ICM

appears to become less responsive to IL6, as measured by STAT3 activity, and, interestingly, the majority of late-stage blastocysts (day 9) scored a 2 or 3 on our STAT3 activity scale, indicating some but not all cells had active STAT3. Because lineage determination has largely finished by this time [73], we speculated that this switch from largely all ICM cells being responsive to IL6 in young blastocysts (day 6.5 and 7) to only some cells in late blastocysts may indicate one lineage retains sensitivity to IL6 while the other loses it. Indeed, we found that very few pSTAT3^{+} cells were also NANOG^{+} in day 9 blastocysts, however the opposite was true for GATA6. Additionally, the majority of blastocysts analyzed for NANOG and pSTAT3 had no colocalization, and again the opposite was true for blastocysts stained for GATA6. This indicated that, after lineage segregation, the EPI appears to lose sensitivity to IL6, while the HYPO retains it. This phenomenon of most, but not all, cytokine-responsive cells being HYPO has also been observed in mice [312]. We cannot confirm though that any of these cells were differentiated EPI or HYPO, because we could not utilize our GATA6 and NANOG antibodies together here due to host isotype overlap with our pSTAT3 antibodies. However, in other studies in this work, we observed, on average, fewer than 1 cells/blastocyst being GATA6 and NANOG positive in day 9 ICMs.

We next wondered if the observed increase in STAT3-unresponsive blastocysts with time indicated that IL6 would not increase cell numbers in late-stage blastocysts. Surprisingly, IL6 increased ICM cell number when supplemented to blastocysts at all the tested timepoints, however the day 7 to 8 and 9 to 10 treatments were only tendencies. This is probably due to the high blastocyst demand in this study for 2 different treatments and 3 time points (essentially 6 groups) driving blastocyst number per group down. After 3 replicates, only 11 to 19 blastocysts total were examined per group. However, the effect of IL6 is still obvious, and refutes our

hypothesis. This result is an interesting contrast to our STAT3 studies. This disparity may be explained by the difference in treatment incubation, 30 minutes versus 24 hours. Perhaps 24 hours is sufficient for IL6 to stimulate production of more IL6R [313], thus increasing its effectiveness via an autocrine mechanism. Other possible explanations might be that IL6 is utilizing a different pathway to increase cell number, or the antibody used to detect $pSTAT3^{Y705}$ may not pick up low levels of STAT3 activity which are still sufficient to increase cell number.

We also asked whether the observed increases in ICM cell number after IL6 treatment may result from a constant presence of IL6 in the culture medium, or it could be a downstream result of earlier programming effects of IL6. When morulae and blastocysts were removed from IL6 treatment on day 7, no effect on ICM, TE or total cell number was observed in day 9 blastocysts, indicating that IL6 must be present at the blastocyst stage in order to stimulate ICM cell number.

Unfortunately, through these studies of later stage blastocysts, we discovered that the medium we use for embryo culture, SOF-BEI, is inadequate to support proper blastocyst development past day 8. To document this specific observation, we examined cell numbers in day 8, 9 and 10 blastocysts. Indeed, ICM cell number decreased from day 8 to 10, while TE number increased. This problem of ICM-loss *in vitro* has been observed by others as well [245,314,315]. Degeneration of the ICM is troublesome and may indicate that some effects observed herein may be the result of cell death. For example, not every day 9 blastocyst analyzed here responded to IL6 (approximately 40% responded), so the ability of IL6 to activate STAT3 in the ICM may be transient, or this may be a result of degrading ICM health. To potentially remedy this issue, we next analyzed the suitability of a complex bovine ESC medium to replace SOF-BEI for studies on blastocysts day 8 and older.

The utilization of this TeSR medium for blastocyst culture significantly improved development. However, in a preliminary test, we discovered that non-blastocysts could not be cultured in this medium, as they all lysed. However, blastocysts thrived in the medium. Blastocysts grown in TeSR from day 7 to 9 or 10 had significantly more cells in both their ICMs and TE than blastocysts grown in SOF-BEI. We even observed several day 10 blastocysts grown in TeSR which had over 1,000 total cells whereas the highest number of cells observed in a day 10 SOF-BEI blastocyst was 289 cells. Additionally, the HYPO/PE in some TeSR blastocysts began to spread out from the ICM, to line the TE (see Fig. 4-5A). The exact day post-fertilization when PE migration begins in vivo is unknown, and this is the first documented incidence of this occurring *in vitro* that we are aware of. We did not observe PE migration in SOF-BEI blastocysts here, nor have we ever seen this in prior experiments not in this work.

However, the ability of IL6 to increase ICM cell number was lost in the TeSR medium, and IL6 treatment even decreased the proportion of TeSR-grown blastocysts which showed evidence of PE migration. One explanation for this loss of effect may be due to the formulation of TeSR used here. This medium contained NODAL, FGF2, and a Wnt inhibitor, IWR1, all bioactive factors which may interact or possibly disrupt IL6's ability to increase ICM cell number. The effect of NODAL and IWR1 have not been individually tested on bovine blastocysts, however, FGFs skew the ICM cell population towards the HYPO fate, albeit when used at a much higher concentration and supplemented prior to blastocyst formation [142]. Additionally, we noticed IL6 decreased the proportion of blastocysts which showed PE migration but did not completely prevent it. We speculated earlier in this work that IL6 acts on EPI-precursors to slow their differentiation. This evidence that IL6 decreased PE migration may suggest then that IL6 holds the ICM, both HYPO/PE and EPI, in a "younger" state. Finally, one other explanation for this

loss of IL6-effect may be that this TeSR medium also contains a relatively high level of bovine serum albumin (BSA) (1.3% w/v) whereas SOF-BEI has nearly a third of that amount (0.4% w/v). BSA is known to bind IL6 in the blood [316], and thus this high concentration of BSA may have sequestered enough IL6 to prevent an increase in ICM cell number. However, the fact that the IL6 effect on ICM cell number was lost in this TeSR formulation indicates that others wishing to study other embryokines and bioactive factors in day 8+ blastocysts may want to utilize a version of this TeSR medium without any potential confounding factors (NODAL, IWR1, etc).

Because we observed no increase in ICM cell number after IL6 treatment of blastocysts grown in TeSR, we hypothesized that these blastocysts would show no pSTAT3^{Y705} response to IL6 treatment on day 9. This hypothesis was incorrect, as IL6 treatment increased the proportion of blastocysts which had at least one pSTAT3^{+} ICM cell in comparison to treatment with the carrier only. In addition to pSTAT3 staining, we also stained half of the blastocysts for NANOG, and the other half to GATA6. While these blastocysts are not directly comparable to the set of blastocysts grown in SOF-BEI, the results we obtained here are remarkably similar. A total of 12.4% of pSTAT3^{+} cells grown in SOF-BEI were also NANOG^{+}, and this was true for 13.3% of the pSTAT3^{+} cells in TeSR blastocysts. Additionally, 93.7% of pSTAT3^{+} cells of blastocysts grown in SOF-BEI were also GATA6^{+}, and 99.2% were the same in TeSR blastocysts. This refutes our hypothesis and complicates the effects of IL6 in TeSR.

In summary, this work provides evidence that IL6 is a potent stimulator of HYPO proliferation. We also show that IL6 appears to slow, but not prevent, EPI precursor differentiation into EPI cells. This information provides evidence that IL6-treated IVP blastocysts are likely to have improved yolk sac development post-transfer, but future studies

will be needed to confirm this. Because IL6 was able to slow EPI differentiation, IL6 may also prove useful in bovine ESC culture. We also show that the ICM-specific, STAT3-response induced by IL6 decreases with time. By day 9, most EPI cells are unresponsive to IL6, as measured by STAT3 activity, while HYPO cells appear to retain sensitivity. These findings are parallel to functions of LIF in murine blastocysts and cell lines. However, some of these findings may be a result of, or influenced by, the inadequacy of *in vitro* culture conditions; we show that ICM cell number decreases with time after day 8, indicating cell death. We assessed the ability of a bovine ESC medium TeSR formulation to support long-term blastocyst growth, and found that it was able to propel development, unlike SOF-BEI. However, the effect of IL6 on ICM cell number was lost in TeSR medium, possibly because of confounding factors (FGF2, NODAL, IWR1). This shows that this TeSR formulation can be used to culture blastocysts past the normal *in vitro* culture period, however it may be inappropriate for certain studies investigating other embryokines.

Acknowledgements

Authors thank Dr. Matthew Utt, Dr. Bo Harstine and Select Sires, Inc. (Plain City, OH) for donating the bovine semen used for this work. The authors also thank graduate and undergraduate students at Virginia Tech for assisting with bovine *in vitro* embryo production. And finally, the authors also thank the USDA-NIFA and Virginia Tech Institute for Critical Technology and Applied Science for fellowship support for LKW.

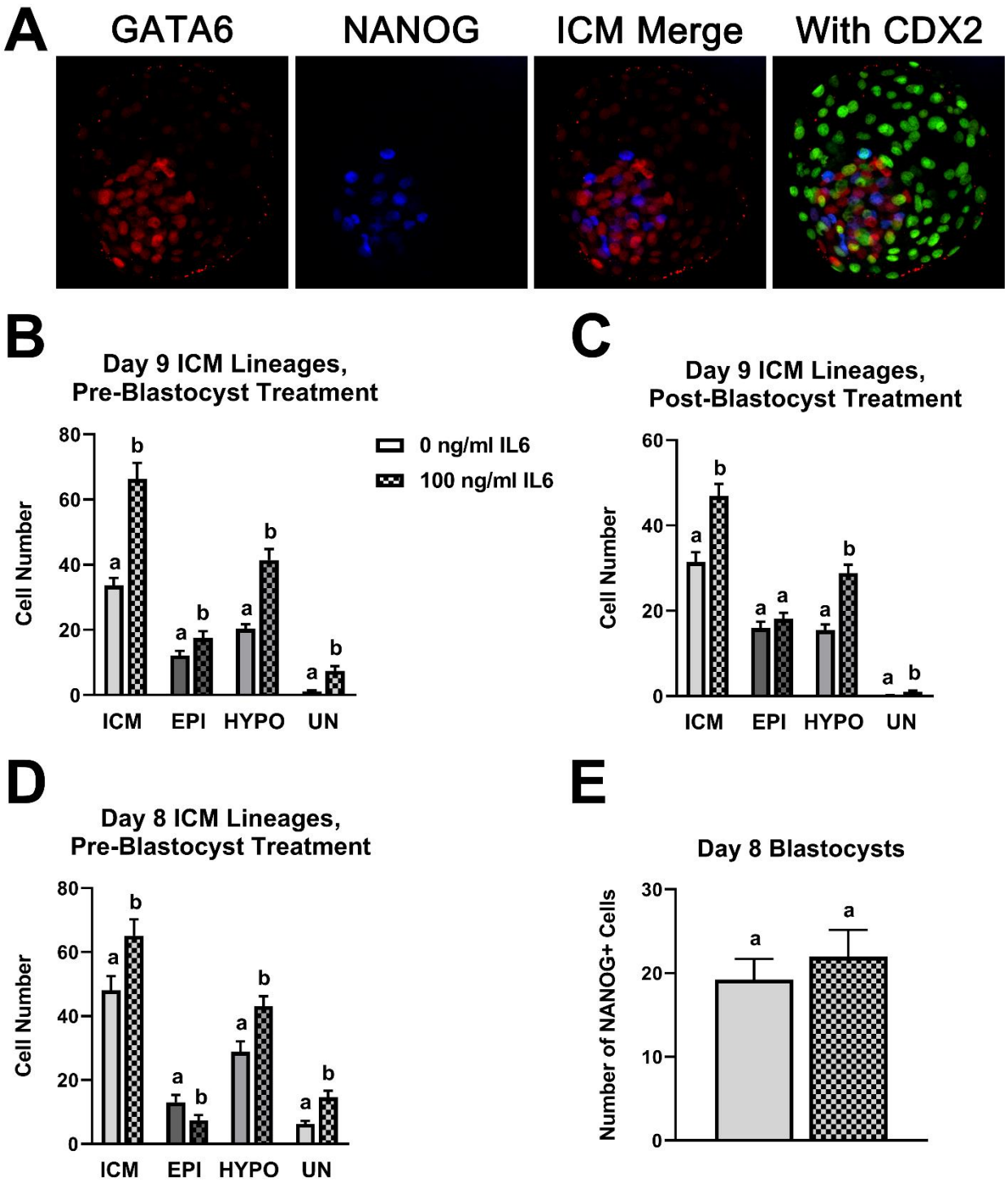

Figure 4-1. Treatment with IL6 increases the number of HYPO and UN cells in the ICM and slows EPI differentiation. *Panel A:* Example of a blastocyst stained for GATA6, NANOG and CDX2. Cells positive for both GATA6 and NANOG were considered UN ICM Cells. *Panel B:* The effect of IL6 on the ICM lineages when supplemented from day 5 to 9. *Panel C:* The effect of IL6 on the ICM lineages when supplemented from day 7 to 9. *Panel D, E:* The effect of IL6

on the ICM lineages when supplemented from day 5 to 8. Different superscripts within each panel indicate differences ($P<0.05$).

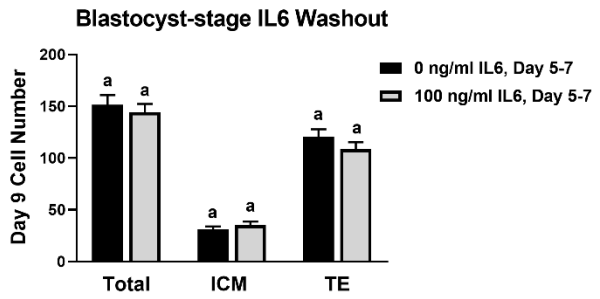

Figure 4-2. IL6 must be present during blastocyst development to increase ICM cell number. Day 9 blastocyst cell numbers after treatment with IL6 from day 5 to 7. Different superscripts within each panel indicate differences (P<0.05).

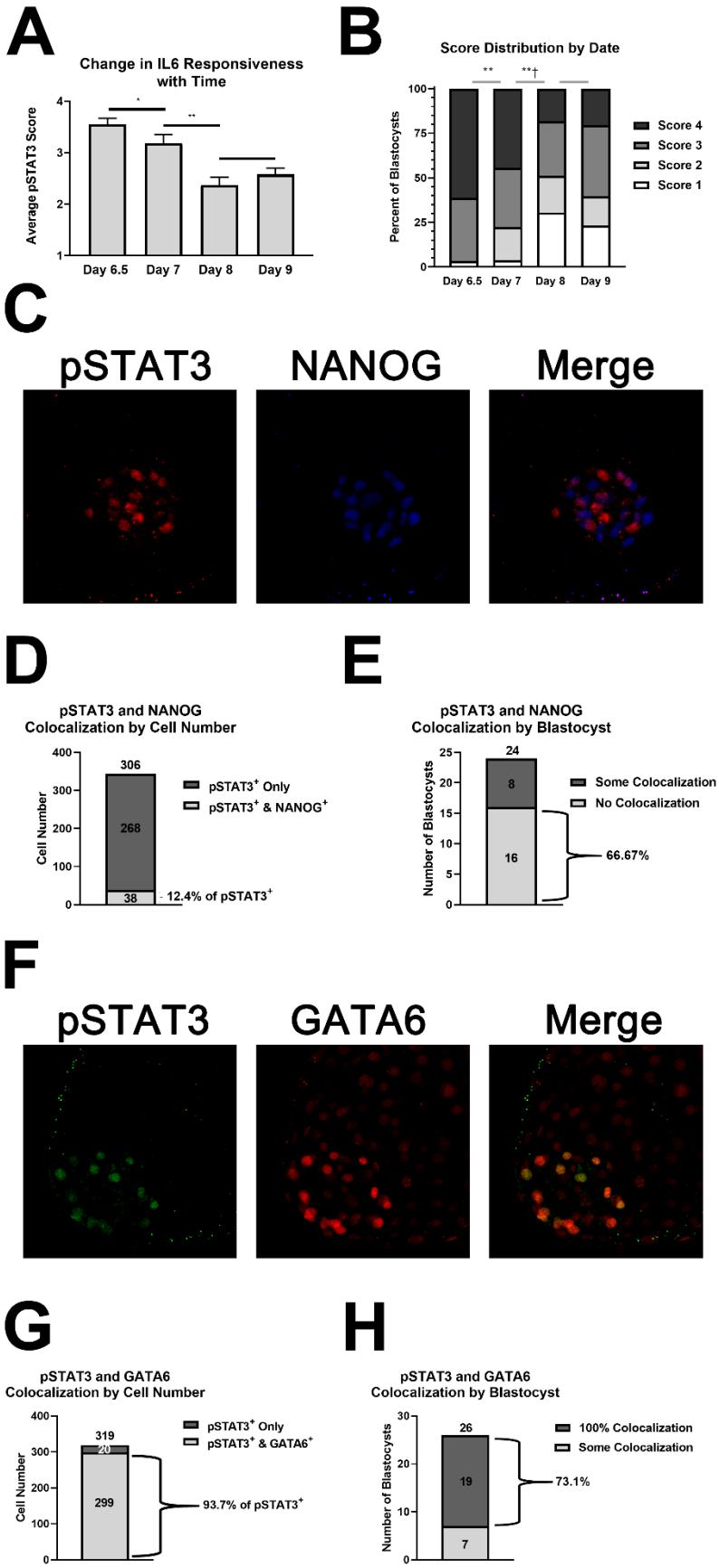

Figure 4-3. IL6's ability to stimulate STAT3 in the ICM wanes with time, and mostly stimulates HYPO cells. *Panel A:* Average STAT3 score of blastocysts treated with 100 ng/ml IL6 for 30 minutes either on day 6.5, 7, 8 or 9. This graph shows means and SEMs, but the data were also analyzed by Fisher's Exact Test (multinomial). We chose to show means and SEMs because they easily and accurately communicate the results. *Panel B:* Distribution of STAT3 scores of day 6.5, 7, 8 or 9 blastocysts treated with 100 ng/ml IL6 for 30 minutes. *Panel C:* Example of a blastocyst stained for $pSTAT3^{Y705}$ (rabbit antibody) and NANOG. *Panel D, E:* Number of cells and blastocysts showing NANOG and $pSTAT3^{Y705}$ colocalization. *Panel F:* Example of a blastocyst stained for $pSTAT3^{Y705}$ (mouse antibody) and GATA6. *Panel G, H:* Number of cells and blastocysts showing GATA6 and $pSTAT3^{Y705}$ colocalization. In panels A and B, a single * indicates a tendency ($0.05<P<0.1$) while two ** indicates a difference ($P<0.05$). In panel B, ** indicates a change in the proportion of rank 4 blastocysts, while a † indicates a change in the proportion of rank 1 blastocysts ($P<0.05$).

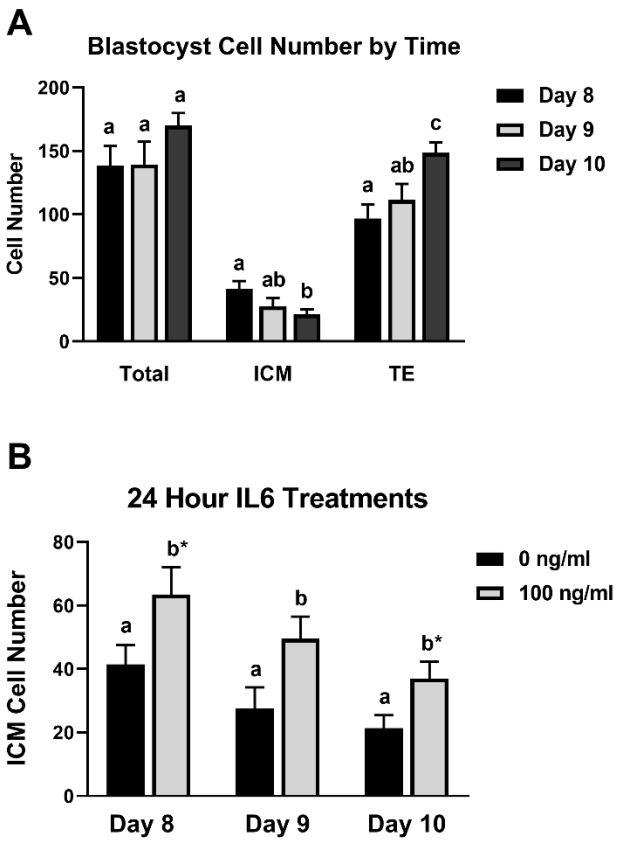

Figure 4-4. The ICM shrinks with time in SOF-BEI, and IL6 can increase ICM cell number in day 7, 8 and 9 blastocysts. *Panel A:* Total, ICM and TE cell numbers of day 8, 9 and 10 blastocysts grown in SOF-BEI. *Panel B:* ICM cell numbers of blastocysts treated with IL6 for 24 hours from day 7 to 8, 8 to 9, or 9 to 10. The days listed on the x-axis indicate the treatment endpoint. Different superscripts within each panel indicate differences ($P<0.05$).

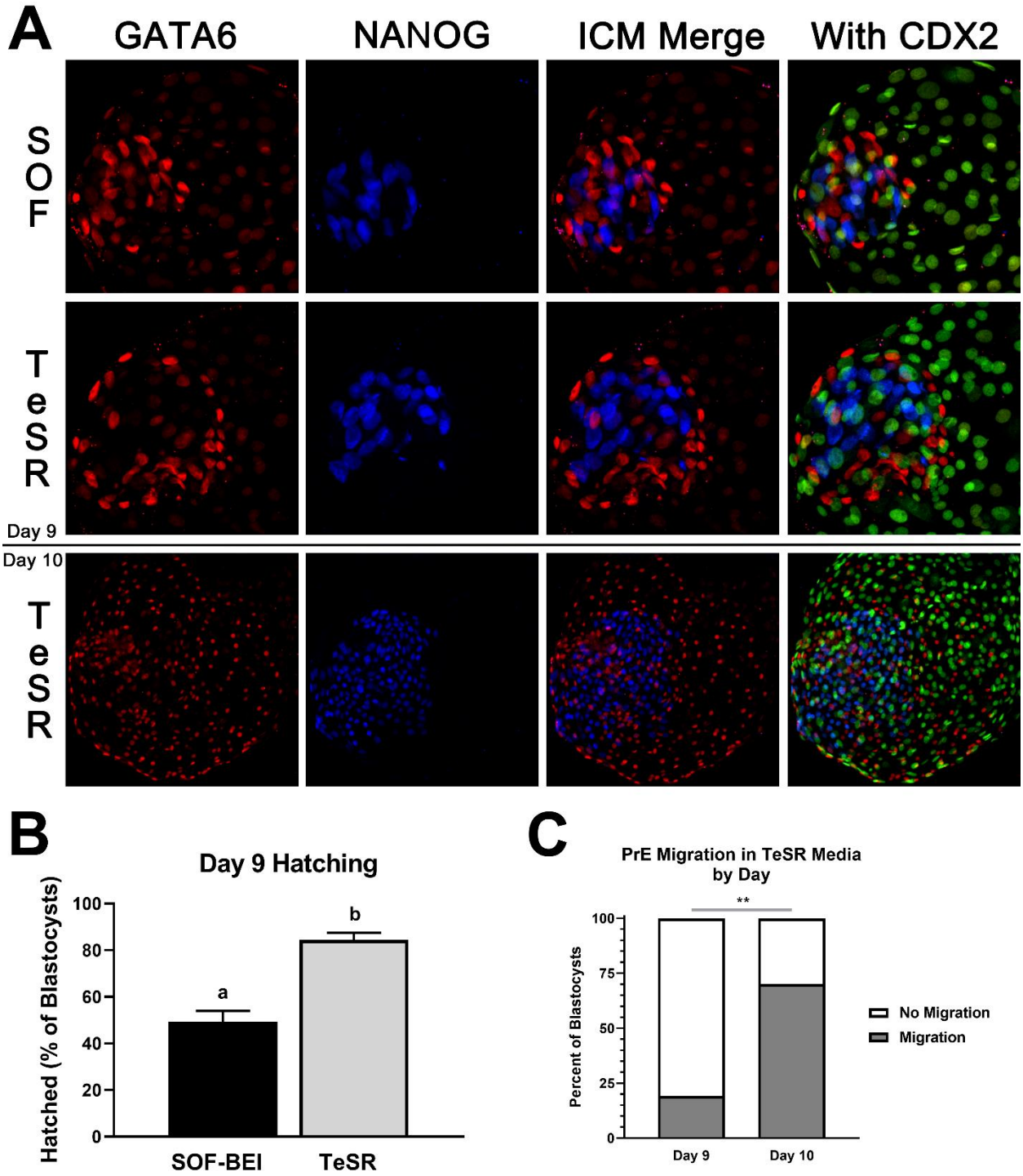

Figure 4-5. Culturing day 7 blastocysts in TeSR medium increases blastocyst cell numbers, hatching, and PE migration. *Panel A:* The first two rows are examples of day 9 blastocysts ICMs grown in either SOF (SOF-BEI) or TeSR. These images were taken at 20x magnification. The third row shows an example of a day 10 blastocyst grown in TeSR. This blastocyst had 995 total cells, shows PE migration ($GATA6^+$ cells spread out from the EPI), and had to be imaged at 10x because of its size. *Panel B:* The effect of medium on the percent of day 9 blastocysts which

were completely hatched from their zona pellucidae. *Panel C:* The percent of blastocysts grown in TeSR medium which showed evidence of PE migration on day 9 and 10. Different superscripts or ** indicate differences within each panel ($P<0.05$).

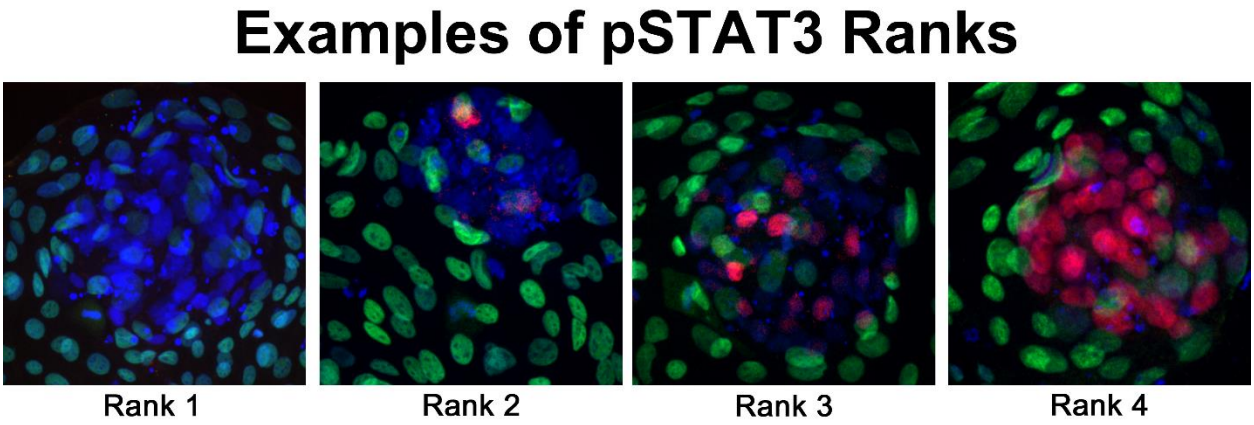

Figure 4-6. Example images of ICM pSTAT3 ranks. Trophectoderm cells are labeled in green (CDX2). ICM cells are blue (DAPI; $CDX2^{-}$). $pSTAT3^{Y705+}$ cells are labeled in red. A rank of 1 indicated no ICM cells were positive for $pSTAT3^{Y705}$. A rank of 2 indicated <5 cells were positive. A rank of 3 indicated >5, but not all, ICM cells were positive. A rank of 4 indicated all ICM cells were positive.

Table 4-1. Day 9 and 10 cell numbers from blastocysts grown in SOF-BEI or TeSR, with or without IL6.

