Stress and Coping Responses in Early Embryonic Development

Naomi Dicks, DVM, MSc.
Department of Animal Science, McGill University, Montreal
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ABSTRACT

Impaired fertility is a developing concern both in humans and animals, necessitating the use of assisted reproductive technologies (ART). *In vitro* embryo production (IVP) is an integral component of ART, however, cellular stress during the IVP process negatively impacts embryo development and quality, leading to lower rates of pregnancy and live births. Understanding the cellular stresses, including endoplasmic reticulum (ER) stress and nuclear stress, that affect the early embryo as well as their coping responses represent a first step towards improving the efficiency of IVP and mitigating problems with fertility. In the first manuscript, poorly developing late-cleaving embryos were found to not only exhibit increased nuclear stress in the form of DNA damage, but they also demonstrated increased ER stress. Remarkably, treatment of these late-cleaving embryos with the natural bile acid and ER stress inhibitor, tauroursodeoxycholic acid (TUDCA), rescued their development to the blastocyst stage, by reducing their level of DNA damage and ER stress. In fact, these TUDCA-treated late-cleaving embryos developed at a similar rate to untreated developmentally-competent early-cleaving embryos. These findings suggest that ER stress is a major determinant of the reduced developmental potential of late-cleaving embryos. Furthermore, these results suggest the ER stress response, or unfolded protein response (UPR), and the response to DNA damage, or genome damage response (GDR), are linked. In the second manuscript, the mechanism of action by which TUDCA exerts its beneficial effects was investigated. Firstly, TUDCA was shown to dramatically reduce embryo development when injected intracellularly, whereas it increased rates of blastocyst development when supplied in the culture medium. This implied TUDCA activated its mode of action indirectly to facilitate development. Naturally, the necessity of the only exclusively membrane-bound bile acid receptor, TGR5, was evaluated. Using DsiRNA targeting TGR5 mRNA, it was shown that TGR5 was not needed for normal preimplantation development, however, it was needed for TUDCA to increase the rate of blastocyst development. In a glucose-induced ER stress model, data demonstrated that TGR5 was needed for TUDCA to reduce cellular stress, in the form of ER and oxidative stress, and improve early embryo development. Finally, attenuation of TGR5 signaling in the model of glucose-induced ER stress resulted in altered mRNA abundance of genes controlling pluripotency, which may have detrimental impacts on embryo viability. In the third manuscript, the ability of TUDCA to relieve

nuclear stress was evaluated. Interestingly, TUDCA rescued the development of UV-exposed embryos to that of the control group. This improvement in development coincided with a reduction in both DNA damage and ER stress in developing embryos. Furthermore, it was determined that TGR5 signaling was needed for these beneficial effects to occur. In fact, embryos exposed to UV radiation showed altered GDR and sustained UPR activity when TGR5 activation was attenuated. These findings demonstrated that coordinated activation of the UPR and GDR occurs through the TGR5 pathway. Together, the results from this thesis provide insight into the cellular stresses affecting early embryo development and the coping responses initiated to alleviate their effects. Furthermore, this thesis identifies an upstream therapeutic target that can potentially be exploited to improve early embryo development and mitigate infertility.

RÉSUMÉ

L'infertilité devient un problème important chez les humains et les animaux, nécessitant l'utilisation de technologies de reproduction assistées (TRA). La production d'embryons *in vitro* (PIV) est un élément intégral de TRA, cependant, le stress cellulaire qui se produit pendant le processus de la PIV a de graves conséquences sur le développement et la qualité de l'embryon, provoquant une diminution des taux de grossesse et naissances vivantes. Une meilleure compréhension du stress cellulaire, incluant le stress du réticulum endoplasmique (RE) et le stress nucléaire, qui affecte l'embryon préimplantatoire, en plus de ses réponses d'adaptation, représente une première étape pour améliorer l'efficacité de la PIV et résoudre les problèmes d'infertilité. Il est bien connu que les embryons préimplantatoires qui subissent leur premier clivage tard ont un développement défavorisé, tandis que ceux qui commencent à diviser tôt développent mieux. Dans le premier manuscrit, les résultats ont démontré que ces embryons défavorisés souffraient non seulement d'une augmentation de stress nucléaire, dans la forme de dommages à l'ADN, mais aussi une augmentation de stress au niveau du RE. Notablement, traitement de ces embryons défavorisés avec l'acide biliaire naturel et l'inhibiteur de stress du RE, l'acide tauroursodésoxycholique (TUDCA), a réussi à augmenter le développement au stade du blastocyste en diminuant le niveau de dommages à l'ADN et le stress du RE. En fait, ces embryons traités avec TUDCA ont récupéré le même taux de développement que les embryons qui commencent à se cliver tôt. Ces observations suggèrent que le stress du RE est un facteur important qui détermine le potentiel développemental des embryons qui se clivent tard. En plus, ces résultats suggèrent que la réponse d'adaptation au stress du RE, ou la réponse aux protéines malformées (UPR), et la réponse d'adaptation aux dommages à l'ADN, ou la réponse au dommage génomique (RDG), sont reliés. Dans le deuxième manuscrit, le mécanisme par lequel TUDCA exerce ses effets bénéfiques a été investigué. Premièrement, l'injection intracellulaire de TUDCA a causé une réduction remarquable dans le développement, tandis que le taux de formation de blastocystes s'est amélioré lorsque TUDCA était incorporé dans le milieu de culture. Ce fait signifiait que TUDCA activait sa mode d'action pour améliorer le développement par un mécanisme indirect. Naturellement, la nécessité du récepteur membranaire d'acides biliaires uniquement lié aux membranes, TGR5, a été évalué. En utilisant l'interférence ARN,

TGR5 a été ciblé, et bien qu'il ne fût pas nécessaire pour le développement préimplantatoire normal, TUDCA en avait besoin pour améliorer le taux de formation de blastocystes. Dans un modèle de stress du RE induit par le glucose, les résultats ont démontré que TGR5 était nécessaire pour la capacité de TUDCA à diminuer le stress du RE, le stress oxydatif, et à améliorer le développement des embryons préimplantatoires. Finalement, l'atténuation de la signalisation de TGR5 dans ce modèle de stress du RE a causé une altération d'abondance de ARNm de gènes qui contrôlent la pluripotence, ce qui pourra avoir de conséquences néfastes sur la viabilité de l'embryon. Dans le troisième manuscrit, la capacité de TUDCA à soulager le stress nucléaire a été évalué. Curieusement, TUDCA a été capable de rétablir le développement d'embryons exposés à la radiation UV à un niveau comparable au groupe de contrôle. Cette amélioration correspondait à une réduction de dommages à l'ADN et le stress du RE dans les embryons en voie de développement. De plus, cette amélioration était dépendante de la signalisation de TGR5. En fait, les embryons exposés à la radiation UV ont démontré une altération de leur RDG et une activité soutenue de la réponse UPR, quand la signalisation de TGR5 a été atténuée. Ces données ont démontré que l'activation coordonnée de la réponse UPR et la RDG se produit par les voies de signalisation de TGR5. Ensemble, les résultats de cette thèse apportent une meilleure connaissance des types de stress qui affectent l'embryon préimplantatoire et les réponses d'adaptation initiés pour apaiser leurs effets. De plus, cette thèse identifie une cible thérapeutique qui pourra être exploitée pour améliorer le développement préimplantatoire et mitiger les problèmes d'infertilité.

