THESIS

DEVELOPMENT OF A BISON-SPECIFIC EMBRYO CULTURE SYSTEM THROUGH TARGETED SUPPLEMENTATION OF MEDIA WITH STAGE-SPECIFIC GROWTH FACTORS

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ABSTRACT

DEVELOPMENT OF A BISON-SPECIFIC EMBRYO CULTURE SYSTEM THROUGH TARGETED SUPPLEMENTATION OF MEDIA WITH STAGE-SPECIFIC GROWTH FACTORS

In vitro embryo production (IVP) offers a practical genetic exchange method for bison herds, eliminating the need for live animal transport and reducing stress on the animals. While successful in cattle, IVP efficiency is lower in bison. This study aims to enhance bison embryo quantity and quality by supplementing IVP media with stage-specific growth factors. Thirteen growth factor receptors (GFRs) were screened in bison and bovine embryos, with six GFRs falling within acceptable ranges. Abattoir-sourced oocytes were used for IVP (4 replicates). GFR expression, notably IGFR2, BMPR2, FGFR1, and EGFR1, peaked in bison embryos at the zygote and 8-16 cell stages, with higher IL6 expression at the morula stage. Bovine embryos displayed highest expression of BMPR2, EGFR, and IGFR1 at zygote and 8-16 cell stages, and of FGFR1 and IGFR2 at zygote stages. Corresponding growth factors were incorporated into bison culture media based on GFR expression. Treatment evaluations, included EGF, IGF1, IGF2, IL6, BMP2, FGF1, and a combination of all GFs to bison culture media. Results revealed that the addition of BMP2 resulted in a decrease in cleavage rates. Notably, EGF, IGF1, and IGF2 enhanced blastocyst rates, with IGF1 significantly higher than the control. Subjective embryo qualitative evaluation showed

an upward trend in the number of high quality expanded blastocysts, and lipid content decreased (IGF1) while cell count increased (IGF1, IL6, BMP2, FGF1) with growth factor supplementation. In conclusion, supplementing IVP media with GFs, particularly IGF1 at 50 ng/mL, significantly improved both quantity and quality of bison embryos. Application of IVP theology has advance the use of assisted reproductive technologies for bison, potentially benefiting other species.

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DEDICATION

I dedicate this thesis to my husband, Javier Humaran, whose unwavering support and encouragement have been my pillars throughout this academic journey. His belief in my capabilities and the countless moments of encouragement propelled me forward during the challenges of research and writing.

To my family and in-laws, I extend my deepest gratitude for their steadfast support from a distance. Without their encouragement, understanding, and belief in my pursuits, the realization of this academic endeavor would have remained an unattainable dream. Their love and encouragement have been a source of strength, making this journey a reality.

This work stands as a tribute to the collective support and belief from my loved ones, without whom this academic achievement would not have been possible.

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Introduction

There are two species of bison in the world, *Bison bison* in North America, and *Bison bonasus*, commonly referred to as the wisent, located in Europe. *Bison bison* is further divided into two sub-species, *Bison bison* bison, known as the plains bison, and *Bison bison athabascae*, recognized as the wood bison (Aune et al., 2017). Both bison species have encountered a reduction in suitable habitats and anthropogenic pressures, culminating in a historical diminution of their populations and fragmentation of their distribution. However, the implementation of conservation initiatives has led to an amelioration of their prospects (Aune et al., 2017; Plumb et al., 2020; Olech and Perzanowski, 2022).

In North America, bison populations are stable, numbering approximately 31,000 individuals distributed across 68 conservation herds, with four herds (2 plains and 2 wood bison herds) surpassing the minimum viable population threshold, as documented by Aune et al. (2017). Notably, these figures do not encompass a significant proportion of bison in the commercial meat industry, estimated at around 400,000 individuals, with some managed within ranching systems that contribute positively to conservation, as reported by National Park Service (2023) and National Bison Association (2023). The persistence of these bison populations is reliant on active human intervention through effective herd management and conservation measures, emphasizing the protection of diverse territories, including national, state, provincial, and tribal lands in the United States and Canada (Aune et al., 2017).

Looking towards the future of bison conservation, restoring bison to larger landscapes, and building genetically robust and sustainable herds are priorities. This strategic direction aligns with the prospective role of bison in mitigating the effects of climate change on North American prairies. According to a recent long term study, bison grazing enriched native plant species twice as much as cattle grazing, and these gains persisted for nearly 3 decades, including during significant drought events. Bison are also a key element of cultural revitalization for many Native American and indigenous communities (Shamon et al., 2022). Bison restoration to native lands may play a key role in the ability of these communities to adapt to ecological and economic challenges through reestablishing food sovereignty and traditional food practices (Shamon et al., 2022).

Building and augmenting herds to achieve these goals will take careful planning and management to ensure herds are genetically sustainable. A population viability study conducted by the United States Department of the Interior, encompassing herds under their purview along with two in Canada, underscores the necessity for ongoing management to avert genetic diversity loss. The study indicated that genetic augmentation, involving the introduction of 2 or 3 genetically unrelated animals every 5 or 10 years, and potentially more frequently for less genetically variable herds, could mitigate these challenges (Hartway et al., 2020). Given the fragmented nature of existing herds and associated barrier to natural gene flow, interventions such as animal translocations or assisted reproductive technologies (ART) utilizing unrelated gametes and embryos are required for reintroducing genetic diversity (Hartway et al., 2020). However, despite the common use of translocations, the transport of large mammals like bison carries inherent risks, including potential injuries and biosecurity concerns (Comizzoli, 2017).

These challenges extend to disease transmission and social dynamics, influencing successful integration and genetic contributions of new members to the herd (personal experience and shared insights of bison herd managers). In contrast to translocations, these risks and challenges can be mitigated through use of ART. Shipping gametes and embryos is logistically easier than shipping live animals (Comizzoli 2017). If gametes and embryos are handled and tested properly, the chance of transmitting disease is negligible, as has been demonstrated by a large body of research on the disease risks associated with ART in livestock (Stringfellow and Givens, 2010; Mapletoft 2013).

Reproductive physiology

From an anatomical and physiological perspective, bison share sufficient similarities with domestic cattle to allow cattle to serve as a model for advancing ART in bison. Nevertheless, it is crucial to acknowledge the myriad of subtleties inherent in bison reproductive cycles, seasonality, and behavior that diverge from those of cattle. These distinctions hold significance for the strategic implementation of ART in bison.

Bison are seasonally polyestrous; estrous cycles range from 19-26 d (Kirkpatrick et al., 1991). Ovarian follicular waves occur every 7 days on average as observed by daily ultrasounds of wood bison heifers. Bison are considered long day breeders with peak breeding occurring July to September, though some breeding does occur in fall months (Vervaecke and Schwarzenberger, 2006). For females the transition from non breeding to breeding season is characterized by short cycles, as determined by fecal progesterone metabolites, with most females conceiving during their second seasonal cycle (Vervaecke and Schwarzenberger, 2006).

Male bison also exhibit reproductive seasonality, though sperm can be collected in all months of the year (Helbig et al., 2007a). The breeding season, or rut, is characterized by high circulating testosterone in bulls, typically double prerut measurements (Helbig et al., 2007; Mooring et al., 2004), and increased testis weight (Helbig et al., 2007). As such, sperm concentration and the number of normal sperm in the ejaculate also peak during the breeding months and may remain elevated for months after (Oct - Dec; Helbig et al., 2007). Hormonal changes are accompanied by rut behaviors which include tending females, threat displays, bellowing, charging, and fighting (Mooring et al., 2004); the rut season lasts 2 months (Roden et al., 2003).

Assisted reproductive technologies

Assisted reproductive technologies holds notable implications for bison conservation, offering a substantial avenue for conserving valuable reproductive material and facilitating the production of viable progeny through the utilization of gametes and embryos sourced from both captive and wild populations. Numerous investigations have centered on strategies aimed at reducing animal manipulation and consequent stress, alongside methodologies adaptable to field environments where interactions with highly valued individuals are feasible. Among the most prevalent ART techniques are sperm collection and preservation, estrus synchronization, artificial insemination, superovulation, and in vitro embryo production (IVP). While various techniques hold significance, this project specifically focuses on in vitro embryo production (IVP), aiming to provide a comprehensive and detailed exploration of this specific methodology.

In vitro embryo production

In vitro embryo production is perhaps the fastest growing ART for bison. Significant improvements have been made since the first report of bison IVP embryos in 2007 (Thundathil et al., 2007). Day 8 bison blastocyst development rates ranged from 5-10%, whereas, on average, bovine blastocyst IVP embryos at day seven post-fertilization usually fluctuate between 30 and 40 percent (Thundathil et al., 2007; Stoecklein, et al., 2021). IVP embryos have been generated for all bison species and subspecies. Despite these successes, there is much room for improvement. As in other species, in vitro embryo production rates in bison can vary due to many factors, including but not limited to seasonality, donor parameters such as age or pregnancy status, and differences in in vitro culture systems. While oocytes can be collected from post-mortem animals and via ovum pick up (OPU) throughout the year, there are some seasonal variations that are worth noting. While seasonal differences have not been noted in the oocyte's ability to undergo maturation, more oocytes are collected per ovary during the breeding season (Cervantes et al., 2016; Palomino et al., 2020).

Although there are seasonal variations in in vitro embryo development rates, competent embryos can be produced from post-mortem oocyte collections during the non breeding season. Benham et al., (2021) reported the birth of a calf from a vitrified embryo generated from oocytes collected postmortem from bison from Yellowstone National Park during the winter (non breeding season). Embryo production rates in this study were low and unaffected by age (juvenile or adult) or pregnancy status of the female (8-9% blastocysts per oocyte) possibly owing to the seasonally induced nutritional stress in this wild population (Benham et al., 2021). Nevertheless, when 15 embryos were warmed and transferred to 10 recipients, one healthy calf was born,

demonstrating the value of collecting oocytes from animals of all ages, regardless of pregnancy status, outside of the optimal season, particularly when they are genetically valuable.

The choice of culture media significantly impacts the efficiency and quality of in vitro-produced (IVP) embryos. Initial attempts to generate bison embryos using a two-step cattle IVP system yielded limited success, prompting exploration of fetal calf serum (FCS) supplementation. Addition of FCS to the first culture medium used for culturing embryos to the 8-cell stage improved cleavage rates, but blastocyst production rates were below 5%. However, when adding 5% FCS to the second culture medium only, used for growth of embryos beyond the 8 cell stage, blastocyst production rates reached 16% on a per oocyte basis (Barfield and Seidel., 2011). Thus, our system has incorporated 5% FCS in the culture medium after the 8-cell stage; a full description of our lab protocol for producing bison embryos can be found in Barfield (2019). It should be noted that the incorporation of FCS into culture media is thoughtfully evaluated due to potential consequences. These include secondary effects and changes in gene expression related to pathways involved in macroautophagy, growth, and development at the blastocyst stage. Additionally, it may be associated with developmental anomalies like fetal overgrowth and placental malformations (Rorie et al., 1994; Thompson et al., 1998)

Significant progress has been made in ART for bison, yet there are still many areas ripe for investigation. In vitro embryo production rates, while improved, remain low, particularly for oocytes collected postmortem. The media protocols employed for bison are predominantly derived from cattle systems, featuring minimal alterations (Barfield, 2019). Exploring the development of a media culture system tailored to bison could potentially offer a solution for augmenting embryo production.

Growth factors

One approach to modifying culture media for bison embryo development is through supplementation with growth factors (GFs). Growth factors play a pivotal role in regulating cellular functions and influencing the overall development of organisms. Thus, exploring the presence and significance of GF becomes essential when tailoring a media system for a particular species. One effective strategy to enhance the culture environment is to identify the specific growth factor receptor (GFR) in the target organism and the supplement the media system accordingly.

Growth factors are peptides and polypeptides categorized into families based on structural attributes. Interacting with specific cell membrane receptors, they initiate intracellular signaling pathways, often involving tyrosine kinase activation or serine/threonine phosphorylation (Giudice & Saleh, 1995). Growth factors possess the capability to regulate cell proliferation, operating in both positive aspects, often referred to as mitogens or promoters of mitosis, and negative aspects, either directly or by initiating the induction of other factors. Growth factors exhibit multifaceted mechanisms of action, functioning across various modes including autocrine, juxtracrine, paracrine, and endocrine, while concurrently exerting their mitogenic attributes (Mckay & Leigh, 1993; Giudice & Saleh, 1995).

Growth factors are produced and secreted by cells from a wide variety of tissues. They play a pivotal role as regulatory agents, serving as biological signals that govern cell growth, differentiation, and specific metabolic processes throughout both pre- and postnatal phases. These factors are synthesized by both normal and neoplastic cells in both in vitro and in vivo

settings. They are necessary constituents in culture media for sustaining and fostering cell survival and growth in vitro, and reducing rates of apoptosis (Pimentel, 1994).

Establishing an optimal environment within the mother's body prior to implantation is crucial to ensure successful pregnancy and subsequent embryo growth. Any disruptions to the mother's physiological balance during this early period can adversely affect embryo survival (Tríbulo et al., 2018). This observation implies that there exist factors produced either by the embryos themselves or within the maternal reproductive tract, that exert influence on the ability to survive before implantation. Growth factors play a regulatory role in the early stages of embryo formation (Pimentel, 1994). Their substantial influence on embryo development has been observed consistently across various species (Pimentel, 1994; Giudice & Saleh, 1995; Tríbulo et al., 2018).