COMPARISON	MEDIA	IL6	DAY	N	ICM	HYPO	EPI	UN	TE	TOTAL
1	SOF-BEI	0 ng/ml	9	26	32.2 ± 4.7 (a)	19.5 ± 2.9 (a)	11.0 ± 2.0 (a)	1.7 ± 0.7 (a)	138.8 ± 11.3 (a)	170.9 ± 15.2 (a)
	TeSR	0 ng/ml	9	36	95 ± 10.1 (b)	63.2 ± 7.0 (b)	29.1 ± 3.8 (b)	2.6 ± 0.7 (a)	182.6 ± 11.3 (b)	277.6 ± 15.5 (b)
2	SOF-BEI	Combined	10	10	39.1 ± 8.8 (a)	22.2 ± 5.2 (a)	16.4 ± 5.5 (a)	0.5 ± 0.3 (a)	187.2 ± 16 (a)	226.3 ± 20.1 (a)
	TeSR	Combined	10	19	135.5 ± 26.3 (b)	98.6 ± 18.2 (b)	35.1 ± 9.4 (a)	1.9 ± 0.8 (a)	588.1 ± 61.7 (b)	723.6 ± 73.2 (b)
3	SOF-BEI	0 ng/ml	9	26	32.2 ± 4.7 (a)	19.5 ± 2.9 (a)	11.0 ± 2.0 (a)	1.7 ± 0.7 (a)	138.8 ± 11.3 (a)	170.9 ± 15.2 (a)
	SOF-BEI	100 ng/ml	9	36	50.6 ± 3.9 (b)	31.9 ± 2.3 (b)	13.0 ± 1.7 (a)	5.7 ± 0.9 (b)	139.5 ± 8.3 (a)	190.1 ± 10.5 (a)
4	TeSR	0 ng/ml	9	36	95 ± 10.1 (a)	63.2 ± 7.0 (a)	29.1 ± 3.8 (a)	2.6 ± 0.7 (a)	182.6 ± 11.3 (a)	277.6 ± 15.5 (a)
	TeSR	100 ng/ml	9	40	107.3 ± 10.6 (a)	72.6 ± 6.7 (a)	27.0 ± 3.7 (a)	7.7 ± 1.4 (b)	215.2 ± 12.2 (b*)	322.4 ± 17.7 (b*)
5	SOF-BEI	Combined	9	62	42.9 ± 3.2 (a)	26.7 ± 2.0 (a)	12.2 ± 1.3 (a)	4.0 ± 0.7 (a)	139.2 ± 6.7 (a)	182.0 ± 8.8 (a)
	SOF-BEI	Combined	10	10	39.1 ± 8.8 (a)	22.2 ± 5.2 (a)	16.4 ± 5.5 (a)	0.5 ± 0.3 (b)	187.2 ± 16 (b*)	226.3 ± 20.1 (a)
6	TeSR	Combined	9	76	101.4 ± 7.3 (a)	68.2 ± 4.8 (a)	28.0 ± 2.6 (a)	5.3 ± 0.9 (a)	199.8 ± 8.5 (a)	301.2 ± 12.1 (a)
	TeSR	Combined	10	19	135.5 ± 26.3 (a)	98.6 ± 18.2 (b*)	35.1 ± 9.4 (a)	1.9 ± 0.8 (b)	588.1 ± 61.7 (b)	723.6 ± 73.2 (b)

In the IL6 column, “Combined” indicates that both the 0 and 100 ng/ml IL6 treatments were grouped together for this specific analysis. Different superscripts within each comparison denote differences. Significance established at $P<0.05$. Asterisks indicate a tendency $0.05<P<0.1$. Data shown are means ± SEMs. For all day 9 groups, 4 replicates were completed. For all day 10 groups, 2 replicates were completed.

Table 4-2. Formulation of the TeSR medium used

Component	Amount	Catalog Information
TeSR-E6	Base, 80ml/100ml needed	Stemcell Technologies; 5946
5x E6 Supplement	1x	Stemcell Technologies; 5946
GABA	10mg/100ml	Sigma; A2129
Glutamax	1x	ThermoFisher; 35050-061
100x MEM NEAA	1x	ThermoFisher; 1140050
Lithium Chloride	4.236 mg/100ml	Sigma; L4408
Chemically-defined lipid concentrate	200 ul/100ml	ThermoFisher; 11905-031
Glutathione	20 mg/100ml	Sigma; G6013
Thiamine	66.7 mg/100ml	Sigma; T1270
1000x Trace elements B	1x	VWR; 89422-908
1000x Trace elements C	1x	VWR; 89422-910
2-Mercaptoethanol	55 µM	ThermoFisher; 21985-023
Pipecolic acid	0.013 mg/100 ml	MP Biomedicals; 0215189880
FGF2	20 ng/ml	R&D Systems; 233-FB-025
IWR1	2.5 µM	Selleckchem; S7086
BSA	13.4 mg/ml	MP Biomedicals; 219989925

Chapter 5: Leukemia inhibitory factor does not stimulate STAT3 activity in the bovine ICM, nor does it increase inner cell mass cell number

Introduction

This group recently reported that supplementation of interleukin-6 (IL6) to in vitro produced (IVP) bovine embryos increases blastocyst inner cell mass (ICM) cell number [8,309]. This finding was significant because bovine IVP embryos are known to have abnormally few cells in the ICM [237], and the ICM often degenerates after transfer [244–246,265,266]. This problem likely contributes to a significant percentage of pregnancy losses of IVP bovine embryos [244]. Additionally, we have shown that IL6 stimulates STAT3 in bovine embryos, and specifically within the ICM cells of blastocysts [309]. STAT3 is a transcription factor, important for the expression of various pluripotency factors and the maintenance of murine embryonic stem cells (ESCs), cells derived from the ICM of murine blastocysts [122,124]. Functional STAT3 is needed to support the bovine blastocyst ICM and was necessary for IL6 to increase ICM cell numbers [289,309]. However, factors other than IL6 are also capable of activating STAT3, indicating other proteins may also substitute for IL6 in IVP bovine blastocyst culture.

IL6 is only one member of the IL6 family of cytokines. Each member of the IL6 family induces intracellular signaling via a hetero-complex of an alpha and a beta receptor. The beta receptor, IL6ST (a.k.a. gp130), is common to all family members, and contains intracellular signal-transducing regions, including binding sites for janus kinases (JAKs), which activate the STATs, including STAT3 [268]. The alpha receptor subunits are ligand specific, though a few are shared by more than one family member [268]. We previously reported on the presence of

other IL6 family alpha receptor subunit transcripts in bovine blastocysts, in which we noted the presence of leukemia inhibitor factor's (LIF) receptor (LIFR) [8]. LIF is known to activate STAT3 in murine ESCs [122], and thus could likely mimic the effects of IL6 if supplemented to bovine IVP embryos.

Recombinant human and murine versions of LIF have previously been supplemented to bovine embryos and have produced disparate effects on blastocyst formation and cell number [58,126–129,131]. The reason for these contradictory results may be in the use of a human or murine LIF, which are only 89 and 73% similar to bovine LIF by amino acid sequence. Due to the recent availability of a recombinant bovine LIF, we decided to test the ability of LIF to activate STAT3 in bovine IVP embryos and increase ICM cell numbers in blastocysts. We hypothesized that LIF would activate STAT3 in ICM cells and increase ICM cell number.

Materials and Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials that followed humane slaughter practices according to USDA guidelines. Unless specified otherwise, reagents were purchased from Thermo Fisher Chemical Company (Waltham, MA).

In vitro Embryo Production

Bovine embryos were produced by in vitro maturation, fertilization and culture procedures described previously [269,309]. Cumulus-oocyte complexes (COCs) were harvested from both beef and dairy ovaries purchased from Brown Packing Company (Gaffney, SC, USA)

and transported to the laboratory in 0.9% [w/v] saline containing antibiotic-antimycotic mix (ABAM; 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B). Upon arrival, COCs were collected by slashing follicles and placed in groups of 20-35 in 500 µl maturation media covered in paraffin oil (Ovoil, Vitrolife, Göteborg, Sweden), as described previously [309]. The COCs were matured for 21 to 24 hours at 38.5°C in 5% CO_2 in humidified air. For fertilization, the COCs were washed in HEPES-SOF before being placed in groups of 150-200 in 3 ml SOF-FERT covered in paraffin oil [73,270]. Sperm were isolated using a BoviPure™ density gradient (Nidacon; Spectrum Technologies Healdsburg, CA, USA), washed once in SOF-FERT, and then added to the fertilization media at a concentration of 1 million sperm/ml fertilization media. Sperm and COC coincubation lasted 14 to 18 hours (overnight) at 38.5°C in 5% CO_2 in humidified air. The following morning, presumptive zygote-cumulus complexes were denuded by gentle pipetting and then washed in HEPES-SOF before being placed in groups of approximately 25 in droplets of 50 µl of SOF-BE1 covered by paraffin oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 in humidified air [146].

Treatment Preparation

A concentrated stock of LIF (recombinant bovine; Kingfisher Biotech, St. Paul, MN) or IL6 (recombinant bovine; Kingfisher Biotech) was prepared for each protein at 10 µg/ml in SOF containing 1% [w/v] bovine serum albumin (BSA). Control treatment stocks did not contain LIF or IL6. All stocks were frozen in single-use aliquots at -80°C. Treatments were administered at various time points, as described below, by the addition of 2 µl of SOF-BE1 containing concentrated treatment or carrier to the existing droplet of SOF-BEI, to achieve an in-droplet final concentration of 100 ng/ml (or 0 ng/ml for controls).

LIF and STAT3 Activation

On day 5, early morulae (n=3-5 embryos/treatment; 2 replicates) were exposed to 0 or 100 ng/ml recombinant bovine LIF for 30 minutes before being fixed for immunofluorescent analysis of active pSTAT3^{Y705} (see below for methods), as previously done with IL6 [309]. Additionally, day 8 (blastocysts) (n=3-5 blastocysts/treatment) were exposed to 0 or 100 ng/ml recombinant bovine LIF for 30 minutes and then were fixed and processed for pSTAT3^{Y705} and CDX2 immunofluorescent staining, as described below.

LIF's Effect on Day 8 Blastocyst Cell Numbers

On day 5, embryos were treated with either 100 ng/ml LIF, 100 ng/ml IL6, or carrier only (n=18-32 embryos/drop; 2-4 drops/treatment/replicate; 7 replicates). Blastocyst formation was recorded on days 7 and 8 post-fertilization. Day 8 blastocysts (n=33-36 blastocysts/treatment over 7 replicates) were processed for immunofluorescent staining of CDX2 and cell counting, as described below.

Immunofluorescence

Bovine embryos were process for STAT3 and/or CDX2 immunostaining as described previously [309]. Briefly, the embryos were washed in PBS-PVP (Dulbecco's PBS (DBPS) containing 0.2% [v/w] polyvinylpyrrolidone) and fixed in 4% [w/v] paraformaldehyde for 15-20 minutes at room temperature. The embryos were then either processed immediately for staining of CDX2 and/or pSTAT3^{Y705} or were stored in PBS-PVP at 4°C for up to one week. One of two different protocols, described below, were used, depending on the target protein(s).

For CDX2 staining alone, the blastocysts were permeabilized in 0.25% Triton-X for 20 minutes, before blocking for 1 hour in 10% [v/v] horse serum. The primary antibody was mouse anti-CDX2 (Biogenex, San Ramon, CA, AM392-5M; sold ready-to-use). The secondary antibody used was a donkey anti-mouse IgG secondary (Alexafluor 488 or 647; 1:200).

For any staining for pSTAT3^{Y705}, including CDX2 and pSTAT3^{Y705} co-staining, the following protocol was used. The embryos were incubated for 5 minutes in room temperature 70% ethanol, before additional permeabilization and blocking in 10% [v/v] horse serum containing 0.5% [v/v] Triton-X for 1 hour. The embryos were then either incubated with either rabbit polyclonal IgG anti-pSTAT3^{Y705} (Cell Signaling Technologies; 9145T; 1:100) alone, or a mix of the same anti-pSTAT3^{Y705} antibody diluted in the CDX2 antibody described above. The secondary antibodies were either used singularly or in a mix, as needed, and were donkey anti-mouse IgG and anti-rabbit IgG Alexafluors (555 for STAT3, and 647 or 488 for CDX2; both 1:200).

At the end of each staining protocol, the embryos were incubated with 1 µg/ml DAPI for 5 minutes and then flattened on a glass slide lined with petroleum jelly, or not flattened for z-series acquisition. Staining was visualized with an Eclipse Ti-E inverted microscope equipped with an X-cite 120 epifluorescence illumination system and DS-L3 digital camera. Images were captured with NIS-Elements Software (Nikon Instruments, Melville, NY). Z-series images were "flattened" into one plane of focus by an extended depth of field module (Nikon Instruments).

Cell Counting

Differential embryo staining was completed using CDX2 and DAPI staining as described above (Wooldridge and Ealy 2019). The program, FIJI (ImageJ) was used to label and record

individual nuclei by utilizing the cell counter plugin to count nuclei [273]. Nuclei positive for CDX2 were considered TE, while $DAPI^+$ $CDX2^-$ nuclei were considered ICM.

qRT-PCR

Pools of 10 untreated embryos were collected on day 5 (early morulae) and 8 (blastocysts) (n=4 replicates; total 4-5 pools per stage). Their zona pellucidae were removed by exposure to acidic Tyrode's solution (Sigma-Aldrich, St. Louis, MO, USA), and the embryos were snap frozen in ≤ 6 µl PBS-PVP and stored at -80°C. Total RNA was extracted by using the Qiagen RNeasy Micro Kit (Qiagen, Germantown, MD). The entire RNA sample was incubated with RNase-free DNAse I (Applied Biosystems, Inc.) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.). Negative controls did not receive reverse transcriptase. For the final reaction, SybrGreen PCR Master Mix (Applied Biosystems, Inc.) was mixed with RT product and 250 nM concentration of forward and reverse primers. For the cycle, an activation/denaturation (95°) step was set for 10 minutes and followed by a two-step amplification sequence set for 50 cycles (95°C for 15 s, 57°C for 1 min). Each sample and primer combination were run in triplicate. The LIFR primer pair (see Supplementary Table 5-1 for primer list) was identified using the Primer-BLAST Program from the National Center for Biotechnology Information (U.S. National Library of Medicine, Bethesda, MD). The IL6ST and SDHA primer pairs were published previously [8,271]. All primers were synthesized by Integrated DNA Technologies (IDT; San Diego, CA). Primer efficiency standard curve analysis was completed to verify adequate primer efficiency (≥ 90% efficiency). Dissociation curve analysis (60 to 95°C) was completed after each PCR amplification to confirm the presence of one amplicon. *SDHA* was used as a housekeeping gene based on previously verified stability

across early embryonic stages [271]. The $2^{[-ddCt]}$ approach was used to generate mRNA abundances relative to the day 5 findings.

Statistical Analyses

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (Proc GLM; SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). Individual comparisons were partitioned further, when necessary, by using the Probability of difference (PDIFF) test of SAS. Relative mRNA abundance data were log-transformed before analysis, but data is presented as fold change with standard error. For each embryo development study, the replicate was used as the experimental unit. This replicate refers to the mean developmental percentages observed on each in vitro embryo production occasion (*i.e.* each IVP run). Replicate was considered a random independent variable for all blastocyst formation analyses. Percentage data (*e.g.* blastocyst formation rates) were arcsine-transformed before analysis but are presented as non-transformed means and SEM. The Tukey honestly significant difference test was used for all blastocyst formation data. Blastomere numbers and differential staining analysis used individual embryos as the experimental unit. Statistical significance was determined at $P \leq 0.05$.

Results

LIF Activates STAT3 in Day 5 but Not Day 8 Embryos

We previously reported that IL6 activates STAT3 in all or most cells of day 5 bovine embryos, and specifically within the ICM of day 8 blastocysts [309]. Because LIF is an IL6 family member and is known to activate STAT3 in mouse ESCs, we utilized a commercially

available recombinant bovine LIF to examine its ability to stimulate STAT3 in day 5 and 8 bovine embryos. Like our previous studies with IL6, LIF stimulated pSTAT3^{Y705} nuclear staining in all blastomeres of day 5 embryos (Fig. 5-1A). However, we could not detect an increase in pSTAT3^{Y705} nuclear localization in the ICM or TE of blastocysts on day 8 with LIF treatment (Fig. 5-1B). For comparison with an IL6-treated blastocyst, see Chapter 3 Figure 3-3.

LIF Increases Day 8 Advanced Blastocyst Formation but Not Cell Number

Because IL6 is known to increase ICM cell number in bovine blastocysts, we next tested the effects of LIF on blastocyst cell number [8,309]. Additionally, we recorded the percentage of embryos which formed blastocysts after treatment for comparison. On day 7, LIF supplementation did not affect blastocyst formation (Fig 5-2A). On day 8, total blastocyst formation was not affected by LIF but the percentage of advanced blastocysts was greater ($P<0.05$) in both LIF and IL6-supplemented embryos than controls (Fig 5-2B). Treatment with LIF did not affect total, ICM or TE blastomere numbers (Fig. 5-2B) in day 8 blastocysts. Supplementation with LIF also did not affect the ICM:TE ratio (Fig. 5-2C). By comparison, supplementation with IL6 increased ($P<0.05$) ICM cell numbers and the ICM:TE ratio but not TE numbers (Fig. 5-2B-C).

Transcripts for LIFR Decrease from Day 5 to Day 8

Next, we speculated that receptor downregulation might be a cause for the loss of pSTAT3^{Y705} induction with LIF treatment between day 5 and 8 embryos. To determine this, we analyzed LIFR and IL6ST transcript abundance in day 5 and 8 embryos. The relative abundance

of *LIFR* mRNA was greatly reduced ($P < 0.05$) between day 5 and 8 (Fig. 5-3). Transcripts for *IL6ST* were unaffected by embryonic stage (Fig. 5-3).

Discussion

This work was undertaken to examine the ability of LIF to active STAT3 and increase ICM cell numbers in IVP bovine embryos, as IL6 has been shown to do [8,309]. Several studies examining the effect of LIF on bovine blastocyst formation and cell number have already been published, however some studies showed positive effects, some showed no effects, and some showed negative effects of LIF [58,126–129,131]. These studies all utilized murine or human variants of LIF, so, in this work, we utilized a commercially available recombinant bovine LIF to clarify the effects of LIF on bovine IVP embryos.

For the first study, we utilized 100 ng/ml LIF because we previously found that 100 ng/ml IL6 was needed to increase ICM cell number, and IL6 and LIF are similar in mass (19.8 kDa versus 20.7 kDa, respectively)[8]. Indeed, we confirmed this amount of LIF was able to activate STAT3 in day 5 early morulae. Unlike at day 5, however, the same dose of LIF did not visibly increase nuclear $pSTAT3^{Y705}$ in blastocyst ICMs. However, 100 ng/ml LIF did not increase day 8 blastocyst cell number, unlike 100 ng/ml IL6. This indicates that LIF cannot substitute for IL6 in bovine embryo culture. It is possible but unlikely that the LIF dose utilized was insufficient to detect an effect. The observance of STAT3 activation in day 5 embryos indicates this dose was biologically active

However, 100 ng/ml LIF did increase the percentage of advanced (expanded and hatched) blastocysts on day 8. Because expansion and hatching are largely driven by the TE, this suggests LIF may have some effect on the TE. However, because we observed no STAT3

activity in the TE after LIF stimulation, LIF is likely working through another pathway, such as the MAPK or PI3K pathways to increase expansion and hatching [121].

The most likely cause of this change in LIF responsiveness was a decrease in receptor abundance. We detected a marked decline in *LIFR* transcript abundance between day 5 and 8 embryos, while IL6ST, the IL6 family common subunit, was unchanged. Additionally, while we previously reported the presence of LIFR transcripts in day 8 blastocysts, the number of transcripts was very low, which is reflective of our findings here [309]. To further complicate matters, it remains unclear if LIF will act similarly in IVP and *in vivo*-produced bovine embryos. Transcripts for the *LIFR* were detected in IVP bovine embryos but were absent in *in vivo* produced embryos [125]. However, we did not assess LIFR protein levels to confirm a decrease in actual receptor abundance, but, taken with the loss of STAT3 activation between day 5 and 8, the protein was likely downregulated. However, we detected an effect of LIF on the percentage of day 8 advanced blastocysts, which indicates the presence of some LIFR subunits. Perhaps LIFR is more abundant in TE rather than ICM cells.

In summary, LIF does not mimic the effects of IL6 on bovine preimplantation embryos. A dose of 100 ng/ml LIF could stimulate pSTAT3^{Y705} in day 5 early morulae, but not in day 8 blastocysts. Additionally, LIF did not increase ICM cell numbers in day 8 blastocysts. This lack of effects of LIF on blastocysts is likely caused by low LIFR abundance at the blastocyst stage.

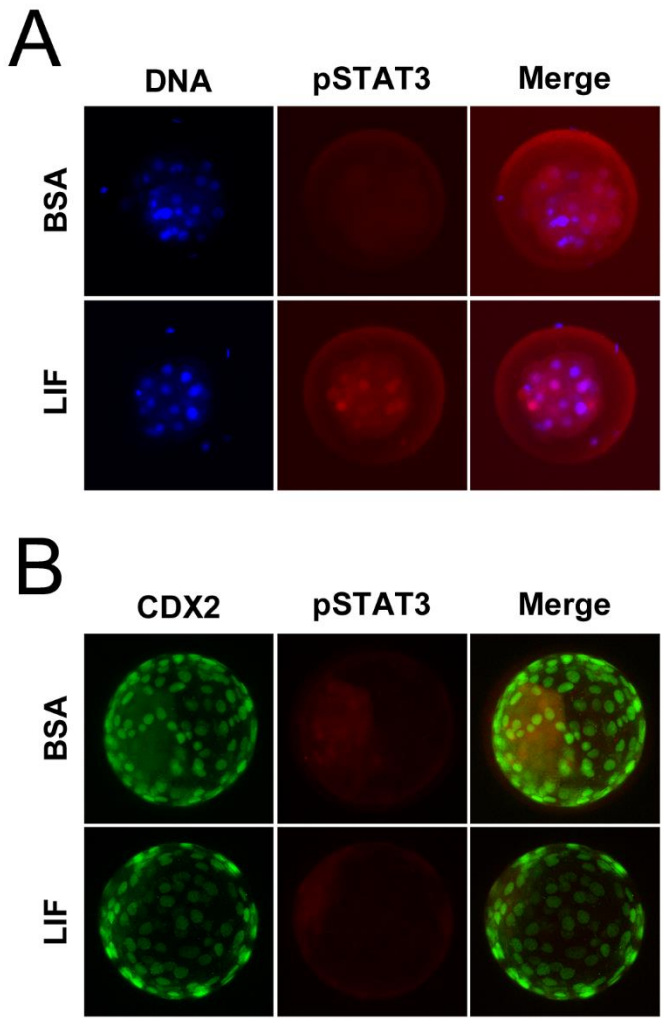

Figure 5-1. STAT3 activation profiles for LIF-treated bovine embryos. *Panels A-B*: Representative images of embryos from day 5 (>8-cell) and day 8 (blastocysts) receiving either 0 (BSA only) or 100 ng/ml LIF for 30 minutes before immunostaining to detect $pSTAT3^{+}$ (red) and total nuclei (DAPI, blue). *Panel A:* Representative day 5 embryos. *Panel B:* Representative day 8 embryos.

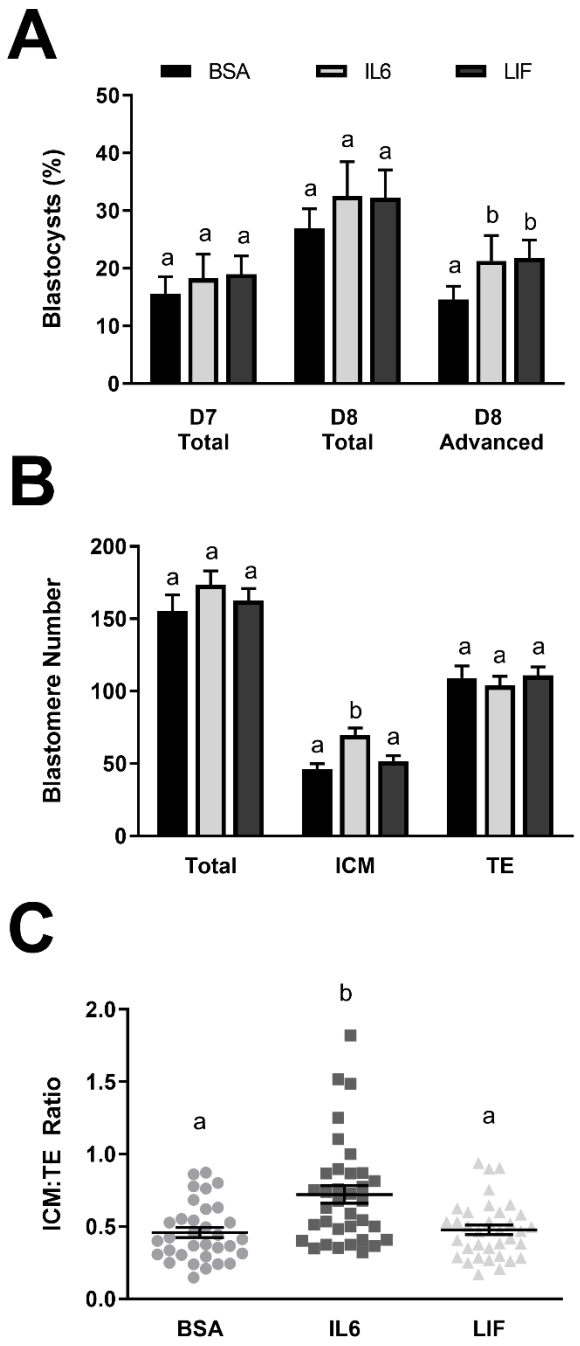

Figure 5-2. Comparison of LIF versus IL6 supplementation on blastocyst formation and cell numbers. *Panel A*: Effects of IL6 and LIF supplementation total blastocyst formation (as a percentage of cleaved embryos) at day 7 and total and advanced blastocysts (includes expanded and hatched blastocysts) formation at day 8. *Panel B*: Effects of IL6 and LIF supplementation on total, ICM and TE cell numbers in day 8 blastocysts. *Panel C*: ICM:TE ratios of day 8 blastocysts. Different superscripts within each panel indicates differences (P<0.05).

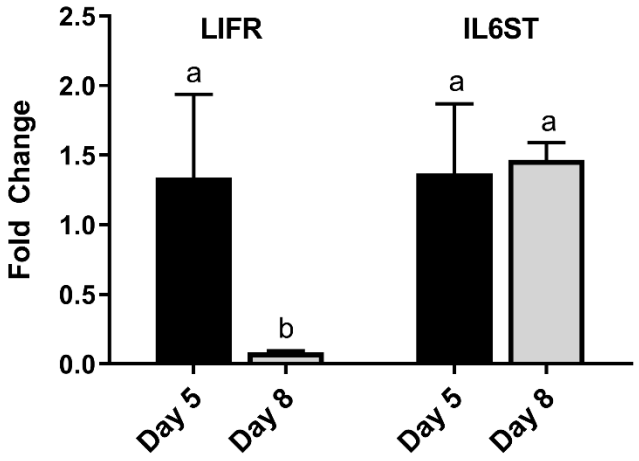

Figure 5-3. Changes in *LIFR* and *IL6ST* transcript abundance between day 5 embryos and day 8 blastocysts.

Table 5-1. Primers used for quantitative RT-PCR.

Gene	Reference or Genbank accession no.	Primer sequence (5′ - 3′)	Product size (bp)
LIFR	NM_001192263.3	**Forward:** AGAGCACTTTTTGGAGCAGGT **Reverse:** AACAAGTACGGGACCGCTTT	70
IL6ST	[8]	**Forward:** GTCTCATGCTCACGGCACTA **Reverse:** CGCGTCTGATTTGCCAACAA	220
SDHA	[271]	**Forward:** GCAGAACCTGATGCTTTGTG **Reverse:** CGTAGGAGAGCGTGTGCTT	185

Chapter 6: JAK2/STAT activity is not required for inner cell mass formation in bovine blastocysts but is required for hypoblast maintenance

Introduction

In vitro produced (IVP) bovine blastocysts are plagued by low pregnancy retention after transfer (~30-40%) [116,264]. This problem, at least in part, is likely attributable to the health of the inner cell mass (ICM), the population of cells in the blastocyst which will produce the calf. In comparison to *in vivo* produced blastocysts, IVP have fewer cells in their ICMs, and this appears to cause problems post-transfer [237]. After transfer to a recipient cow, the cells of the ICM will form what is known as the embryonic disk (ED) around day 12. Detection of EDs via stereomicroscopy is alarmingly low (25-83%) in IVP bovine embryos examined at day 14-16 [59,244–246,265,266,317], and conceptuses with poor quality or no ED fail to establish pregnancy at a much higher rate than conceptuses with quality EDs [244]. Unfortunately, the specific factors controlling ICM formation and maintenance in bovine embryos are largely unknown, thus limiting our ability to improve IVP ICM health. However, we recently uncovered that IL6 nearly doubles the blastomere count of the ICM in IVP blastocysts [8,309]. Further, immunofluorescent analysis revealed that IL6 induced activation of the Janus-activated kinase and Signal transducer and activator of transcription 3 (JAK/STAT3) pathway specifically within the ICM of blastocysts [309]. We also found that, if this pathway is inhibited, resultant blastocysts have zero to very few ICM cells, indicating that the STAT3 pathway is very important for bovine ICM development, but the exact time period in which STAT3 activity is required, is unknown [309].