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Firstly, I would like to thank my supervisor, Dr. Vilceu Bordignon, and my co-supervisor, Dr. Luis B. Agellon, for their support and guidance throughout my PhD. I am quite confident that I would never have started, nor completed, my doctoral studies without the continual motivation, optimism, and both academic and personal support provided by Dr. Bordignon. I must also thank Dr. Agellon for imparting his methodical approaches and critical thinking, which helped me through difficult portions of my thesis and greatly affected the scholar I have become. I hope you have both enjoyed the journey as much as I have!

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis aimed to investigate cellular stress and coping responses in the developing embryo to better understand preimplantation embryo development and mitigate infertility. More specifically, ER stress and the UPR, as well as DNA damage and the GDR, were investigated, along with the potential link between the two coping responses.

Evidence for links between the UPR and GDR coping responses were presented in a review entitled, "Endoplasmic reticulum stress, genome damage and cancer", and published in the journal, "Frontiers in Oncology" in February 2015. This review paper was referenced throughout this thesis and was presented in Chapter 3.

In the first manuscript (Chapter 5), ER stress was shown to be a determining factor differentiating developmentally-incompetent late-cleaving embryos from developmentally-competent early-cleaving embryos. In addition, this study showed that the developmental potential of TUDCA-treated late-cleaving embryos could be rescued to levels similar to that of untreated early-cleaving embryos. Finally, this study suggested a link between the UPR and GDR given TUDCA was shown to not only decrease ER stress in late-cleaving embryos, but DNA damage as well.

In the second manuscript (Chapter 6), TGR5 mRNA and protein were shown to be present in cleaved porcine embryos for the first time. More importantly, the TGR5 receptor was shown to be needed for TUDCA to improve preimplantation embryo development. This beneficial effect on development coincided with a TGR5-mediated reduction in both ER and oxidative stress in developing embryos.

In the third manuscript (Chapter 7), TUDCA was shown to reduce DNA damage and ER stress and improve early embryo development when subjected to nuclear stress. These changes were also found to be mediated by the TGR5 receptor. In addition, disrupted TGR5 signaling was shown to alter the GDR, favoring the error-prone NHEJ repair pathway, as well as cause

sustained UPR and altered cell survival signaling. Together these findings confirmed coordinated activation of the UPR and GDR coping responses occurs through activation of TGR5 pathways.

In conclusion, results from this thesis demonstrated that increased ER stress and DNA damage negatively affect the developmental potential of preimplantation embryos, while activation of TGR5 signaling can mitigate UPR and promote physiological GDR necessary for improved embryo development. These findings elaborate our understanding of cellular stress during IVP and identify a potential therapeutic target to improve embryo development and mitigate infertility.

PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was written in manuscript-based format based on the guidelines set by McGill University's department of Graduate and Postdoctoral Studies. The thesis was written by Naomi Dicks with editorial support by Dr. Vilceu Bordignon and Dr. Luis B. Agellon.

The review paper (Chapter 3), entitled "Endoplasmic reticulum stress, genome damage, and cancer" was published in the journal "Frontiers in Oncology" in February 2015 (doi.org/10.3389/fonc.2015.00011). Naomi Dicks was responsible for writing the review manuscript, in collaboration with Dr. Karina Gutierrez and Dr. Werner Glanzner. Dr. Marek Michalak contributed scientific expertise during the manuscript preparation. Dr. Vilceu Bordignon and Dr. Luis B. Agellon contributed throughout the entire process of manuscript preparation, including editorial support.

The first manuscript (Chapter 5), entitled "Relief of endoplasmic reticulum stress enhances DNA damage repair and improves development of pre-implantation embryos" was published in the journal "PLOS One" in November 2017 (doi.org/10.1371/journal.pone.0187717). Naomi Dicks carried out the experiments, data collection and analysis, as well as wrote the manuscript. Dr. Rodrigo C. Bohrer and Dr. Karina Gutierrez helped with embryo production, qPCR and immunofluorescence staining. Dr. Marek Michalak provided expertise to support the project, while Dr. Vilceu Bordignon and Dr. Luis B. Agellon conceived the experimental design, aided with data analysis and presentation, as well as edited the manuscript.

The second manuscript (Chapter 6), entitled "The TGR5 receptor is needed for tauroursodeoxycholic acid-mediated reduction of ER and oxidative stress and improvement of early embryo development" is in preparation for submission to the "FASEB Journal". Naomi Dicks was responsible for all the experiments, data collection and analysis, as well as writing the manuscript. Dr. Karina Gutierrez, Luke Currin, Dr. Mariana Priotto de Macedo and Dr. Werner Glanzner helped with embryo production. Dr. Marek Michalak provided expertise to support the study. Both Dr. Vilceu Bordignon and Dr. Luis B. Agellon designed the experiments, helped with data analysis and edited the manuscript.

The third manuscript (Chapter 7), entitled “Tauroursodeoxycholic acid acts via TGR5 receptor to facilitate DNA damage repair and improve early embryo development” is in preparation for submission to the journal “Molecular Reproduction and Development”. Naomi Dicks performed all the experiments, data collection and analysis, as well as preparation of the manuscript. Dr. Karina Gutierrez, Luke Currin, Dr. Mariana Priotto de Macedo and Dr. Werner Glanzner helped with the production of embryos. Dr. Marek Michalak once again provided expertise to support the study. Experimental design, help with data analysis and manuscript editing was performed by Dr. Vilceu Bordignon and Dr. Luis B. Agellon.