Both the oviduct and endometrium of cattle display a noteworthy array of genes that are transcribed into signaling mRNA within the initial week post-ovulation (Tríbulo et al., 2018). Other studies reveal the presence of epidermal growth factor (EGF), transforming growth factor (TGF), as well as their shared receptors and corresponding mRNAs in the oviductal epithelium across multiple species (Giudice & Saleh, 1995). Many of these proteins likely have important roles in early embryo development. In fact, some of the proteins identified in Tríbulo et al., (2018) that are produced by the oviduct and endometrium have been shown to impact how cattle embryos develop.

Growth factors are organized into "superfamilies," each consisting of various subfamilies of proteins. These growth factors act as signals for transmembrane receptors, and each superfamily corresponds to its own set of related receptors. There is a strong specificity in

receptor binding within these superfamilies, but there are instances where multiple family members can bind to a single receptor, and a single family member may bind to multiple receptors (Zhang et al., 2019). Several growth factor families have been found to impact embryo development including EGF, IGF, FGF, TGF, IL6, LIF, and G-CSF, which will be reviewed here.

Epidermal growth factor (EGF) is a 53-amino acid cytokine (6.2 kDa) secreted by ectodermic cells, monocytes, kidneys, and duodenal glands (Leung, 2007). It plays crucial roles in cellular processes, including cell proliferation, survival, adhesion, migration, and differentiation (Leung, 2007; Richani & Gilchrist, 2018; Murphrey et al., 2023).

Fibroblast growth factors (FGF) constitute a diverse group of growth factors crucial for various essential cellular functions during embryonic development, such as proliferation, migration, angiogenesis, differentiation, and cell survival. In both mice and humans, there are 22 identified FGF ligands. Among these, the 18 canonical mammalian FGFs are categorized into six subfamilies based on sequence similarity and evolutionary relationships. These subfamilies include five paracrine subfamilies (FGF1, FGF2, FGF4, FGF7, FGF8) and one endocrine subfamily, FGF9 (Okumu et al., 2014; Xie Y. S. et al., 2020). FGF1 has been localized in the primate and ovine uterus, where its levels increase in response to estradiol (E2) treatment and are associated with mitogenic activity, and it has also been detected in bovine placentomes (Okumu et al., 2014). FGF2 is a pivotal player in numerous cellular processes, encompassing cell proliferation, apoptosis, migration, embryonic development, differentiation, wound healing, and notably, angiogenesis. It also holds significance in the growth and maturation of ovarian follicles, as demonstrated by increased follicular development and substantially elevated estradiol production when human ovarian tissue is exposed to FGF2 in vitro (Sherbet, 2011; Okumu et al.,

2014). Additionally, during early pregnancy, FGF2 is expressed by the ovine endometrium and conceptus, with peri-attachment conceptuses possessing multiple types of FGFRs. Furthermore, FGF2 mRNA has been detected in the bovine endometrium, suggesting its potential role in this context (Okumu et al., 2014).

Insulin like growth factor (IGF) is a polypeptide growth factor family that shares structural similarities with insulin. The two principal forms are insulin-like growth factor I (IGF1) and insulin-like growth factor II (IGF2), although several variants are recognized (Neirijnck et al., 2019). Specifically, the insulin-like family of growth factors plays a central role in regulating cell metabolism, growth, proliferation, differentiation, and survival, impacting virtually every organ. These components are significant participants in an intricate network of biochemical events that connect metabolic pathways, mitogenic processes, and reproductive functions (Neirijnck et al., 2019).

IGF1 has been linked to various reproductive traits, including age at first calving, conception rate during the initial service, twin ovulations, and early preimplantation embryo development (Velazquez et al., 2008). IGF2 produces a polypeptide that is present in large amounts in fetal tissues and the fetal bloodstream. It stands as one of the most influential growth factors during embryonic development, influencing the metabolism, growth, survival, and specialization of a broad range of cell types (Sandovici et al., 2022). The biological effects of IGFs are mediated through receptors, including insulin-like growth factor receptor I (IGFRI), insulin-like growth factor receptor II (IGFRII), and the insulin receptor (Sherbet, 2011). IGFI/IGFRI role is pivotal in FSH-triggered AKT activation, leading to the expression of steroidogenic enzymes and estradiol production in granulosa cells in both humans and rodents (Neirijnck et al., 2019).

Transforming growth factor -β (TGF-β) family is a group of pleiotropic cytokines that play crucial roles in tissue development and growth (Ingman & Robertson, 2002). They are particularly abundant in mammalian reproductive tissues, where they contribute to post-natal and adult tissue development and remodeling. This family includes well-known members like TGF-β1, TGF-β2, and TGF-β3, as well as other important growth factors like bone morphogenetic proteins (BMPs), inhibins, activin (ACV), growth and differentiation factors (GDFs), Vg-1, and anti-Müllerian hormone (AMH) among others (Shull & Doetschman, 1994; Ingman & Robertson, 2002; Sherbet, 2011).

TGF-β ligands have a pivotal role in regulating a wide range of cellular functions, including proliferation, differentiation, migration, adhesion, survival, and apoptosis. They exert their influence on both normal cellular processes and disease related pathways. In addition to their general functions, inhibin and activin, two peptide growth factors, play a critical role in controlling the release of follicle-stimulating hormone (FSH) from the anterior pituitary gland (Sherbet, 2011). Ovarian TGF-β is specifically involved in supporting folliculogenesis and the development of mature oocytes by enhancing FSH activity and promoting estradiol production. Furthermore, after ovulation, TGF-β likely contributes to the growth and function of the corpus luteum, with luteal cells and macrophages producing TGF-β1 and TGF-β2 (Ingman & Robertson, 2002).

TGF-β1 plays a significant role in reproductive function, influencing various stages from germ cell migration in embryonic development to the regulation of ovarian and testicular function in adulthood. Specifically, it affects ovarian function, as demonstrated through molecular techniques like Northern blotting, reverse transcriptase-PCR, and immunohistochemical analysis, which have identified mRNA transcripts for TGF-β1, TGF-β2, and TGF-β3, as well as corresponding

proteins for TGF-β1 and TGF-β2 in ovarian tissue (Shull & Doetschman, 1994). Both TGF-β1 and TGF-β2 proteins are found in the developing oocyte and embryos with TGF-β2 being most abundant prior to fertilization and diminished in 4- and 8-cell embryos (Ingman & Robertson, 2002).

BMPs have been acknowledged as vital regulators in the initial phases of embryonic development and play a prominent role in the differentiation of bone, cartilage, and connective tissues (Chen et al., 2004; Sherbet, 2011). Their importance goes beyond these functions, as they exert significant regulatory control over the differentiation, growth, and apoptosis of diverse cell types, encompassing epithelial, mesenchymal, hematopoietic, and neuronal cells. Furthermore, BMPs play a crucial role in the development of vital organs and systems, including the nervous system, heart, kidneys, lungs, skin, and gonads (Sherbet, 2011).

Activin Type IIB (ACV2) has been recognized for its involvement in early embryo development and its traditional role in regulating FSH secretion (Yoshioka et al., 1998). In a study conducted by Refaat et al. (2004), the examination of inhibin, activin, and their receptors in the fallopian tubes of premenopausal women revealed the presence of mRNA, suggesting potential activin synthesis by the uterine tube epithelium. This implies a role in the paracrine regulation of tubal epithelial cell function and embryonic activity. Furthermore, activin receptors have been identified in bovine oocytes and embryos throughout various developmental stages (Yoshioka et al., 1998).

Platelet-derived growth factors (PDGF) are potent inducers of mesenchymal cell proliferation and play a role in guiding the migratory behavior and differentiation of these cells during both developmental and adult stages. Additionally, in the context of embryonic

development, PDGFs facilitate progress beyond the 8-cell stage. The advancement to the morula stage is similar between embryos exposed to PDGF and those exposed to oviductal cells (Thibodeaux et al., 1993).

Platelet activating factor (PTAF) originates from various cell types, such as neutrophils, mononuclear phagocytes, and other inflammatory cells, in multiple species, including humans. PAF has been associated with a range of biological processes, including ovulation, sperm motility, fertilization, implantation, fetal lung maturation, and parturition, among others (Frenkel et al., 1996). In research conducted by Frenkel et al., (1996), they concluded that PTAF, generated by the developing embryo, plays a functional role in the process of implantation.

Interleukins (IL) serve as regulators of immune responses, influencing the differentiation of lymphocytes and hematopoietic stem cells, as well as affecting cell proliferation and mobility. These cytokines have a paracrine impact on nearby cells within the microenvironment and can stimulate the transcription of specific target genes (Sherbet, 2011). Members of the IL family, including IL-6, IL-11, IL-27, and leukemia inhibitory factor (LIF), have pivotal functions in different pregnancy stages, contributing significantly to anti-inflammatory processes. Notably, IL-6 is produced by glandular and luminal epithelial cells of the endometrium consistently throughout the menstrual cycle, with heightened secretion during the mid-secretory phase and early pregnancy (Pantos et al., 2022).

Leukemia inhibitory factor (LIF) is a member of the IL-6 pro-inflammatory cytokines, plays a crucial role in regulating embryo implantation in mice, and is believed to be necessary in these functions for various mammalian species, including humans. (Rosario & Stewart, 2016). LIF is synthesized within the glandular epithelium (GE) and subsequently released into the uterine

lumen (Kimber, 2005; Nicola & Babon, 2015). LIF can exhibit paradoxical and contrasting effects in various cell types, which can include both stimulation and inhibition of cell proliferation, differentiation, and survival (Nicola & Babon, 2015; Rosario & Stewart, 2016). Despite its complex effects on various cell types, LIF plays distinct and essential roles in maternal receptivity for blastocyst implantation, placental development, and nervous system development. In vitro experiments have shown that LIF promotes the development and differentiation of blastocysts. Additionally, LIF plays a significant role in the development of trophoblast cells and the placenta in both mice and humans. (Kimber, 2005). LIF has practical applications as well, as it is used to maintain the self renewal and totipotency of embryonic stem cells and induced pluripotent stem cells (Nicola & Babon, 2015). It is expressed in various embryonic and adult tissues with particularly high levels in the uterus (Kimber, 2005).

Colony stimulating factor (CSF) is produced by diverse cell types within the female reproductive system, including fibroblasts, granulosa cells, macrophages, natural killer cells, epithelial apical cells, and trophoblasts. Its presence has been identified as crucial for normal blastocyst development, as well as for ensuring the subsequent fetal viability and health in mice. (Ziebe et al., 2013; Ding et al., 2022). This family includes factors such as macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF; Ding et al., 2022). G-CSF plays a vital role in sustaining pregnancy through the regulation of immune responses in macrophages, lymphocytes, and Th2 cells (Ding et al., 2022).

These GFs elicit their effect by binding to specific receptors that govern numerous downstream signaling pathways. Figure 1, is a comprehensive overview of the signaling pathways

activated by the GF discussed here, primarily provided for reference, encompassing the MAP kinase pathway, SMAD kinase pathway, PI3K/Akt kinase pathway, and JAK-STAT pathway. These downstream pathways collectively influence nuclear activities, such as the regulation of gene expression, metabolism, cell division, cellular morphology, and cell survival (Qi & Elion, 2005; Morrison, 2012). Table 1 explains the predominant pathway associated with each growth factor family.

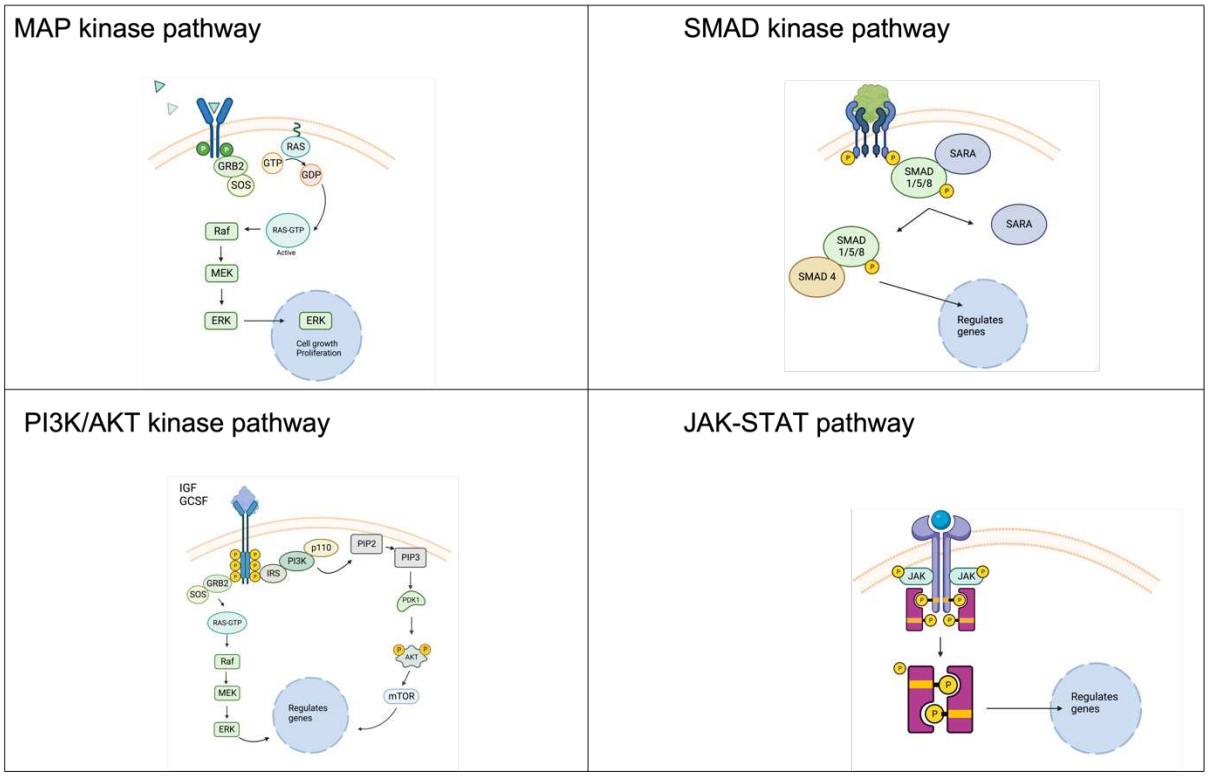

Figure 1. Different signaling pathways activated by various growth factors, such as the MAP Kinase Pathway, SMAD Kinase Pathway, PI3K/Akt Kinase Pathway, and JAK-STAT Pathway. Figure created in BioRender.com.