In mice, the relationship of STAT3 and the ICM is well-characterized. STAT3-null embryos are early embryonic lethal, degenerating soon after the blastocyst stage [243,248]. Under closer examination, these STAT3-null embryos initially have an apparently normal ICM, however it rapidly degenerates, indicating that STAT3 is required for ICM maintenance, and not formation, in mice [248]. Our previous report on bovine embryos showed little evidence that a normal ICM ever formed with STAT3 inhibition; all blastocyst stages observed (regular, expanded, or hatched) had only a few or zero ICM cells [309]. If STAT3 were only involved in ICM maintenance, like in the mouse, then we would expect a decrease in ICM number with advancing blastocyst stage after AZD1480 treatment. Because of these observations, we hypothesize that STAT3 is required for ICM formation in bovine blastocysts. Additionally, we have found that IL6 largely stimulates STAT3 in hypoblast (HYPO) cells rather than epiblast (EPI) cells in later-stage blastocysts (Wooldridge and Ealy, Chapter 4, unpublished). Thus, we also hypothesize that STAT3 is required for HYPO maintenance. This work was designed to test these hypotheses.

Materials and Methods

No animals were used in these studies. Instead, studies were completed on slaughterhouse-derived materials from a commercial slaughterhouse that followed humane slaughter practices according to USDA guidelines. Reagents were purchased from ThermoFisher Chemical Company (Waltham, MA), unless otherwise specified.

In vitro Embryo Production

Bovine blastocysts were produced by in vitro maturation, fertilization and culture procedures described previously [8,310]. In brief, cumulus-oocytes complexes (COCs) were collected into

BoviPlus Oocyte Washing Medium (Minitube USA Inc., Verona, WI) by slashing follicles. For maturation, COCs were cultured in groups of 10 in 50 µl of maturation medium [8] covered in light mineral oil (Cooper Surgical Inc., Trumbull, CT, USA) for 21-24 hours, in 5% CO2 in humidified air at 38.5°C. After maturation, the COCs were washed in HEPES-SOF [73] before being placed in groups of 150-200 in 3 ml SOF-FERT [270] under light mineral oil. Spermatozoa were isolated from pooled semen from four Holstein bulls (donation from Select Sires, Plain City, OH, USA) using a biphasic gradient (40 and 80% [v/v] Bovipure™; Nidacon; Spectrum Technologies, Healdsburg, CA, USA). After washing once in SOF-FERT, sperm were added to the dish containing COCs at 1 million sperm/ml medium. Fertilization co-incubation occurred overnight (14-18 hours) in humidified air at 5% CO2 at 38.5°C. After denuding by gentle pipetting in HEPES-SOF, presumptive zygotes were placed in groups of approximately 25 in drops of 50 µl SOF-BEI [146] under light mineral oil. The embryos were then incubated in 5% CO2, 5% O2 and 90% N2 in humidified air at 38.5°C. Day of fertilization was designated as day 0. A stock solution of 100 mM AZD1480 (JAK 2 inhibitor; S2162; Selleck Chemicals, Houston, TX, USA) in DMSO was prepped beforehand and stored in single-use aliquots at -80°C.

ICM Formation Study

On day 4 post-fertilization, existing droplets of embryos in SOF-BEI were supplemented with either 1 µM AZD1480 or carrier only (0.001% DMSO). Treatments were administered to existing droplets via addition of 2 µl of treatment-concentrated SOF-BEI to achieve an in-drop concentration of 0 or 1 µM AZD1480. The embryos were then incubated until day 7 and 8 post-fertilization, when samples were collected for fixation and immunofluorescent analysis of SOX2,

an ICM marker [284], and CDX2, a TE marker [283,318], as described below. On each date, the sampled embryos were separated by stage of development. On day 7, morulae, early blastocysts (blastocoel cavity <50% of interior), regular blastocysts (blastocoel at least 50% of interior cavity, but no diameter expansion) and expanded blastocysts (diameter expansion) were sampled. On day 8, regular and expanded blastocysts were sampled. This study was replicated twice, with 21-45 embryos analyzed per treatment.

HYPO Maintenance Study

For this study, AZD1480 was administered to day 8 blastocysts. Blastocysts were produced as described above. On day 8 post-fertilization, treatments of 5 µM AZD1480 or carrier only (0.005% DMSO) were added to existing drops via 2 µl of treatment-concentrated SOF-BEI. After 18 hours, expanded and hatched blastocysts were collected and fixed for NANOG, GATA6 and CDX2 immuno-staining, as described below. This study was replicated 3 times, and 29-34 blastocysts were analyzed per treatment.

Whole ICM Maintenance Study

For this study, blastocysts were produced as described above, and treatment began on day 6.5 or 7.5. On day 6.5, a portion of blastocysts were collected and moved to new 50 µl droplets of SOF-BEI containing either 3 µM AZD1480 or carrier only (0.003% DMSO). The following day, day 7.5, the remaining blastocysts were collected and moved to new 50 µl droplets of SOF-BEI (separate from the day 6.5 droplets) containing the same treatments. Each droplet housed 5-10 blastocysts.

Within each start date, the treatment groups were as follows: 1) DMSO for 24 hours, 2) AZD1480 for 24 hours, 3) DMSO for 48 hours, 4) AZD1480 for 48 hours, 5) DMSO for 24 hours, followed by a washout and culture in fresh SOF-BEI (no treatment) for 24 hours, and 6) AZD1480 for 24 hours, followed by a washout and culture in fresh SOF-BEI (no treatment) for 24 hours. In total, there were 12 treatment groups, 6 per start date.

At the end of treatment, blastocysts were collected and fixed for NANOG, GATA6 and CDX2 immuno-staining, as described below. This study was replicated three times, with 14-25 blastocysts analyzed per treatment.

Immunofluorescence

Prior to fixation, embryos were collected and washed twice in Dulbecco's Phosphate Buffered Saline (DPBS), with 0.2% [w/v] polyvinylpyrrolidone (PBS-PVP). For fixation, the embryos were incubated in 4% [w/v] paraformaldehyde for 20 minutes at room temperature. After washing three more times in PBS-PVP, the embryos were either stored in PBS-PVP at 4°C for up to one week or were processed immediately. Depending on the study, one of the two protocols described below was used. For both protocols, the blocking buffer used was 10% horse serum, and the antibody buffer used was 1% horse serum. Additionally, following any antibody incubation step, the embryos were washed three times in wash buffer (DPBS containing 0.1% [w/v] BSA and 0.1% [v/v] Tween20).

For CDX2, NANOG and GATA6 triple-staining, blastocysts were first permeabilized for 30 minutes with 0.5% Triton-X in DPBS and blocked for one hour. Due to host-isotype overlap of the CDX2 and NANOG antibodies, two rounds of primary and secondary antibody incubations were completed. For the first round, a mix of rabbit polyclonal IgG anti-GATA6 (Cell Signaling

Technology, Danvers, MA; 5851T; 1:500) and mouse monoclonal IgG1 anti-NANOG (eBioscience; 14-5768-82; 1:200) was used for 1 hour at room temperature, and followed by incubation with a mix of donkey anti-rabbit IgG and donkey anti-mouse IgG secondary antibodies (Alexafluor 647 for NANOG, and 555 for GATA6; both 1:200). For the second round, mouse monoclonal IgG1 anti-CDX2 antibody (Biogenex, San Ramon, CA, AM392-5M, sold ready-to-use) was used for 1 hour at room temperature, followed by incubation with a donkey anti-mouse IgG secondary (Alexafluor 488; 1:500).

For SOX2 and CDX2 staining, embryos were first permeabilized for 20 minutes in 0.25% Triton-X in DPBS and then blocked for one hour. After blocking, the embryos were incubated in a mix of anti-CDX2 (same as above) and monoclonal rat anti-SOX2 antibodies (Thermofisher; 14-9811-80; 1:100 in CDX2 antibody solution) for either 1 hour at room temperature, or overnight at 4°C. After washing, the embryos were incubated with a mix of donkey anti-rat IgG and anti-mouse IgG secondary antibodies (Alexafluor 488 for SOX2 and 647 for CDX2; both 1:200).

Following the above protocols, the embryos were incubated with 1 µg/ml DAPI for 5 minutes at room temperature, washed in PBS-PVP, and flattened on a glass slide lined with petroleum jelly. Staining was visualized with an Eclipse Ti-E inverted microscope equipped with an X-cite 120 epifluorescence illumination system and DS-L3 digital camera. Images were captured with NIS-Elements Software (Nikon Instruments, Melville, NY), and cell counting was completed with the cell counter plugin in the program FIJI (ImageJ) [273]. Cells dual positive for GATA6 and NANOG were considered undifferentiated ICM (UN) cells. In studies utilizing the CDX2, NANOG and GATA6 staining protocol, some secondary mis-binding occurred between the CDX2 secondary and the NANOG primary, due to host-isotype overlap. To ensure our cell

counts were accurate, cells which were $CDX2^+$:$NANOG^+$ were identified and labeled as EPI or UN, and not TE cells.

Statistical Analyses

All cell count analyses were completed by least-squares ANOVA using the general linear model (Proc GLM) of the Statistical Analysis System (SAS for Windows; SAS Institute Inc., Cary, NC, USA). For all data, the individual blastocyst was considered the experimental unit. Replicate was considered a random independent variable. Individual comparisons were partitioned further by using the Probability of Difference (PDIFF) test. Statistical significance was determined at $P \leq 0.05$.

Results

JAK2/STAT activity is Not Required for ICM Formation

Due to the severe effects of JAK2 inhibition on bovine ICM cell number, we hypothesized that JAK2/STAT is important for ICM formation in cattle [289,309]. To test this hypothesis, we treated IVP bovine embryos with either 1 µM AZD1480 or carrier only (DMSO) from day 4 to 7 or 8 post-fertilization and examined ICM (SOX2) and TE (CDX2) marker localization in morulae, early blastocysts, regular blastocysts and expanded blastocysts.

For the control embryos, SOX2 staining was present and vibrant in all cells of morulae, while CDX2 was weakly visible in some cells (Fig. 6-1). In early blastocysts, SOX2 was again visible in all cells, though somewhat weaker staining was observed in TE cells and CDX2 staining was considerably more robust than at the morula stage and localized to only some cells, the presumptive TE (Fig. 6-1). In regular blastocysts, SOX2 was still generally present in all cells,

though the staining was now considerably weaker in TE cells, whereas CDX2 staining was more vibrant in TE (Fig. 6-1). Finally, expanded blastocysts showed little to no SOX2 staining in TE cells but maintained vibrant staining in the ICM, while CDX2 staining was exclusive to the TE (Fig. 6-1).

When we examined the JAK inhibited embryos, we observed remarkably similar SOX2 and CDX2 staining patterns, indicating that JAK2/STAT inhibition does not inhibit ICM formation. However, we did note JAK inhibited embryos had fewer ICM cells. To confirm, we counted the number of $SOX2^{+}$:$CDX2^{-}$ cells in regular and expanded day 7 and 8 blastocysts and found that 1 µM AZD1480 did indeed reduce ($P<0.05$) ICM cell number at both time points (Fig. 6-2A). Examples of blastocysts treated with AZD1480 can be seen in Fig. 6-2B.

JAK2/STAT Activity is Necessary for Hypoblast Maintenance

Because we previously observed that IL6 increased HYPO cell numbers and primarily activated STAT3 in HYPO cells (Wooldridge and Ealy, Chapter 4, unpublished), we hypothesized that JAK2/STAT inhibition in blastocysts with committed ICM cells (day 8) would result in a loss of only hypoblast cells. To test this hypothesis, we treated day 8 blastocysts with 5 µM ADZ1480 or carrier only (DMSO) for 18 hours. Remarkably, JAK2/STAT inhibition resulted in a decrease ($P<0.01$) in ICM, HYPO and total cell numbers (Fig. 6-3). However, the EPI cell population was unaffected ($P>0.05$) and the number of UN cells was increased ($P<0.05$) after JAK inhibition (Fig. 6-3). TE cell number was also reduced ($P<0.01$) with AZD1480 treatment (115.2 ± 5.4 vs. 130.2 ± 7.5 cells in the controls).

JAK2/STAT Inhibition Predominantly Affects Hypoblast Cells

Because our previous results indicate near-total ICM annihilation after JAK2 inhibition [309], and our evidence herein that late JAK2 inhibition only affects HYPO cells, we next hypothesized that earlier JAK inhibition would disrupt EPI maintenance if supplemented before lineage commitment. To test this hypothesis, we collected early- (day 6.5) and late- (day 7.5) stage blastocysts and treated them with 3 µM AZD1480 or DMSO for 24 or 48 hours. We utilized 3 µM in this study rather than 5 µM because we became aware of some evidence that 5 µM can inhibit aurora kinases [319], so we decreased the dose. For these analyses, pairwise comparisons were made between treatments at each timepoint.

In all situations, treatment with AZD1480 reduced ($P<0.05$) ICM and hypoblast cell number (Fig. 6-4A-B). EPI cell number was unaffected by treatment in the day 6.5 dataset and after 24 hours of treatment in the day 7.5 blastocysts, but a decrease ($P<0.05$) in EPI cell number was observed at the 48-hour timepoint in the day 7.5 dataset (Fig. 6-4C). Treatment with AZD1480 increased UN cell number ($P<0.01$) after 48 hours of treatment in the day 7.5 group but had no effect at any other time (Fig. 6-4D). Additionally, AZD1480 decreased ($P<0.05$) TE cell number only in day 6.5 blastocysts treated for 48 hours but had no effect at any other time (Fig. 6-4E). An example of a blastocyst treated with DMSO verses a blastocyst treated with AZD1480 can be seen in Figure 6-5.

Blastocyst Age has Some Impact on Cell Numbers

We next utilized the ICM maintenance dataset to analyze the effect of blastocyst age on cell number by comparing day 7.5, 8.5 and 9.5 blastocysts (sampling age) treated with only DMSO (24 or 48 hours). Unlike above, this analysis combined blastocysts from both treatment start-

dates, based on sampling age. We have previously reported that ICM cell number declines after day 8 in IVP embryos grown in SOF-BEI (Wooldridge and Ealy, Chapter 4, unpublished), thus we were concerned some effects observed herein may be due, at least in part, to blastocyst age.

There was an overall effect of age on ICM, HYPO, and UN cell number, but there was no effect on TE. Additionally, age affected total and EPI cell number. In more detail, ICM cell number decreased ($P<0.05$) from day 7.5 to 8.5, 8.5 to 9.5, and 7.5 to 9.5 (Fig. 6-6). HYPO cell number decreased ($P<0.05$) from day 7.5 to days 8.5 or 9.5, but only tended ($P=0.09$) to decrease from day 8.5 to 9.5 (Fig. 6-6). EPI cell number did not differ from day 7.5 to 8.5 but decreased ($P<0.05$) from day 8.5 to 9.5 (Fig. 6-6). UN cell number decreased ($P<0.01$) after day 7.5, but there was no difference between day 8.5 and day 9.5 blastocysts (Fig. 6-6).

Older Blastocyst HYPO Cells Exhibit Impaired Recovery After JAK Inhibition

In addition to the treatments detailed above in the ICM maintenance study, some blastocysts were treated with DMSO or 3 µM AZD1480 for 24 hours, washed in standard SOF-BEI, and cultured in new 50 µl drops of SOF-BEI with no DMSO or AZD1480 for an additional 24 hours. This treatment was added to determine if AZD1480-treated blastocyst ICMs could ‘recover’ after treatment, or if the EPI might be lost independently of the presence of AZD1480, possibly due to the absence of HYPO cells. To analyze this data and account for differences simply caused by removal of DMSO, the average ICM, HYPO and EPI cell number was calculated for each treatment by exposure time (DMSO 48 hour, DMSO wash, AZD1480 48 hour and AZD1480 wash) per replicate. Then the average number of cells in the 48 hour groups were subtracted from the wash groups within their respective treatment to generate the average difference in cell

number between the DMSO and AZD1480 groups. An ANOVA was then performed on the differences in cell number.

No differences in the change in cell number after washout were observed between the control and AZD1480 groups in the day 6.5 dataset (Fig. 6-7A). In the day 7.5 dataset, there were no differences in ICM or EPI cell number recovery between treatments (Fig. 6-7B). However, AZD1480-treated blastocysts tended (P=0.09) to recover fewer HYPO cells than the DMSO group (Fig. 6-7B).

Discussion

Previous work indicated that the JAK2/STAT pathway is critical for proper bovine ICM development [289,309]. Inhibition of the pathway decimates the ICM, but whether it is necessary for ICM formation or simply its maintenance in cattle was unknown. This work was undertaken to pinpoint this role.

In order to determine if JAK2/STAT inhibition disrupts ICM formation, we examined ICM and TE marker localization from the morula to expanded blastocyst stages. If ICM formation was indeed prevented, then the cells trapped in the ICM area should aberrantly express TE markers [320]. We selected a lower concentration of AZD1480 (1 µM) than we previously reported [309] because 5 µM severally decreased blastocyst formation. Prior to use of this lower dose, we confirmed that it was sufficient to block most STAT3 activity after 100 ng/ml IL6 treatment (See Figure 6-8). To begin, we first characterized SOX2 and CDX2 localization in morulae, early blastocysts, regular blastocysts and expanded blastocysts. While SOX2 is a known ICM marker [284], and CDX2 is a known TE marker [283], the exact localization of these markers during the morula to expanded blastocyst stages has not been reported.

Unfortunately, we found that SOX2 is present in TE precursors in morulae, and in TE in blastocysts, however staining grew weaker and weaker from the early to expanded blastocyst stages, and it was often absent in expanded blastocyst TE. Because SOX2 was still present in the TE in most stages, it wasn't an ideal ICM marker, however we could not find a suitable substitute which marks both EPI and HYPO cells. In the end, the SOX2 and CDX2 markers appeared to behave the same in JAK inhibited embryos as compared to the controls, indicating that JAK2/STAT isn't required for bovine ICM formation, however this may need to be confirmed in the future with more robust markers.

We next confirmed our hypothesis that JAK2/STAT inhibition in late blastocysts (day 8) would result in a loss of HYPO cells without affecting EPI. We selected day 8 as the start point, because day 8 blastocyst ICMs should be largely committed to EPI or HYPO [73]. Intriguingly, after only 18 hours of JAK inhibition, the HYPO population was nearly completely lost, and the number of EPI cells was unaffected. This indicates that the HYPO cell population is very sensitive to JAK2/STAT perturbation, while the EPI is insensitive, in late-stage blastocysts.

At this point, we still had not answered what happened to the ICM in our previous study [309]. In these blastocysts, JAK2 inhibition left ICMs of only 0-4 cells, which indicates cells were lost from both lineages. Herein however we could not disrupt ICM formation or EPI maintenance with JAK2/STAT inhibition in day 8 blastocysts. Thus, we next hypothesized that JAK inhibition might only affect EPI cells if administered early, before ICM cells have committed to the HYPO or EPI fate. This led us to design a study starting JAK inhibition at two start-points, day 6.5, and day 7.5. Day 6.5 blastocysts are relatively young, and the majority of their ICMs should be UN [73]. We included day 7.5 blastocysts because we expected these blastocysts to behave the same as our day 8 inhibited study.

In both cohorts, JAK inhibition resulted in a loss of ICM cells, but the loss was mainly of HYPO cells. Additionally, we found that this effect of JAK2/STAT inhibition on HYPO cell number is largely irreversible, particularly in older blastocysts. After 24 hours of AZD1480 treatment and 24 hours of washout, ICM and HYPO cell numbers increased slightly in AZD1480-washout blastocysts, however this increase was caused by a removal of DMSO, and not AZD1480 (See Fig. 6-7). Interestingly though, in the day 7.5 blastocyst cohort, AZD1480-washout blastocysts tended to recover fewer HYPO cells than the DMSO washout group. Taken together, these data indicate the JAK2/STAT activity is critical for HYPO maintenance, and even short-term inhibition of this pathway in older blastocysts may have insurmountable effects on the HYPO.

The effect of JAK2/STAT inhibition on the EPI population, however, is uncertain. We detected some loss of EPI cells with longer AZD1480 treatment, but this effect was inconsistent and may be attributable to blastocyst age or length of time in culture. In the day 6.5 cohort, both treatments had similar numbers of EPI cells after 24 and 48 hours of treatment. Thus, our hypothesis that early JAK2 inhibition would result in a loss of EPI cells was incorrect. In contrast, the day 7.5 cohort showed reduced EPI cell number after 48 hours of treatment. However, we also showed that time decreased EPI cell number in day 9.5 DMSO-treated blastocysts. We have previously reported that blastocyst culture past day 8 in SOF-BEI has negative effects on the ICM (Wooldridge and Ealy, Chapter 4, unpublished). Therefore, blastocyst age may play some part in this loss of EPI cells in the day 7.5 cohort. Perhaps EPI cells are more sensitive to AZD1480 after longer in vitro culture. In conclusion, we cannot be certain about direct effects of JAK2/STAT inhibition on the EPI population in the bovine.

Interestingly though, JAK inhibition increased UN cell numbers in day 7.5 and 8 blastocysts. This increase, while significant, was not massive. Perhaps JAK inhibition can slow ICM differentiation somewhat. Although, to make things more confusing, IL6, which activates STAT3, also increases the number of UN cells (Wooldridge and Ealy, Chapter 4, unpublished).

In some but not all instances, AZD1480 treatment reduced TE cell number. Initially, in STAT3-null mouse embryos, the ICM degenerates before the whole embryo dies [248]. Perhaps this TE loss is a sign of early embryonic lethality in the bovine as well. However, because we inconsistently detected this effect, it may be caused by chance.

In order to determine the role of the JAK2/STAT pathway in bovine embryos, we utilized a JAK2 inhibitor, AZD1480, in all studies in this work. We, and others, previously used this inhibitor and have validated its ability to block STAT3 activation [289,309]. While this does not achieve specific STAT3 inhibition, we suspect the results presented here are a result of just that. These results are similar to STAT3-null mouse embryos, and no other STAT knockout, in mice, results in ICM degradation [243,248,321–328]. We attempted to utilize a STAT3-specific inhibitor, known as Stattic, but could not successfully block IL6-induced STAT3 activity with the range of concentrations we tested (0.5-10 µM). We abandoned its use due to toxicity effects.

In the end, we find it to be clear that JAK2, and likely STAT3 inhibition, is essential for maintenance of the HYPO lineage, and dispensable for ICM formation. It is possible that JAK activity is also necessary, either directly or indirectly, for EPI maintenance, but we cannot be entirely sure from the studies herein. Future studies using STAT3 knockouts would be best for confirming the exact roles of STAT3 on the EPI and HYPO population. This work dissects the importance of the JAK2/STAT pathway in bovine embryos and may prove useful as the framework for utilizing STAT3 manipulation to improve IVP bovine ICM health.

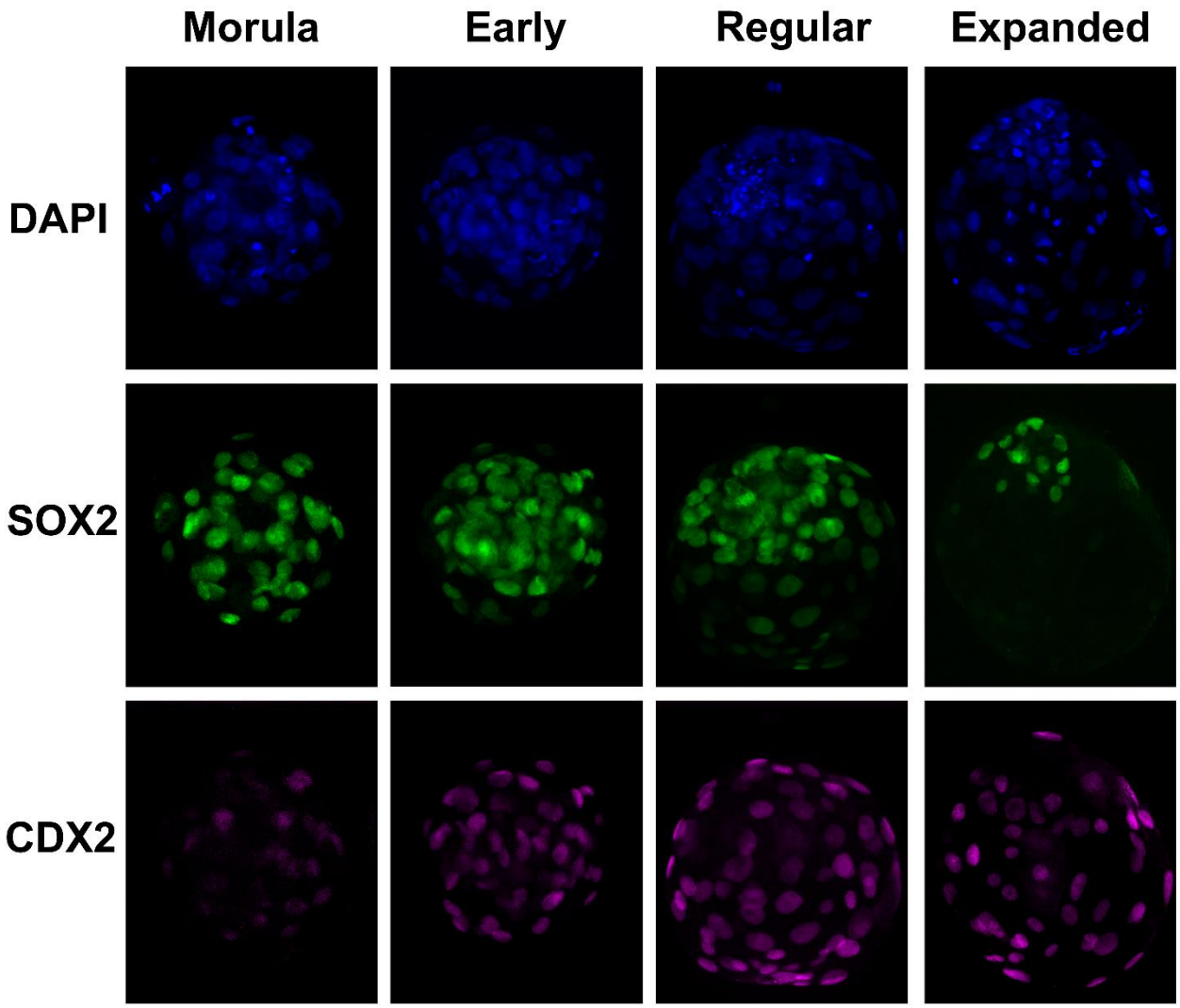

Figure 6-1. Localization of SOX2 and CDX2 in the morula to expanded blastocyst stages in bovine blastocysts. Morula and blastocysts received 0.001% DMSO from day 4-7 or 4-8. Each column shows a representative embryo of the labeled stage, while each row shows the indicated immunofluorescent label.

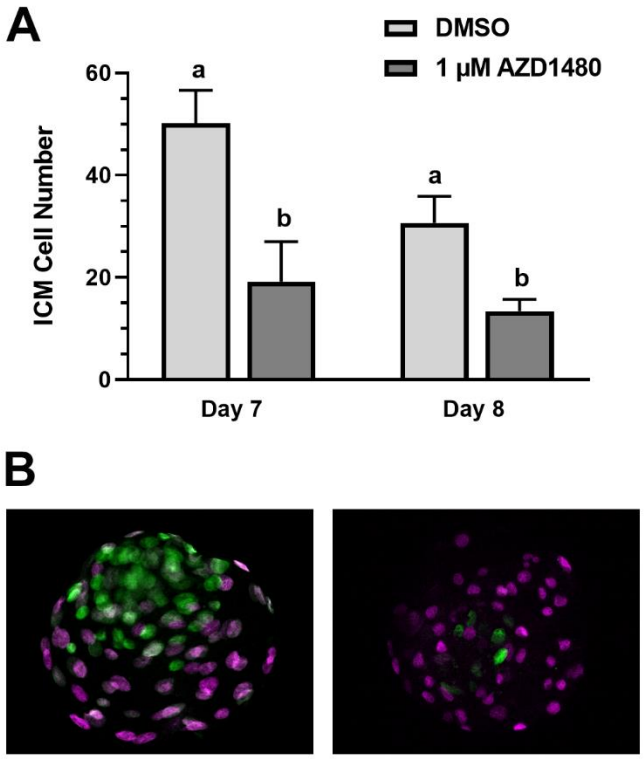

Figure 6-2. JAK2/STAT inhibition does not disrupt bovine ICM formation but does reduce ICM cell number. Blastocysts received 1 μM AZD1480 from day 4-7 or 4-8. *Panel A*: Average cell number of regular and expanded blastocysts sampled on day 7 or 8. Different superscripts indicate differences (P<0.05). *Panel B*: Examples of blastocysts treated with AZD1480. SOX2 is labeled in green and CDX2 is labeled in purple.