LIST OF ABBREVIATIONS

18S – 18S ribosomal subunit

53BP1 – tumour suppressor p53 binding protein 1

ACTB – beta actin

ADN – acide désoxyribonucléique (DNA, in French)

AKT – AKT serine/threonine kinase 1 (Protein kinase B)

ANOVA – analysis of variance

AP-1 – activated protein 1

ART – assisted reproductive technology

ASK1 – apoptosis signaling kinase 1

ATF4 – activating transcription factor 4

ATF6 – activating transcription factor 6

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia and Rad-3 related protein

ATRIP – ATR interacting protein

Bax – Bcl-2 associated X protein

Bcl-2 – B-cell lymphoma 2

Bim – Bcl-2-like protein 11

BRCA1 – breast cancer type 1 susceptibility protein

BRCA2 – breast cancer type 2 susceptibility protein

BSA – bovine serum albumin

c-Abl – Abelson tyrosine-protein kinase 1

CC3 – cleaved caspase-3

CCT – cytidine triphosphate phosphocholine cytidylytransferase

Cdc2 – cell division cycle 2 protein

Cdc25 – cell division cycle 25 protein

Cdk2 – cyclin-dependent kinase 2

CDP – cytidine diphosphate

CDX2 – caudal-type homeobox protein 2

Chk1 – checkpoint kinase 1

Chk2 – checkpoint kinase 2

CHOP – C/EBP homologous protein

CK – choline kinase

COC – cumulus oocyte complex

CPT – choline phosphotransferase

CREB – cAMP response element binding protein

Cs-DNAPK – catalytic subunit DNA-activated protein kinase

D3 – day 3

D5 – day 5

D7 – day 7

DAPI – 4'6-diamidino-2-phenylindole

dbcAMP – dibutyryl cyclic adenosine monophosphate

DMEM/F12 – Dulbecco modified Eagle's medium/nutrient mixture F12

DNAPK – DNA-activated protein kinase

DSB – double-strand break

DsiRNA – dicer substrate small interfering RNA

DsiRNA – dicer-substrate short interfering RNA

EDEM – ER degradation enhancing alpha-mannosidase-like

eIF2α - elongation initiation factor 2-alpha

ER – endoplasmic reticulum

ERAD – ER associated degradation

ERK1/2 – extracellular signal-regulated kinase ½

ERO1α - ER oxidoreductin 1-alpha

Erp57 – ER resident protein 57

ERSE – ER stress response element

FSH – follicle stimulating hormone

GADD153 – growth arrest and DNA damage inducible protein 153

GADD34 – growth arrest and DNA damage inducible protein 34

GDR – genome damage response

GPBAR1 – G-protein-coupled bile acid receptor 1

GPCR – G-protein coupled receptor

GRP78 – glucose regulated protein 78

GRP94 – glucose regulated protein 94

GSK-3 – glycogen synthase kinase 3

H2A – histone H2A

H2AX – H2A histone family member X

γH2AX – H2A histone family member X, phosphorylated at serine 139

H3K14 – histone 3 lysine 14

H3K27 – histone 3 lysine 27

H3K9 – histone H3 lysine 9

HDAC – histone deacetylase

HDAC1 – histone deacetylase 1

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1 – hypoxia inducible factor 1

HMGN2 – high mobility group nucleosome binding protein 2

HR – homologous recombination

ICSI – intracytoplasmic sperm injection

IgG – immunoglobulin G

IP_3 – inositol-(1,4,5)-triphosphate

IRE1α – inositol requiring enzyme 1-alpha

IVC – in vitro culture

IVC – in vitro culture

IVF – in vitro fertilization

IVM1 – in vitro maturation medium 1

IVM2 – in vitro maturation medium 2

IVP – in vitro embryo production

JAB1 – c-Jun activation domain binding protein 1

JNK – c-Jun-N-terminal kinase

KEAP1 – Kelch-like ECH-associated protein 1

Ku70 – X-ray repair cross-complementing protein 6 (XRCC6)

Ku80 – X-ray repair cross-complementing protein 5 (XRCC5)

LH – luteinizing hormone

LIG4 – ligase IV

MAPK – mitogen-activated protein kinase

Mdm2 – mouse double minute 2 homolog

MFN2 – mitofusin 2

miR – micro RNA

MMR – mismatched repair

Mre1 – meiotic recombination 11 homolog 1

MRN – Mre11-Rad50-Nsb1

NANOG – homeobox transcription factor Nanog

NBS1 – Nijmegen breakage syndrome protein 1

NF-κB – nuclear factor kappa light chain enhancer of activated B cells

NHEJ – non-homologous end-joining

NRF2 – nuclear factor erythroid-2-related factor 2

OCT4 – octamer binding protein 4

ORAI1 – calcium release-activated calcium modulator 1

PA – parthenogenetically-activated

PARP1 – poly-(ADP ribose) polymerase 1

PBA – 4-phenyl butyrate

PBS – phosphate buffered saline

PDI – protein disulfide isomerase

PERK – protein kinase RNA-like ER kinase

PI3K – phosphatidylinositol 3-kinase

PIKK – phosphatidyl inositol 3 kinase-related kinase

PIP_2 – phosphatidylinositol-4,5-bisphosphate

PIV – production *in vitro* d'embryons (*in vitro* embryo production, in French)

PKA – protein kinase A

PMCA – plasma membrane calcium ATPase

PZM-3 – porcine zygote medium 3

qPCR – quantitative real-time polymerase chain reaction

RAD50 – DNA repair protein RAD50

RAD51 – RAD51 recombinase

RAD52 – recombination protein RAD52

RE – réticulum endoplasmique (endoplasmic reticulum, translated in French)

RDG – réponse au dommage génomique (genome damage response, in French)

ROS – reactive oxygen species

Rpd3 – Reduced potassium dependency 3

SAPK – stress-associated protein kinase

SCNT – somatic cell nuclear transfer

SERCA – sarco/endoplasmic reticulum calcium ATPase

si-CTRL – Scrambled control DsiRNA

si-TGR5 – TGR5 DsiRNA

SOCE – store-operated calcium entry

SOD1 – superoxide dismutase 1

SOD2 – superoxide dismutase 2

SOX2 – (sex determining region Y)-box 2

STIM1 – stromal interaction molecule 1

TBM – Tris-buffered medium

TBM-Fert – Tris-buffered medium for fertilization

TBS-T – Tris-buffered saline with 0.1% Tween

TCM-199 – Tissue culture medium 199

TGR5 – Takeda-G-protein-receptor 5

TP53 – tumour suppressor protein 53

TRA – technologies de reproduction assistées (assisted reproductive technologies, in French)