Table 1. Growth factor families and their corresponding activating pathways.

Growth factor Family	Activating pathway	Reference
EGF	MAPK	Stone et al., 2023
FGF		

FGF1 FGF2	MAPK PI3K/AKT	Xie Y. S. et al., 2020
IGF IGFI IGFII	 MAPK PI3K/AKT JAK/STAT	 Sherbet, 2011 Neirijnck et al., 2019
TGFB TGF-β1 TGF-β2 BMP2 ACV	 JAK/STAT pathway JAK/STAT pathway SMAD	 Ingman & Robertson, 2002 Yoshioka et al., 1998 Shull & Doetschman, 1994
IL6	JAK/STAT3 MAPK	Sherbet, 2011 Wooldridge et al., 2019
LIF	JAK/STAT3 PI3K/AKT	Rosario & Stewart, 2016 Kimber, 2005
PDGF PTAF	 PI3K/AKT JAK/STAT3	 Fischer et al., 2007 Sherbet, 2011
G-CSF	PI3K/AKT	Ding et al., 2022

The GFs play a pivot role in regulating cellular functions and influencing overall organism development. Various growth factor families, including EGF, FGF, IGF, TGF, IL6, LIF, and G-CSF, play multifaceted roles in regulating cell proliferation, differentiation, and survival, with specific implications for embryonic development. Subsequent chapters will explore growth factor receptors (GFR) in different embryo stages, aiming to identify the specific GF required for bison embryo development. This investigation is crucial for tailoring a media system that fosters optimal conditions for embryo growth, with the ultimate goal of incorporating these identified growth factors into the culture media to potentially enhance the efficiency and quality of in vitro-produced bison embryos.

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Chapter 2
Growth factor receptors in bison and bovine embryos

Introduction

Assisted reproductive technologies have revolutionized breeding programs in various animal species, yet the application in bison remains relatively underexplored. As the global population of bison faces challenges related to habitat loss, climate change, and genetic diversity, there is an urgent need to establish effective strategies for the conservation and management of these iconic species. This thesis addresses this critical gap by delving into the potential utilization of ART in bison, in particular, the study focuses on incorporation of growth factor supplementation to enhance the success of IVF in bison.

Growth factors are proteins capable of stimulating cellular growth, proliferation, and differentiation and play a central role in coordinating specific cellular reactions within a biological framework. Their significance extends to the regulation of a wide range of physiological processes, including apoptosis, immune and hematopoietic responses, morphogenesis, angiogenesis, metabolism, and wound healing (Pimentel, 1994; Zhang et al., 2019; Stone et al., 2023). Natural signaling molecules like hormones, growth factors, peptides, and neurotransmitters interact with specific receptors on the cell's surface, kickstarting a process called signal transduction. This process transfers signals from the outside to the inside of the cell, causing changes in gene activity and various cell responses (Pimentel, 1994; Stone et al., 2023).

Cellular responses to growth factors are initiated when these factors bind to transmembrane receptor proteins, which can take various forms, including those with intrinsic tyrosine kinase activity, G protein-linked receptors, and receptors incorporating ion channels in

their structure (Pimentel, 1994). The binding site for peptide/protein growth factors is located on the outer cell membrane surface, known as the extracellular domain. Phosphorylation cascades involving enzymes with kinase or phosphatase activity are central to the transmission of growth signals (Stone et al., 2023).

As discussed in Chapter 1, there are a variety of growth factors and their receptors that have been found to play important roles in female reproduction, follicle development, embryo development and implantation (Pimentel, 1994; Zhang et al., 2019). For example, EGF receptors (EGFR) in the mural layer and cumulus cells of follicles are activated in response to LH and lead to cumulus expansion, enhances oocyte developmental competence, and ultimately facilitates ovulation (Schneider & Eckhard, 2009). IGF1 has been found to contribute to oocyte maturation and in vitro embryo development in mammals (Neirijnck et al., 2019). In bovine oocytes and embryos, mRNA for TGF-β receptors (activin β A subunits, follistatin, and both type I and type II activin) have been identified, spanning from the immature oocyte stage to the hatched blastocyst stage (Yoshioka et al., 1998). The presence of mRNA activin receptor-IIB in the mouse ovary suggests that activin and inhibin act as localized regulators of ovarian function (Lu et al., 1993). Platelet-derived growth factor receptor (PDGFR) is found in stromal cells around oocyte clusters and primordial follicles, followed by expression in theca cells in the rat ovary (Sleer & Taylor, 2007). This suggests that PDGFR is likely expressed by cells surrounding pregranulosa cells, potentially acting as precursors to the later-developing theca cells. Leukemia inhibitory factor receptor (LIFR) mRNA has been verified in human oocytes and in vitro cultured preimplantation embryos through RT-PCR analysis, as demonstrated by van Ejik et al. in 1996. The G-CSF receptor (G-CSFR), is broadly expressed in T and B cells, granulosa-lutein cells, and endothelial cells.

Moreover, its presence has been verified in invading extravillous trophoblasts and stromal cells within the decidua (Ding et al., 2022).

Collectively, research on GFR highlights their ubiquitous presence in a diverse array of reproductive tissues, gametes, and embryos. The extensive distribution of GFR underscores their potential significance in regulating crucial processes across various stages of reproductive development. In this study, we tested the hypothesis that bison embryos have different expression patterns of GFRs than cattle embryos at the same stages of embryo development. We screened for the presence of 13 GFR in both bison and bovine embryos across developmental stages, including the 1-cell embryo (zygote), 8-16 cell embryo, morula, blastocyst, and expanded blastocyst. By systematically assessing GFR, we hope to streamline experiments that involve supplementing bison embryo culture medium with growth factors by only targeting growth factors for which there are receptors present at the different stages of development. The overall goal is to formulate a bison- specific culture media system that will enhance the in-vitro development and quality of in vitro-produced bison embryos.

Materials and methods:

Collection of bovine and bison granulosa cell

Ovaries were collected from a local slaughterhouse and transported to the lab in sterile saline at 25-28°C within 2 h of collection. Cumulus oocyte complexes (COCs) were aspirated using an 18-gauge needle and vacuum pump set to 50mm Hg, (Pioneer Pro Pump, Life Global Group, LLC, Guliford, CT, USA). The aspirated follicular fluid was collected in a 50 mL conical tube. To separate the granulosa cells, the upper fraction was transferred to a 15 mL tube, while the oocyte and tissue pellet were left behind. The collected follicular fluid underwent centrifugation at 500 x

g for 7 minutes, leading to the isolation of granulosa cells from the remaining fluid, which was subsequently decanted. To ensure optimal cell purity, 200 µl of RBC lysis buffer was added to the granulosa cell pellet and resuspended for a period of 3 minutes. After resuspension, 2 mL of holding media was added, followed by another centrifugation at 500 x *g* for 5 minutes. The supernatant was carefully removed, and this process was repeated until the highest purity of cells was achieved, typically requiring approximately three repetitions. Purity was assumed when only white cells were visible, 3 mL of 1 x PBS was added to eliminate any remaining RBC lysis buffer. Subsequently, another centrifugation at 500 x *g* for 5 minutes was performed, and the supernatant was again removed. This purification process was repeated using 2 mL of 1 x PBS. The purified granulosa cells were then stored at -80°C until they were ready for use.

In vitro embryo production

Ovaries were collected as mentioned. All media used for COC and embryo handling and culture were prepared as described by Barfield (2019). Recovered COCs with a minimum of three layers of intact cumulus cells were washed multiple times through H-CDM-M to remove debris and COCs placed in 4 well dishes containing 1 mL CSU chemically defined medium (see appendix 2) for in vitro maturation of oocytes (IVM) at 38.5°C in 5% CO_2 and air for ≥ 5 h. Oocytes were matured in IVM medium supplemented with 15 ng/mL NIDDK-oFSH-20, 1 µg/mL USDA-LH-B-5, 1 µg/mL estradiol 17β, 50 ng/µL epidermal growth factor, and 0.1 mM cysteamine for 23 h (Barfield 2019).

After maturation, COCs were fertilized in 4-well culture dishes, each containing 430 µL of equilibrated CSU chemically defined medium for in vitro fertilization (FCDM)/well (see appendix 2). Approximately 50 mature COCs were moved to each well in 20 µL of medium, and fertilized

with 0.5 x 10^6 sperm/mL of frozen-thawed bison sperm from a proven bull that had been washed through a 45/90 Percoll® gradient. The COCs and sperm were co-incubated for 18 h at a temperature of 38.5°C in a humid environment with 5% CO_2 in air.

Following the 18 h period, potential zygotes were stripped of cumulus cells through gentle micropipetting using a STRIPPER® equipped with a 150 µm stripper tip (Origio, CooperGenomics, Denmark). Denuded zygotes underwent washing through a series of HCDM-1 drops and were then transferred to wells containing 500 µL of equilibrated CSU chemically defined medium for in vitro culture of early embryos (CDM-1, see appendix 2). These early embryos were cultured for 56 h at 38.5°C in a humid atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

After culture in CDM-1 for 56 h embryos were evaluated for cell cleavage and embryos that possessed 4 blastomeres or more were transferred to 500 µL of equilibrated CSU chemically defined medium for in vitro culture of late embryos (CDM-2, see appendix 2), supplemented with 5% FCS per well (up to 30 embryos/well). These embryos were cultured for an additional 96-120 h at 38.5°C in a humid environment of 5% CO_2, 5% O_2, and 90% N_2, following the protocol outlined by Barfield in 2019. On day 7 and 8 post-fertilization, embryos were assessed to determine their development stage according to IETS standards (Stringfellow & Givens, 2010; Barfield and Demetrio, 2021).

Collection of embryos for PCR

During the above IVP process, a subset of embryos were collected as summarized in Table 2; varying cell quantities per stage accounted for differences in RNA concentration during isolation. All embryos were washed in 3-4 drops of DPBS-PVA, transferred to 100µL of RLT Buffer + 0.01% mercaptoehtahnol, and frozen at -80°C.

Table 2. Embryo stage and time of collection for Polymerase Chain Reaction (PCR).

Stage of embryos	Time of collection	No. of embryos collected*
1 cell embryo	20 h p.i.	200
8 + cell embryo	56 h p.i.	120
Morula	6 d p.i.	80
Blastocyst	7 d p.i.	20
Expanded blastocyst	7 d p.i.	20

*Embryos were collected over 4 replicates, p.i.= post insemination.

Isolation of RNA

RNA isolated for this study was extracted using the miRNeasy® Mini Kit for granulosa cell samples and the RNeasy® Plus Micro Kit for embryonic samples, both of which were procured from Qiagen. Comprehensive protocols for RNA isolation from granulosa cells and embryos are available on the manufacturer's official website.

First-Strand cDNA

For the synthesis of first-strand cDNA, we employed the SuperScript™ III First-Strand Synthesis Super Mix provided by Invitrogen, following the manufacturer's recommended protocol. This protocol is designed to convert a range of RNA quantities, from 0.1 picograms (pg) to 5 micrograms (µg) of total RNA, into first-strand cDNA. The RNA sample was combined with 1 µL of primer (0.5 µL of Oligo(dT) and 0.5 µL of random hexamers), and RNase-free water was added to achieve a final volume of 8 µL. This mix was incubated in a thermal cycler at 65°C for 5 minutes. Following this initial step, 10 µL of 2X First-Strand reaction buffer and 2 µL of the Enzyme mix were added to the reaction mixture. Subsequently, the new mixture underwent a series of thermal cycles, which included incubation at 25°C for 5 minutes, followed by 50 minutes at 50°C,

and a final step at 85°C for 5 minutes. The resulting cDNA synthesis reaction was then stored at -20°C until further use.