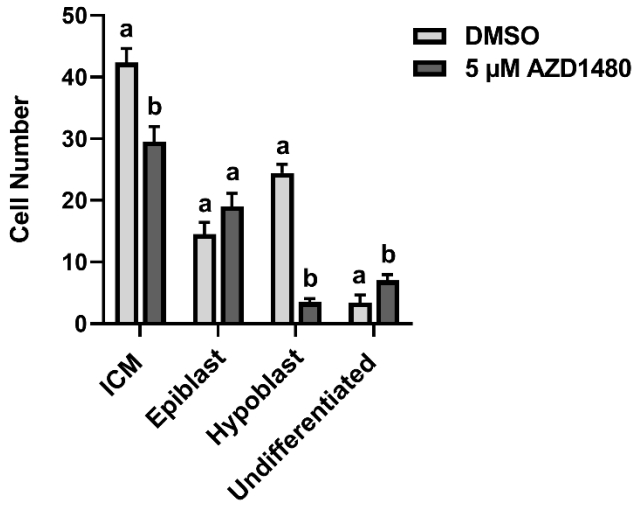

Figure 6-3. JAK2/STAT inhibition after the ICM lineages are committed reduces HYPO but not EPI cell number. Blastocysts received DMSO or 5 µM AZD1480 for 18 hours starting at day 8. Different superscripts indicate differences (P<0.05).

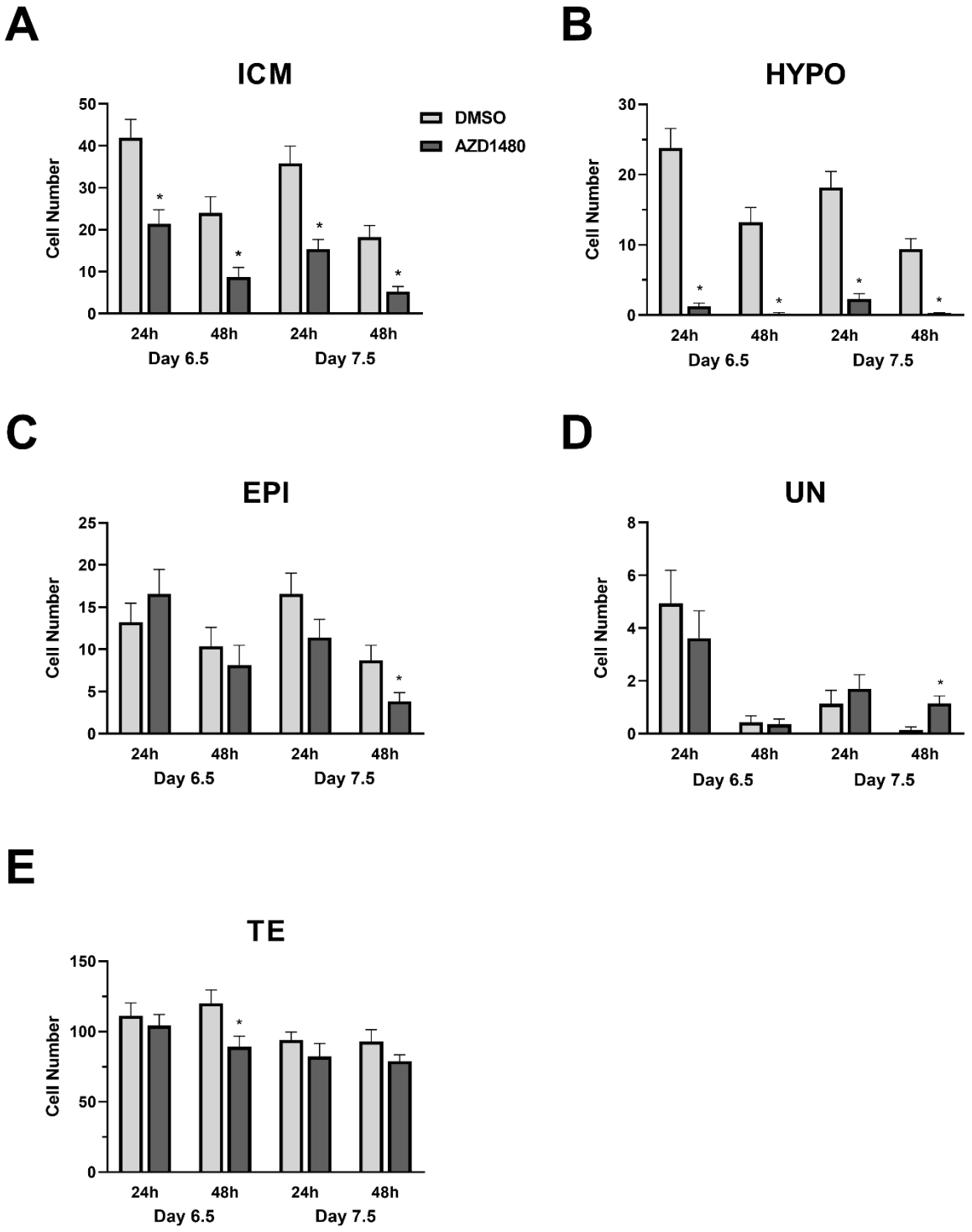

Figure 6-4. JAK2/STAT inhibition disrupts bovine ICM maintenance. Blastocysts received DMSO or 3 µM AZD1480 for 24 or 48 hours starting on day 6.5 or 7.5. *Panel A*: Average ICM cell number of blastocysts from the day 6.5 and 7.5 datasets. *Panel C*: Average HYPO cell number of blastocysts from the day 6.5 and 7.5 datasets. *Panel E*: Average EPI cell number of

blastocysts from the day 6.5 and 7.5 datasets. An asterisk indicates a difference ($P<0.05$) between the DMSO and AZD1480 groups within each panel.

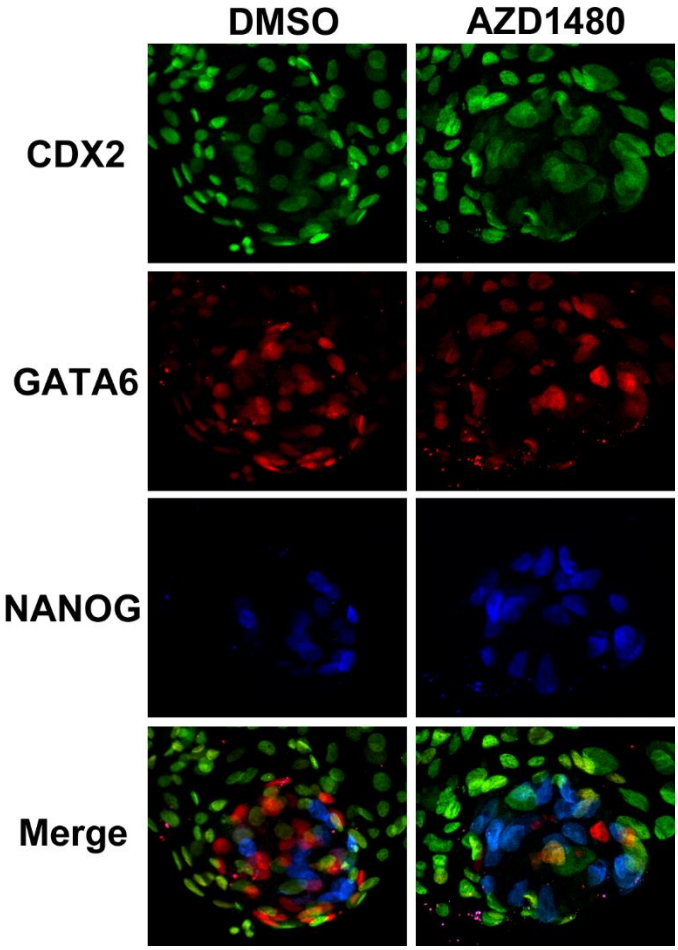

Figure 6-5. Examples of blastocysts treated with DMSO or AZD1480. Blastocysts were stained for CDX2 (TE marker), GATA6 (HYPO marker), and NANOG (EPI Marker).

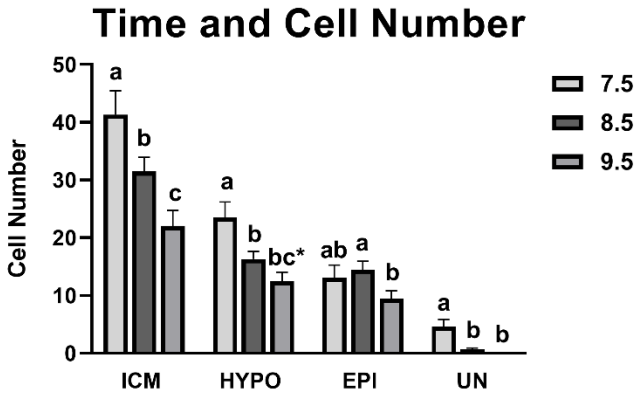

Figure 6-6. ICM, HYPO, EPI and UN cell number decreases with blastocyst age. Blastocysts from the ICM maintenance study which were treated with DMSO for 24 or 48 hours were analyzed by age at time of sampling. Different superscripts indicate differences (P<0.05). An asterisk indicates a statistical tendency (0.05<P<0.1).

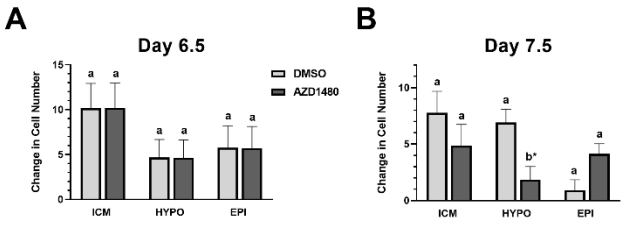

Figure 6-7. Blastocyst ICMs do not recover after AZD1480 treatment. Blastocysts on day 6.5 or 7.5 were treated with DMSO or 3 µM AZD1480 for 24 hours, before being cultured in standard SOF-BEI for an additional 24 hours. *Panel A*: The difference in average ICM, HYPO and EPI cell number between the washout and 48-hour treatment groups in the day 6.5 dataset. *Panel B*: The difference in average ICM, HYPO and EPI cell number between the washout and 48-hour treatment groups in the day 7.5 dataset. Different superscripts within each panel indicate differences ($P<0.05$). An asterisk indicates a statistical tendency ($0.05<P<0.1$).

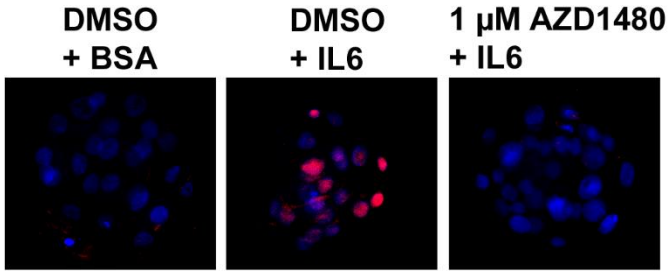

Figure 6-8. 1 µM AZD1480 is sufficient to block IL6-induced STAT3 activation. Day 5 early morulae were treated with DMSO or 1 µM AZD1480 for 4 hours prior to treatment with 100 ng/ml IL6 or carrier (BSA) for 30 minutes. Nuclei are labeled blue (DAPI), and pSTAT3^{Y705} is labeled in red using an immunostaining protocol described previously [309].

Chapter 7: Zinc Supplementation During In Vitro Embryo Culture Increases Inner Cell Mass and Total Cell Numbers in Bovine Blastocysts

Introduction

The *in vitro* production (IVP) of bovine embryos has become a popular way to generate a large number of embryos from genetically elite cows and heifers [329]. These embryos may also be used for impregnating cows with poor fertilization rates and for circumventing the detrimental effects of heat stress and other environmental stresses [330]. In 2016, approximately one-million IVP embryo transfers were completed worldwide, with 400,000 IVP embryos transferred to cattle in North America (IETS Data Retrieval Committee Report, December 2017).

Despite the increasing use of IVP, the ability of transferred IVP embryos to maintain pregnancies (*i.e.* embryo competency) remains inferior to that of embryos produced *in vivo* [264,330]. Culture media used for maturing and fertilizing oocytes and developing embryos is undoubtedly a major contributor to this reduced post-transfer competency. In recent years, bovine embryos have primarily been cultured in media formulated specifically for mammalian embryos. Synthetic oviduct fluid (SOF)-based media formulations are popular in bovine IVP systems because its formulation is based on a biochemical analysis of energy sources, amino acids, pH buffers, and various salts found within ovine oviducts [285]. Several modifications to the original SOF formulation (*e.g.* amino acids, citrate, adjusted glucose concentrations) allow for bovine blastocyst development in the absence of serum [146,331,332].

Unfortunately, several micronutrients (*e.g.* vitamins, trace minerals) that exist in oviductal and uterine secretions are not present in SOF and other embryo media formations.

Trace mineral salt supplementation is a well-established component to optimizing fertility and improving oocyte quality and embryo and fetal development [333–335]. Trace mineral supplementation can also improve ovum pickup success in cattle [336]. The omission of these micronutrients in embryo media formulations likely reflects that these media originally were developed to be used in combination with fetal bovine serum (FBS), which contains many of these micronutrients. The absence of serum may also reduce the incidence and severity of large offspring syndrome in calves produced from IVP embryos, although other factors also influence the occurrence of large offspring [330,337]. Many present-day embryo culture media use bovine serum albumin (BSA) in place of serum to provide a protein source that is semi-defined, but BSA, unlike serum, lacks zinc.

Zinc is a trace mineral that is essential for various physiological actions. Over 300 metalloproteins and >2,000 transcription factors depend on zinc for chemical reactions or protein stability for numerous cellular activities, including cell proliferation, DNA methylation, DNA repair and apoptosis [249–251]. Zinc is present at concentrations of 0.8-0.9 µg/ml (12 to 14 µM) in bovine serum and 0.16 to 4.3 µg/ml (2.4 to 65.0 µM) in bovine uterine lumen fluid, depending on the method of fluid capture [338,339].

We propose that the absence of zinc in bovine embryo media formations has deleterious consequences on IVP embryo development and competency. Zinc supplementation is beneficial during *in vitro* maturation [252–254], but no reports exist that describe whether supplementing zinc after fertilization influences bovine embryo development. The objective of this work was to examine the effects of zinc supplementation to BSA-containing, FBS-lacking SOF (SOF-BE1) on IVP bovine embryo development and on total, inner cell mass (ICM) and trophectoderm (TE) cell numbers in blastocysts.

Materials and Methods

No animals were used for this work. All studies were completed on slaughterhouse-derived materials from a commercial slaughterhouse that followed humane slaughter practices according to USDA guidelines. Unless specified otherwise, reagents were purchased from ThermoFisher Chemical Company (Waltham, MA).

In vitro Embryo Production

Bovine embryos were produced by *in vitro* maturation, fertilization and culture procedures described previously [8,310]. In brief, cumulus-oocyte complexes (COCs) obtained from beef and dairy cow ovaries purchased from Brown Packing Co. (Gaffney, SC) were matured for 21 to 24 hours in tissue culture medium 199 containing Earle's salts supplemented as described previously [8]. Matured COCs and isolated sperm (four Holstein bulls, donation from Select Sires, Plain City, OH, USA) were co-incubated in SOF-based fertilization medium (SOF-FERT) [73,270]. The time of fertilization was designated as day 0. After 16 h, presumptive zygotes were denuded by gentle pipetting and placed in 50 µl drops of SOF-BE1 (23-26 zygotes/drop) [146].

Zinc Supplementation

On day 1 post-fertilization, zinc sulfate solution (2 M in water; Sigma-Aldrich, St. Louis, MO) was added to SOF-BE1 at 0, 2, 20 or 40 µM. Embryos were cultured until day 8 post-fertilization (1-3 drops/replicate; 7 replicates, 325-401 total zygotes/treatment). Cleavage was assessed on day 3 (percentage of zygotes that formed ≥ 2-cell stage embryos) and blastocyst formation was determined on days 7 and 8 (percentage of cleaved embryos that formed

blastocysts). Regular blastocysts were defined as embryos containing a blastocoel cavity that did not exhibit expansion in diameter or zona pellucida thinning. Advanced blastocysts included expanded blastocysts (expansion in diameter with zona pellucida thinning), hatching blastocysts (zona pellucida hatching), and hatched blastocysts (expanded blastocysts lacking a zona pellucida).

Differential Cell Labeling in Blastocysts

In 5 of the 7 replicates, all blastocysts were collected from each treatment (total 22-57 blastocysts/treatment) and fixed and immunostained as described previously [8]. In brief, immunostaining involved incubation with an anti-Caudal-type Homeobox-2 (CDX2) antibody (Biogenex, San Ramon, CA; sold ready-to-use) followed by washing and incubation with donkey anti-mouse Alexa Fluor 488 or 647. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; 1µg/ml). After washing, embryos were flattened between a glass slide and cover slip. Fluorescent markers were visualized by using an Eclipse Ti-E inverted microscope equipped with an X-Cite 120 epifluorescence illumination system. Images were captured with DS-L3 digital camera and assembled with NIS-Elements Software (Nikon Instruments, Melville, NY). Cell counting was completed by using the cell counter plugin in the program FIJI (ImageJ) to label and record individual nuclei stained with CDX2, a TE-specific marker, or that lacked CDX2 staining (ICM cells) [273].

Statistical Analysis

All analyses were completed by least-squares ANOVA using the general linear model of the Statistical Analysis System (SAS for Windows, version 9.4; SAS Institute Inc., Cary, NC, USA). Main effects tested were treatment (zinc concentration) and replicate. The Dunnett's Test was used to contrast each zinc treatment group with the control. Replicate was used as the

experimental unit for embryo development studies. Percentage data were arcsine-transformed before analysis but are presented in figures as non-transformed means and SEM. Embryo was used as the experiment unit for cell number data. In all studies, statistical significance was determined at $P \leq 0.05$.

Results

Effects of Zinc Supplementation on Embryo Development

Supplementation with zinc began immediately after fertilization (day 1; 16 h after initial exposure to spermatozoa). On day 3 post-fertilization, zinc supplementation did not affect cleavage rates (Fig. 7-1A). On day 7 and 8 (Fig. 7-1B & C), supplementation with 2 or 20 μM zinc did not affect total, regular or advanced blastocyst formation. However, exposure to 40 μM zinc reduced ($P < 0.05$) the percentage of total blastocysts on both days 7 and 8. This treatment also reduced ($P < 0.05$) the percentage of regular blastocysts on day 7 and the percentage of advanced blastocysts on day 8.

Zinc Supplementation Increases ICM and Total Cell Numbers in Blastocysts

Differential cell staining was used to examine TE, ICM and total cell numbers in day 8 blastocysts exposed to the zinc treatments (Fig. 7-2). Supplementation with 2 μM zinc increased ($P < 0.05$) total and ICM cell numbers but had no effect on TE cell numbers (Fig. 7-2A). No effects on cell numbers were detected in embryos supplemented with 20 or 40 μM zinc. No differences in the ICM:TE ratio was observed in any of the treatment groups (Fig. 7-2B).

Discussion

Zinc plays essential roles in DNA repair and oxidative stress responses, so its absence in bovine embryo media may be detrimental to IVP bovine embryos. This is the first report we are aware of that describes the effects of physiological concentrations of zinc during bovine embryo culture. A previous report examining zinc and bovine embryo development supplemented non-physiological amounts of zinc (≥150 µM zinc) and found it to be cytotoxic [340]. The zinc concentration range examined here covered the range found in bovine serum (12-14 µM) and uterine fluid (2.4-65.0 µM) [338,339]. This work identified that a much lower concentration of 40 µM zinc was cytotoxic to bovine embryos, which is below some reported concentrations in uterine fluid. The SOF medium formation used for this work contained BSA, which is a prominent zinc carrier in the bloodstream [341], but uterine fluid likely contains more zinc binding factors to prevent high zinc concentrations from being cytotoxic to an embryo. The lowest zinc concentration tested (2 µM) was included in the present work in part because it represented the lower range of zinc concentrations found in the bovine uterus. Also, this concentration approximated the amount of zinc supplied in medium containing 10% FBS (various serum supplements contain 12-14 µM zinc before dilution).

The principal highlight of this work was observing improvements in ICM and total cell numbers with 2 µM zinc supplementation. The improvement in total cell numbers were caused primarily by the increase in ICM numbers, although a small, non-statistically significant improvement in TE numbers also was observed. No differences in the ratio of ICM:TE cells were observed, likely because of the broad distribution of ratios observed in all treatment groups. Greater zinc concentrations did not affect total, ICM or TE cell numbers. We did not examine why this biphasic response in ICM numbers was observed but predict that the greater zinc

concentrations could have low-level cytotoxic effects on embryonic cells [342]. This idea is consistent with the poor development of embryos exposed to 40 µM zinc.

The increase in ICM cell numbers observed in this work is noteworthy because a small ICM size is commonly associated with poor post-transfer embryo competency. The ICM develops into the embryonic disk, and this structure will produce the fetus as well as the yolk sac and allantois [343]. Reductions in embryonic disk size and the absence of embryonic disks have been noted in IVP bovine conceptuses [59,244,265,266,317,344]. The roles that zinc play in mediating bovine ICM and embryonic disk development are not clear, but there is evidence in the mouse embryo that zinc plays a role in yolk sac development. Culturing mouse embryos in the absence of zinc does not affect *in vitro* development rates, but pregnancy losses after transfer were observed and linked to impaired yolk sac development and egg cylinder formation [345].

None of the zinc concentrations tested in this work influenced cleavage or blastocyst rates. This is in contrast to work in the pig, where zinc supplementation during embryo culture improved both cleavage and blastocyst rates [346]. That study noted improvements in both parthenogenetically activated and fertilized embryos in a narrow window of zinc concentrations, where 12 µM zinc (0.8 µg/ml) increased cleavage and blastocyst rates but supplementing ≤ 6 µM or ≥ 18 µM did not affect either parameter [346]. It is not clear if the lack of positive effects on cleavage rates and blastocyst development in this study reflect species differences in embryonic responses to zinc or if a smaller range of zinc concentrations than tested here is needed to detect changes. It is interesting to note that zinc supplementation during bovine COC maturation improved subsequent blastocyst development [252–254]. Perhaps the timing of zinc supplementation is an important contributor to observing positive effects of zinc on cleavage and

blastocyst rates. More work is needed to explore how zinc and potentially other trace minerals may be used to improve the quantity and quality of blastocysts from IVP systems.

In summary, this work provides evidence that the addition of 2 µM zinc sulfate but not 20 or 40 µM zinc improves ICM and total cell numbers in IVP bovine blastocysts. This could lead to improved bovine embryo media formulations for greater post-transfer embryo competency.

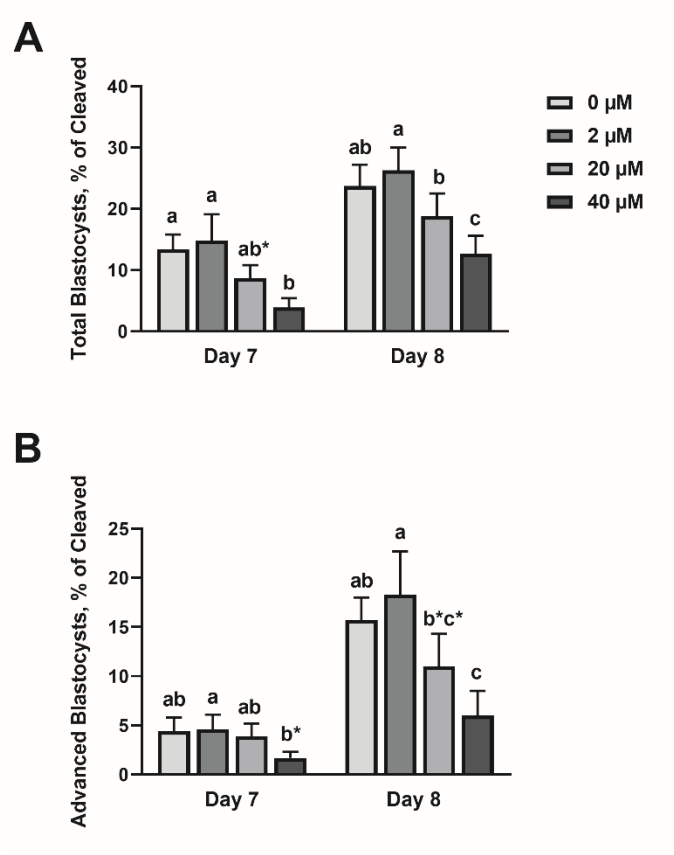

Figure 7-1. Effects of zinc supplementation during in vitro bovine embryo culture on cleavage rate and blastocyst development. Bovine zygotes were cultured in SOF-BE1 containing 0, 2, 20 or 40 µM zinc sulfate from day 1 to 8 post-fertilization (23-26 zygotes/drop, 1-3 drops/replicate, 7 replicates). *Panel A:* Percentage of zygotes that cleaved to at least the 2-cell stage. *Panel B and C:* The percentage cleaved embryos that generated blastocysts at day 7 *(Panel B*) and day 8 (*Panel C*). Blastocysts are categorized as total (all blastocysts), regular (presence of a blastocoel cavity but no expansion or zona pellucida thinning), or advanced (expansion and zona pellucida thinning with or without hatching). Means and SEMs are shown. The asterisks (*****) indicates increases in responses relative to the control value and the hashtag (#) indicates decreases in responses relative to the control ($P < 0.05$).

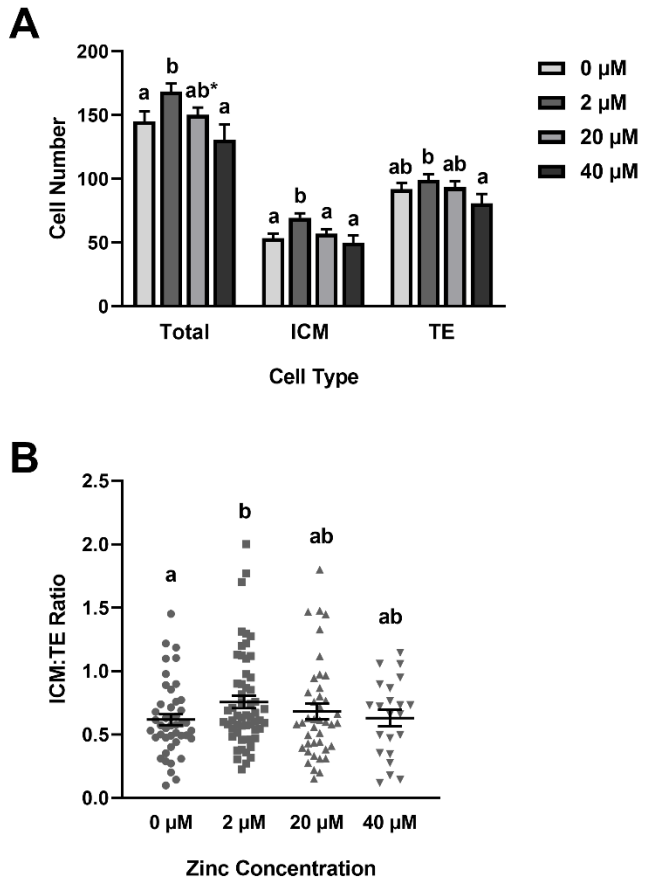

Figure 7-2. Effects of zinc supplementation during in vitro bovine embryo culture on total, ICM and TE cell numbers in day 8 blastocysts. Bovine zygotes were cultured in SOF-BE1 containing 0, 2, 20 or 40 µM zinc sulfate from day 1 to 8 post-fertilization (23-26 zygotes/drop, 1-3 drops/replicate, 5 replicate studies). On day 8, differential fluorescence staining was completed on blastocysts to identify TE and ICM cells. *Panel A:* Cell counts in each treatment (means and SEMs). *Panel B*: Treatment mean and SEMs (bars) and individual (dots) ICM:TE ratios for blastocysts. The asterisks (*****) indicates increases in responses relative to the control value and the hashtag (#) indicates decreases in responses relative to the control ($P < 0.05$).

Chapter 8: Conclusions and Implications

While in vitro fertilization and embryo production techniques have existed for cattle for decades, the percentage of zygotes which develop and survive to calving are low, approximately 10 percent (See Figure 1-1). This low efficiency is, at least in part, a result of the use of inadequate embryo culture media. Embryo culture media may be improved by increasing the complexity to better mimic true oviductal fluid. The goals of this work were to improve production and/or quality of IVP bovine blastocysts through the addition of IL6 or zinc to a common synthetic oviduct fluid (SOF) formulation, and to test the use of a complex bovine ESC media as a replacement for the more simplex SOF.