TRAF2 – TNF receptor-associated factor 2

TUDCA – tauroursodeoxycholic acid

UDCA – ursodeoxycholic acid

UPR – unfolded protein response

XBP1 – X-box binding protein 1

XBP1s – spliced X-box binding protein 1

XBP1u – unspliced X-box binding protein 1

XIAP – X-linked inhibitor of apoptosis

XRCC4 – X-ray repair cross-complementing protein 4

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CHAPTER 1

INTRODUCTION

The use of assisted reproductive technologies (ART) to combat human infertility has increased steadily in the past twenty-five years (Stephen et al., 2016). Impaired fertility is a developing concern due to many causes, including delayed age of reproduction (Mathews and Hamilton, 2009; Stephen and Chandra, 1998), as well as an increased incidence of diseases that negatively affect fertility, most notably, diabetes and metabolic syndrome (Aguilar et al., 2015; Glueck et al., 2003; Kasturi et al., 2008; Legro et al., 2014). The increased use of germ-cell toxic cancer therapies has also negatively affected fertility (Lee et al., 2006; Maltaris et al., 2007; Meirow and Schenker, 1995). ART, including *in vitro* fertilization (IVF), artificial insemination and *in vitro* embryo production (IVP), represent important tools to mitigate problems with fertility, not only in humans, but in animals as well. Veterinary use of ART has been performed to select genetically valuable production and performance animals, create transgenic animal models for translational research (Dicks, 2015; Gutierrez et al., 2015; Prather et al., 2013), as well as promote conservation of threatened or endangered species (Andrabi and Maxwell, 2007; Selvaraj et al., 2011; Stanton et al., 2019).

In vitro embryo production represents an integral component of ART, however, attempts to simulate the *in vivo* environment to support early embryo development have proven less than optimal. Regrettably, increased cellular stress during the IVP process negatively affects embryo development and quality (Karja et al., 2006; Kitagawa et al., 2004; Michalak and Gye, 2015; Sturmey et al., 2009; Yoon et al., 2014), leading to impaired implantation and failed pregnancies. While *in vivo*-derived preimplantation embryos will naturally suffer from cellular stress, including stress within the endoplasmic reticulum (ER) (Zhang et al., 2012a), this is amplified during IVP (Canepa et al., 2014). Increased ER stress leads to activation of the ER stress response, otherwise known as the unfolded protein response, or UPR (Groenendyk et al., 2013). Unfortunately, the IVP process not only increases UPR activity, but it also activates responses to another contributor of cellular stress, DNA damage or nuclear stress (Zheng et al., 2005). Interestingly, late-cleaving embryos, which have poorer development compared to their early-

cleaving counterparts (Booth et al., 2007; Coutinho et al., 2011; Isom et al., 2012; Lonergan et al., 1999; Lundin et al., 2001; Sakkas et al., 1998; van Soom et al., 1997), exhibit increased DNA damage (Bohrer et al., 2015). Furthermore, embryos with impaired DNA damage repair capability have reduced development to the blastocyst stage as well as reduced quality (Bohrer et al., 2018). These findings illustrate the importance of mitigating cellular stress and promoting appropriate stress coping responses during IVP to improve the efficiency of viable embryo production. Interestingly, it has been documented in numerous species that inhibition of ER stress with the natural bile acid tauroursodeoxycholic acid (TUDCA) can rescue early embryo development (Kim et al., 2012; Lin et al., 2016; Song et al., 2011; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b) and even improve rates of implantation and live births (Lin et al., 2015).

TUDCA has been shown in multiple disease models to mitigate activation of the UPR (Ben Mosbah et al., 2010; Malo et al., 2010) and this has equally been shown during early embryo development (Lin et al., 2016; Yoon et al., 2014; Zhang et al., 2012a). While the beneficial effects of TUDCA are well known, the mechanism and mode of action by which it acts is poorly understood, particularly in the context of embryo development. TUDCA can reduce apoptosis (Kim et al., 2012; Lin et al., 2016; Zhang et al., 2012b) and stimulate survival signaling pathways (Schoemaker et al., 2004), however, how it activates this mode of action within the cell is not known. This is particularly intriguing, given bile acids are toxic to cells that do not normally metabolize them, such as enterocytes and hepatocytes that function during enterohepatic recirculation (Torchia et al., 2001). The toxic nature of bile acids intracellularly suggests the mechanism by which TUDCA exerts its beneficial effect is mediated by a cell surface receptor, with the only exclusively membrane-bound bile acid receptor, Takeda-G-protein-receptor (TGR5), being the prime candidate (Duboc et al., 2014). While evidence suggests TUDCA requires the TGR5 receptor to reduce inflammation in microglial cells (Yanguas-Casas et al., 2017), it is unknown if the same applies during early embryo development. In addition, while TGR5 mRNA has been shown to be expressed in multiple tissues, including the ovary (Duboc et al., 2014), it is not known if it is even present in the oocyte or embryo.

Understanding the different cellular stresses that affect early embryo development, as well as the stress coping responses they activate, is critical for improving the efficiency of IVP and mitigating problems with fertility. While cleavage kinetics is known to affect embryo development, it is not known whether ER stress plays a role in determining the developmental potential of these embryos. Furthermore, the mechanism of action by which TUDCA mitigates UPR activity and improves embryo development is not clear. Finally, while TUDCA prevents activation of the UPR, its effect on nuclear stress has not been examined. This thesis aimed to investigate these questions through three main objectives: 1) determine if ER stress affects cleavage kinetics and the development of early embryos; 2) determine if TUDCA requires the TGR5 receptor to activate its mode of action within the embryo cell or blastomere and improve preimplantation development; and 3) determine if TUDCA can alleviate nuclear stress and affect both the GDR and UPR.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Embryonic mortality is an important element affecting fertility in humans and domestic animals. IVP is an important aspect of ART that is increasingly utilized to mitigate fertility problems in humans. Recently, the global prevalence of infertility has been estimated at 9%, with over 50% of infertile couples seeking medical assistance (Boivin et al., 2007). The number of infertile women is projected to increase, in part due to delayed reproduction to a later age (Mathews and Hamilton, 2009; Stephen and Chandra, 1998). With decreased ovarian reserve at an older age and higher risk of infertility, the demand for assisted reproductive technologies has increased from 1.0 to 5.2% from 1995 to 2010 (Leridon and Slama, 2008; Stephen et al., 2016).