PCR amplification reaction

To generate a PCR product, a master mix was formulated by combining the following components: 10 µL of Go Taq Green Master Mix (Promega), 1 µL of forward primer, 1 µL of reverse primer, 6 µL of water, and 2 µL of cDNA. This master mix underwent a thermal cycling program as follows: A) initial denaturation at 95°C for 2 minutes; B) amplification consisting of 40 cycles, including 1) denaturation at 95°C for 30 seconds, 2) annealing at 55°C for 30 seconds, and 3) extension at 72°C for 1 minute; C) a final extension step at 72°C for 10 minutes; and D) storage at 4°C indefinitely.

PCR amplification reactions were carried out using a 2% agarose gel, composed of 100 mL of 1x TAE Buffer (Invitrogen), 2 grams of agarose (Sigma-Aldrich), and 3 µL of Ethidium Bromide (Fisher Bioreagents). The prepared agarose gel was positioned within a running chamber, and 10 µL of the PCR product was added to each well. The running chamber was operated at 120 volts for a duration of 30 minutes.

Real-Time PCR (RT-qPCR)

A master mix was prepared by combining the following constituents: 10 µL of iQ™ SYBR® Green Supermix (Bio-Rad), 0.5 µL of the forward primer, 0.5 µL of the reverse primer, and 7 µL of water for each sample. Subsequently, 18 µL of this master mix was dispensed into each well of a 96-well plate, followed by the addition of 2 µL of cDNA. As a control for each primer, 2 µL of water was included.

Primer selection

Table 3 displays the sequences of the primers used for this study. These primers were custom designed through the National Center for Biotechnology Information (NCBI) and subsequently acquired from Integrated DNA Technologies (IDT). The primer pair for PDHA was utilized as a control for primer validation, while the primer pairs for β-actin and GAPDH were included as controls for RTq-PCR.

Table 3. Gene, primer sequences, GenBank accession Number, product size, and annealing temperature used for analysis by RTq-PCR.

Gene	Specie primer designed	Primers Sequence (5'->3')	GenBank Accession No.	Product size	Annealing temperature (°C)
EGFR	Bison bison bison	F: ATTGATGGCCCTCACTGCGT R: GACGGGATCTTGGGCCCTTT	XM_010842153.1	179	62.2-62.21
FGFR1	Bison bison bison	F: CCGTCAAATGGATGGCACCG R: CCCAAAAGACCACACGTCGC	XM_010838846.1	74	61.99-62.11
FGFR2	Bison bison bison	F: TCACACTCACAACCAATGAGGAAT R: TCCTGAAAGAAGGGAAGAGAGACG	XM_010859237.1	114	60.45-61.59
IGFR1	Bos taurus	F: CGAGTGGAGAAATCTGCGGG R: GGTAGCTGCGGTAGTCCTCG	NM_001244612.1	138	60.81-62.06
IGFR2	Bos taurus	F: CCACAACGGAGCCTCGTACT R: CTGGCTCTCTCCGAGCAGTC	NM_174352.2	78	61.87-62
TGFBR1	Bison bison bison	F: TCCCCGAGACAGGCCATTTG R: GGCCAGATGATGGCTTTCCAAC	XM_010847106.1	128	62.2-62.11
TGFB2	Bison bison bison	F: GCAACGCAGTGGGAGAAGTGA R: TCTGGTCGGCCTCGATCTCT	XM_010835564.1	115	63.16-62.27
ACVR2	Bos taurus	F: CGAAGGAAGGAGCGGGAAGG R: CCCCGAACCGGCGCA	NM_174495.2	173	62.58-62.4
BMPR2	Bison bison bison	F: AGGATGCTGACAGGAGACCG R: ACAGCTCCATATCGACCCCG	XM_010860989.1	134	61.33-61.46
LIFR	Bison bison bison	F: TCCTGGTTGCTGATTGGAGAACT R: CCAGCCAGGATGGTCGTTTC	XM_010853752.1	81	61.84-61.03
IL6R	Bison bison bison	F: CGCTTGGTGGTGGATGTTCC R: GGACTCGGGGGAATACTGGC	XM_010838440.1	186	61.59-62.04
PTAFR	Bison bison bison	F: CCCTGGGGTCATGGCATTTC R: AGGATCCTGTCTGTTCCTGTCC	XM_010830158.1	158	61.04-61.16
GCSFR	Bison bison bison	F: CCAGGACAAATGCCAGACGC R: GGGGCTCCAGTTTCACAACG	XM_010841154.1	200	61.93-61.52

F: Forward, R: Reverse

Primer efficiency

Using granulosa cells, primer efficiencies were assessed for both bison and bovine, using RT-qPCR analysis, employing standard curves. To evaluate the efficiency of the primers, a series of dilutions (1X, 2X, 4X, 8X, and 16X) were prepared. The primer efficiency was calculated from the slope of the standard curve using the formula: efficiency (%) = (10^(-1/slope) - 1) × 100%. Efficiency values falling within the 90% to 110% range were considered acceptable for both species. This method allowed for the rigorous assessment of primer performance, ensuring that only primers with efficient amplification properties were utilized in subsequent experiments.

Statistics

RT-qPCR data from bison and bovine embryos screened for the 13 GFRs underwent analysis through the ΔΔCt method. Statistical comparisons involved two dimensions: between species (Bison vs. Bovine) and within species at varying embryo stages. To conduct these comparisons, a two-way ANOVA followed by Tukey's post-hoc test was applied, with bovine embryos serving as the reference for species comparisons.

Results

Primer validation

A total of 13 primers, with PDHA serving as the control, were evaluated. Notably, successful amplification was observed for all 13 primers, including the control PDHA, as evidenced by the presence of bands in the gel electrophoresis analysis (Fig 2). This validation underscores the robust performance of these primers across the samples tested.

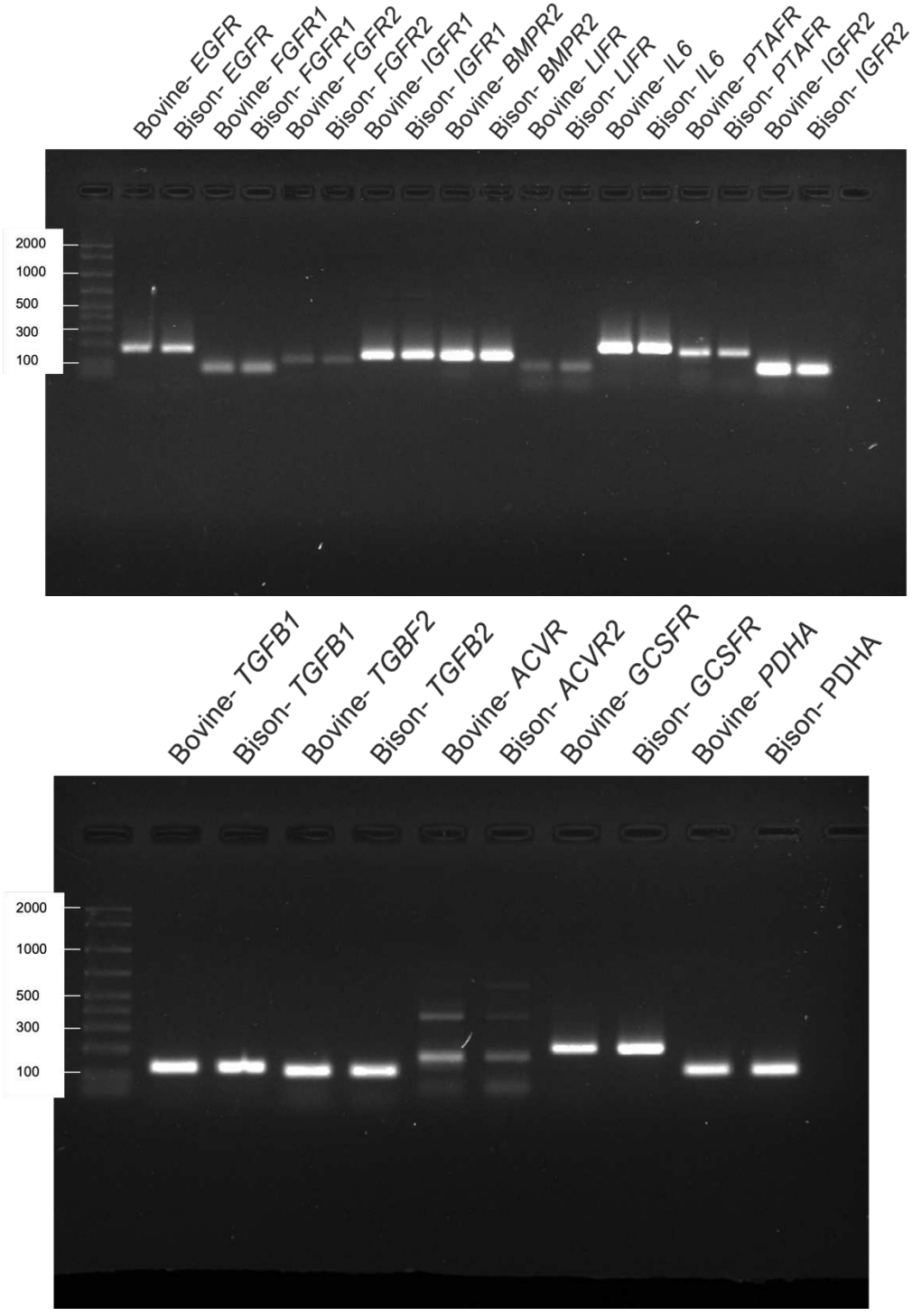

Figure 2. Primers amplification with bison and bovine granulosa cells by PCR. PDHA was used as a control.

Primer efficiency

Thirteen primers were assessed for primer efficiency in the study. Among these, IGFR2, IL6R, BMPR2, FGFR1, EGFR, and IGFR1 exhibited efficiencies falling within the accepted range of 90% to 110%. This successful validation confirms their appropriateness for precise gene expression quantification in both bison and bovine granulosa cells. The remaining genes did not meet the necessary primer efficiency criteria, which led to their exclusion from the analysis. For detailed efficiency values and quantification results, see Table 4.

Table 4. Primer efficiency values for genes in both Bison and Bovine samples.

	Bison	Bovine
GAPDH	90.1	89.3
B-Actin	102.1	90.0
EGFR	92.5	81.1
FGFR1	99.7	86.9
FGFR2	-	-
IGFR1	94.8	77.7
IGFR2	116.6	94.2
LIFR	-	-
PTAFR	-	93.4
IL6R	106.6	94.1
BMPR2	94.4	103.6
ACVR2	9.1X10^13	159.6
GCSFR	40.7	-
TGFB1	68.4	113.7
TGFB2	69.4	50.0

The slope of the standard curve values between 90-110% were considered acceptable.

Detection of growth factor receptors in bison and bovine embryos

When analyzing bison embryo stages with the zygote as the reference point, the highest expression levels of IGFR2, BMPR2, FGFR1, EGFR, and IGFR1 occurred during the zygote and 8-16

cell embryo stages. Notably, morulae stage bison embryos exhibited significantly elevated expression of IL6R (Figure 3).

Within bovine embryos, the highest expression levels of BMPR2, EGFR, and IGFR1 were observed during the zygote and 8-16 cell embryo stages. Additionally, zygotes displayed notably elevated expression of FGFR1 and IGFR2, while 8-16 cell embryos exhibited significantly increased expression of IL6R (Figure 4).

In comparing bovine embryos to bison embryos, significant variations in gene expression were observed (Figure 5). In bison embryos, notably higher fold changes were detected in several genes, including BMPR2 in 8-16 cell embryos ($p<0.05$) and morulae ($p<0.00001$), FGFR1 in 8-16 cell embryos ($p<0.00001$), EGFR in 8-16 cell embryos ($p<0.001$) and blastocysts ($p<0.00001$), and IGFR1 in 8-16 cell embryos ($p<0.001$), morulae ($p<0.00001$), blastocysts ($p<0.05$), and expanded blastocysts ($p<0.001$). Notably, there were no significant differences in the expression of IL6R between the two species. Additionally, the fold change in IGFR2 was consistently lower in bison embryos across all developmental stages.