Initial studies examined the effects of IL6 on blastocyst formation and cell number. Both are important targets for improving IVF efficiency; improving blastocyst production could mean increased calf production directly through a sheer increase in number of embryo transfers, while improved blastocyst cell number may achieve the same through increased embryo quality and survivability. No dose or supplementation scheme improved blastocyst production in group-cultured embryos under standard conditions. However, IL6 improved embryo survival when culture conditions were sub-standard. Thus, IL6 is unlikely to improve blastocyst production in normal commercial bovine in vitro embryo production systems (Figure 8-1).

IL6's effects on blastocyst cell number, however, were much more promising. Doses of at least 100 ng/ml IL6 supplemented from days 1-8, 3-8 or 5-8 post-fertilization increased blastocyst total and ICM cell numbers. This increase in ICM cell number was large, and in many cases IL6-treated blastocysts had twice as many ICM cells as the controls. The TE, however were unaffected by IL6 in day 8 blastocysts, indicating IL6 acts specifically on ICM cells. IL6 is the first, and currently only, known factor which increases total cell number by specifically

targeting the ICM (See Figure 8-2). Because bovine IVP blastocysts are known to have fewer ICM cells than in vivo produced, this finding was exciting, and could indicate that IL6 may improve IVP embryo quality and survivability post-transfer. In theory, having more cells in the ICM indicates the ICM is more likely to thrive and develop into a calf. However, future studies are needed to confirm this.

As anticipated, IL6 stimulated the JAK/STAT3 pathway in bovine embryos, and specifically within the ICM cells of blastocysts. In contrast, inhibition of the JAK2/STAT3 pathway decimated the ICM population and produced presumably non-viable blastocysts with less than 10% the normal ICM cell number. This result is similar to what happens in STAT3-null mouse blastocysts, which are early embryonic lethal. Thus, the necessity of STAT3 appears to be evolutionarily conserved in the mouse and bovine, but we cannot yet be entirely sure. The use of gene-editing to knockout STAT3 in bovine embryos would be necessary to confirm this.

Further observation of blastocysts revealed IL6 largely targets the hypoblast population, and approximately doubles their cell number. Additionally, revealed through RNA sequencing, IL6 increased transcripts of two primitive endoderm (PE) markers, PDGFRA and HNF4A, in blastocysts. The hypoblast/PE gives rise to the yolk sac, a transient but crucial extraembryonic membrane which functions in gas exchange, nutrient absorption and early embryonic blood cell development. Bovine yolk sac development and health are understudied, but these results may indicate that IL6 will improve yolk sac development of IVP embryos. In turn, IL6 may then improve pregnancy retention, as some evidence exists that IVP embryos have defective yolk sac development, which likely contributes to embryonic mortality. We are not aware of other factors known to stimulate hypoblast proliferation in bovine blastocysts, and thus, IL6 may prove to be an invaluable tool in studying bovine yolk sac development (Figure 8-2).

In addition to stimulating hypoblast proliferation, IL6 also had some interesting effects on the epiblast. If supplemented prior to blastocyst formation, IL6 stimulated a small but significant increase in epiblast cell number. This indicates IL6 may also have some effects on the future fetus and/or amnion and extraembryonic mesoderm. Upon closer examination though, IL6 initially decreased the number of epiblast cells before increasing them, by apparently holding the cells in an undifferentiated state. This indicates that IL6 may also slow epiblast differentiation. A major goal of bovine research is to efficiently produce bovine embryonic stem cells (ESCs), especially those with an early-ICM phenotype (capable of differentiating into hypoblast or epiblast). Because IL6 both increases ICM cell number and appears to slow ICM cell differentiation, it may prove to be a useful tool in bovine ESC culture as well.

However, because IL6 increases ICM cell number, one concern of IL6-treatment is the possibility of increased incidence of large offspring syndrome. The concern is that having more cells in the ICM, the portion of the blastocyst which will produce the fetus, will lead to larger calves. However, I do not believe this is likely to be an issue. First, IL6's ICM proliferative effects are largely specific to the hypoblast. The hypoblast will only give rise to the yolk sac, while the epiblast will form the embryo proper and other extraembryonic membranes. Thus, IL6's may not affect fetal size, at least directly. Second, IVP bovine blastocysts are known to have abnormally fewer cells in their ICMs, and thus IL6 may be restoring them to a normal cell number. IVP embryos are known to produce smaller fetuses early in gestation, which overgrow later in gestation [234,344]. Because IL6 produces blastocysts with more ICM cells, perhaps this biphasic growth pattern will be abolished. Although, because IL6 largely increases ICM cell number through the HYPO population, if alterations in fetal growth are observed, it may be an

indirect effect of altered yolk sac development. Future studies should examine in utero fetal and extraembryonic development, as well as calving outcomes.

Because our RNA sequencing data revealed the presence of LIF's receptor, LIFR, on blastocysts, and because LIF is known to activate STAT3 in murine ESCs, I also tested the ability of LIF to mimic the effects of IL6 on bovine blastocysts. Previous work in other laboratories utilizing human and murine variants of LIF had shown disparate results on the subject, and so I utilized a recombinant bovine protein for these studies. Unfortunately, LIF was unable to activate STAT3 in day 8 blastocysts and did not produce an increase in ICM cell number. These data indicate that LIF is not suitable as a substitute for IL6 in bovine IVP embryo production. However, LIF did increase the percentage of advanced (expanded and hatched) at day 8, but it did not improve the total percentage of blastocysts. This, taken together with our RNA-sequencing data, suggests that IL6 may be the predominant family member controlling STAT3 activity and ICM cell number in bovine blastocysts. However, future studies should also examine IL11, CNTF and IL27, as our RNA-sequencing study also detected the presence of their receptors in blastocysts.

The next set of studies dissected the necessity of STAT3 for bovine ICM development. These studies revealed STAT3 is not required for ICM formation, but is necessary for ICM maintenance in the bovine, particularly of the hypoblast. These results are consistent with our findings that IL6 largely stimulates STAT3 activity in hypoblast cells. The effect of STAT3 inhibition on the epiblast, however, remains unclear. Regardless, because the hypoblast was rapidly lost after brief JAK 2 inhibition, and because hypoblast cell number largely did not recover after removal of the inhibitor, it seems unlikely that these embryos would develop and establish a pregnancy after transfer to a recipient, though an embryo transfer experiment would

be needed to confirm this. It will also be interesting to see if STAT3 knockout bovine embryos also rapidly lose the hypoblast, and what effects this might have on epiblast and conceptus development. These studies also highlight the importance of STAT3 activity in the blastocyst-stage bovine embryo and show that STAT3 should be a target for future studies of yolk sac development.

In addition to IL6 and LIF, I also tested the ability of zinc to improve in vitro bovine blastocyst production and morphology. The addition of zinc to bovine SOF is not commonly practiced. In some formulations, serum is used as a source of zinc, however many embryo culture media do not contain serum, and thus are zinc-free. Zinc is an important component of many proteins and enzymes, especially those involved in DNA repair. Without zinc, IVP bovine embryos may be more susceptible to DNA damage and cell loss. This study showed that addition of a physiological amount of zinc results in an increase in the total number of cells in blastocysts by increasing ICM cell number. I did not have time to dissect the mechanism of this effect, but zinc supplementation should be a serious consideration for bovine embryo culture media formulations. In the future, studies will be needed to determine if zinc-containing media produces more embryos competent to maintain a pregnancy than zinc-free media.

Another shortcoming of in vitro bovine embryo culture is the inability to culture embryos longer than 7-9 days. Prior to this work, others observed that extended culture of blastocysts results in a loss of the ICM lineages, thus producing TE vesicles. In contrast, murine and human embryos have been cultured successfully for considerably longer periods. Indeed, I confirmed this problem of bovine in vitro embryo production on several occasions in this work. By analyzing blastocyst cell number, I found that ICM cell number decreases after 8 days in culture, while TE cell numbers generally increased. The formulation of SOF used herein is fairly

common, and similar to most bovine SOF formulations, thus this problem is not limited to this laboratory. This loss of the ICM severely limits our ability to study preimplantation bovine embryos.

In attempt to remedy this problem of inability to culture bovine embryos long-term in vitro, I tested the efficacy of a bovine ESC TeSR media to support blastocyst culture beyond day 8. With this media, blastocyst and ICM development past day 8 was significantly improved. However, this exact formulation was unusable as a normal culture media because non-blastocysts would lyse in it. Amazingly, I also observed evidence of PE migration out from the ICM in day 9 and 10 blastocysts grown in this media, a phenomenon which had not yet been described in IVP bovine embryos. However, I also found that IL6 could not further increase ICM cell numbers in this TeSR media. This indicates that exogenous IL6 may only increase ICM cell number in suboptimal conditions, or IL6's effect may have been negated by some bioactive factor present in the TeSR media that is not in SOF-BEI. In the end, this TeSR formulation will be a powerful tool for extended culture and observation of bovine embryos, however this exact formulation will not benefit embryo transfers as it cannot be used until the blastocyst stage.

In conclusion, these studies provide evidence for new tools for improving IVP bovine embryo development, particularly through improving ICM cell number. IL6 may have several uses, such as improving pregnancy retention of IVP embryos, study of the hypoblast/PE in vitro, and culture of bovine ESCs, but future studies will be needed to confirm this. These studies also highlight the inadequacies of current formulations of bovine embryo culture media and indicate that modifications as simple as the addition of zinc can considerably improve blastocyst morphology. Continued research on the formulation of bovine embryo culture media and the supplementation of embryokines is warranted.

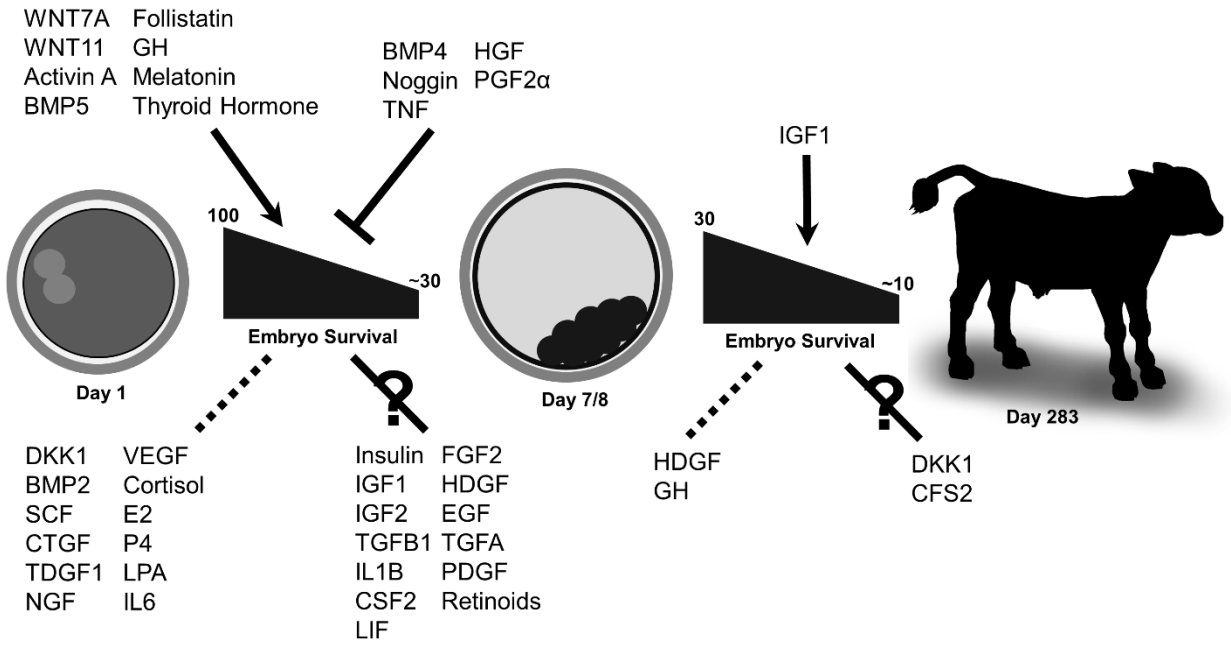

Figure 8-1. IL6 is among the factors which do not affect embryo survival to the blastocyst stage in vitro. The arrow indicates those factors improve emrbyo survival to the indicated day of development. The barred line indicates those factors decrease embryo survival to the indicated day of development. The dashed line indicates those factors have no effect on embryo survival to the indicated day of development. The line with a "?" indicates less then 75% of studies report the same effect for those factors.

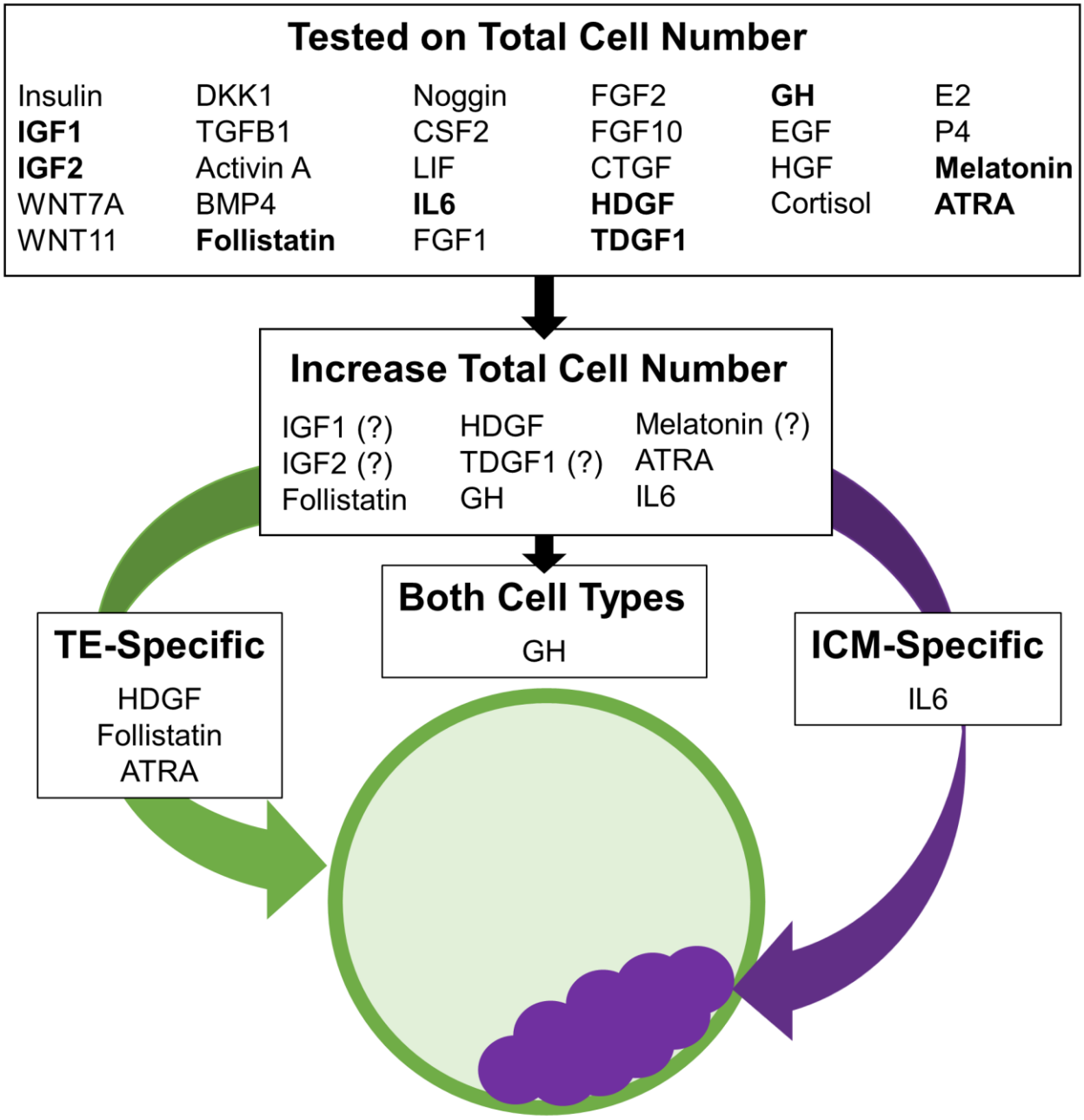

Figure 8-2. IL6 is the only factor currently known to increase blastocyst total cell numbers via the ICM. From Chapter 1, of all the factors tested at least once on total cell number, only 9 increased total cell number in over half of the studies. Of these, IL6 is the only one which is known to increase total cell number by specifically increasing ICM cell number. In the "Increased Total Cell Number" box, a (?) indicates it is unknown which cell type of the blastocyst is affected by this factor.

References

[1] Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following in vitro fertilization in the cow. 1982; 27:147–158.

[2] Viana J. 2017 Statistics of embryo production and transfer in domestic farm animals 2018.

[3] Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003; 59:651–674.

[4] Baldassarre H, Bordignon V. Laparoscopic ovum pick-up for in vitro embryo production from dairy bovine and buffalo calves. Anim Reprod 2018; 15:191–196.

[5] Hansen PJ, Block J. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. Reprod Fertil Dev 2004; 16:1–14.

[6] Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, Santos GMG, Sanches B V, Porcionato JPF, Vieira PHS, Faifer FS, Sterza FAM, Schenk JL, et al. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 2010; 74:1349–1355.

[7] M N-PV, Hansen PJ, Negrón-Pérez V, Hansen PJ. Role of {Yes-Associated} Protein 1, Angiomotin and Mitogen Activated Kinase Kinase 1/2 in development of the bovine blastocyst. Biol Reprod 2018; 98:170–183.

[8] Wooldridge LK, Ealy AD. Interleukin-6 increases inner cell mass numbers in bovine embryos. BMC Dev Biol 2019; 19.

[9] Zolini A, Erly C-T, de King A, Hansen PJ, Torres CA, Block J. Effect of addition of l-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro. Theriogenology 2019; 133:135–143.

[10] Zuo Z, Niu Z, Liu Z, Ma J, Qu P, Qiao F, Su J, Zhang Y, Wang Y. The effects of glycine-glutamine dipeptide replaced L-glutamine on bovine parthenogenetic and {IVF} embryo development. Theriogenology 2019; 141:82–90.

[11] Santos JEP, Thatcher WW, Chebel RC, Cerri RLA, Galvão KN. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. 2004; 82–83:513–535.

[12] Oliveira C, Quintão C, de Freitas C, dos Camargo A, Serapião R, de Camargo L. Post implantation development reveals that biopsy procedure can segregate “healthy” from “unhealthy” bovine embryos and prevent miscarriages. Anim Reprod Sci 2017; 184:51–58.

[13] An L, Marjani SL, Wang Z, Liu Z, Liu R, Xue F, Xu J, Nedambale TL, Yang L, Tian CX, Su L, Du F. Magnesium is a critical element for competent development of bovine embryos. Theriogenology 2019; 140:109–116.

[14] Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep 1998;

3:155–163.

[15] Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002; 67:767–775.

[16] Hansen PJ, Dobbs KB, Denicol AC. Programming of the preimplantation embryo by the embryokine colony stimulating factor 2. Anim Reprod Sci 2014; 149:59–66.

[17] Tokarz VL, MacDonald PE, Klip A. The cell biology of systemic insulin function. J Cell Biol 2018; 217:1–17.

[18] Duan C, Ren H, Gao S. Insulin-like growth factors {(IGFs),} {IGF} receptors, and {IGF-binding} proteins: Roles in skeletal muscle growth and differentiation 2010; 167:344–351.

[19] Baker J, Liu J-P, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth 1993; 75:73–82.

[20] Liu J-P, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I {(Igf-1)} and type 1 {IGF} receptor {(Igf1r)}. Cell 1993; 75:59–72.

[21] Mathews LS, Hammer RE, Behringer RR, D'ercole AJ, Bell GI, Brinster RL, Palmiter RD. Growth Enhancement of Transgenic Mice Expressing Human {Insulin-Like} Growth Factor I* 1988; 123:2827–2833.

[22] Smith LC, Therrien J, Filion F, Bressan F, Meirelles F V. Epigenetic consequences of artificial reproductive technologies to the bovine imprinted genes {SNRPN,} {H19/IGF2,} and {IGF2R} 2015; 6:58.

[23] Geisert RD, Lee C-Y, Simmen FA, Zavy MT, Fliss AE, Bazer FW, Simmen R. Expression of Messenger {RNAs} Encoding Insulin-like Growth {Factor-I,} {-II,} and Insulin-like Growth Factor Binding Protein-2 in Bovine Endometrium during the Estrous Cycle and Early Pregnancy 1991; 45:975–983.

[24] Pushpakumara PG, Robinson RS, Demmers KJ, Mann GE, Sinclair KD, Webb R, Wathes DC. Expression of the insulin-like growth factor {(IGF)} system in the bovine oviduct at oestrus and during early pregnancy 2002; 123:859–868.

[25] Rhoads ML, Meyer JP, Kolath SJ, Lamberson WR, Lucy MC. Growth Hormone Receptor, {Insulin-Like} Growth Factor {(IGF)-1,} and {IGF-Binding} Protein-2 Expression in the Reproductive Tissues of Early Postpartum Dairy Cows 2008; 91:1802–1813.

[26] Robinson RS, Mann GE, Gadd TS, Lamming GE, Wathes DC. The expression of the {IGF} system in the bovine uterus throughout the oestrous cycle and early pregnancy 2000; 165:231–243.

[27] Tríbulo P, Siqueira LGB, Oliveira LJ, Scheffler T, Hansen PJ. Identification of potential embryokines in the bovine reproductive tract. J Dairy Sci 2018; 101:690–704.

[28] Khan D, Fournier É, Dufort I, Richard FJ, Singh J, Sirard M-A. Meta-analysis of gene

expression profiles in granulosa cells during folliculogenesis. Reproduction 2016; 151:R103–R110.

[29] Schultz GA, Hogan A, Watson AJ, Smith RM, Heyner S. Insulin, insulin-like growth factors and glucose transporters: temporal patterns of gene expression in early murine and bovine embryos 1992; 4:361–371.

[30] Wang LM, Feng HL, Ma YZZ, Cang M, Li HJ, Yan Z, Zhou P, Wen JX, Bou S, Liu DJ. Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. Anim Reprod Sci 2009; 114:99–108.

[31] Yaseen MA, Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Changes in the relative abundance of {mRNA} transcripts for insulin-like growth factor {(IGF-I} and {IGF-II)} ligands and their receptors {(IGF-IR/IGF-IIR)} in preimplantation bovine embryos derived from different in vitro systems 2001; 122:601–610.

[32] Byrne AT, Southgate J, Brison DR, Leese HJ. Regulation of apoptosis in the bovine blastocyst by insulin and the insulin-like growth factor {(IGF)} superfamily 2002; 62:489–495.

[33] Mihalik J, Rehák P, Koppel J. The influence of insulin on the in vitro development of mouse and bovine embryos. Physiol Res Acad Sci Bohemoslov 2000; 49:347–354.

[34] Wydooghe E, Heras S, Dewulf J, Piepers S, den Abbeel VE, Sutter DP, Vandaele L, Soom VA. Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. Reprod Fertil Dev 2014; 26:717.

[35] Matsui M, Takahashi Y, Hishinuma M, Kanagawa H. Insulin and insulin-like growth factor-I(IGF-I) stimulate the development of bovine embryos fertilized in vitro. J Vet Med Sci 1995; 57:1109–1111.

[36] Augustin R, Pocar P, Wrenzycki C, Niemann H, Fischer B. Mitogenic and anti-apoptotic activity of insulin on bovine embryos produced in vitro. Reproduction 2003; 126:91–99.

[37] Liu Z, Foote RH, Yang X. Development of early bovine embryos in co-culture with ksom and taurine, superoxide dismutase or insulin. Theriogenology 1995; 44:741–750.

[38] Byrne AT, Southgate J, Brison DR, Leese HJ. Regulation of apoptosis in the bovine blastocyst by insulin and the insulin-like growth factor (IGF) superfamily. Mol Reprod Dev 2002.

[39] Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. J Reprod Fertil 1996; 106:299–306.

[40] Khurana NK, Niemann H. Energy Metabolism in Preimplantation Bovine Embryos Derived In Vitro or In Vivo1. Biol Reprod 2000; 62:847–856.

[41] Wang LM, Wen JX, Yuan JL, Cang M, Liu DJ. Knockdown of {IGF-IR} by {siRNA} injection during bovine preimplantation embryonic development. Cytotechnology 2011; 64:165–172.

[42] Moreira F, Paula-Lopes FF, Hansen PJ, Badinga L, Thatcher WW. Effects of growth hormone and insulin-like growth factor-I on development of in vitro derived bovine embryos. Theriogenology 2002.

[43] Sirisathien S, HJ H-F, Brackett BG. Influences of epidermal growth factor and insulin-like growth {factor-I} on bovine blastocyst development in vitro. Anim Reprod Sci 2003; 77:21–32.

[44] Sirisathien S, Brackett BG. TUNEL analyses of bovine blastocysts after culture with EGF and IGF-I. Mol Reprod Dev 2003.

[45] Sakagami N, Umeki H, Nishino O, Uchiyama H, Ichikawa K, Takeshita K, Kaneko E, Akiyama K, Kobayashi S, Tamada H. Normal calves produced after transfer of embryos cultured in a chemically defined medium supplemented with epidermal growth factor and insulin-like growth factor I following ovum pick up and in vitro fertilization in Japanese black cows. J Reprod Dev 2012; 58:140–146.

[46] Xie M, McCoski SR, Johnson SE, Rhoads ML, Ealy AD, R MS, Johnson SE, Rhoads ML, Ealy AD. Combinatorial effects of epidermal growth factor, fibroblast growth factor 2 and insulin-like growth factor 1 on trophoblast cell proliferation and embryogenesis in cattle. Reprod Fertil Dev 2015; 29:419.

[47] Prelle K, Stojkovic M, Boxhammer K, Motlik J, Ewald D, Arnold GJ, Wolf E. Insulin-like growth factor I (IGF-I) and long R3IGF-I differently affect development and messenger ribonucleic acid abundance for IGF-binding proteins and type I IGF receptors in in vitro produced bovine embryos. Endocrinology 2001.

[48] Makarevich AV V, Markkula M. Apoptosis and Cell Proliferation Potential of Bovine Embryos Stimulated with Insulin-Like Growth Factor I During In Vitro Maturation and Culture1. Biol Reprod 2002; 66:386–392.

[49] Jousan FD, Hansen PJ. Insulin-like growth {factor-I} promotes resistance of bovine preimplantation embryos to heat shock through actions independent of its anti-apoptotic actions requiring {PI3K} signaling. Mol Reprod Dev 2006; 74:189–196.

[50] Moss JI, Pontes E, Hansen PJ. Insulin-like growth factor-1 protects preimplantation embryos from anti-developmental actions of menadione. Arch Toxicol 2009; 83:1001–1007.

[51] Fabian D, Il'ková G, Rehák P, Czikková S, Baran V, Koppel J. Inhibitory effect of IGF-I on induced apoptosis in mouse preimplantation embryos cultured in vitro. Theriogenology 2004; 61:745–55.

[52] Herrler A, Krusche CA, Beier HM. Insulin and Insulin-like Growth Factor-I Promote Rabbit Blastocyst Development and Prevent Apoptosis1. Biol Reprod 1998; 59:1302–1310.

[53] Spanos S, Becker DL, Winston RML, Hardy K. Anti-Apoptotic Action of Insulin-Like Growth Factor-I During Human Preimplantation Embryo Development. Biol Reprod 2000; 63:1413–1420.

[54] Block J, Wrenzycki C, Niemann H, Herrmann D, Hansen PJ. Effects of insulin-like

growth factor-1 on cellular and molecular characteristics of bovine blastocysts produced in vitro. Mol Reprod Dev 2008; 75:895–903.

[55] Jousan FD, Hansen PJJ. Insulin-like Growth Factor-I as a Survival Factor for the Bovine Preimplantation Embryo Exposed to Heat Shock1. Biol Reprod 2004; 71:1665–1670.

[56] Jousan FD, Oliveira LJ, Hansen PJ. Short-term culture of in vitro produced bovine preimplantation embryos with insulin-like growth factor-I prevents heat shock-induced apoptosis through activation of the phosphatidylinositol 3-kinase/Akt pathway. Mol Reprod Dev 2008; 75:681–688.

[57] Bonilla AQS, Oliveira LJ, Ozawa M, Newsom EM, Lucy MC, Hansen PJ. Developmental changes in thermoprotective actions of insulin-like growth factor-1 on the preimplantation bovine embryo. Mol Cell Endocrinol 2011; 332:170–179.

[58] Kocyigit A, Cevik M. Effects of leukemia inhibitory factor and insulin-like growth factor-I on the cell allocation and cryotolerance of bovine blastocysts. Cryobiology 2015; 71:64–69.

[59] Block J, Fischer-Brown AE, Rodina TM, Ealy AD, Hansen PJ. The effect of in vitro treatment of bovine embryos with IGF-1 on subsequent development in utero to Day 14 of gestation. Theriogenology 2007; 68:153–161.