Delayed reproduction is not the only cause for increased infertility as increases in cancer and aggressive therapies also play a role. While chemotherapy and other treatments have allowed cancer patients to live longer, negative sequelae such as reproductive dysfunction or even failure have resulted (Lee et al., 2006; Maltaris et al., 2007; Meirow and Schenker, 1995). Clinicians are more and more cognizant of the need to preserve reproductive potential prior to cancer therapy (Zavras et al., 2016). Depending on the patient and type of neoplasia, various strategies can be employed including assisted reproductive technologies, such as *in vitro* oocyte maturation and embryo production (Prasath et al., 2014; Zavras et al., 2016).

In addition, there is a high prevalence of metabolic syndrome in current society, characterized by obesity, impaired glucose metabolism, hyperlipidemia, and hypertension, all which have been associated with poor fertility (Glueck et al., 2003; Kasturi et al., 2008). It is suggested that metabolic syndrome is ultimately the result of insulin resistance (Kasturi et al., 2008), which is also a key factor involved in polycystic ovarian disease (Diamanti-Kandarakis and Dunaif, 2012), the most common cause of infertility in women (Apridonidze et al., 2005). Thirty-four percent of women suffering from polycystic ovarian disease concurrently suffer from metabolic syndrome (Legro et al., 2014). Interestingly, cellular stress has been implicated in metabolic

syndrome (Delibegovic et al., 2009; Hebert and Molinari, 2007). In fact, metabolic syndrome has been shown to cause increased oxidative stress, a contributor to cellular stress, in oocytes of mice suffering from the disease (Delibegovic et al., 2009; Hebert and Molinari, 2007; Hou et al., 2016; Ou et al., 2012). Furthermore, cellular stress affecting mouse sperm has been shown to affect development of the embryos and subsequently cause metabolic syndrome in the offspring (Lane et al., 2014). Unfortunately, recent studies indicate the prevalence of metabolic syndrome is rising (Aguilar et al., 2015). As the number of individuals suffering from this condition continues to grow, the demand for facilitated reproductive technologies will only increase, necessitating improved efficacy of IVP.

Improving the efficiency of IVP is not only important in human reproduction, but animal reproduction as well. ART is highly utilized in agriculture for the production of high quality livestock and its use continues to grow. Furthermore, development of transgenic animal research models relies on successful IVP, particularly large animal models, which are necessary to bridge the gap between animal and human translational studies (Dicks, 2015; Gutierrez et al., 2015; Prather et al., 2013). Finally, assisted reproductive technology is also important for the conservation of endangered species (Andrabi and Maxwell, 2007; Selvaraj et al., 2011; Stanton et al., 2019). Given the limited and valuable nature of the reproductive material in these animals, efficient and successful IVP is essential (Andrabi and Maxwell, 2007).

Improving the efficiency of IVP lies in a better understanding of early embryonic development and the cellular stresses that impede normal embryo growth. There are many types of cellular insults that can affect embryo development, including heat stress (Sakatani et al., 2004) and oxidative stress (Karja et al., 2004). ER stress and nuclear stress, in the form of DNA damage, also contribute to cellular stress and it appears they may be linked (Dicks et al., 2015). ER stress has been investigated in early development, and multiple reports indicate a negative correlation between it and embryo development rate and quality (Karja et al., 2006; Kim et al., 2012; Kitagawa et al., 2004; Lin et al., 2016; Wang et al., 2013; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b) DNA damage has similarly been associated with poor development of early embryos (Bohrer et al., 2015). Furthermore, ER stress has been implicated in many diseases such as inflammatory disease, cardiovascular disease, neurodegenerative disease, cancer, and most

notably, metabolic disease (Hebert and Molinari, 2007), which has negative implications on fertility (Glueck et al., 2003; Kasturi et al., 2008).

It is not surprising cellular stress has a negative impact on the developing embryo. Throughout its development, the embryo must contend with a multitude of constraints and needs, including high energy requirements to carry out the necessary functions essential to a dividing cell: DNA repair and replication, protein synthesis, calcium homeostasis, lipid synthesis and membrane trafficking, to name a few (Bohrer, 2016; Crosby et al., 1988; Latham et al., 1991; Leclerc et al., 2012; Lim and Hasty, 1996; Pratt, 1980; Sasaki-Osugi et al., 2013). These processes rely heavily on a functioning ER. Various cellular insults, including DNA damage, can affect the ER and result in increased ER stress, impairing the ability of the organelle to carry out its functions. When the ER is stressed, coping mechanisms can lead to recovery of cellular homeostasis and function, or programmed cell death if the insults are extensive (Dicks et al., 2015; Groenendyk et al., 2013). While the stress coping response may be successful in re-establishing homeostasis, the cell may acquire either a normal adaptation for survival, or a pathological one. With a pathological adaptation, the cell achieves an altered metabolic state, whereby it survives, but at the detriment of the tissue or organism (Dicks et al., 2015; Groenendyk et al., 2013). As the developing embryo is subjected to many insults that disrupt cellular homeostasis, there is a fine balance occurring between survival and death, as well as normal or altered cell function, which ultimately impacts the viability and health of the embryo (Figure 2.2.1). The challenge is to minimize these cellular perturbations and tip the balance towards recuperation of normal cellular homeostasis and hence, normal embryo development (Figure 2.2.1). To accomplish this, a clearer understanding of the impact of cellular stress, including ER and nuclear stress, on IVP is necessary. With this knowledge, *in vitro* produced embryos will have a greater chance of successful development.