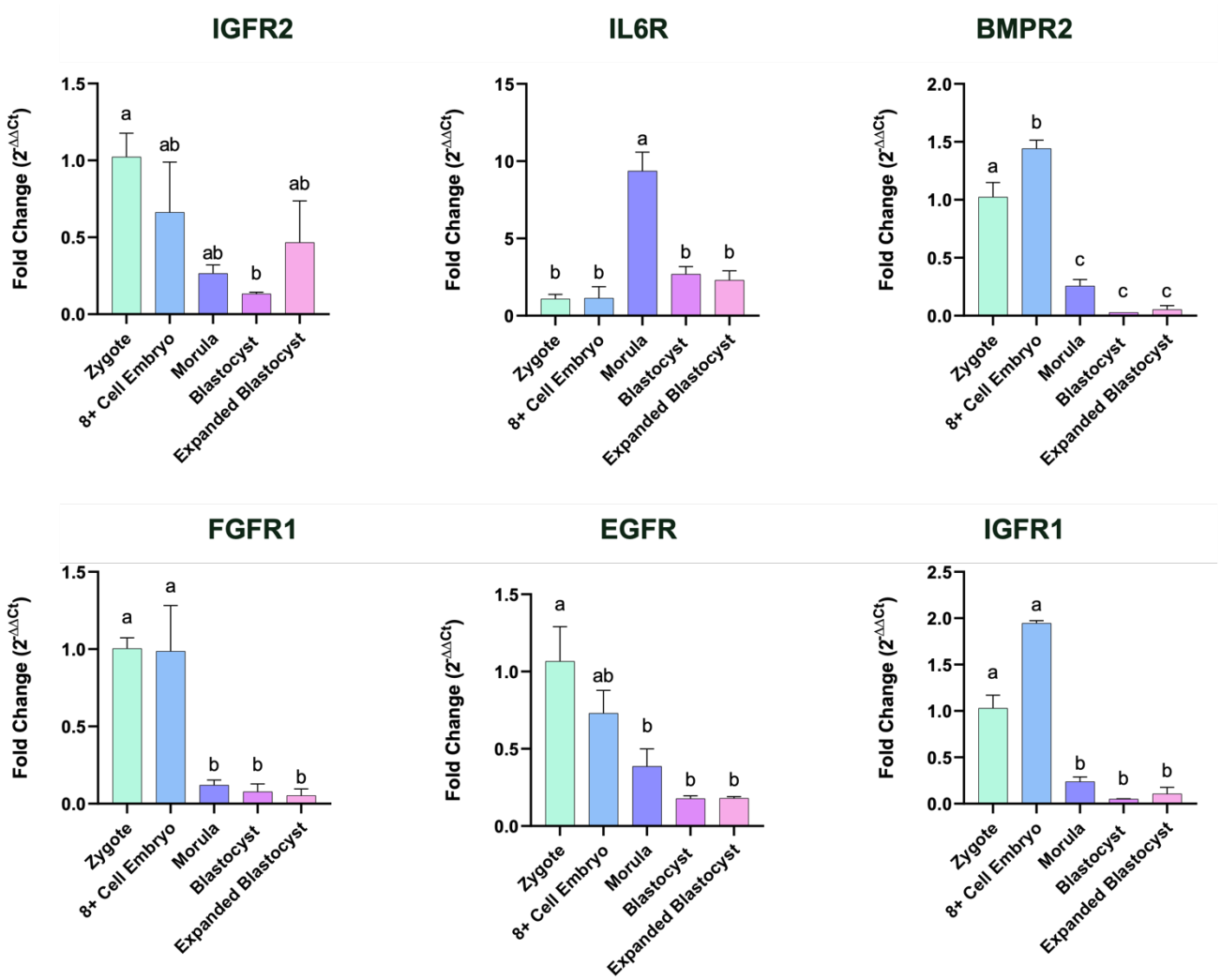

Figure 3. Growth factor expression in bison embryos by stage.
The data reflects relative abundance values calculated through the ΔΔCt method. Stage-wise comparisons were conducted using the zygote as the baseline, and statistical significance was determined based on $p < 0.05$.

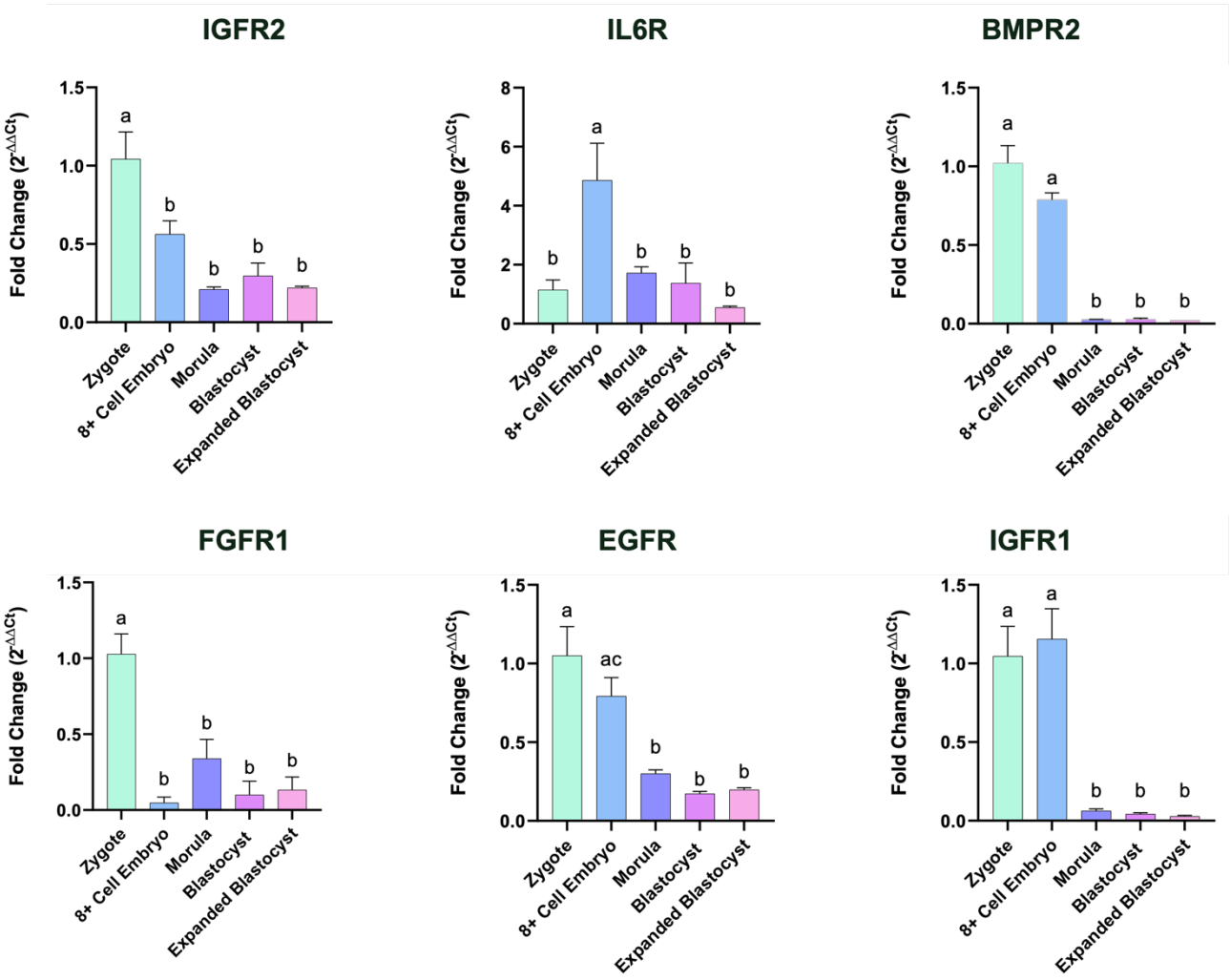

Figure 4. Growth factor expression in bovine embryos by stage.

The data reflects relative abundance values calculated through the ΔΔCt method. Stage-wise comparisons were conducted using the zygote as the baseline, and statistical significance was determined based on a $p < 0.05$

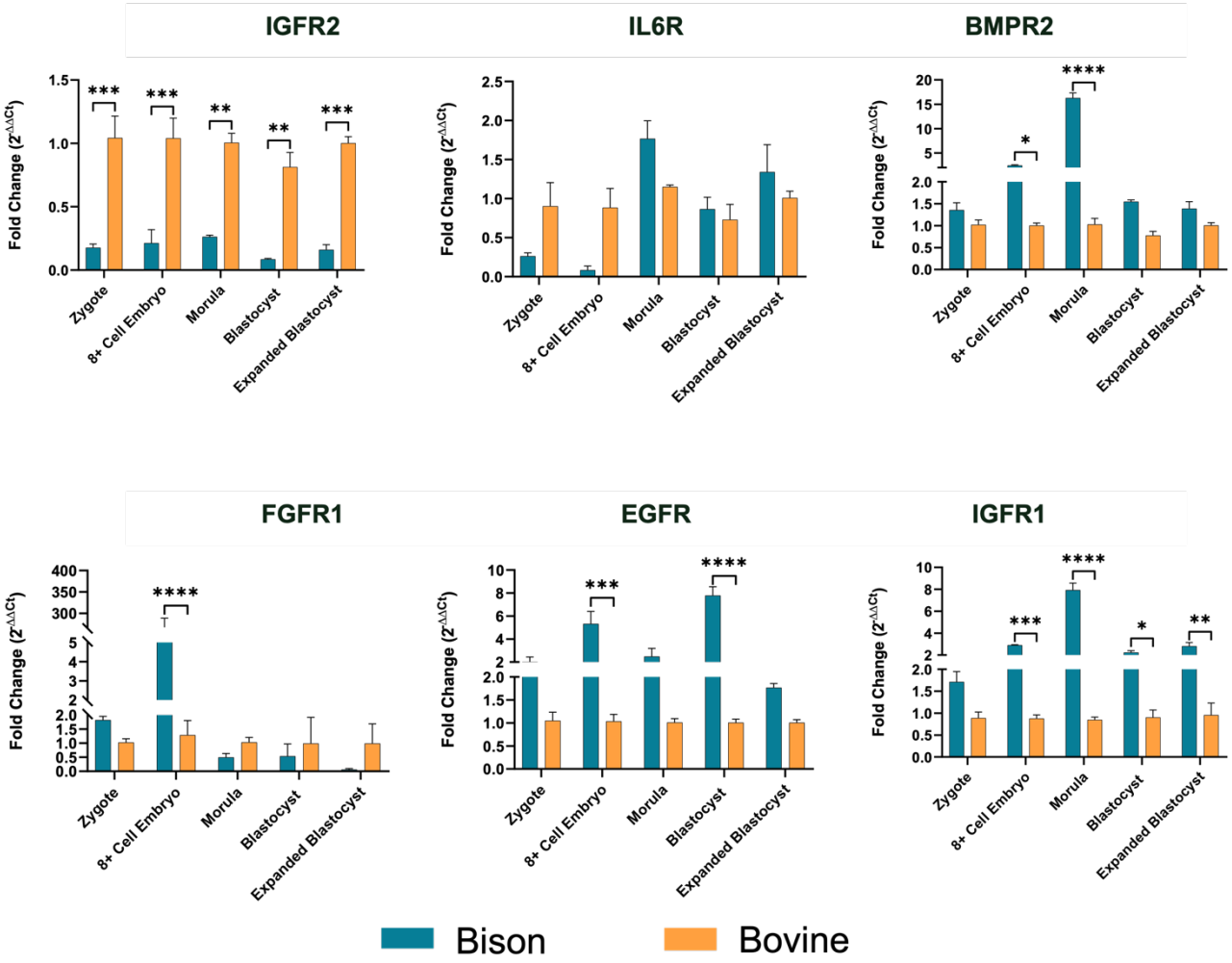

Figure 5. Comparison of GFR expression in bison and bovine embryos.
The data reflects relative abundance values calculated through the ΔΔCt method. Stage-wise comparisons were conducted using the bovine zygotes as the baseline, and statistical significance was determined based on a $p < 0.05$.
*, $p < 0.05$; **, $p<0.001$); ***, $p<0.0001$; **** $p<0.00001$

Discussion

In this study, we found 6 growth factor receptors that were differentially expressed between bison and cattle preimplantation embryos. While we had planned to screen 13 growth factor receptors (GFRs) to determine their presence in both bison and bovine embryos, our primer efficiencies for 7 of the GFR were not reliable enough to produce accurate measurements. We acknowledge that the issue with primer inaccuracies may have arisen from the potential presence of primer dimers in our experimental setup.

However, six genes, namely IGFR2, IL6R, BMPR2, FGFR1, EGFR, and IGFR1, met the primer efficiency criteria, and have been identify in bison and bovine embryos at different stages. Our finding of EGFR in bovine and bison IVF embryos reveals significant EGFR expression during the zygote and 8-16 cell embryo stages in both species, consistent with a role in support of early embryo development. This pattern aligns with similar findings in other species (Schneider & Eckhard, 2009).

Kliem et al., (1998) emphasized the significance of the EGFR system in bovine preimplantation development, detecting EGFR mRNA and protein in various stages of bovine blastocysts, implying active EGFR signaling pathways. Our findings complement this by revealing high EGFR expression during the zygote and 8-16 cell embryo stages in bovine embryos. Additionally, Thouas et al., (2015) provided insights into EGFR in human preimplantation embryos, demonstrating its presence from the earliest stages of embryogenesis, specifically in the oocyte and eight-cell stage embryos. Paria et al., (1994) demonstrated the importance of EGF in the spotted skunk, revealing EGFR presence in both the uterus and blastocysts during delayed implantation, suggesting its role in blastocyst activation and implantation. Comparing these

findings with our results, which indicate high EGFR expression in bovine and bison embryos, underscores a conserved EGFR role in regulating early embryo developmental processes across species.

Our results indicate presence of IGFR1 and IGFR2 in bovine and bison embryos wich aligns with existing literature, emphasizing the significance of IGF receptor expression in early embryonic development. We found high expression of IGFR1 and IGFR2 during the zygote and 8-16 cell embryo stages in both species. Previous research by Lonergan et al., (2000) detected IGFR1 in 2-cell bovine embryos. Additionally, Thouas et al., (2015) highlighted IGFR1 and IGFR2 expression in all stages of preimplantation human embryonic development, confirming the conservation of IGFR expression across species.