[60] Block J, Drost M, Monson RL, Rutledge JJ, Rivera RM, Paula-Lopes FF, Ocon OM, Krininger CE, Liu J, Hansen PJ. Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed, lactating cows. J Anim Sci 2003; 81:1590–1602.

[61] Flood MR, Gage TL, Bunch TD. Effect of various growth-promoting factors on preimplantation bovine embryo development in vitro. Theriogenology 1993; 39:823–833.

[62] Neira JA, Tainturier D, Peña MA, Martal J. Effect of the association of IGF-I, IGF-II, bFGF, TGF-β1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. Theriogenology 2010; 73:595–604.

[63] Steinhart Z, Angers S. Wnt signaling in development and tissue homeostasis. Development 2018; 145:dev146589.

[64] Niehrs C. The complex world of {WNT} receptor signalling. Nat Rev Mol Cell Bio 2012; 13:767–779.

[65] Denicol AC, Dobbs KB, M MK, Carambula SF, Loureiro B, Hansen PJ. Canonical {WNT} signaling regulates development of bovine embryos to the blastocyst stage. Sci Rep-Uk 2013; 3:1266.

[66] Tribulo P, Moss JI, Ozawa M, Jiang Z, Tian X, Hansen PJ. {WNT} regulation of embryonic development likely involves pathways independent of nuclear {CTNNB1}. Reproduction 2017; 153:405–419.

[67] Biechele S, Cockburn K, Lanner F, Cox BJ, Rossant J. Porcn-dependent Wnt signaling is not required prior to mouse gastrulation 2013; 140:2961–2971.

[68] Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. Requirement for {β-Catenin} in {Anterior-Posterior} Axis Formation in Mice 2000; 148:567–578.

[69] Lim K, Gupta M, Lee S, Jung Y, Han D, Lee H. Possible involvement of Wnt/β-catenin signaling pathway in hatching and trophectoderm differentiation of pig blastocysts 2013; 79:284-290.e2.

[70] Xie H, Tranguch S, Jia X, Zhang H, Das SK, Dey SK, Kuo CJ, Wang H. Inactivation of nuclear Wnt-β-catenin signaling limits blastocyst competency for implantation 2008; 135:717–727.

[71] Tribulo P, da Leão B, Lehloenya KC, Mingoti G, Hansen PJ. Consequences of endogenous and exogenous {WNT} signaling for development of the preimplantation bovine embryo†. Biol Reprod 2017; 96:1129–1141.

[72] Tríbulo P, Jumatayeva G, Lehloenya K, Moss JI, M N-PV, Hansen PJ. Effects of sex on response of the bovine preimplantation embryo to insulin-like growth factor 1, activin A, and {WNT7A.}. Bmc Dev Biol 2018; 18:16.

[73] Denicol AC, Block J, Kelley DE, Pohler KG, Dobbs KB, Mortensen CJ, Ortega SM, Hansen PJ. The {WNT} signaling antagonist Dickkopf-1 directs lineage commitment and promotes survival of the preimplantation embryo. Faseb J 2014; 28:3975–3986.

[74] Tríbulo P, Rabaglino M, Bo M, de Carvalheira LR, Bishop J V, Hansen TR, Hansen PJ. Dickkopf-related protein 1 is a progestomedin acting on the bovine embryo during the morula-to-blastocyst transition to program trophoblast elongation. Sci Rep-Uk 2019; 9:11816.

[75] Massagué J. {TGFβ} signalling in context. Nat Rev Mol Cell Bio 2012; 13:616–630.

[76] Katagiri T, Watabe T. Bone Morphogenetic Proteins. Csh Perspect Biol 2016; 8:a021899.

[77] Namwanje M, Brown CW. Activins and Inhibins: Roles in Development, Physiology, and Disease. Csh Perspect Biol 2016; 8:a021881.

[78] Wijayarathna R, de Kretser DM. Activins in reproductive biology and beyond. Hum Reprod Updat 2016; 22:342–357.

[79] Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, et al. Bone Morphogenetic Protein {(BMP)} signaling in development and human diseases. Genes Dis 2014; 1:87–105.

[80] Barrera AD, García E V, Miceli DC. Effect of exogenous transforming growth factor β1 {(TGF-β1)} on early bovine embryo development. Zygote Camb Engl 2018; 26:1–10.

[81] Roelen BA, Eijk VMJ, Rooijen VMA, Bevers MM, Larson JH, Lewin HA, Mummery CL. Molecular cloning, genetic mapping, and developmental expression of a bovine transforming growth factor beta {(TGF-beta)} type I receptor. Mol Reprod Dev 1998; 49:1–9.

[82] Ibrahim LA, Rizo JA, Fontes PLP, Lamb GC, Bromfield JJ. Seminal plasma modulates

expression of endometrial inflammatory meditators in the bovine†. Biol Reprod 2019; 100:660–671.

[83] Keefer CL, Stice SL, Paprocki AM, Golueke P. In vitro culture of bovine IVM-IVF embryos: Cooperative interaction among embryos and the role of growth factors. Theriogenology 1994.

[84] Lee ES, Fukui Y. Effect of various growth factors in a defined culture medium on in vitro development of bovine embryos matured and fertilized in vitro. Theriogenology 1995.

[85] Yang BK, Yang X, Foote RH. Effect of growth factors on morula and blastocyst development of in vitro matured and in vitro fertilized bovine oocytes. Theriogenology 1993; 40:521–530.

[86] Yoshioka K, Takata M, Taniguchi T, Yamanaka H, Sekikawa K. Differential expression of activin subunits, activin receptors and follistatin genes in bovine oocytes and preimplantation embryos. Reprod Fertil Dev 1998; 10:293.

[87] Gandolfi F, Modina S, Brevini TAL, Passoni L, Petraglia F, Lauria A, Artini P. Activin {?A} subunit is expressed in bovine oviduct. Mol Reprod Dev 1995; 40:286–291.

[88] Kannampuzha-Francis J, Tribulo P, Hansen PJ. Actions of activin A, connective tissue growth factor, hepatocyte growth factor and teratocarcinoma-derived growth factor 1 on the development of the bovine preimplantation embryo 2016.

[89] Lee K-B, Bettegowda A, Wee G, Ireland JJ, Smith GW. Molecular Determinants of Oocyte Competence: Potential Functional Role for Maternal {(Oocyte-Derived)} Follistatin in Promoting Bovine Early Embryogenesis. Endocrinology 2009; 150:2463–2471.

[90] Park JE, Oh HJ, Hong SG, Jang G, Kim MK, Lee BC. Effects of activin A on the in vitro development and {mRNA} expression of bovine embryos cultured in chemically-defined two-step culture medium. Reprod Domest Anim Zuchthygiene 2010; 45:585–593.

[91] Trigal B, Gómez E, Díez C, Caamaño JN, Martín D, Carrocera S, Muñoz M. In vitro development of bovine embryos cultured with activin A. Theriogenology 2010; 75:584–588.

[92] Yoshioka K, Kamomae H. Recombinant human activin A stimulates development of bovine one-cell embryos matured and fertilized in vitro. Mol Reprod Dev 1996; 45:151–156.

[93] Lee K-B, Folger JK, Rajput SK, Smith GW. Temporal regulation of {mRNAs} for select bone morphogenetic proteins {(BMP),} {BMP} receptors and their associated {SMAD} proteins during bovine early embryonic development: effects of exogenous {BMP2} on embryo developmental progression. Reprod Biol Endocrin 2014; 12:67.

[94] Jiang Z, Sun J, Dong H, Luo O, Zheng X, Obergfell C, Tang Y, Bi J, R O, Ruan Y, Chen J, Tian XC. Transcriptional profiles of bovine in vivo pre-implantation development. {BMC} Genomics 2014; 15:756.

[95] García E V, Valdecantos PA, Barrera D, Mariela R-O, Miceli DC. Bone morphogenetic

proteins in the bovine oviduct: Differential expression of {BMP-5} in the isthmus during the estrous cycle. Theriogenology 2014; 81:1032–1041.

[96] Rosa I, Camargo LSA, Pereira MM, Rafael F-M, Paz DA, Salamone DF. Effects of bone morphogenic protein 4 {(BMP4)} and its inhibitor, Noggin, on in vitro maturation and culture of bovine preimplantation embryos. Reprod Biol Endocrinol Rb E 2011; 9:18.

[97] García E V., Miceli DC, Rizo G, Valdecantos PA, Barrera AD. Effect of early addition of bone morphogenetic protein 5 (BMP5) toembryo culture medium on invitro development and expression of developmentally important genes in bovine preimplantation embryos. Theriogenology 2015; 84:589–599.

[98] Phillips DJ, de Kretser DM. Follistatin: A Multifunctional Regulatory Protein. Front Neuroendocr 1998; 19:287–322.

[99] Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS. The Structure of the {Follistatin:Activin} Complex Reveals Antagonism of Both Type I and Type {II} Receptor Binding. Dev Cell 2005; 9:535–543.

[100] Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A. Multiple defects and perinatal death in mice deficient in follistatin. Nature 1995; 374:360–363.

[101] Killeen AP, Morris DG, Kenny DA, Mullen MP, Diskin MG, Waters SM. Global gene expression in endometrium of high and low fertility heifers during the mid-luteal phase of the estrous cycle. 2014.

[102] Zhenhua G, Rajput SK, Folger JK, Di L, Knott JG, Smith GW. Pre- and {Peri-/Post-Compaction} Follistatin Treatment Increases In Vitro Production of Cattle Embryos. PLoS One 2017; 12:e0170808.

[103] Groppe J, Greenwald J, Wiater E, Joaquin R-L, Economides AN, Kwiatkowski W, Affolter M, Vale WW, Belmonte J, Choe S. Structural basis of {BMP} signalling inhibition by the cystine knot protein Noggin. Nature 2002; 420:636–642.

[104] Zimmerman LB, M J-EJ, Harland RM. The Spemann Organizer Signal noggin Binds and Inactivates Bone Morphogenetic Protein 4. Cell 1996; 86:599–606.

[105] McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. Noggin-mediated antagonism of {BMP} signaling is required for growth and patterning of the neural tube and somite. Genes Dev 1998; 12:1438–1452.

[106] Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, JA M, AP M, Harland RM, Rossant J, Robertis DEM. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 2000; 403:658–661.

[107] Brunet LJ, A MJ, P MA, Harland RM. Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton. Science (80-) 1998; 280:1455–1457.

[108] Marcelino J, Sciortino CM, Romero MF, Ulatowski LM, Ballock RT, Economides AN, Eimon PM, Harland RM, Warman ML. Human disease-causing {NOG} missense mutations: effects on noggin secretion, dimer formation, and bone morphogenetic protein binding. Proc Natl Acad Sci {USA} 2001; 98:11353–11358.

[109] Pennington KA, Ealy AD. The expression and potential function of bone morphogenetic proteins 2 and 4 in bovine trophectoderm. Reprod Biol Endocrinol 2012; 10:12.

[110] Dinarello CA. Overview of the {IL-1} family in innate inflammation and acquired immunity. Immunol Rev 2017; 281:8–27.

[111] Gerard N, Caillaud M, Martoriati A, Goudet G, Lalmanach AC. The interleukin-1 system and female reproduction. J Endocrinol 2004; 180:203–212.

[112] Correia-Álvarez E, Gómez E, Martín D, Carrocera S, Pérez S, Otero J, Peynot N, Giraud-Delville C, Caamaño JN, Sandra O, Duranthon V, Muñoz M. Expression and localization of interleukin 1 beta and interleukin 1 receptor (type I) in the bovine endometrium and embryo. J Reprod Immunol 2015; 110:1–13.

[113] Paula-Lopes FF, de Moraes AAS, Edwards JL, Justice JE, Hansen PJ. Regulation of Preimplantation Development of Bovine Embryos by Interleukin-1β1. Biol Reprod 1998; 59:1406–1412.

[114] Becher B, Tugues S, Greter M. {GM-CSF:} From Growth Factor to Central Mediator of Tissue Inflammation. Immunity 2016; 45:963–973.

[115] de Moraes AAS, Hansen PJ. Granulocyte-Macrophage Colony-Stimulating Factor Promotes Development of in Vitro Produced Bovine Embryos1. Biol Reprod 1997; 57:1060–1065.

[116] Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQS, Hansen PJ. {Colony-Stimulating} Factor 2 {(CSF-2)} improves development and posttransfer survival of bovine embryos produced in vitro. Endocrinology 2009; 150:5046–5054.

[117] Dobbs KB, Khan FA, Sakatani M, Moss JI, Ozawa M, Ealy AD, Hansen PJ. Regulation of pluripotency of inner cell mass and growth and differentiation of trophectoderm of the bovine embryo by colony stimulating factor 2. Biol Reprod 2013; 89:141.

[118] Hickman CF, Ainslie A, Ealy AD, Ashworth CJ, Rooke JA. Effect of Ovine {Granulocyte-Macrophage} Colony-stimulating Factor on Bovine In Vitro Embryo Development and Blastocyst Interferon-τ Secretion. Reprod Domest Anim 2010; 46:608–615.

[119] Zolini AM, Block J, Rabaglino MB, Tribulo P, Hoelker M, Rincon G, Bromfield JJ, Hansen PJ. Molecular fingerprint of female bovine embryos produced in vitro with high competence to establish and maintain pregnancy†. Biol Reprod 2019.

[120] Dobbs KB, Rodriguez M, Sudano MJ, Ortega SM, Hansen PJ. Dynamics of {DNA} Methylation during Early Development of the Preimplantation Bovine Embryo. PLoS One 2013; 8:e66230.

[121] Nicola NA, Babon JJ. Leukemia inhibitory factor {(LIF).} 2015; 26:533–544.

[122] Yoshida K, Chambers I, Nichols J, Smith A, Saito M, Yasukawa K, Shoyab M, Taga T, Kishimoto T. Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mech Dev 1994; 45:163–171.

[123] Williams LR, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988; 336:684–687.

[124] Nichols J, Chambers I, Smith A. Derivation of Germline Competent Embryonic Stem Cells with a Combination of Interleukin-6 and Soluble Interleukin-6 Receptor 1994; 215:237–239.

[125] Eckert J, Niemann H. mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-β in bovine embryos derived in vitro or in vivo. Mol Hum Reprod 1998; 4:957–965.

[126] Fukui Y, Matsuyama K. Development of in vitro matured and fertilized bovine embryos cultured in media containing human leukemia inhibitory factor. Theriogenology 1994; 42:663–673.

[127] Sirisathien S, Hernandez-Fonseca HJ, Bosch P, Hollet BR, Lott JD, Brackett BG. Effect of leukemia inhibitory factor on bovine embryos produced in vitro under chemically defined conditions. Theriogenology 2003; 59:1751–1763.

[128] Rodríguez A, De Frutos C, Díez C, Caamaño JN, Facal N, Duque P, García-Ochoa C, Gómez E. Effects of human versus mouse leukemia inhibitory factor on the in vitro development of bovine embryos. Theriogenology 2007; 67:1092–1095.

[129] Vejlsted M, Avery B, Gjorret JO, Maddox-Hyttel P. Effect of leukemia inhibitory factor (LIF) on in vitro produced bovine embryos and their outgrowth colonies. Mol Reprod Dev 2005; 70:445–454.

[130] Yamanaka M, Kudo T, Kimura N, Amano T, Itagaki Y. Effect of Bovine Leukemia Inhibitory Factor on Hatching and Numbers of Inner Cell Mass and Trophectoderm of Bovine Intact and Biopsied Blastocysts. Nihon Chikusan Gakkaiho 1999; 70:444–450.

[131] Kocyigit A, Cevik M. Leucemia inhibitory factor; investigating the time-dependent effect on viability of vitrified bovine embryos. Reprod Domest Anim 2017; 52:1113–1119.

[132] Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev 2014; 25:453–472.

[133] Hansen PJ, Soto P, Natzke RP. Mastitis and Fertility in Cattle - Possible Involvement of Inflammation or Immune Activation in Embryonic Mortality*. Am J Reprod Immunol 2004; 51:294–301.

[134] Jackson LR, Farin CE, Whisnant S. Tumor necrosis factor alpha inhibits in vitro bovine embryo development through a prostaglandin mediated mechanism. J Anim Sci Biotechnol 2012; 3.

[135] Lennartsson J, Rönnstrand L. Stem cell factor receptor/c-Kit: From basic Science to clinical implications. Physiol Rev 2012; 92:1619–1649.

[136] Łupicka M, Bodek G, Shpigel N, Elnekave E, Korzekwa AJ. Identification of pluripotent cells in bovine uterus: In situ and in vitro studies. Reproduction 2015; 149:317–327.

[137] Dhali A, Anchamparuthy VM, Butler SP, Pearson RE, Gwazdauskas FC. In vitro development of bovine embryos cultured with stem cell factor or insulin-like growth {factor-I} following {IVF} with semen of two bulls having different field fertility. Anim Reprod Sci 2009; 116:188–195.

[138] Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip Rev Dev Biol 2015; 4:215–266.

[139] Kang M, Piliszek A, Artus J, Hadjantonakis A-K. {FGF4} is required for lineage restriction and salt-and-pepper distribution of primitive endoderm factors but not their initial expression in the mouse 2013; 140:267–279.

[140] Krawchuk D, Nobuko H-Y, Anani S, Yamanaka Y. {FGF4} is a limiting factor controlling the proportions of primitive endoderm and epiblast in the {ICM} of the mouse blastocyst 2013; 384:65–71.

[141] Yamanaka Y, Lanner F, Rossant J. {FGF} signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. Development 2010; 137:715–724.

[142] Kuijk EW, van Tol LTA, de Velde H, Wubbolts R, Welling M, Geijsen N, Roelen BAJ. The roles of {FGF} and {MAP} kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development 2012; 139:871–882.

[143] Yang QE, Fields SD, Zhang K, Ozawa M, Johnson SE, Ealy AD. Fibroblast Growth Factor 2 Promotes Primitive Endoderm Development in Bovine Blastocyst Outgrowths1. Biol Reprod 2011; 85:946–953.

[144] Ozawa M, Yang Q-E, Ealy AD. The expression of fibroblast growth factor receptors during early bovine conceptus development and pharmacological analysis of their actions on trophoblast growth in vitro. Reproduction 2013; 145:191–201.

[145] Cooke FNT, Pennington KA, Yang Q, Ealy AD. Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm. Reproduction 2009; 137:259–269.

[146] Fields SD, Hansen PJ, Ealy AD. Fibroblast growth factor requirements for in vitro development of bovine embryos. Theriogenology 2011; 75:1466–1475.

[147] Larson RC, Ignotz GG, Currie WB. Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. Development 1992.

[148] Ramazani Y, Knops N, Elmonem MA, Nguyen TQ, Arcolino F, van den Heuvel L, Levtchenko E, Kuypers D, Goldschmeding R. Connective tissue growth factor {(CTGF)} from basics to clinics. Matrix Biol 2018; 68–69:44–66.

[149] Enomoto H, Yoshida K, Kishima Y, Kinoshita T, Yamamoto M, Everett AD, Miyajima A, Nakamura H. Hepatoma-derived growth factor is highly expressed in developing liver and promotes fetal hepatocyte proliferation. Hepatology 2007; 36:1519–1527.

[150] Everett AD. Identification, cloning, and developmental expression of hepatoma-derived growth factor in the developing rat heart. Dev Dynam 2001; 222:450–458.

[151] Everett AD, Stoops T, A MC. Nuclear Targeting Is Required for Hepatoma-derived Growth Factor-stimulated Mitogenesis in Vascular Smooth Muscle Cells. J Biol Chem 2001; 276:37564–37568.

[152] Oliver JA, Q A-A. An endothelial growth factor involved in rat renal development. J Clin Invest 1998; 102:1208–1219.

[153] Zhao J, Yu H, Lin L, Tu J, Cai L, Chen Y, Zhong F, Lin C, He F, Yang P. Interactome study suggests multiple cellular functions of hepatoma-derived growth factor {(HDGF)}. J Proteomics 2011; 75:588–602.

[154] Chen S-C, Hu T-H, Huang C-C, Kung M-L, Chu T-H, Yi L-N, Huang S-T, Chan H-H, Chuang J-H, Liu L-F, Wu H-C, Wu D-C, et al. Hepatoma-derived growth factor/nucleolin axis as a novel oncogenic pathway in liver carcinogenesis. Oncotarget 2015; 6:16253–16270.

[155] Gómez E, Correia-Álvarez E, Caamaño JN, Díez C, Carrocera S, Peynot N, Martín D, C G-D, Duranthon V, Sandra O, Muñoz M. Hepatoma-derived growth factor: from the bovine uterus to the in vitro embryo culture. Reprod Camb Engl 2014; 148:353–365.

[156] Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, Martín D, Carrocera S, Gómez E. Proteome of the Early {Embryo–Maternal} Dialogue in the Cattle Uterus. J Proteome Res 2011; 11:751–766.

[157] Gómez E, Carrocera S, Martin D, MJ S-C, A G-A, Murillo A, Muñoz M. Hepatoma-derived growth factor: Protein quantification in uterine fluid, gene expression in endometrial-cell culture and effects on in~vitro embryo development, pregnancy and birth. Theriogenology 2017; 96:118–125.

[158] Kato T. Biological roles of hepatocyte growth factor-Met signaling from genetically modified animals (Review). Biomed Reports 2017; 7:495–503.

[159] Nakamura T, Sakai K, Nakamura T, Matsumoto K. Hepatocyte growth factor twenty years on: Much more than a growth factor. J Gastroenterol Hepatol 2011; 26:188–202.

[160] Organ SL, Tsao MS. An overview of the c-MET signaling pathway. Ther Adv Med Oncol 2011; 3:S7–S19.

[161] Dreux AC, Lamb DJ, Modjtahedi H, Ferns GAA. The epidermal growth factor receptors and their family of ligands: Their putative role in atherogenesis. Atherosclerosis 2006; 186:38–53.

[162] Normanno N, Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor {(EGFR)} signaling in cancer. Gene 2006; 366:2–16.

[163] Herbst RS. Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys 2004; 59:S21–S26.

[164] Lim K, Jang G, Ko K, Lee W, Park H, Kim J, Lee S, Hwang W, Lee B, Kang S. Improved in vitro bovine embryo development and increased efficiency in producing viable calves using defined media. Theriogenology 2007; 67:293–302.

[165] Lott WM, Anchamparuthy VM, ML M, Mullarky IK, Gwazdauskas FC. Influence of cysteine in conjunction with growth factors on the development of in vitro-produced bovine embryos. Reprod Domest Anim Zuchthygiene 2010; 46:585–594.

[166] Ahumada CJ, Salvador I, Cebrian-Serrano A, Lopera R, Silvestre MA. Effect of supplementation of different growth factors in embryo culture medium with a small number of bovine embryos on in vitro embryo development and quality. Animal 2013.

[167] Saeed-Zidane M, Tesfaye D, Shaker YM, Tholen E, Neuhoff C, Rings F, Held E, Hoelker M, Schellander K, Salilew-Wondim D. Hyaluronic acid and epidermal growth factor improved the bovine embryo quality by regulating the DNA methylation and expression patterns of the focal adhesion pathway. PLoS One 2019; 14.

[168] Minchiotti G. Nodal-dependant Cripto signaling in {ES} cells: from stem cells to tumor biology. Oncogene 2005; 24:5668–5675.

[169] Yan Y-T, Liu J-J, Luo Y, E C, Haltiwanger RS, Abate-Shen C, Shen MM. Dual Roles of Cripto as a Ligand and Coreceptor in the Nodal Signaling Pathway. Mol Cell Biol 2002; 22:4439–4449.

[170] Chu J, Ding J, Jeays-Ward K, Price SM, Placzek M, Shen MM. Non-cell-autonomous role for Cripto in axial midline formation during vertebrate embryogenesis. Development 2005; 132:5539–5551.

[171] Spike BT, Kelber JA, Booker E, Kalathur M, Rodewald R, Lipianskaya J, La J, He M, Wright T, Klemke R, Wahl GM, Gray PC. CRIPTO/GRP78 signaling maintains fetal and adult mammary stem cells ex vivo. Stem Cell Reports 2014; 2:427–439.

[172] Ding J, Yang L, Yan Y-T, Chen A, Desai N, Anthony W-B, Shen MM. Cripto is required for correct orientation of the anterior–posterior axis in the mouse embryo. Nature 1998; 395:702–707.

[173] Johnson SE, Rothstein JL, Knowles BB. Expression of epidermal growth factor family gene members in early mouse development. Dev Dynam 1994; 201:216–226.

[174] Singh B, Carpenter G, Coffey RJ. EGF receptor ligands: recent advances. F1000Research 2016.

[175] Kumar V, Bustin SA, McKay IA. Transforming growth factor alpha. Cell Biol Int 1995.

[176] Humphreys RC, Hennighausen L. Transforming growth factor alpha and mouse models of human breast cancer. Oncogene 2000.

[177] Chen PH, Chen X, He X. Platelet-derived growth factors and their receptors: Structural and functional perspectives. Biochim Biophys Acta - Proteins Proteomics 2013.

[178] Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 2008.

[179] Viuff D, Hyttel P, Greve T, Eriksen T, Alexandersen S. Transcription and localization of growth factor mRNA in the bovine oviduct. Mol Reprod Dev 1995; 42:28–34.

[180] Eckert J, Niemann H. Effects of platelet-derived growth factor (PDGF) on the in vitro

production of bovine embryos in protein-free media. Theriogenology 1996.

[181] Mitsiadis TA, Pagella P. Expression of nerve growth factor (NGF), TrkA, and p75NTR in developing human fetal teeth. Front Physiol 2016.

[182] Manca A, Capsoni S, Di Luzio A, Vignone D, Malerba F, Paoletti F, Brandi R, Arisi I, Cattaneo A, Levi-Montalcini R. Nerve growth factor regulates axial rotation during early stages of chick embryo development. Proc Natl Acad Sci U S A 2012.

[183] Miralles F, Philippe P, Czernichow P, Scharfmann R. Expression of nerve growth factor and its high-affinity receptor Trk-A in the rat pancreas during embryonic and fetal life. J Endocrinol 1998.

[184] Wheeler EF, Bothwell M. Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. J Neurosci 1992.

[185] Wiesmann C, De Vos AM. Nerve growth factor: Structure and function. Cell Mol Life Sci 2001.

[186] Li C, Ma Y, Yi K, Wang C, Li W, Liu Z, Sun L, Chen S, Yu J, Li H, Chen L, Zhou X. The interactions between nerve growth factor and gonadotrophins in bovine oviduct. Anim Reprod Sci 2014; 149:117–123.

[187] Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9:669–676.

[188] Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes and Cancer 2011; 2:1097–1105.

[189] Luo H, Kimura K, Aoki M, Hirako M. Vascular Endothelial Growth Factor (VEGF) Promotes the Early Development of Bovine Embryo in the Presence of Cumulus Cells. vol. 64. 2002.

[190] Bergan-Roller HE, Sheridan MA. The growth hormone signaling system: Insights into coordinating the anabolic and catabolic actions of growth hormone. Gen Comp Endocrinol 2018; 258:119–133.

[191] Izadyar F, Van Tol HTA, Hage WG, Bevers MM. Preimplantation bovine embryos express mRNA of growth hormone receptor and respond to growth hormone addition during in vitro development. Mol Reprod Dev 2000; 57:247–255.

[192] Kölle S, Sinowatz F, Boie G, Lincoln D, Palma G, Stojkovic M, Wolf E. Topography of growth hormone receptor expression in the bovine embryo. Histochem Cell Biol 1998; 109:417–419.

[193] Kölle S, Stojkovic M, Boie G, Wolf E, Sinowatz F. Growth hormone inhibits apoptosis in in vitro produced bovine embryos. Mol Reprod Dev 2002; 61:180–186.

[194] Iwata H, Ohota M, Hashimoto S, Kimura K, Isaji M, Miyake M. Stage-Specific Effect of Growth Hormone on Developmental Competence of Bovine Embryos Produced In-Vitro.

vol. 49. 2003.

[195] Shirazi SN, Friedman AR, Kaufer D, Sakhai SA. Glucocorticoids and the Brain: Neural Mechanisms Regulating the Stress Response. In: Wang J-C, Harris C (eds.), Glucocorticoid Signaling: From Molecules to Mice to Man. New York, NY: Springer New York; 2015:235–252.

[196] Kuo T, McQueen A, Chen T-C, Wang J-C. Regulation of Glucose Homeostasis by Glucocorticoids. In: Wang J-C, Harris C (eds.), Glucocorticoid Signaling: From Molecules to Mice to Man. New York, NY: Springer New York; 2015:99–126.

[197] Siemieniuch MJ, Majewska M, Takahashi M, Sakatani M, Łukasik K, Okuda K, Skarzynski DJ. Are glucocorticoids auto- and/or paracrine factors in early bovine embryo development and implantation? Reprod Biol 2010; 10:249–256.