2.2 Figure

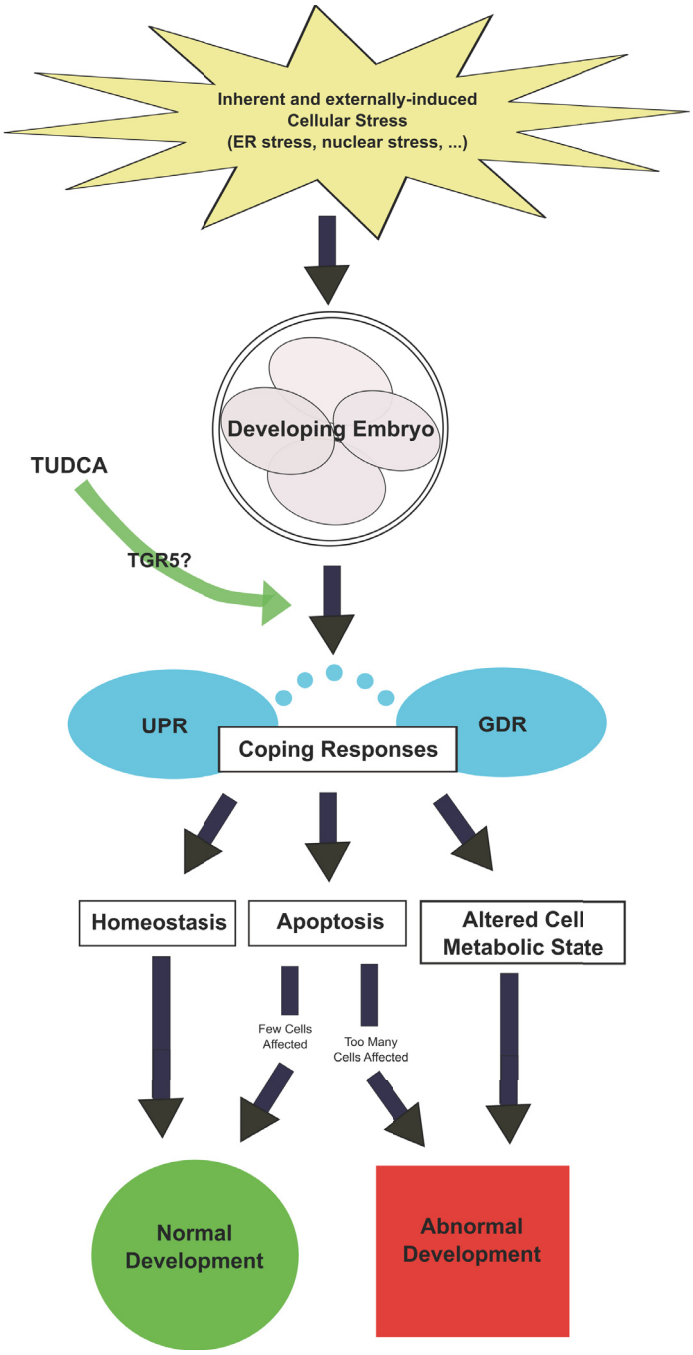

Figure 2.2.1. Consequences of Cellular Stress during Early Embryonic Development

Schematic diagram depicting the cellular stresses, coping responses, and potential consequences that occur during *in vitro* early embryonic development. UPR = unfolded protein response; GDR = genome damage response; TUDCA = tauroursodeoxycholic acid; TGR5 = Takeda G-protein coupled receptor 5 (or G-protein coupled bile acid receptor 1, GPBAR1);

2.3 Early Embryo Development

2.3.1 The privileged embryo

It is evident that increased cellular stress negatively affects cellular function and this can result in a number of consequences for the cell and organism (Figure 2.2.1). This is no different to cells during early embryonic development. However, the developing embryo represents a unique period of plasticity in the life of an organism, such that the consequences of individual cellular dysfunction differ from that of the adult animal. It has been shown that embryos that have cells removed at early stages of development can compensate and continue to develop normally (Hardy et al., 1990; Roudebush et al., 1990). This window of plasticity does not remain open indefinitely, however, with impaired development occurring if cells are removed at later stages, such as the morula (Krzyminska et al., 1990; Van Blerk et al., 1991). Ultimately, this provides the embryo with a unique ability to salvage normal development early on if individual cells dysfunction and undergo apoptosis, as long as a sufficient number of viable cells remain (Roudebush et al., 1990) (Figure 2.2.1). If the cellular dysfunction is rampant and few normal cells remain, however, the embryo will not be able to compensate and development will arrest (Figure 2.2.1).

2.3.2 Embryo cleavage kinetics affect development

During embryo development, the timing of cell divisions as well as the activation of the zygotic genome follows a unique program specific to each species (Memili et al., 2000; Duranthon et al., 2008). The porcine and human embryo share similar cleavage kinetics as well as timing of zygotic genome activation, making the pig a suitable model for human pre-implantation development, while these important events occur much more quickly in the mouse (Memili et al., 2000; Duranthon et al., 2008). Embryos that more faithfully follow the ideal pattern of cleavage for their species, are more likely to develop to term (Alikani et al., 2000). Interestingly, during IVP, numerous studies have noted distinct populations of embryos that exhibit differential developmental competence and quality, based on total cell number (Booth et al., 2007; Coutinho et al., 2011; Isom et al., 2012; Lonergan et al., 1999; Lundin et al., 2001; Sakkas et al., 1998; van Soom et al., 1997). It appears that not all embryos develop equally, with some not developing at all, and others developing, but with a reduced number of cells. This is not surprising, given the potential stresses and outcomes possible for the developing embryo (Figure 2.2.1). These studies showed that embryo cleavage kinetics affect embryo development, with early-cleaving embryos

having an advantage over those that cleave late (Booth et al., 2007; Coutinho et al., 2011; Isom et al., 2012; Lonergan et al., 1999; Lundin et al., 2001; Sakkas et al., 1998; van Soom et al., 1997). Early-cleaving embryos are generally defined as those that begin dividing within the first 24 hours after fertilization or activation, whereas those that divide after 24 hours are considered late-cleaving embryos (Isom et al., 2012; Lechniak et al., 2008). Increased blastocyst rates from embryos undergoing their first cell division sooner have been shown in multiple studies using bovine *in vitro* fertilization (IVF) (Henrique Barreta et al., 2012; Lonergan et al., 1999; van Soom et al., 1997). In fact, Lonergan and colleagues demonstrated that the time of first cleavage had a major impact on blastocyst development (Lonergan et al., 1999). Similar results have also been obtained in buffalo embryos (Rajhans et al., 2010; Totey et al., 1996). Porcine embryos derived by somatic cell nuclear transfer (SCNT), IVF and parthenogenetic activation (PA) all had impaired development to the blastocyst stage when their initial cleavage was late (Isom et al., 2012). Accordingly, porcine embryos that cleaved early were more likely to develop into blastocysts, and also their quality was improved (Coutinho et al., 2011). This correlates with a study in 2007 done by Booth and colleagues, where they found cell number at day 1 and day 2, as well as cleavage time, were positive predictors of porcine blastocyst development (Booth et al., 2007). Similarly, late-cleaving human embryos were less likely to develop into normal blastocysts (Alikani et al., 2000). While most of these studies focus on pre-implantation development and do not have data on post-implantation viability, there is evidence in humans that these early-cleaving embryos result in more successful pregnancies (Lundin et al., 2001; Sakkas et al., 1998). Human embryos derived by IVF or intracytoplasmic sperm injection that cleaved within 27 hours and were transferred to patients had increased pregnancy rates compared to those that divided later (Lundin et al., 2001; Sakkas et al., 1998). This study is supported by results from Luna and colleagues in 2008, where they showed early-cleaving human embryos exhibited better quality and greater clinical potential for successful pregnancy (Luna et al., 2008). Early work with hamster embryos similarly showed improved implantation and pregnancy rates in embryos that exhibited early cleavage (McKiernan and Bavister, 1994). These differences in developmental competence indicate inherent variations in the developing embryos, which may be explained by variations in cellular stress.