Our study reveals high FGFR1 expression during the zygote and 8-16 cell embryo stages in bison embryos and high in only zygote stage bovine embryos, which is consistent with previous research. Kurowski et al., (2019) demonstrated FGFR1 importance in trophectoderm development and trophoblast stem cell maintenance in mice, with implications for early pregnancy outcomes. Kang et al., (2017) suggested distinct roles for FGFR1 and FGFR2 in mouse blastocysts, which aligns with our results showing high FGFR1 expression in zygotes (bison and cattle) and 8-16 cell (bovine). Deng et al., (1994) found that FGFR1 was necesary for proper embryonic cell proliferation and axial organization during early post implantation development in mice. Moreover, the literature review by Thouas et al., (2015) highlights the presence of FGFR1 protein in trophectoderm cells of human blastocysts, providing further evidence for the preservation of FGFR1 involvement in early human embryonic development. Collectively these

findings enhance our understanding of FGFR1 significance in early embryogenesis across species and its potential implications in embryonic development.

The limitations in primer efficiencies hindered our ability to assess seven other GFRs, including TGF-β and BMPs, recognized for their crucial roles in folliculogenesis, mature oocyte development, and early embryonic development (Ingman & Robertson, 2002; Chen et al., 2004; Sherbet, 2011). Yoshioka et al. (1998) identified the significance of activin β subunits, follistatin, and activin receptors in bovine oocytes and embryos. Similarly, PDGFs, instrumental in advancing beyond the 8-cell stage, were not tested in our study, but have shown comparable progression to the morula stage (Thibodeaux et al., 1993). GCSF, is essential for normal blastocyst development and fetal viability (Pantos et al., 2022; Kimber, 2005; Ziebe et al., 2013; Ding et al., 2022). Although these GFRs were not included due to primer limitations, their known functions emphasize the potential impact of their differential expression on embryonic development. Although we could not test for seven of the genes, they may still play a role in bison embryo development. Future research will focus on redesigning of these primers for screening in bison embryos.

In our analysis, we observed differential expression in 6 GFRs between bison and cattle embryos. Noteworthy variations include elevated BMPR2 in 8-16 cell embryos and morulae, FGFR1 in 8-16 cell embryos, EGFR in 8-16 cell embryos and blastocysts, and IGFR in 8-16 cell embryos, morulae, blastocysts, and expanded blastocysts among bison embryos. No significant distinctions were found in IL6R expression between the two species. Furthermore, IGFR2 consistently exhibited lower fold changes in bison embryos across all developmental stages. These significant disparities highlight the distinct molecular profile of bison embryos, providing

potential insights on the inadequacy of employing a medium originally designed for bovine embryos in the IVP for bison.

In conclusion the identification of differential expression in GFRs during bison preimplantation embryo development provides valuable insights for the establishment of a species-specific embryo culture system. Leveraging the observed high expression of certain GFRs, such as IGFR1, IGFR2, BMPR2, FGFR1, and EGF, in the zygote and 8-16 cell stages, their respective GF can be strategically incorporated into our culture medium up to 8-16 cell stage. Likewise, GFRs expressed in the morula, blastocyst, and expanded blastocyst stages, like IL6R, suggest a potential benefit at formulation of culture medium for embryos past the 16 cell stage enriched with these growth factors. This tailored approach aims to optimize the culture environment, enhancing the conditions for bison embryonic development and contributing to the advancement of ART for the conservation and management of this iconic species.

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Chapter 3
Supplementation of validated growth factor on embryo culture media

Introduction

In recent years, advancements in ART have propelled research in the field of bovine embryo culture, focusing on the supplementation of culture media with various GFs to enhance developmental outcomes. Several studies have investigated the impact of individual or multiple growth factors on bovine embryo culture, with promising results.

Xie et al., 2017 reported significant increases in bovine blastocyst development (*P<0.05)*, expansion (*P<0.05)*, and hatching (*P<0.05)*, depending on supplementation with GF individually or in combination. Studies by Yang et al., (2022) showed that supplementation of bovine IVM medium with 50 ng/mL EGF, 100 ng/mL insulin like growth factor 1 (IGF1), and 25 µg/mL Cx37, significantly increased nuclear maturation, blastocyst development, blastocyst rate, and blastocyst total cell number when compared with control group ($p < 0.05$). Similar results were obtained by Yuan et al., (2017) and Stoecklein et al., (2021) when IVM medium was supplemented with 40 ng/mL fibroblast growth factor 2 (FGF2), 20 ng/mL leukemia inhibitory factor (LIF), and 20 ng/mL IGF1, in combination called “FLI” medium. This medium increased nuclear maturation of oocytes in cumulus-oocytes complexes derived from immature pig ovaries from 55% in controls to 89% in the FLI treatment ($p<0.05$; Yuan et al., 2017). After IVF, FLI-treated oocytes exhibited elevated blastocyst rates, with a statistically significant difference observed (38% in the control group compared to 50% in the FLI treatment, $p<0.05$; Yuan et al., 2017). FLI treatment was also tested with bovine in vitro maturation medium (Stoecklein et al., (2021). In this study, there was no effect in cleavage percentage (day 3 development rates) between the FLI treatment (83.7%)

and the control group (82.4%). However, there was an increase in the proportion of embryos that developed to blastocyst stage in the FLI treatment (38.6%) vs control (27.2%). The supplementation of FLI during the culture period increased development to the blastocyst stage, cytoskeleton integrity, decreased lipid content in blastocysts, survival following slow freezing, and decreased post-thaw cell apoptosis (Yuan et al., 2017; Stoecklein et al., et al., 2021).

Individually and in combination, EGF and IGF1 have been added to culture media for buffalo and bovine in vitro embryo production. These growth factors enhanced nuclear maturation and embryo development for both species. While the inclusion of growth factors individually led to improvements in embryo production, the most favorable outcomes were achieved when the growth factors were used in combination (Lee & Fukui, 1995; Purohit et al., 2005). The effect of supplementing bovine IVP media with EGF and IGF1 on the blastocyst rate was as follows: EGF 14.6% (*P<0.01)*; IGF1 8.7%; EGF + IGF1 25.9% (*P<0.001)*; control 5.2% (Purohit et al., 2005). Neira et al., (2010), found that simultaneous supplementation of GF and cytokines at 50 ng/mL in bovine culture media resulted in significant increases in percentages of blastocyst (45%) and hatched blastocysts (22%) compared to the control medium (blastocyst 24%, hatched blastocysts 12%) on day 8 after fertilization.

In a study by Flood et al., (1992) all GFs were uniformly added at a concentration of 10 ng/mL. There was no improvement in blastocyst rates compared to the control (control 12%; EGF 17%; TGFα 14%; TGFβ1 3%, IGF1 12%, IGF2 11%, bFGF 12%). It may be that the chosen concentration was too low to initiate an effect. This underscores the significance of identifying the right concentration, specific GFs, and optimal supplementation timing necessary to formulate an appropriate medium for successful in vitro embryo production.

Supplementation of media with GF, specially FLI, can reduce embryo lipid content (Yuan et al. 2017). Lipids, play a crucial role in cellular energy storage, signaling transduction, and membrane structure. In the context of mammalian gamete and embryo cryopreservation, phospholipids (PLs), represent the primary constituents of the plasma membrane and stand out as particularly susceptible to freezing-induced damage among all chemical constituents (Zhu et al., 2023). Observationally, species characterized by abundant lipids in their oocytes and embryos, such as pigs and domestic cats, exhibit reduced cryoresistance (Pereira & Marques, 2008, Galliguis et al., 2013, Zhu et al., 2023), thus reducing lipids may have benefits for viability after thawing. Approaches to diminish intracellular lipid content in bovine IVP, has enhanced their tolerance to micromanipulation and cryopreservation (Pereira & Marques, 2008).

The supplementation with GFs such as IGF1, IGF2, EGF, IL6, FGF1, and BMP2 at a concentration of 50 ng/mL, as identified in Chapter 2, into the culture media during in vitro production (IVP), represents a targeted enhancement approach to improve both the yield and quality of bison embryos. By supplementing the media with these specific GFs during their respective stages, we are poised to establish a nurturing milieu conducive to embryonic growth and differentiation. This tactic is predicated on the premise that the synergistic action of the introduced GFs will exert a beneficial influence on essential molecular and cellular pathways, thereby optimizing embryonic development trajectories.

Materials and methods

In vitro embryo production

The IVP protocol used for this experiment is detailed in Materials and Methods of Chapter 2. Based on results presented in Chapter 2, the GFRs expressed in the zygote and 8-16 cell

embryos are IGFR1, IGFR2, EGFR, FGFR1, and BMP2. IL6R emerged as the singular GFR with high expression at the morula stage. No GFRs were highly expressed in later stages of development. Growth factors corresponding to the identified GFRs were incorporated into CDM-1 when highly expressed in zygotes or 8-16 cell embryos. In contrast, for GFRs highly expressed in morula, blastocyst, or expanded blastocyst, the corresponding GFs were added to CDM-2. This strategic allocation aimed to customize the growth factor composition to align with specific developmental stages based on the observed GFR expression profiles. The preparation of GF prior to supplementation is described in Table 5.

Table 5. Growth factors used for media supplementation.

Growth Factor	Reconstitution	Biotechne® catalog number
Recombinant Human IGF1/IGF2 Protein, CF	Sterile PBS	292-G2-050
Recombinant Human IGF1/IGF1 Protein, CF	Sterile PBS	291-G1-200
Recombinant Human EGF Protein, CF	Sterile PBS	236-EG-200
*Recombinant Human/Mouse/Rat BMP-2 Protein, CF	4 mM HCL	355-BM-010/CF
Recombinant Human FGF acidic/FGF1 (aa 16-155) Protein, CF	Sterile PBS	232-FA-025/CF
Recombinant Human IL-6 (HEK293-expressed) Protein, CF	Sterile PBS	7270-IL-010/CF

* Hydrochloric acid (HCl) was employed for the reconstitution of BMP2. The introduction of this GF into CDM-1 resulted in the acidification of the media. Sodium hydroxide (NaOH) was utilized to raise the pH. All GF were obtained from Biotechne®

GF addition to culture media

After fertilization, embryos were cultured in CDM-1 for 56h with 50 ng/mL as follows: Control, EGF, IGF1, IGF2, BMP2, FGF1, and All GF (combination of all GFs; plates were prepared ≥3h before use). On day 3, our standard CDM-2 plates were prepared ≥3h before use, supplemented with 5% FCS per well per bison IVP protocol (Barfield 2019). IL6 was added to one well and all GF well. All other embryos were transferred to wells without any GFs. All embryos

were cultured for 96-120h. On day 7.5, embryo evaluation included assessing developmental stage and embryo grade following IETS standards (Stringfellow & Givens, 2010). Blastocysts and expanded blastocysts from each treatment were selected for lipid count and cell count assessment. Details of the GFs supplementation can be found in Table 6.

Table 6. IVP with the supplementation of GFs.

Treatment	GFs added at culture media	No. of oocytes per treatment
Control	-	268
EFG	CDM-1	380
IGF1	CDM-1	262
IGF2	CDM-1	374
BMP2	CDM-1	265
FGF1	CDM-1	219
IL6	CDM-2	331
All GFs	CDM-1, IL6 at CDM-2	267

Oocytes were randomly assigned to each experimental group, ensuring only grade 1 and 2 oocytes were collected, across at least 4 replicates. Growth factors were supplemented at a concentration of 50 ng/mL.

Lipid staining of embryo

A Bodipy 493/503 staining solution was prepared using 1mg of diluted in dimethyl sulfoxide (DMSO) to attain a concentration of 1mg/mL. Following this, a 60uL aliquot of the 1mg/mL stock solution was combined with 940uL of PBS (60ug/mL) and stored at -20°C. The final concentration of Bodipy 493/503 in the staining solution for embryos was adjusted to 1ug/mL in PBS.

Nuclear staining preparation (DAPI Solution)

A DAPI stock solution was prepared by dissolving 5mg of DAPI in deionized water to achieve a concentration of 5mg/mL. The resulting stock solution was stored at -20°C until used. Upon thawing on day of use, an intermediate solution with a concentration of 300uM was created by mixing 2.1uL of the DAPI stock solution with 10uL of DPBS-PVP. To obtain the final working solution with a concentration of 500nM, 4uL of the intermediate solution was combined with 2,396uL of DPBS-PVP.

Embryo staining protocol

The embryo staining protocol was adapted from by Yang et al., 2010, and Dunning et al., 2014. Embryos from each treatment were initially fixed in 4% paraformaldehyde (PFA) for 30 minutes. Following fixation, embryos underwent a thorough washing process in 300uL PBS-PVP drops, with three sequential washes lasting 5 minutes each.

Lipid staining

Embryos were permeabilized for 30 minutes in a solution containing 0.5% Triton X100 in PBS-PVP at room temperature. Subsequently, embryos were stained with Bodipy 493/503 (1ug/mL in PBS) for 1 hour in the dark. Post-staining, embryos were washed again in a 300ul PBS-PVP drop before proceeding to cell count staining.