[198] Lamy J, Liere P, Pianos A, Aprahamian F, Mermillod P, Saint-Dizier M. Steroid hormones in bovine oviductal fluid during the estrous cycle. Theriogenology 2015; 86:1409–1420.

[199] Banliat C, Dubuisson F, Corbin E, Beurois J, Tomas D, Le Bourhis D, Salvetti P, Labas V, Mermillod P, Saint-Dizier M. Intraoviductal concentrations of steroid hormones during in vitro culture changed phospholipid profiles and cryotolerance of bovine embryos. Mol Reprod Dev 2019; 86:661–672.

[200] Hamilton KJ, Hewitt SC, Arao Y, Korach KS. Estrogen Hormone Biology. Current Topics in Developmental Biology, vol. 125. Academic Press Inc.; 2017:109–146.

[201] Levin ER. Integration of the Extranuclear and Nuclear Actions of Estrogen. Mol Endocrinol 2005; 19:1951–1959.

[202] Gomes WR, Erb RE. Progesterone in Bovine Reproduction: A Review. J Dairy Sci 1965; 48:314–330.

[203] Lonergan P, Forde N, Spencer T. Role of progesterone in embryo development in cattle. Reprod Fertil Dev 2016; 28:66–74.

[204] Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D, Lonergan P. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction 2009; 138:507–517.

[205] Larson JE, Krisher RL, Lamb GC. Effects of supplemental progesterone on the development, metabolism and blastocyst cell number of bovine embryos produced in vitro. Reprod Fertil Dev 2011; 23:311–318.

[206] Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, Fougerou C. Melatonin: Pharmacology, Functions and Therapeutic Benefits. Curr Neuropharmacol 2017; 15:434–443.

[207] Pandi-Perumal SR, Srinivasan V, Maestroni GJM, Cardinali DP, Poeggeler B, Hardeland R. Melatonin. FEBS J 2006; 273:2813–2838.

[208] Sampaio R V, Conceição DSB, Miranda MS, Sampaio L de FS, Ohashi OM. MT3

melatonin binding site, MT1 and MT2 melatonin receptors are present in oocyte, but only MT1 is present in bovine blastocyst produced in vitro. Reprod Biol Endocrinol 2012; 10:103.

[209] Wang F, Tian X, Zhang L, Gao C, He C, Fu Y, Ji P, Li Y, Li N, Liu G. Beneficial effects of melatonin on in vitro bovine embryonic development are mediated by melatonin receptor 1. J Pineal Res 2014; 56:333–342.

[210] Wang F, Tian XZ, Zhou YH, Tan DX, Zhu SE, Dai YP, Liu GS. Melatonin improves the quality of in vitro produced (IVP) bovine embryos: Implications for blastocyst development, cryotolerance, and modifications of relevant gene expression. PLoS One 2014; 9.

[211] Pang YW, Sun YQ, Sun WJ, Du WH, Hao HS, Zhao SJ, Zhu H Bin. Melatonin inhibits paraquat-induced cell death in bovine preimplantation embryos. J Pineal Res 2016; 60:155–166.

[212] Papis K, Poleszczuk O, Wenta-Muchalska E, Modlinski JA. Melatonin effect on bovine embryo development in vitro in relation to oxygen concentration. J Pineal Res 2007; 43:321–326.

[213] Jerome A, Srivastava N. Prostaglandins vis-à-vis bovine embryonic mortality: a review Bovine Embryonic mortality Prostaglandins Luteolysis Cyclooxygenase 2012.

[214] Zhang J, Gong Y, Yu Y. PG F2α Receptor: A Promising Therapeutic Target for Cardiovascular Disease. Front Pharmacol 2010; 1.

[215] Grycmacher K, Boruszewska D, Sinderewicz E, Kowalczyk-Zięba I, Staszkiewicz-Chodor J, Woclawek-Potocka I. Prostaglandin F2α (PGF2α) production possibility and its receptors expression in the early- and late-cleaved preimplantation bovine embryos. BMC Vet Res 2019; 15.

[216] Scenna FN, Edwards JL, Rohrbach NR, Hockett ME, Saxton AM, Schrick FN. Detrimental effects of prostaglandin F2α on preimplantation bovine embryos. Prostaglandins Other Lipid Mediat 2004; 73:215–226.

[217] Lin ME, Herr DR, Chun J. Lysophosphatidic acid (LPA) receptors: Signaling properties and disease relevance. Prostaglandins Other Lipid Mediat 2010; 91:130–138.

[218] Torres AC, Boruszewska D, Batista M, Kowalczyk-Zieba I, Diniz P, Sinderewicz E, Saulnier-Blache JS, Woclawek-Potocka I, Lopes-Da-Costa L. Lysophosphatidic acid signaling in late cleavage and blastocyst stage bovine embryos. Mediators Inflamm 2014; 2014.

[219] Woclawek-Potocka I, Komiyama J, Saulnier-Blache JS, Brzezicka E, Bah MM, Okuda K, Skarzynski DJ. Lysophosphatic acid modulates prostaglandin secretion in the bovine uterus. Reproduction 2009; 137:95–105.

[220] Ghyselinck NB, Duester G. Retinoic acid signaling pathways. Dev 2019; 146.

[221] Das BC, Thapa P, Karki R, Das S, Mahapatra S, Liu TC, Torregroza I, Wallace DP, Kambhampati S, Van Veldhuizen P, Verma A, Ray SK, et al. Retinoic acid signaling

pathways in development and diseases. Bioorganic Med Chem 2014; 22:673–683.

[222] Mamo S, Ponsuksili S, Wimmers K, Gilles M, Schellander K. Expression of retinoid X receptor transcripts and their significance for developmental competence in in vitro-produced pre-implantation-stage bovine embryos. Reprod Domest Anim 2005; 40:177–183.

[223] Mohan M, Malayer JR, Geisert RD, Morgan GL. Expression Patterns of Retinoid X Receptors, Retinaldehyde Dehydrogenase, and Peroxisome Proliferator Activated Receptor Gamma in Bovine Preattachment Embryos1. Biol Reprod 2002; 66:692–700.

[224] Mohan M, Malayer JR, Geisert RD, Morgan GL. Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos. Mol Reprod Dev 2001; 60:289–296.

[225] Gómez E, Rodríguez A, Muñoz M, Caamaño JN, Carrocera S, Martín D, Facal N, Díez C. Development and quality of bovine morulae cultured in serum-free medium with specific retinoid receptor agonists. Reprod Fertil Dev 2008; 20:884–91.

[226] Rodríguez A, Diez C, Ikeda S, Royo LJ, Caamaño JN, Alonso-Montes C, Goyache F, Alvarez I, Facal N, Gomez E. Retinoids during the in vitro transition from bovine morula to blastocyst. Hum Reprod 2006; 21:2149–2157.

[227] Rodríguez A, Díez C, Caamaño JN, de Frutos C, Royo LJ, Muñoz M, Ikeda S, Facal N, Álvarez-Viejo M, Gómez E. Retinoid receptor-specific agonists regulate bovine in vitro early embryonic development, differentiation and expression of genes related to cell cycle arrest and apoptosis. Theriogenology 2007; 68:1118–1127.

[228] Litwiniuk M, Krejner-Bienias A, Gauto AR, Grzela T. Hyaluronic Acid in Inflammation and Tissue Regeneration Proteasome inhibitors in tumor biology View project Limb Salvage View project. n.d.

[229] Furnus CC, Valcarcel A, Dulout FN, Errecalde AL. The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. Theriogenology 2003; 60:1633–1644.

[230] Furnus CC, De Matos DG, Martínez AG. Effect of hyaluronic acid on development of in vitro produced bovine embryos. Theriogenology 1998; 49:1489–1499.

[231] Brent GA. Mechanisms of thyroid hormone action. J Clin Invest 2012; 122:3035–3043.

[232] Rho NY, Ashkar FA, Revay T, Madan P, Rho GJ, King WA, Favetta LA. De novo transcription of thyroid hormone receptors is essential for early bovine embryo development in vitro. Reprod Fertil Dev 2018; 30:779–788.

[233] Ashkar FA, Semple E, Schmidt CH, St. John E, Bartlewski PM, King WA. Thyroid hormone supplementation improves bovine embryo development in vitro. Hum Reprod 2010; 25:334–344.

[234] Vailes MT, R MS, Wooldridge LK, Reese ST, Pohler KG, Roper DA, Mercadante VR, Ealy AD, McCoski SR, Wooldridge LK, Reese ST, Pohler KG, et al. {Post-Transfer} Outcomes in Cultured Bovine Embryos Supplemented with Epidermal Growth Factor,

Fibroblast Growth Factor 2, and {Insulin-Like} Growth Factor 1. Theriogenology 2018; 124:1–8.

[235] Moreno D, Neira A, Dubreil L, Liegeois L, Destrumelle S, Michaud S, Thorin C, Briand-Amirat L, Bencharif D, Tainturier D. In vitro bovine embryo production in a synthetic medium: Embryo development, cryosurvival, and establishment of pregnancy. Theriogenology 2015; 84:1053–1060.

[236] Ushijima H, Akiyama K, Tajima T. Transition of Cell Numbers in Bovine Preimplantation Embryos: In Vivo Collected and In Vitro Produced Embryos. vol. 54. 2008.

[237] Iwasaki S, Yoshiba N, Ushijima H, Watanabe S, Nakahara T. Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. J Reprod Fertil 1990; 90:279–284.

[238] Scheller J, Chalaris A, Dirk S-A, Stefan R-J. The pro- and anti-inflammatory properties of the cytokine interleukin-6 2011; 1813:878–888.

[239] Mamo S, Mehta JP, Forde N, P M, Lonergan P. Conceptus-endometrium crosstalk during maternal recognition of pregnancy in cattle. Biol Reprod n.d.; 87:1-9,6.

[240] Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. Fine mapping of genome activation in bovine embryos by {RNA} sequencing. Proc Natl Acad Sci U S A 2014; 111:4139–4144.

[241] Mathialagan N, Bixby JA, Roberts RM. Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. 1992; 32:324–330.

[242] Hosseini, Dufort I, Caballero J, Moulavi F, Ghanaei HR, Sirard MA. Transcriptome profiling of bovine inner cell mass and trophectoderm derived from in vivo generated blastocysts 2015; 15:1–13.

[243] Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. P Natl Acad Sci Usa 1997; 94:3801–3804.

[244] Fischer-Brown AE, Lindsey BR, Ireland FA, Northey DL, Monson RL, Clark SG, Wheeler MB, Kesler DJ, Lane SJ, Weigel KA, Rutledge JJ, Fischer-Brown A, et al. Embryonic disc development and subsequent viability of cattle embryos following culture in two media under two oxygen concentrations. Reprod Fertil Dev 2004; 16:787–793.

[245] Machado GM, Ferreira AR, Pivato I, Fidelis A, Spricigo JF, Paulini F, Lucci CM, Franco MM, Dode MA. Post-hatching development of in vitro bovine embryos from day 7 to 14 in vivo versus in vitro 2013; 80:936–947.

[246] Alexopoulos NI, French AJ. The prevalence of embryonic remnants following the recovery of post-hatching bovine embryos produced in vitro or by somatic cell nuclear transfer. 2009; 114:43–53.

[247] Shen XH, Cui XS, Lee SH, Kim NH. Interleukin-6 enhances porcine parthenote development in vitro, through the {IL-6/Stat3} signaling pathway. J Reprod Dev 2012; 58:453–460.

[248] Do D V, Ueda J, Messerschmidt DM, Lorthongpanich C, Zhou Y, Feng B, Guo G, Lin PJ, Hossain MZ, Zhang W, Moh A, Wu Q, et al. A genetic and developmental pathway from {STAT3} to the {OCT4-NANOG} circuit is essential for maintenance of {ICM} lineages in vivo. Genes Dev 2013; 27:1378–1390.

[249] McCall KA, Huang C, Fierke CA. Function and mechanism of zinc metalloenzymes. J Nutr 2000; 130:1437S–46S.

[250] Dreosti IE. Zinc and the gene. Mutat Res Fundam Mol Mech Mutagen 2001; 475:161–167.

[251] Yamasaki S, Kumiko S-S, Hasegawa A, Suzuki T, Kabu K, Sato E, Kurosaki T, Yamashita S, Tokunaga M, Nishida K, Hirano T. Zinc is a novel intracellular second messenger. J Cell Biol 2007; 177:637–645.

[252] Picco SJ, Anchordoquy JM, de Matos DG, Anchordoquy JP, Seoane A, Mattioli GA, Errecalde AL, Furnus CC. Effect of increasing zinc sulphate concentration during in vitro maturation of bovine oocytes. Theriogenology 2010; 74:1141–1148.

[253] Anchordoquy JM, Picco SJ, Seoane A, Anchordoquy JP, Ponzinibbio M V, Mattioli GA, Peral García P, Furnus CC. Analysis of apoptosis and DNA damage in bovine cumulus cells after exposure in vitro to different zinc concentrations . Cell Biol Int 2011.

[254] Anchordoquy JM, Anchordoquy JP, Sirini MA, Picco SJ, P P, Furnus CC. The importance of having zinc during in vitro maturation of cattle cumulus–oocyte complex: role of cumulus cells. Reprod Domest Anim 2014; 49:865–874.

[255] Palma GA, Müller M, Brem G. Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced in vitro. J Reprod Fertil 1997.

[256] Sirisathien S, Brackett BG. {TUNEL} analyses of bovine blastocysts after culture with {EGF} and {IGF-I}. Mol Reprod Dev 2003; 65:51–56.

[257] Bonilla AQS, Ozawa M, Hansen PJ. Timing and dependence upon mitogen-activated protein kinase signaling for pro-developmental actions of insulin-like growth factor 1 on the preimplantation bovine embryo. Growth Horm IGF Res 2011.

[258] Herrler A, Lucas-Hahn A, Niemann H. Effects of insulin-like growth factor-I on in-vitro production of bovine embryos. Theriogenology 1992.

[259] Feldman B, Poueymirou W, Papaioannou V, T D, Goldfarb M. Requirement of {FGF-4} for postimplantation mouse development 1995; 267:246–249.

[260] Arman E, Rebecca H-K, Chen Y, Heath JK, Lonai P. Targeted disruption of fibroblast growth factor {(FGF)} receptor 2 suggests a role for {FGF} signaling in pregastrulation mammalian development 1998; 95:5082–5087.

[261] den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta M, Klein B, Helmgaard L, Arce J-C. Association between blastocyst morphology and outcome of single-blastocyst transfer 2013; 27:353–361.

[262] Kovacic B, Vlaisavljevic V, Reljic M, Mojca C-S. Developmental capacity of different morphological types of day 5 human morulae and blastocysts 2004; 8:687–694.

[263] Richter KS, Harris DC, Daneshmand ST, Shapiro BS. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape 2001; 76:1157–1167.

[264] Peterson AJ, Lee RS. Improving successful pregnancies after embryo transfer. Theriogenology 2003; 59:687–697.

[265] Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR, Anderson GB. Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. Mol Reprod Dev 2002; 63:318–328.

[266] Loureiro B, Block J, Favoreto MG, Carambula S, Pennington KA, Ealy AD, Hansen PJ. Consequences of conceptus exposure to colony-stimulating factor 2 on survival, elongation, interferon-τ secretion, and gene expression 2011; 141:617–624.

[267] Kimber SJ. Leukaemia inhibitory factor in implantation and uterine biology 2005; 130:131–145.

[268] Heinrich PC, Behrmann I, Gerhard Mü-N, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the {gp130/Jak/STAT} pathway. Biochem J 1998; 334:297–314.

[269] Zhang K, Hansen PJ, Ealy AD. Fibroblast growth factor 10 enhances bovine oocyte maturation and developmental competence in vitro. Reproduction 2010; 140:815–826.

[270] Sakatani M, Alvarez N V, Takahashi M, Hansen PJ. Consequences of physiological heat shock beginning at the zygote stage on embryonic development and expression of stress response genes in cattle 2012; 95:3080–3091.

[271] Goossens K, Poucke M, Soom A, Vandesompele J, Zeveren A, Peelman LJ. Selection of reference genes for quantitative real-time {PCR} in bovine preimplantation embryos 2005; 5:1–9.

[272] Yang Q-E, Ozawa M, Zhang K, Johnson SE, Ealy AD. The requirement for protein kinase C delta {(PRKCD)} during preimplantation bovine embryo development 2014; 28:482–490.

[273] Schindelin J, Ignacio A-C, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White D, et al. Fiji: an open-source platform for biological-image analysis 2012; 9:676–682.

[274] Madeja ZE, Sosnowski J, Hryniewicz K, Warzych E, Pawlak P, Rozwadowska N, Plusa B, Lechniak D. Changes in sub-cellular localisation of trophoblast and inner cell mass specific transcription factors during bovine preimplantation development 2013; 13:32.

[275] Abe H, Otoi T, Tachikawa S, Yamashita S, Satoh T, Hoshi H. Fine structure of bovine morulae and blastocysts in vivo and in vitro 1999; 199:519–527.

[276] Graf A, Krebs S, Mari H-B, Zakhartchenko V, Blum H, Wolf E. Genome activation in bovine embryos: Review of the literature and new insights from {RNA} sequencing

experiments 2014; 149:46–58.

[277] Fischer C, Drillich M, Odau S, Heuwieser W, Einspanier R, Gabler C. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis 2010; 22:818–829.

[278] Healy LL, Cronin JG, Sheldon MI. Polarized Epithelial Cells Secrete Interleukin 6 Apically in the Bovine Endometrium 2015; 92.

[279] Kuijk EW, Puy L, Tol H, Oei C, Haagsman HP, Colenbrander B, Roelen B. Differences in early lineage segregation between mammals 2008; 237:918–927.

[280] M N-PV, Dorianmarie V-F, Hansen PJ. Role of chemokine {(C-C} motif) ligand 24 in spatial arrangement of the inner cell mass of the bovine embryo† 2017; 96:948–959.

[281] Simmet K, Zakhartchenko V, Julia P-M, Blum H, Klymiuk N, Wolf E. {OCT4/POU5F1} is required for {NANOG} expression in bovine blastocysts 2018; 115:201718833.

[282] Valleh M, Hyttel P, Rasmussen M, Strøbech L. Insulin-like growth factor 2: A modulator of anti-apoptosis related genes {(HSP70,} {BCL2-L1)} in bovine preimplantation embryos. Theriogenology 2014; 82:942–950.

[283] Wu X, Song M, Yang X, Liu X, Liu K, Jiao C, Wang J, Bai C, Su G, Liu X, Li G. Establishment of bovine embryonic stem cells after knockdown of {CDX2} 2016; 6:28343.

[284] Goissis MD, Cibelli JB. Functional Characterization of {SOX2} in Bovine Preimplantation Embryos. Biol Reprod 2014; 90:1–10.

[285] Tervit HR, Whittingham DG, Rowson LEA. Successful culture in vitro of sheep and cattle ova. Reproduction 1972; 30:493–497.

[286] Biggers JD. {IVF} and embryo transfer: historical origin and development. Reprod Biomed Online 2012; 25:118–127.

[287] Tríbulo P, Ballesteros BBH, Ruiz A, Tríbulo A, Tríbulo RJ, Tríbulo HE, Bo GA, Hansen PJ. Consequences of exposure of embryos produced in vitro in a serum-containing medium to dickkopf-related protein 1 and colony stimulating factor 2 on blastocyst yield, pregnancy rate, and birth weight. J Anim Sci 2017; 95:4407–4412.

[288] Ataie-Kachoie P, Pourgholami MH, Richardson DR, Morris DL. Gene of the month: Interleukin 6 {(IL-6)} 2014; 67:932–937.

[289] Meng F, B F-G, Turner P, Henderson H, Oback B. Signal Inhibition Reveals {JAK/STAT3} Pathway as Critical for Bovine Inner Cell Mass Development. Biol Reprod 2015; 93:132.

[290] Schmitt RM, Bruyns E, Snodgrass HR. Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. Gene Dev 1991; 5:728–740.

[291] Conover JC, Ip NY, Poueymirou WT, Bates B, Goldfarb MP, TM D, Yancopoulos GD. Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. Dev

Camb Engl 1993; 119:559–565.

[292] Robinson MD, Smyth GK. Moderated statistical tests for assessing differences in tag abundance. Bioinformatics 2007; 23:2881–2887.

[293] Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing 1995; 57:289–300.

[294] Starr R, Willson TA, Viney EM, Murray LJL, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ. A family of cytokine-inducible inhibitors of signalling. Nature 1997; 387:43206.

[295] Bogliotti Y, Wu J, Vilarino M, Okamura D, Soto D, Zhong C, Sakurai M, Sampaio R, Suzuki K, Belmonte J, Ross P. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. Proc Natl Acad Sci 2018; 115:2090–2095.

[296] Ho L-J, Luo S-F, Lai J-H. Biological effects of interleukin-6: Clinical applications in autoimmune diseases and cancers 2015; 97:16–26.

[297] Oliveira LJ, Mansourri-Attia N, Fahey AG, Browne J, Forde N, Roche JF, Lonergan P, Fair T. Characterization of the Th Profile of the Bovine Endometrium during the Oestrous Cycle and Early Pregnancy. PLoS One 2013; 8:e75571.

[298] Alberto ML V, Meirelles F V, Perecin F, Ambrósio CE, Favaron PO, Franciolli ALR, Mess AM, dos Santos JM, Rici REG, Bertolini M, Miglino MA. Development of bovine embryos derived from reproductive techniques. Reprod Fertil Dev 2012; 25:907–917.

[299] Mess A, Carreira A, de Oliveira C, Fratini P, Favaron P, da Barreto R, Pfarrer C, Meirelles F, Miglino M. Vascularization and {VEGF} expression altered in bovine yolk sacs from {IVF} and {NT} technologies. Theriogenology 2017; 87:290–297.

[300] Zhang S, Liu M-G, Kano A, Zhang C, Fu X-Y, Barnstable CJ. {STAT3} activation in response to growth factors or cytokines participates in retina precursor proliferation 2005; 81:103–115.

[301] Brocke-Heidrich K, Kretzschmar AK, Pfeifer G, Henze C, Löffler D, Koczan D, Thiesen H-J, Burger R, Gramatzki M, Horn F. Interleukin-6–dependent gene expression profiles in multiple myeloma {INA-6} cells reveal a Bcl-2 family–independent survival pathway closely associated with Stat3 activation. Blood 2004; 103:242–251.

[302] Eulenfeld R, Dittrich A, Khouri C, Muller PJ, Mutze B, Wolf A, Schaper F. Interleukin-6 signalling: more than Jaks and {STATs}. Eur J Cell Biol 2012; 91:486–495.

[303] Johnson DE, A OR, Grandis JR. Targeting the {IL-6/JAK/STAT3} signalling axis in cancer. Nat Rev Clin Oncol 2018; 15:234–248.

[304] Gjørret JO, Knijn HM, Dieleman SJ, Avery B, Larsson L-II, Poul M-H. Chronology of apoptosis in bovine embryos produced in vivo and in vitro. 2003; 69:1193–1200.

[305] Knijn HM, Gjørret JO, Vos P, Hendriksen P, van der Weijden BC, Poul M-H, Dieleman SJ. Consequences of in vivo development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos 2003; 69:1371–1378.

[306] Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, Roelen J. Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. Theriogenology 2005; 63:2254–2268.

[307] Ealy AD, Wooldridge LK, McCoski SR. Board invited review: Post-transfer consequences of in vitro-produced embryos in cattle. J Anim Sci 2019; 97.

[308] Farin PW, Slenning BD, Britt JH. Estimates of pregnancy outcomes based on selection of bovine embryos produced in vivo or in vitro 1999; 52:659–670.

[309] Wooldridge LK, Johnson SE, Cockrum RR, Ealy AD. Interleukin-6 requires JAK to stimulate inner cell mass expansion in bovine embryos. Reproduction 2019; 158:303–312.

[310] Rivera RM, Hansen PJ. Development of cultured bovine embryos after exposure to high temperatures in the physiological range. 2001; 121:107–115.

[311] Ludwig TE, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA. Feeder-independent culture of human embryonic stem cells. Nat Methods 2006; 3:637–646.

[312] Morgani SM, Brickman JM. {LIF} supports primitive endoderm expansion during pre-implantation development. Development 2015; 142:3488–3499.

[313] Wolf J, Rose-John S, Garbers C. Interleukin-6 and its receptors: a highly regulated and dynamic system. Cytokine 2014; 70:11–20.

[314] Alexopoulos NI, Vajta G, Poul M-H, French AJ, Trounson AO. Stereomicroscopic and histological examination of bovine embryos following extended in vitro culture 2005; 17:799–808.

[315] Brandão DO, Poul M-H, Lvendahl P, Rumpf R, Stringfellow D, Callesen H. Post hatching development: a novel system for extended in vitro culture of bovine embryos. Biol Reprod 2004; 71:2048–2055.

[316] May LT, Viguet H, Kenney JS, Ida N, Allison AC, Sehgal PB. High levels of ‘complexed’ interleukin-6 in human blood. 1992; 267:19698–19704.

[317] Fischer-Brown A, Monson R, Parrish J, Rutledge J. Cell allocation in bovine embryos cultured in two media under two oxygen concentrations. Zygote Camb Engl 2002; 10:341–348.

[318] Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, Pfeffer PL. Trophectoderm Lineage Determination in Cattle. Dev Cell 2011; 20:244–255.

[319] Derenzini E, Lemoine M, Buglio D, Katayama H, Ji Y, Davis RE, Sen S, Younes A. The JAK inhibitor AZD1480 regulates proliferation and immunity in Hodgkin lymphoma. Blood Cancer J 2011; 1:e46.

[320] Cockburn K, Biechele S, Garner J, Rossant J. The Hippo pathway member Nf2 is required for inner cell mass specification. Curr Biol Cb 2013; 23:1195–1201.

[321] Meraz MA, White JM, Sheehan KCF, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, et al. Targeted disruption

of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 1996; 84:431–442.

[322] Durbin JE, Hackenmiller R, Simon MC, Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996; 84:443–450.

[323] Thierfelder WE, Van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DAA, Doherty PC, Grosveld GC, Ihle JN. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 1996; 382:171–174.

[324] Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4- deficient mice. Nature 1996; 382:174–177.

[325] Takeda H, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura SI, Nakanishi K, Yoshida N, Kishimoto T, Akira S. Essential role of Stat6 in IL-4 signalling. Nature 1996; 380:627–630.

[326] Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci U S A 1997; 94:7239–7244.

[327] Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 1997; 11:179–186.

[328] Akira S. Functional roles of STAT family proteins: Lessons from knockout mice. Stem Cells 1999; 17:138–146.

[329] van Leeuw WAM, Mullaart E, de Roos APW, Merton JS, den Daas JHG, Kemp B, de Ruigh L. Effects of different reproduction techniques: {AI,} moet or {IVP,} on health and welfare of bovine offspring. Theriogenology 2000; 53:575–597.

[330] Ealy AD, Wooldridge LK, R, McCoski S. Post-transfer Consequences of In {Vitro-Produced} Embryos in Cattle. J Anim Sci 2019.

[331] Takahashi Y, First NL. In vitro development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992; 37:963–978.

[332] Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced Rates of Cleavage and Development for Sheep Zygotes Cultured to the Blastocyst Stage in Vitro in the Absence of Serum and Somatic Cells: Amino Acids, Vitamins, and Culturing Embryos in Groups Stimulate Development1. Biol Reprod 1994; 50:390–400.

[333] Apgar J. Zinc and Reproduction. Annu Rev Nutr 1985.

[334] Tian X, Diaz FJ. Acute dietary zinc deficiency before conception compromises oocyte epigenetic programming and disrupts embryonic development. Dev Biol 2013; 376:51–61.

[335] Wilde D. Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. Anim Reprod Sci 2006.

[336] Dantas FG, Reese ST, Filho RVO, Carvalho RS, Franco GA, Abbott CR, Payton RR,

Edwards JL, Russell JR, Smith JK, Pohler KG. Effect of complexed trace minerals on cumulus-oocyte complex recovery and in vitro embryo production in beef cattle. J Anim Sci 2019; 97:1478–1490.

[337] Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006; 65:178–191.

[338] Wiebold JL. Embryonic mortality and the uterine environment in first-service lactating dairy cows. Reproduction 1988; 84:393–399.

[339] Alavi-Shoushtari S, Rezaie S, Pak M, Alizadeh S, Abedizadeh R, Khaki A. Copper and zinc concentrations in the uterine fluid and blood serum during the bovine estrous cycle. Vet Res Forum Int Q J 2012; 3:199–203.

[340] Stephenson JL, Brackett BG. Influences of zinc on fertilisation and development of bovine oocytes in vitro. Zygote Camb Engl 1999; 7:195–201.

[341] Handing KB, Shabalin IG, Kassaar O, Khazaipoul S, Blindauer CA, Stewart AJ, Chruszcz M, Minor W. Circulatory zinc transport is controlled by distinct interdomain sites on mammalian albumins. Chem Sci 2016; 7:6635–6648.