2.3.3 Cellular stress and cleavage kinetics

While it is clear that cleavage kinetics affect embryo development, the exact nature of this effect is not fully understood. Studies have suggested various factors that may differentiate the early- and late-cleaving embryos, including differences in glucose consumption (Lee et al., 2015), mRNA abundance (Gutierrez-Adan et al., 2004; Isom et al., 2012), amino acid metabolism (Booth et al., 2007; Lee et al., 2015), and apoptosis (Coutinho et al., 2011). Nuclear stress in the form of DNA damage, a contributor to cellular stress, has been more recently implicated in embryo cleavage kinetics, with late-cleaving embryos exhibiting increased DNA damage and reduced development and quality (Bohrer et al., 2015). Given the importance of DNA damage repair in the early embryo (Jaroudi and SenGupta, 2007), increased DNA damage or impaired ability to deal with DNA damage may be an important determining factor differentiating early- and late-cleaving embryos.

Accordingly, Bohrer and colleagues have shown that more developmentally competent early-cleaving porcine PA embryos have decreased DNA damage during early pre-implantation development (Bohrer et al., 2015). They showed that at days 3 and 5 of development, these embryos had reduced phosphorylated H2AX foci, a marker of DNA damage, expression of DNA damage response genes, as well as reduced expression of checkpoint control genes (Bohrer et al., 2015). Similarly, Barreta and colleagues have demonstrated that late-cleaving embryos had upregulation of DNA damage response genes 53BP1 and RAD52 (Henrique Barreta et al., 2012). Furthermore, late-cleaving embryos have increased H3K14 acetylation and high mobility group nucleosome binding protein 2 (HMGN2), which both make DNA more accessible for DNA damage repair (Bastos et al., 2008). Finally, less DNA fragmentation (van Soom et al., 1997) and chromosomal abnormalities (Magli et al., 2007) have been linked to early-cleaving embryos.

Given that nuclear and ER stress contribute to cellular stress and may be linked (Dicks et al., 2015), it is expected that poorly developing late-cleaving embryos exhibiting increased DNA damage may also have increased ER dysfunction. Interestingly, ER stress has been implicated in polycystic ovarian syndrome (Banuls et al., 2017) and IVP embryos from women suffering from this disease also show delayed cleavage during early development (Wissing et al., 2014). While increased ER stress has been shown to negatively impact early embryo development in numerous

species (Kim et al., 2012; Lin et al., 2016; Zhang et al., 2012a; Zhang et al., 2012b), it has never been specifically shown whether it also affects embryo cleavage kinetics.

2.4 Cellular Stress in the Embryo: Causes, Consequences and Coping Responses

2.4.1 Causes of Cellular Stress

As previously mentioned, early embryonic development represents a very demanding period of growth with high risk of cellular stress, which can only be increased by removing the embryo from its natural environment during IVP. Various inciting events may result in cellular stress. ER dysfunction represents a source and a consequence of cellular stress, whether the instigating event initiated in the ER, or at the level of another cellular compartment or organelle, such as the nucleus in the form of DNA damage. Since the ER plays an important role in cell metabolism, regardless of the stage of organismal development, it is important to understand its functions to appreciate the numerous cellular stressors that may impact it.

2.4.1.1 *The role of the ER and its importance in development*

The ER has a myriad of essential cellular functions and is arguably one of the most important organelles of the cell. Its importance can largely be attributed to its significant role in protein synthesis, calcium homeostasis, and lipid synthesis, however its functions do not stop there (Cao and Kaufman, 2012; Carreras-Sureda et al., 2017; Groenendyk et al., 2013; Hebert and Molinari, 2007; Stutzmann and Mattson, 2011). The ER is responsible for membrane trafficking, controlling the distribution of various molecules to different parts of the cell (Groenendyk et al., 2013; Stutzmann and Mattson, 2011). With its extensive reticular network(Agellon and Michalak, 2017), the ER also plays a role in signaling to other cellular compartments like the nucleus, cytoplasm, mitochondria and plasma membrane (Carreras-Sureda et al., 2017; Groenendyk et al., 2013; Stutzmann and Mattson, 2011). With the induction of coping responses, the ER is also able to regulate gene expression and energy metabolism in the face of changing environmental conditions (Cribb et al., 2005; Groenendyk et al., 2013). With these numerous cellular roles, it is no wonder that stress causing ER dysfunction can have serious consequences on cell viability. The cells of the embryo are no exception, and arguably, dysfunction at this stage can have even greater effects, including arrested development and death of the organism.

2.4.1.1.1 *Protein metabolism*

The ER has an important role in the synthesis and quality control of secretory and membrane-bound proteins. These proteins are not only synthesized in the ER, but they are also folded into their mature form and modified post-translationally, prior to exportation. Without proper folding and post-translational modifications, nascent proteins are not functional and can actually form aggregates that can be detrimental to the cell and the organism (Hebert and Molinari, 2007). New proteins are synthesized by ribosomes along the rough ER and interact with various chaperones, folding proteins and other enzymes as they enter the ER lumen (Groenendyk et al., 2013; Hebert and Molinari, 2007). The 78kDa glucose-regulated protein, GRP78, often termed the "master regulator" of the UPR, is an important chaperone protein that binds misfolded proteins and redirects them to other chaperones that can facilitate their proper folding (Beggah et al., 1996; Cabral et al., 2002). As they are being translated, nascent proteins are glycosylated by oligosaccharide transferase, allowing them to interact with lectin chaperone proteins (Parodi et al., 1972). Calnexin and calreticulin are lectin chaperones that bind monoglucosylated nascent proteins and facilitate their folding through interaction with other enzymes, including the oxidoreductases protein disulfide isomerase (PDI) and Erp57 (Frickel et al., 2002; Hebert and Molinari, 2007; Kozlov et al., 2006). These enzymes participate in protein folding by catalyzing disulfide bond formation and isomerization (Hebert and Molinari, 2007). PDI also acts as a chaperone by preventing aggregation of misfolded proteins that do not have any disulfide bonds (Cai et al., 1994).