Cell count staining

After lipid staining, embryos were incubated in a 300uL DAPI final solution for 10 minutes. The final washing step involved three drops of 300uL PBS-PVP each before embryos were ready for mounting. Fluorescence microscopy was employed to evaluate blastocyst stage bison embryos, highlighting lipid content through Bodipy and total cell count using DAPI.

Table 7. Media used to stain and fix embryos.

Name	Company	Catalog number
BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-*s*-Indacene)	Invitrogen™	D3922
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen™	D1306
Paraformaldehyde (16%) Grade 15710	Electron Microscopy Sciences™	15710

Statistics

The data collected from eight distinct treatments in bison in vitro production (IVP) include crucial variables like cleavage rate, blastocyst rate, stage rate, embryo grading, lipid count, and total cell count. For statistical analysis, a one way ANOVA was utilized, and standard error was calculated with SEM, ensuring precision in the reported variability.

Results

Each treatment group was evaluated for cleavage, blastocyst rate, and embryo grade. As shown in Figure 6A, cleavage results for most treatments did not significantly differ from the control, except for BMP2, which exhibited a significant decrease ($p<0.001$), Figure 6.

Blastocyst rates were higher for the EGF, IGF1, and IGF2 treatments, with IGF1 showing a significantly higher rate than the control ($p < 0.05$), as shown in Figure 7. Both Figure 7A, illustrating blastocyst/oocytes, and Figure 7B, presenting the results in blastocyst/cleavage oocyte show a significant decrease in blastocyst rates for IL6 and All GF treatments. It is noteworthy that these trends align with the cleavage rates observed in the study.

When evaluating embryo grade in comparison to the control, no significant differences were observed between embryo grades and treatments (Fig 8).

Although no substantial differences were observed, there was a slight uptick in the occurrence of grade 1 and 2 expanded blastocysts, as well as grade 2 blastocysts in certain treatments relative to the control, when the media were enriched with growth factors (Fig 8A and B).

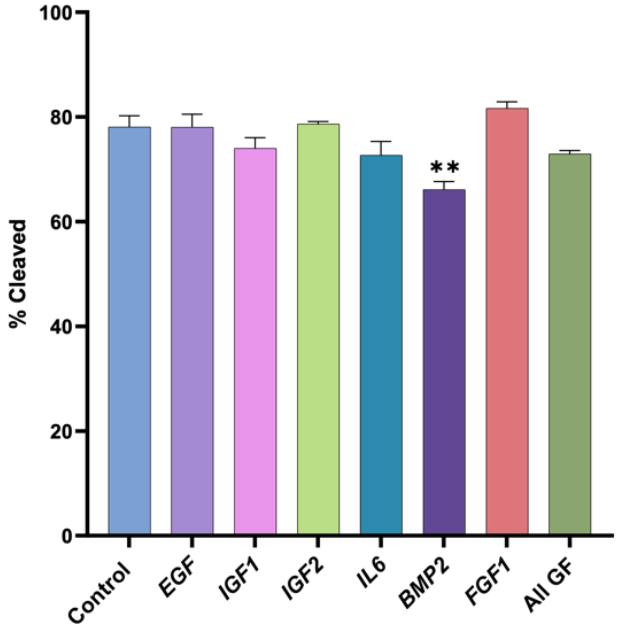

Figure 6. Cleavage rates in IVP media supplemented with GFs.
Impact of culture with GF (control vs. treatments) on cleavage stage: analysis conducted 56 h post fertilization. Among the treatments, only BMP2 exhibited a significant reduction in the percentage embryos cleaved; **, $p<0.001$).

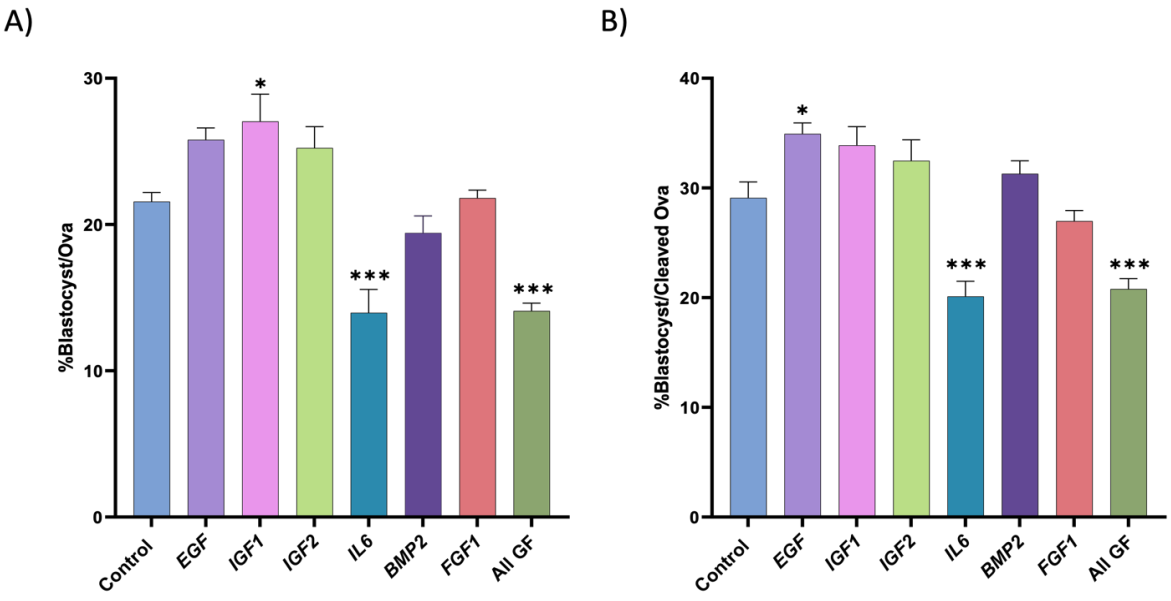

Figure 7. Blastocyst rates in IVP media supplemented with GF.
Impact of culture with GF (control vs. treatments) on blastocyst rates: assessment 166 hours after fertilization (minimum 4 replicates). A) Displays the ratio of blastocysts to oocytes. B) Presents the ratio of blastocysts to cleaved oocytes; $p < 0.05$; ***, $p<0.000$

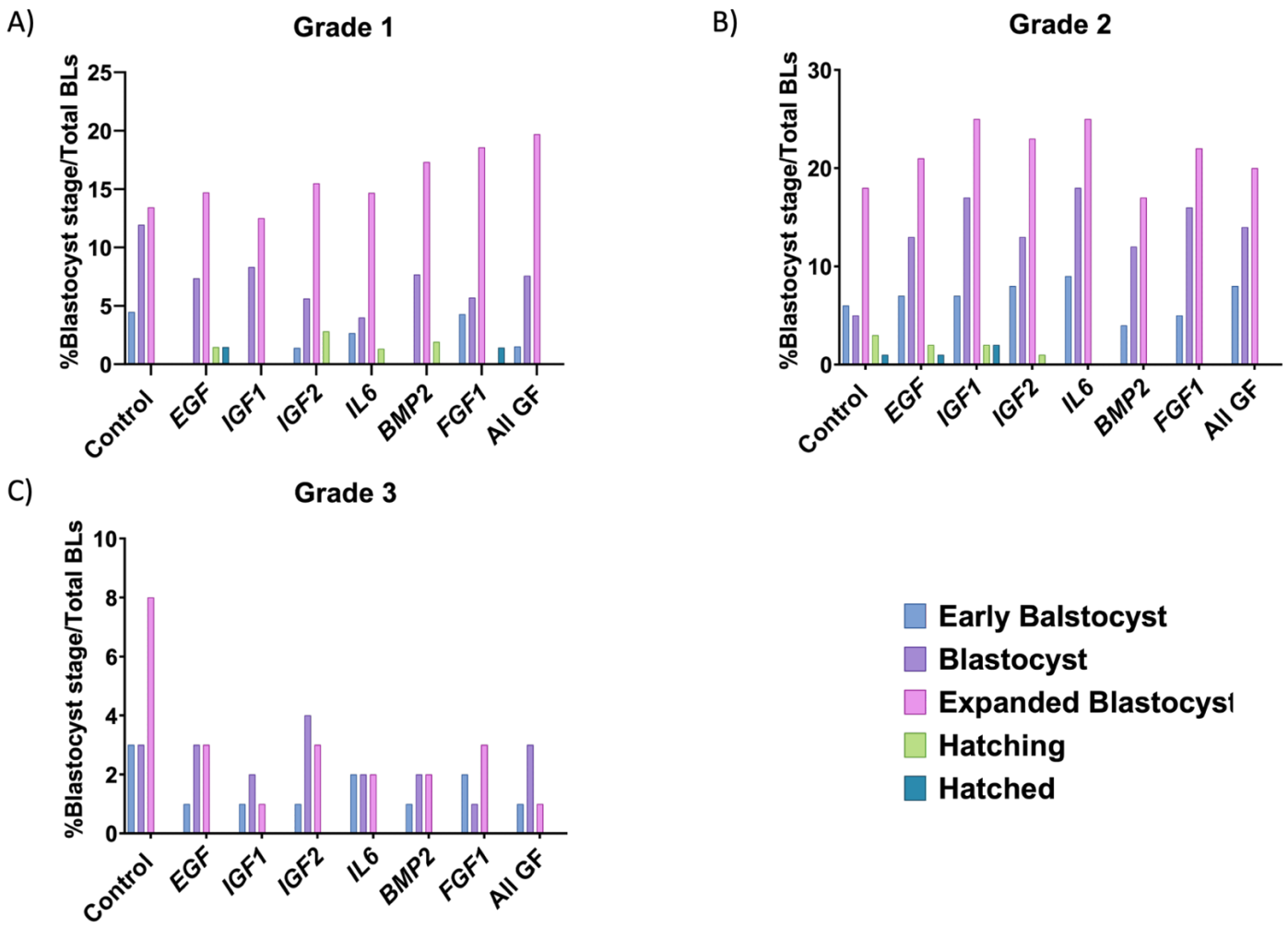

Figure 8. IETS embryo grading results in IVP media supplemented with GF.
Impact of Embryo Culture Conditions (Control vs. Treatments) on IETS embryo grading: assessments conducted at 7.5 days post fertilization (minimum 4 replicates). A) Illustrates percentage grade 1 embryos relative to all embryos produced for given treatment, B) Shows percentage of grade 2 embryos per treatment, C) Shows percentage of grade 3 embryos per treatment.

Embryos underwent lipid staining across three replicates, totaling 18-31 embryos per treatment. The analysis revealed a general decrease in lipid content for most treatments, with IGF1 notably displaying a statistically significant reduction in lipids (Fig 9). Additionally, embryos collected over three replicates (14-27 embryos per treatment), were subjected to total cell count analysis. The results indicated an overall increase in cell numbers for all treatments, with IGF1, IL6, BMP2, and FGF1 demonstrating notably higher cell counts (Fig 10). Figure 11 contains images of embryos subjected to lipid and total cell count staining.

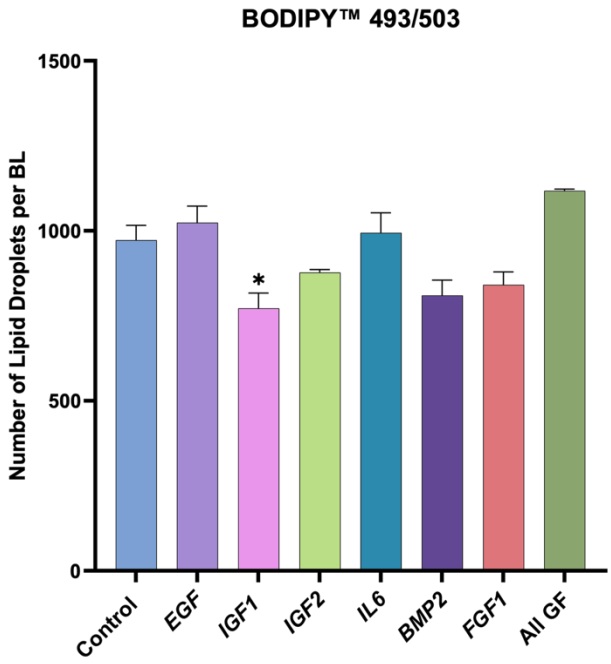

Figure 9. Lipid droplets per blastocyst cultured in GF supplemented media.
Impact of growth factor supplemented culture media on lipid droplet counts: embryos were collected at day 7.5 for Bodipy staining (3 replicates n=8-31). Notably, culture in IGF1 treatment resulted in a significant reduction in the number of lipid droplets compared to the control;* $p < 0.05$.