[342] Borovanský J, Riley PA. Cytotoxicity of zinc in vitro. Chem-Biol Interact 1989; 69:279–291.

[343] Maddox-Hyttel P, Alexopoulos NI, Vajta G, Lewis I, Rogers P, Cann L, Callesen H, P T-N, Trounson A. Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. Reproduction 2003; 125:607–623.

[344] Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Kominek DJ, Moyer AL, Famula TR, Sainz RD, Anderson GB. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. Theriogenology 2002; 58:973–994.

[345] Hanna LA, Clegg MS, Momma TY, Daston GP, Rogers JM, Keen CL. Zinc influences the in vitro development of peri-implantation mouse embryos. Birth Defects Res Part Clin Mol Teratol 2003; 67:414–420.

[346] Jeon Y, Yoon J, Cai L, Hwang S-U, Kim E, Lee E, Jeung E, Hyun S-H. Effect of zinc on in~vitro development of porcine embryos. Theriogenology 2015; 84:531–537.

[347] dos Santos EC, Varchetta R, de Lima CB, Ispada J, Martinho HS, Fontes PK, Nogueira MFG, Gasparrini B, Milazzotto MP. The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. Theriogenology 2019.

[348] Cavalcanti CM, Campelo IS, Silva MMAS, Albuquerque JVS, Melo LM, Freitas VJF. Efficiency of different incubation systems for the in vitro production of bovine embryos. Zygote 2018.

Appendix A: Experimental Units and Analyzing Blastocyst Formation

In Chapter 1, I noted many disparate outcomes between and within laboratories studying the same bioactive molecule. I believe some of this disparity may be because many authors utilized only one droplet of embryos per treatment per replicate and/or utilized a low total number of oocytes or embryos. I have noted that blastocyst production varies widely within (droplet to droplet) and across replicates in my own experiments. This problem likely causes both Type I and Type II errors. I will illustrate herein how the natural amount of variation from droplet to droplet of bovine IVP embryos makes it difficult to accurately assess differences caused by treatments, and why a large 'n' is crucial if we are to accurately assess whether or not some factor actually affects blastocyst formation.

To start, I collected and analyzed blastocyst formation data from five replicates of IVF which were conducted by myself in 2019. This data is from replicates which were discarded for various reasons (study terminated, embryos only used for a test, etc.) and never published, and no droplet received any treatment. In this data of 5 IVF replicates, I have a total of 31 droplets of embryos, and 3-13 droplets per replicate. In each droplet of 50 µl of culture medium was 24-30 putative zygotes, which were cultured to day 8 post-fertilization and the number of blastocysts was recorded per droplet. The average blastocyst formation percentage of all these droplets was 24% of the putative zygotes cultured, but each droplet ranged from 12-48%. In order to illustrate the problem with utilizing a low number of putative zygotes per treatment, I analyzed my data with Excel and SAS. Using the RANDBETWEEN function in Excel, each droplet was assigned a random number of 1 or 2, and I ensured that each IVF replicate had at least one droplet with a

‘1’ and a ‘2’. From here forward, I considered ‘1’ and ‘2’ to be different treatments, even though they were not.

First, a single ‘1’ and ‘2’ droplet was randomly selected from each replicate to produce sample sizes representative of the those observed in many studies reviewed in Chapter 1 (<150 zygotes/treatment). In this situation, group 1 had a total of 130 putative zygotes analyzed and a mean blastocyst formation rate (out of putative zygotes) of 16.93%. Group 2 had 126 zygotes and a mean blastocyst formation rate of 29.1%. I arcsine transformed this data and then analyzed it by lsmeans ANOVA using PROC GLM of SAS. In this situation, the difference between group 1 and 2 was nearly statistically significant, with a P=0.0569. Yet, there is no difference between these groups. This would have created a Type I error and illustrates just how dangerous it is to utilize only one droplet of embryos per treatment per week, especially with a low number of replicates.

Next, I analyzed the whole of group 1 and 2, to examine what happens when a sufficient number of droplets are used. Now, group 1 had an ‘n’ of 380 zygotes from 15 drops, and a mean blastocyst formation rate of 28.3%. Group 2 had 406 zygotes from 16 drops, and an average rate of 27.2%. When analyzed by ANOVA in the same way, there no longer was a difference (P=0.26), as there should be. You might be asking then, is this variance just a problem with our lab? I do not think so, as I will explain in the next paragraph.

I quickly scanned the literature for recent studies in other laboratories which produced IVP bovine blastocysts and reported their mean, SEM or STD and number of replicates, and compared their mean and STD to mine. In my data which I have been discussing here, my average blastocyst formation percentage was 24%, with a standard deviation of 7%. In the first study I looked at, which was published by Zolini and colleagues at the University of Florida [9],

their mean blastocyst percentage for their controls was 23%, and I calculated their STD to be 5%. In a second study, dos Santos and colleagues in Brazil had a mean control blastocyst percentage of 26%, and a STD of 5% [347]. Finally, I looked at one more study for good measure, this one performed by Cavalcanti and colleagues in Brazil. They reported a control blastocyst percentage of 34%, and a STD of 10% [348]. Our mean of 24% and STD of 7% is very similar to these numbers. So, I conclude that this variation in blastocyst production is normal for bovine IVP embryos. Therefore, all of us as researchers need to be careful to plan appropriately for it. We, in our own lab, regularly utilize the Tukey adjustment after ANOVA on blastocyst formation data, which is a very stringent honestly significant difference test, in effort to avoid this issue.

So, the question now is, what 'n' is sufficient? Well, this will depend on the methods used to calculate the percent of blastocyst formed (experimental unit of droplet or replicate, or blastocyst formation out of cleaved or putative zygotes), as these differences will affect your mean and STD. One thing is very important though: at least 2 droplets per treatment per replicate is a necessity. To give an example of what 'n' is needed for blastocyst formation studies, I generated a table illustrating what 'n' would be needed to detect increases of 5, 10, 15 or 20% in the treatment group utilizing means and STDs from our data (See Table A-1). To do this, I utilized a free online software (https://clincalc.com/stats/samplesize.aspx), and set study group design to "Two independent study groups", primary endpoint was set to "Continuous", the enrollment ratio was 1, alpha was 0.05, and the power was 80%. I did this for all four analysis methods: blastocyst formation out of zygotes by droplet or week, and blastocyst formation out of cleaved embryos by droplet or week. I hope that this information will be useful for others who are currently planning or conducting experiments. I urge reviewers and editors to scrutinize

manuscripts which utilize experimental units below these numbers, however the actual ‘n’ needed may differ depending on a study’s actual mean and STD.

To finish, I must also discuss the method of analyzing blastocyst formation as binomial data. While this has been a very common practice, I do not believe it is appropriate, unless individually cultured embryos are being analyzed, or if the total number of zygotes per treatment is large (multiple thousands). Binomial analysis is inappropriate for group cultured embryos because each droplet of media and the group of embryos within it will be different. Binomial analysis cannot account for this nor the natural and chaotic variation of IVP bovine blastocyst formation. I also analyzed our “small n” data of group 1 and 2 with a Chi-square (PROC FREQ in SAS), and found that the groups were different (P=0.02), even though they aren’t. Thus, I urge researchers to minimize binomial analyses, and urge reviewers and editors to be scrutinous of this method.

Table A-1. Experimental units for analyzing bovine blastocyst formation.

Table A-1		
Calculations out of Zygotes		
Analysis by Droplet, For 2 Groups (1 treatment, 1 control)		
Mean	27	
STD	12	
% Increase in Treatment	**Experimental Unit Needed/Group (Droplets)**	**At 25 embryos/well**
5%	90	2,250 zygotes/group
10%	23	575 zygotes/group
15%	10	250 zygotes/group
20%	6	150 zygotes/group
Analysis by Week, For 2 Groups (1 treatment, 1 control)		
Mean	24	
STD	7	
% Increase in Treatment	**Experimental Unit Needed/Group (Weeks)**	**At a minimum of 2 drops of 25 zygotes/week**
5%	31	1,550 zygotes/group
10%	8	400 zygotes/group
15%	3	150 zygotes/group
20%	2	100 zygotes/group
Calculations out of Cleaved		
Analysis by Droplet, For 2 Groups (1 treatment, 1 control)		
Mean	31	
STD	13	
% Increase in Treatment	**Experimental Unit Needed/Group (Droplets)**	**At 25 embryos/well**
5%	106	2,650 zygotes/group
10%	27	675 zygotes/group
15%	12	300 zygotes/group
20%	7	175 zygotes/group
Analysis by Week, For 2 Groups (1 treatment, 1 control)		
Mean	28	
STD	8	
% Increase in Treatment	**Experimental Unit Needed/Group (Weeks)**	**At a minimum of 2 drops of 25 zygotes/week**
5%	40	2,000 zygotes/group
10%	10	500 zygotes/group
15%	4	200 zygotes/group
20%	3	150 zygotes/group

This information was generated using the stated means and STDs generated by my blastocyst formation data in Appendix A. A free online software found at https://clincalc.com/stats/samplesize.aspx was used to generate the experimental units needed. In this software, study group design was set to “Two independent study groups”, primary endpoint was set to “Continuous”, the enrollment ratio was 1, alpha was 0.05, and the power was 80%

Appendix B: Embryo Treatment by Culture Drop Injection

Procedure with calculation example

1. Determine the total dilution needed. *I.e.* 1:100, 1:1000, etc.
2. Determine the amount that will be injected into the existing droplet to determine the total drop volume after injection.
3. Divide the #1 by #3 to figure out the needed dilution before injection.
    a. Example: Stock IL6 is 10 µg/ml and want to make drops of 100 ng/ml
    b. 10 µg/ml = 10,000 ng/ml
        i. 10,000/100 = 100
        ii. So overall a 1:100 dilution will be needed (answer to #1)
    c. Will be doing a 2 µl injection into a 50 µl drop. This is a 2:52 then or 1:26. (answer to #2)
    d. Divide 100/26 = 3.85
        i. So, a 1:3.85 dilution is needed before injection
4. Dilute treatment as needed in cryovials, and place in incubator with a loose cap for at least 30 minutes before use.
5. Add determined amount to each drop, and carefully swirl the tip around in the drop to mix.

Appendix C: Removing the Zona Pellucida & Snap-freezing Bovine Embryos

Materials Needed:

All materials must be sterile!

0.2 or 1.5 ml Tubes

35 mm plates

4 well Nunc Plates

Mineral or Paraffin oil

2% [w/v] Protease in Dulbecco's PBS (stored in single use aliquots at -20°C)

DPBS with 0.2% [w/v] PVP (PBS-PVP)

Liquid Nitrogen and Dewar Flask

Wiretrol

Water

Zona Pellucida Removal Instructions:

About 1 hours prior to working with embryos:

1. Thaw 2% Protease by placing upright in incubator or on slide warmer.
2. Remove 2% protease from heater after thawed.
    a. Be careful to keep upright and do not allow the precipitate to mix!
3. Carefully pipet 30-40 µl drops of protease solution onto a 35 mm plate.
    a. Avoid pulling up the precipitate as much as possible
    b. It will be impossible to avoid it completely
4. Cover with oil.
5. Make 4 well PBS-PVP wash plates.

To remove the zona pellucida:

6. Remove embryos from culture droplet and place in protease droplet.
7. Watch carefully under microscope and remove as soon as the zona pellucida is gone.

    a. Most will take 15-30 seconds, though some take longer.
8. Wash 3-4 times in PBS-PVP.

Snap-Freezing Instructions:

About 15 minutes prior to working with embryos:

1. Fill each of the 4 wells of 1 nunc plate per embryo treatment group with 500 µl PBS-PVP/well.
2. Pipette 4 µl of PBS-PVP or water into the bottom of each tube to be used for snap-freezing. (0.2 or 1.5 ml)
    a. It is very important that the drop stays at the bottom of the tube!
3. Set plate and tubes at room temperature.
4. Wipe the wire of a Wiretrol with ethanol before adding the glass capillary.
5. Wipe work surfaces with ethanol.
    a. This is mainly important if the embryos will be used for RNA isolation.
6. Fill a Dewar flask partially with liquid nitrogen.

When working with embryos:

7. Remove zona, if needed, as described above.
8. Wash embryos through 4 washes of PBS-PVP.
    a. Gently swirl for a few seconds for each wash.
    b. Be careful to avoid any kind of debris, including scratching the plate.
9. Carefully pull the needed embryos into <1 µl in Wiretrol.
    a. The white line indicates 5 µl.
10. Place the embryos in the droplet of PBS-PVP in the bottom of a tube.
    a. The tip of the Wiretrol MUST be submerged in the drop, but do not place it up against the plastic, or you will smash the embryos!
    b. The embryos will stick to the Wiretrol and not come out if you do not stick the Wiretrol in the drop.
11. Partially submerge just the bottom of the tube in liquid nitrogen.
    a. Hold it carefully with fingers, but do not lean over the tube.
        i. Tubes can explode if liquid nitrogen gets into the tube.

b. Do not let the cap become submerged to avoid liquid nitrogen getting in the tube.
    i. Only the portion of the tube containing the droplet needs to be submerged.

12. Hold for about 10 seconds.
13. Quickly transfer the tube to a container in the -80°C.
14. Repeat as needed.

Appendix D: Immunolocalization Protocols for SOX2, Cleaved Caspase 3, Ki67, Tead4, GATA6, NANOG and CDX2 in Bovine Preimplantation Embryos

Solutions to Prep Prior to Staining

Universal Solutions

1. PBS-PVP (0.2%)
    a. 0.2g polyvinyl pyrrolidone in 100ml Dulbecco's PBS (DPBS)
2. 4% Paraformaldehyde
    a. From 16% stock (Thermofisher #28906), 1ml in 3ml PBS-PVP
    b. Best used fresh, but ok for 1 week.
    c. Store at room temp, protected from light.
3. DAPI Nuclear Stain
    a. 1 µl stock (1 µg/ml) in 1ml PBS/PVP
    b. Prepare daily immediately before use
4. Antibody Buffer
    a. 100 µl Horse Serum in 9.9 ml DPBS
    b. Make 1ml aliquots and freeze.
    c. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
5. Wash Buffer
    a. 25 µl Tween20 and 0.025g BSA to 25ml DPBS.
6. 10% Horse Serum Blocking Buffer
    a. 1ml Horse Serum in 9ml DPBS
    b. Make 1ml aliquots and freeze.
    c. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
7. 10% Slowfade (Optional; Only necessary for samples which may be stained hours before they are imaged)
    a. 10 µl Slowfade in 90 µl PBS-PVP

Permeabilizations Solutions – Prep at least one day in advance

8. 0.25% Triton-X
    a. 0.25ml Triton X-100 in 100ml DPBS
9. 0.5% Triton-X
    a. 0.5ml Triton-X in 100ml DPBS

Antibodies

Primary	Company: Catalog#	Dilution	Host Isotype	Secondary Match
SOX2	ThermoFisher: 14-9811-80	1:100	Rat Monoclonal IgG2a, kappa	Any Anti-Rat IgG or IgG2a, kappa
Cleaved Caspase-3	Cell Signaling Technology: 9664S	1:100	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
Ki67	Thermofisher; 14-5698-80	1:500	Rat Monoclonal IgG2b	Any Anti Rat IgG or IgG2b
Tead4	Abcam; ab50945* discontinued. Try Ab151274	1:100	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
GATA6	Cell Signaling Technology; 5851T	1:500	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
NANOG	eBioscience; 14-5768-82	1:200	Mouse Monoclonal IgG1	Any Anti-Mouse IgG or IgG1
CDX2	Biogenex; AM392-5M	Use as is	Mouse Monoclonal IgG1	Any Anti-Mouse IgG or IgG1

1. For Secondary Antibody Selection:
    a. Thermofisher's Donkey Alexafluors work well. Use at 1:200. Goat antibodies also work, though can be a bit noisy, presumably because of bovine and caprine similarity.
2. If targeting 2-3 of these proteins, the primaries and secondaries can be mixed if they are different host or isotypes. Different isotypes may be used together so long as the secondaries are specific to those isotypes (i.e. not general IgG).

<u>Plates Needed</u>

1. 2x 96 well plates: one for wash steps, one for antibody/DAPI incubations
    a. Use 30-200 µl volumes.
    b. For wash steps, designate the plate for use only with a single protocol.
        i. These plates may be reused multiple times (washing with water and drying in between).

    c. For the Antibody/DAPI plate
        i. Each well should NOT be reused.
        ii. Mark each well after use and toss plate after all have been used.
2. 1x 4 well plates: for PFA
    a. PFA plates can be reused a few times.
    b. 400-600 µl/well

Protocol

Fixation

1. Wash embryos twice in PBS-PVP
2. Using a 4 well plate, place the embryos in 4% PFA. Incubate for 20 minutes at room temperature.
3. Wash embryos 3 times with new PBS-PVP.
4. Use immediately, or store at 4°C in PBS-PVP for up to 7 days.

Permeabilization

1. Use method 1 or 2, depending on the antibody:
    a. Option 1: For SOX2, Cleaved Caspase-3, ki67, CDX2, or Tead4:
        i. Place embryos in 0.25% Triton-X and incubate for 20 min at room temperature.
    b. Option 2: For GATA6 or NANOG:
        i. Place embryos in 0.5% Triton-X and incubate for 30 min at room temperature.
            1. Less-aggressive protocols may also work. Never tried.
2. Wash embryos twice with wash buffer.

Blocking

1. Place embryos in blocking buffer and incubate for 1 hour at room temp.

Antibodies

1. Place embryos in antibody solution and incubate 1 hour at RT to overnight at 4°C.
    a. Use 25-50 µl amounts, as antibody is expensive.
2. Wash 3 times with wash buffer.

3. Start dark room work. Place embryos in secondary antibody and incubate 1 hour at room temp.
4. Wash embryos 3 times with wash buffer.

<u>DAPI</u>

1. Place embryos in DAPI solution. Incubate for 5 minutes at room temperature.
2. Place embryos in 10% Slow fade or PBS-PVP.
    a. Slow fade is only needed if imaging will be hours-days after slides are made.

<u>Slide Mounting</u>

1. Using a small paint brush, line cover slip with a thin layer of petroleum jelly.
2. Place single embryos in small drops in a line inside the jelly square.
    a. Recommended to do 8 max per slide.
3. Carefully lower the coverslip on to the slide
    a. Watch carefully under a microscope!
    b. Mark embryo's location GENTLY with a fine tip BLACK sharpie.
        i. Colored sharpies will mess with fluorescence.
4. Seal with nail polish.

Appendix E: Immunolocalization Protocols for pSTAT3^{Y705} and its Combination with GATA6, NANOG or CDX2 in Bovine Preimplantation Embryos

Solutions to Prep Prior to Staining

1. PBS-PVP (0.2%)
    a. 0.2g polyvinyl pyrrolidone in 100ml Dulbecco's PBS (DPBS)
2. 4% Paraformaldehyde
    a. From 16% stock (Thermofisher #28906), 1ml in 3ml PBS-PVP
    b. Best used fresh, but ok for 1 week.
    c. Store at room temp, protected from light.
3. DAPI Nuclear Stain
    a. 1 µl stock (1 µg/ml) in 1ml PBS/PVP
    b. Prepare daily immediately before use
4. Antibody Buffer
    a. 100 µl Horse Serum in 9.9 ml DPBS
    b. Make 1ml aliquots and freeze.
    c. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
5. Wash Buffer
    a. 25 µl Tween20 and 0.025g BSA to 25ml DPBS.
6. 10% Horse Serum Blocking Buffer with Triton-X
    a. 1ml Horse Serum in 9ml DPBS
    b. Make 1ml aliquots and freeze.
        i. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
    c. Add 1-5 µl Triton-X, allow to dissolve.
7. 70% Ethanol
8. 10% Slowfade (Optional; Only necessary for samples which may be stained hours before they are imaged)
    a. 10 µl Slowfade in 90 µl PBS-PVP

Antibodies

Primary	Company: Catalog#	Dilution	Host Isotype	Secondary Match
pSTAT3^{Y705} option 1	Cell Signaling Technologies; 9145T	1:100	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
pSTAT3^{Y705} option 2	Santa Cruz Biotech; sc-8059	1:100	Mouse Monoclonal IgG2b	Any Anti-Mouse IgG or IgG2b
NANOG	eBioscience; 14-5768-82	1:200	Mouse Monoclonal IgG1, kappa	Any Anti-Mouse IgG or IgG1, kappa
GATA6	Cell Signaling Technology; 5851T	1:500	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
CDX2	Biogenex; AM392-5M	Use as is	Mouse Monoclonal IgG1, kappa	Any Anti-Mouse IgG or IgG1, kappa

1. For Secondary Antibody Selection:
    a. Thermofisher's Donkey Alexafluors work well. Use at 1:200. Goat antibodies also work, though can be a bit noisy, presumably because of bovine and caprine similarity.
2. For pSTAT3^{Y705} use only: option 1 is recommended (brighter staining).
3. For staining with NANOG, GATA6 or CDX2:
    a. For NANOG: Pair with pSTAT3^{Y705} option 1
    b. For GATA6: Pair with pSTAT3^{Y705} option 2
    c. For CDX2: Pair with pSTAT3^{Y705} option 1
    d. Using the combinations in a a,b or c, the primary and secondary antibodies can be mixed.

<u>Plates Needed</u>

1. 2x 96 well plates: one for wash steps, one for antibody/DAPI incubations
    a. Use 30-200 µl volumes.
    b. For wash steps, designate the plate for use only with a single protocol.
        i. These plates may be reused multiple times (washing with water and drying in between).
        ii. Using different antibodies may contaminate samples.
    c. For the Antibody/DAPI plate
        i. Each well should NOT be reused.
        ii. Mark each well after use and toss plate after all have been used.

2. 2x 4 well plates
    a. 1 for PFA
        i. PFA plates can be reused a few times.
        ii. 400-600 µl/well
    b. 1 for 70% Ethanol
        i. Can be reused
        ii. 400-600 µl/well

Protocol

<u>Fixation</u>

1. Wash embryos twice in PBS-PVP
2. Using a 4 well plate, place the embryos in 4% PFA. Incubate for 20 minutes at room temperature.
3. Wash embryos 3 times with new PBS-PVP.
4. Use immediately, or store at 4°C in PBS-PVP for up to 7 days.

<u>Permeabilization & Lipid Removal</u>

1. Using a 4 well plate, place embryos in 70% ethanol, and incubate for 5-10 minutes.
2. Quickly wash once in blocking buffer.
    a. PBS-PVP only will cause the embryos to float, and they will be difficult to pick up.
    b. Use only blocking buffer with Triton-X!
    c. BE VERY CAREFUL ON THIS STEP! The embryos 'jump' when moved out of ethanol, and it is very easy to lose an embryo. Count them to be sure you have them all. Wash the well 1-2 times with blocking buffer if needed.
3. Wash twice in Wash Buffer.

<u>Blocking</u>

1. Place embryos in blocking buffer and incubate for 1 hour at room temp.

<u>Antibodies</u>

1. Place embryos in antibody solution and incubate 1 hour at RT to overnight at 4°C.
    a. Use 25-50 µl amounts, as antibody is expensive.

2. Wash 3 times with wash buffer.
3. Start dark room work. Place embryos in secondary antibody and incubate 1 hour at room temp.
4. Wash embryos 3 times with wash buffer.

<u>DAPI</u>

1. Place embryos in DAPI solution. Incubate for 5 minutes at room temperature.
2. Place embryos in 10% Slow fade.

<u>Slide Mounting</u>

1. Using a small paint brush, line cover slip with a thin layer of petroleum jelly.
2. Place single embryos in small drops in a line inside the jelly square.
    a. Recommended to do 8 max per slide.
3. Carefully lower the coverslip on to the slide
    a. Watch carefully under a microscope!
    b. Mark embryo's location GENTLY with a fine tip BLACK sharpie.
        i. Colored sharpies will mess with fluorescence.
4. Seal with nail polish.

Appendix F: Immunolocalization Protocols for GATA6, NANOG and CDX2 in Bovine Preimplantation Embryos

Solutions to Prep Prior to Staining

1. PBS-PVP (0.2%)
    a. 0.2g polyvinyl pyrrolidone in 100ml Dulbecco's PBS (DPBS)
2. 4% Paraformaldehyde
    a. From 16% stock (Thermofisher #28906), 1ml in 3ml PBS-PVP
    b. Best used fresh, but ok for 1 week.
    c. Store at room temp, protected from light.
3. DAPI Nuclear Stain
    a. 1 µl stock (1 µg/ml) in 1ml PBS/PVP
    b. Prepare daily immediately before use
4. Antibody Buffer
    a. 100 µl Horse Serum in 9.9 ml DPBS
    b. Make 1ml aliquots and freeze.
    c. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
5. Wash Buffer
    a. 25 µl Tween20 and 0.025g BSA to 25ml DPBS.
6. 10% Horse Serum Blocking Buffer
    a. 1ml Horse Serum in 9ml DPBS
    b. Make 1ml aliquots and freeze.
    c. Thaw to use, meanwhile store at 4°C, discard after 24 hours.
7. 10% Slowfade (Optional; Only necessary for samples which may be stained hours before they are imaged)
    a. 10 µl Slowfade in 90 µl PBS-PVP
8. 0.5% Triton-X Permeabilization Solution
    a. 0.5ml Triton-X in 100ml DPBS

Antibodies

Primary	Company: Catalog#	Dilution	Host Isotype	Secondary Match
GATA6	Cell Signaling Technology; 5851T	1:500	Rabbit Polyclonal IgG	Any Anti-Rabbit IgG
NANOG	eBioscience; 14-5768-82	1:200	Mouse Monoclonal IgG1, kappa	Any Anti-Mouse IgG or IgG1, kappa
CDX2	Biogenex; AM392-5M	Use as is	Mouse Monoclonal IgG1, kappa	Any Anti-Mouse IgG or IgG1, kappa

1. For Secondary Antibody Selection:
    a. Thermofisher's Donkey Alexafluors work well. Use at 1:200. Goat antibodies also work, though can be a bit noisy, presumably because of bovine and caprine similarity.
    b. Use 488, 555 and 647 antibodies.
2. Beware, the NANOG and CDX2 antibodies have host isotype overlap. This requires a protocol with two rounds of primary and secondary antibody incubations, described below.

<u>Plates Needed</u>

1. 2x 96 well plates: one for wash steps, one for antibody/DAPI incubations
    a. Use 30-200 µl volumes.
    b. For wash steps, designate the plate for use only with a single protocol.
        i. These plates may be reused multiple times (washing with water and drying in between).
        ii. Using different antibodies may contaminate samples.
    c. For the Antibody/DAPI plate
        i. Each well should NOT be reused.
        ii. Mark each well after use and toss plate after all have been used.
2. 1x 4 well plates: for PFA
    a. PFA plates can be reused a few times.
    b. 400-600 µl/well

Protocol

Fixation

1. Wash embryos twice in PBS-PVP
2. Using a 4 well plate, place the embryos in 4% PFA. Incubate for 20 minutes at room temperature.
3. Wash embryos 3 times with new PBS-PVP.
4. Use immediately, or store at 4°C in PBS-PVP for up to 7 days.

Permeabilization

1. Place embryos in 0.5% Triton-X permeabilization buffer for 30 minutes.
2. Wash 2 times with wash buffer.

Blocking

1. Place embryos in blocking buffer and incubate for 1 hour at room temp.

Antibodies

1. Place embryos in a primary antibody mix of NANOG and GATA6. Incubate for 1 hour at room temp or overnight at 4C*.
    a. 1.25 µl NANOG, 0.25 µl GATA6 in 248.5 µl antibody buffer
    b. Overnight at 4°C increases nonspecific staining of NANOG, so it is preferable to do 1 hour at room temp.
2. Wash 3-6 times with wash buffer.
3. **Start dark room work.** Place embryos in mix of 2 secondary antibodies and incubate 1 hour at room temp.
    a. 1:200 for both secondaries.
4. Wash embryos 3-6 times with wash buffer.
5. Place embryos in CDX2 antibody solution. Incubate for 1 hour at room temp.
6. Wash 3-6 times with wash buffer.
7. Place embryos in secondary antibody solution and incubate 1 hour at room temp.
    a. Use the remaining anti-mouse alexafluor at 1:500.
8. Wash embryos 3-6 times with wash buffer.

DAPI

1. Place embryos in DAPI solution. Incubate for 5 minutes at room temperature.
2. Place embryos in 10% Slow fade.

Slide Mounting

1. Using a small paint brush, line cover slip with a thin layer of petroleum jelly.
2. Place single embryos in small drops in a line inside the jelly square.
    a. Recommended to do 8 max per slide.
3. Carefully lower the coverslip on to the slide
    a. Watch carefully under a microscope!
    b. Mark embryo's location GENTLY with a fine tip BLACK sharpie.
        i. Colored sharpies will mess with fluorescence.
4. Seal with nail polish.