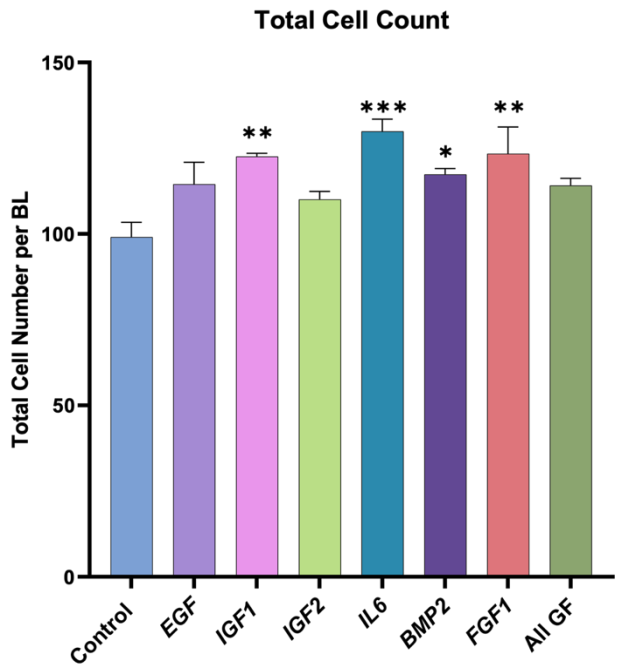

Figure 10. Total cell count results in IVP supplemented with GF.
Impact of growth factor supplemented culture media on total cell count: embryos were collected across 3 replicates and subjected to DAPI staining at day 7.5. While all treatments exhibited higher total cell counts compared to the control, only the IGF1, IL6, BMP2, and FGF1 treatments significantly reduced lipid droplets in comparison to the control group; * $p < 0.05$; **, $p<0.001$; ***, $p<0.0001$.

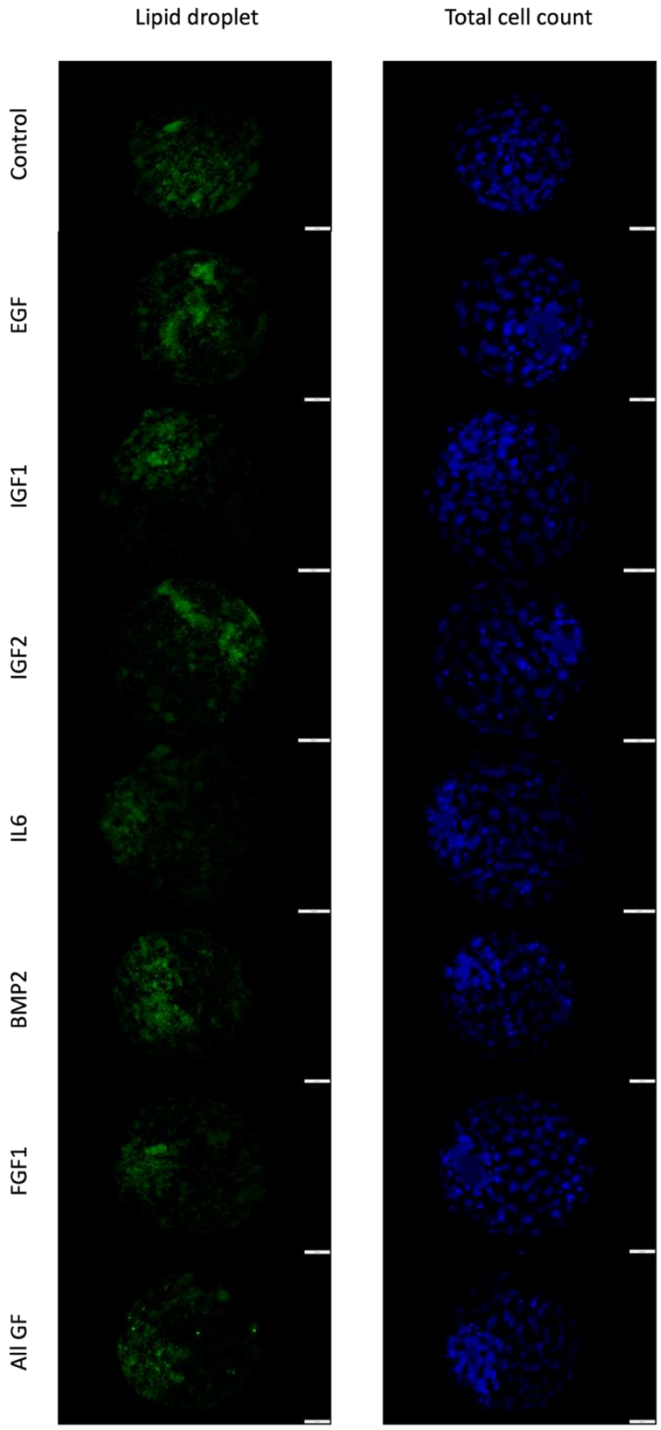

Figure 11. Embryos total cell count and lipid droplet images per treatment.
Blastocyst stage bison embryos collected from the control and treatment groups, illustrating with Bodipy staining (on the left) and DAPI staining (on the right) by fluorescence microscopy. These stains were utilized to assess lipid content and to count the total number of cells, respectively.

Discussion

The findings of this study support the hypothesis that supplementation of IVP media with growth factors tailored to specific stages of embryo development enhances both the quantity and quality of bison embryos. Growth factors were added at a concentration of 50 ng/mL. This concentration aligns with the findings of Neira et al., (2010), who employed a similar concentration when adding various GFs to the culture media for bovine embryo development. Among the growth factors used (EGF, IGF1, IGF2, IL6, BMP2, and FGF1), IGF1 emerged as the most beneficial GF tested, exhibiting a significantly higher blastocyst rate compared to the control. Our findings are similar to the observations made by Wooldridge et al., (2019) regarding the impact of interleukin-6 (IL6) on in vitro produced bovine embryos. Our analysis reveals an overall increase in cell numbers across all treatments, with IL6 demonstrating particularly elevated cell counts. Wooldridge et al., (2019) documented a similar trend, noting that IL6 supplementation led to augmented inner cell mass (ICM) cell numbers in various blastocyst stages. Despite variations in species and growth factor compositions, the consistent positive effect on cell numbers suggests a generalizable pattern highlighting the role of IL6 in fostering cell proliferation during embryo development.

The study by Neirijnck et al., (2019) provides valuable insights into the impact of IGF1 on meiotic progression and blastocyst development in bovine embryos. Our research aligns with their findings, revealing a notable rise in blastocyst rates with IGF1 supplementation. Neirijnck et al., (2019) underscored the mitogenic and anti-apoptotic activities associated with IGF1, aligning well with our results that exhibit a statistically significant reduction in lipid content and an

increase in total cell count. These combined outcomes indicate enhanced cell proliferation, reinforcing the positive influence of IGF1 on bovine embryo development.

Furthermore, Stoecklein et al. (2021) reported a notable decrease in lipid content at the blastocyst stage when FGF2, LIF, and IGF1 were incorporated into the maturation medium, in contrast to the control group. Their findings emphasize the potential of these GF to address challenges associated with the cryosurvival of IVP bovine embryos, contributing to improved embryo development and quality. Consistent with this, our own observations revealed an enhancement in embryo quality, characterized by reduced lipid content and an increased cell count, specifically with IGF1 supplementation. This underscores the importance of specific growth factors in modulating lipid levels during embryo development, suggesting potential benefits for the cryopreservation of bison embryos, particularly in scenarios where lipid content needs to be minimized.

Consistent with earlier studies on bovine embryos, our results reflect similar patterns where supplementation with EGF led to higher blastocyst rates (Thibodeaux et al., 1993), and the proliferative effects prompted by EGF, FGF2, and IGF1 in bovine trophoblast cells were also noted (Xie et al., 2017). These insights, together with our findings, jointly emphasize the beneficial influence of GF supplementation on blastocyst development, as well as the critical functions of FGF and BMP in directing differentiation and cellular proliferation. (Sherbet 2011; Yang et al., 2022). This alignment strengthens the argument that specific growth factors, particularly IGF1 in our case, play crucial roles in improving both the quantity and quality of developing embryos.

In conclusion, our study demonstrates that supplementing IVP media with GFs, notably IGF1 at a concentration of 50ng/mL, significantly enhances both the quantity and quality of bison

embryos. IGF1 emerges as particularly effective, elevating blastocyst rates, reducing lipid content, and increasing cell count. Our results provide valuable insights for refining ART and cryopreservation protocols, with implications for optimizing the reproductive success of bison and potentially other species.

Conclusion

The culmination of this thesis marks a pivotal advancement in the field of bison conservation through ART. It explores the natural reproductive mechanisms of the bison and the environmental and anthropogenic challenges they face, which necessitate innovative conservation strategies. By detailing the physiological similarities and differences between bison and cattle. There is a necessity for a species-specific culture system, paving the way for a more effective application of ART in bison.

One approach to modifying culture media for bison embryo development is through supplementation with GFs. Growth factors play a pivotal role in regulating cellular functions and influencing the overall development of organisms. Through RTq-PCR, it was revealed that IGFR2, BMPR2, FGFR1, EGFR, and IGFR1, are expressed differently across various embryonic stages in bison, offering new insights into the species-specific developmental requirements.

The practical application of the research demonstrates the effectiveness of supplementing bison embryo culture media with specific GF. The experimental results showed that growth factors corresponding to the receptors identified in Chapter 2 significantly improved embryo development. Treatments with IGF1, IL6, BMP2, and FGF1 increased the total cell count and IGF1 notably reduced lipid content. The results presented not only have profound implications for the conservation of bison but also offer a blueprint for the application of similar methodologies to

other species where standard protocols fall short. This work stands as a testament to the importance of species-specific research in the conservation and advancement of biodiversity.

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Appendix 1

Stock Solution for IVF

Stock	Formula / Comp	Quantity (g)	H2O mL (up to)	0.9% Saline ml (up to)	Phenol Red (g)
Ala-Glu	Alanyl- Glutamine	0.434	20		
B	$NaHCO_3$	2.100	100		0.005
BSA	Bov. Ser. Albumin	2.000	19.3		
C	$CaCl_2.2H_2O$	0.588	20		
CAFN	Caffeine	0.194	20		
CYSTMN	Cysteamine	0.011		10	
CDM	NaCl	8.298			
	KCl	0.895			
	KH_2PO_4	0.272			
	Na-Citrate.$2H_2O$	0.294			
	Total		200		
EAA'S	BME AA sol 50X				
EDTA	$C_{10}H_{16}N_2O_8$	0.037	100		
F	D-Fructose	0.721	20		
G	D-Glucose	1.261	20		
Gentamicin	Gentamicin				
GLYCN	Glycine	0.736	20		
H(1:1)	HEPES free ac.	2.383			
	HEPES Na salt	2.603			
	Total		100		0.005
HP-MAT	Heparin Na Salt	0.020		2	
HP-FERT	Heparin Na Salt	0.020		20	
MI	Myo-Inositol	0.998	20		
L-L	Sodium L-lactate	1.121	10		
MS	$MgSO_4$	0.120	20		
NaCl	NaCl	5.844	20		
NEAA'S	MEM AA sol 100X				
P	Na-Pyruvate	0.110	20		
T	Taurine	0.025	20		

* All stocks should be filtered before use, except Cysteamine, Gentamicin, HP-Mat and HP-Fert.
* All stocks should be stored at 4°C, except Cysteamine and Taurine that are stored at -20°C.
* Alanyl-Glutamine and Sodium L-Lactate are light sensitive, so they should be covered with foil during storage.

Appendix 2

Media formulation

Component	HCDM-M	IVM	FCDM	HCDM-1	CDM-1	HCDM-2	CDM-2
CDM	78.5	78.5	78.5	78.5	78.5	78.5	78.5
B	5.0	25.0	25.0	5.0	25.0	5.0	25.0
C	2.0	2.0	2.0	2.0	2.0	2.0	2.0
G	0.88	0	0.88	0	0	0	0
F	0	2.0	0	0.5	0.5	2.0	2.0
Glycn	4.90	4.90	4.9	4.9	4.9	4.9	4.9
ALA-GLU	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hepes (1:1)	20.0	20.0	20.0	20.0	20.0	20.0	20.0
L-L	10.0	10.0	10.0	10.0	10.0	5.0	5.0
P	0.5	0.5	0.5	0.5	0.5	0.2	0.2
MS	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MI	0	2.77	0	0	2.77	0	2.77
NEAAs	1%	1%	1%	1%	1%	1%	1%
EAAs	0	0	0	0	0	2%	2%
T	0	0.1	0	0	0.1	0	0
BSA-FAF	2.5%	5%	5%	2.5%	5%	2.5%	5%
NaCl	22.5	0	14.0	22.5	1.0	25.6	5.0
HP–Mat	20µg/mL	0	0	0	0	0	0
HP–Fert	0	0	2µg/mL	0	0	0	0
Cafn	0	0	2.0	0	0	0	0
EDTA	0	0	0	0	0.01	0	0
FCS	0	0	0	0	0	0	5%
Gentamicin	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL	25 µg/mL
Osmolality	275 mOsm	275 mOsm	300 mOsm	275 mOsm	275 mOsm	275 mOsm	275 mOsm
pH	7.3–7.4	7.3–7.4	7.5–7.6	7.3–7.4	7.3–7.4	7.3–7.4	7.3–7.4

For IVM, on the day of maturation the following hormones are added to the medium: 1 µL/mL of IVM should be added of 15 ng/µL ovine FSH stock, 1 µg/mL bovine LH stock, and 0.1 µg/µL Estradiol 17β; 10 µL/mL of IVM should be added of 50 ng/µL epidermal growth factor stock and 10 mM cysteamine stock (see Table 1). For example, if you are making 20 mL of IVM you would add 20 µL of the FSH, LH, and E2 stocks and 200 µL of EGF and cysteamine stocks Heparin, gentamicin, and FCS are added after the media are filtered through a 0.22 µm membrane filter