Calnexin and calreticulin chaperone proteins are almost identical, with calnexin having a transmembrane domain that restricts it to interact with membrane-associated nascent proteins, while calreticulin is free to interact with nascent proteins in the ER lumen (Hebert and Molinari, 2007; Krause and Michalak, 1997). Newly synthesized proteins are constantly being cycled such that they are first de-glucosylated, and hence released by lectin chaperones. As they are released by their chaperone, they can be exported from the ER if they have acquired their native form, or they may be re-glucosylated by glucosyltransferase, so they can once again bind with a chaperone and another attempt at proper folding can occur (Caramelo et al., 2004; Hebert and Molinari, 2007). This cycle of de-glucosylation and re-glucosylation continues until the protein attains its native form, or it is terminally misfolded and subsequently marked for degradation (Hebert and Molinari, 2007).

Progressive de-mannosylation (Cabral et al., 2001; Helenius, 1994) of proteins by ER degradation enhancing α-mannosidase-like (EDEM) proteins targets them for exportation into the cytoplasm and proteasomal degradation (Olivari et al., 2005). These EDEM proteins equally prevent aggregation of misfolded proteins by preventing them from interacting with each other (Hosokawa et al., 2006; Olivari et al., 2006). In addition, the ER plays an important role in the regulation of autophagy, a protective mechanism by which misfolded protein aggregates are engulfed and fused with lysosomes for degradation (La Rovere et al., 2016; Senft and Ronai, 2015). Based on these functions, it is clear that the ER not only plays a vital role in secretory and membrane protein synthesis, but also in the maintenance of protein quality control. While embryos may not be transcriptionally active early in development, prior to zygotic genome activation, the level of protein synthesis remains high and thus a functional ER is essential (Crosby et al., 1988; Latham et al., 1991). Furthermore, the importance of maintaining protein quality control is evident given the induction of autophagy has been shown to facilitate development and quality of IVF bovine embryos, as well as reduce their levels of ER stress (Song et al., 2012).

2.4.1.1.2 *Calcium homeostasis*

Another essential function of the ER is the maintenance of calcium homeostasis. The concentration of calcium within the ER lumen is 4 to 5 times that of the cytoplasm, creating a necessary gradient for various calcium signaling processes in the cell (Clapham, 2007; Stutzmann and Mattson, 2011). While the ER lumen serves as a calcium reservoir for cellular signaling, it also functions as a calcium sink, given its ability to sequester the mineral and buffer it with binding proteins such as calreticulin and GRP78 (Lievremont et al., 1997; Stutzmann and Mattson, 2011). The buffering ability of calcium-binding proteins within the ER is essential for maintaining homeostasis, as illustrated by the impaired cardiac development and embryonic lethality seen in the calreticulin gene-inactivated mouse (Mesaeli et al., 1999), as well as embryonic lethality seen in the GRP78 gene-inactivated mouse (Luo et al., 2006). Interestingly, rescue of calcium-binding function in the calreticulin gene-inactivated mice by overexpression of calcineurin, abrogated embryonic lethality and allowed normal cardiac development (Guo et al., 2002). Surprisingly, calnexin, the membrane-bound counterpart of calreticulin that also exhibits calcium-binding properties, appears less essential for survival, at least in mice development,

given gene inactivation results in viable, yet neurologically impaired, offspring (Kraus et al., 2010). These ER resident calcium-binding proteins differ from cytosolic calcium-binding proteins given they have low binding affinity, but high capacity (Krebs et al., 2015). While the cytosolic calcium-binding proteins contain EF-hand domains capable of binding calcium, some ER proteins have also been shown to contain them (Krebs et al., 2015).

The high concentration of calcium in the ER and low concentration in the cytosol is maintained by the sarcoplasmic ER calcium ATPase, or SERCA, pump, as well as the plasma membrane calcium ATPase, or PMCA, pump (Clapham, 2007; Stutzmann and Mattson, 2011). The SERCA pump represents the primary means of maintaining the calcium gradient, transferring two calcium ions to the ER lumen per ATP consumed (Krebs et al., 2015). The PMCA functions on a lower scale to export calcium to the extracellular space, exchanging calcium and ATP on a 1:1 basis (Krebs et al., 2015). The ER can sense changes to calcium concentrations in the cellular compartments, so that it may respond and return the calcium gradient to homeostatic levels. The ER membrane-associated protein, stromal interaction molecule 1, or STIM1, is a EF-hand containing calcium sensor that can interact with channels on the plasma membrane to regulate calcium levels (Clapham, 2007; Krebs et al., 2015). Specifically, under conditions of calcium depletion in the ER, STIM1 on the ER membrane can activate ORAI1 calcium channels on the plasma membrane, thereby facilitating calcium entry into the cytoplasm, a process known as store-operated calcium entry, or SOCE (Clapham, 2007; Krebs et al., 2015). Once in the cytoplasm, calcium can subsequently be pumped back into the ER via the SERCA pump (Clapham, 2007; Stutzmann and Mattson, 2011). Without this ability to maintain calcium homeostasis, the cell would not be able to carry out various functions since its signaling processes rely on fluctuations in cytosolic calcium levels. For example, activation of receptor tyrosine kinases or G-protein coupled receptors on the plasma membrane activate a cascade which ultimately produces inositol (1,4,5) triphosphate (IP_3), the ligand for the IP_3 receptor on the ER membrane which allows calcium release into the cytoplasm (Clapham, 2007). Without a concentration gradient, there is no flow of calcium and complete dysregulation of cellular signaling occurs. In addition, pulsed calcium release by the IP_3 receptor from the ER to mitochondria also promotes oxidative phosphorylation, ATP production and cell survival (Joseph et al., 2019; Sano et al., 2012; Scorrano et al., 2003). Finally, maintenance of calcium

homeostasis in the cell is essential for other functions of the ER. High calcium levels in the ER stabilize interactions between chaperone proteins such as GRP78 and newly synthesized proteins, promoting their proper folding (Krebs et al., 2015). PDIs also bind calcium, which facilitate their function in protein isomerization and folding (Krebs et al., 2015).

The importance of calcium homeostasis within the cell cannot be underscored, and this is true for oocytes and embryos as well (Carvacho et al., 2018). In fact, calcium oscillations that occur during oocyte fertilization initiate development and rely on the IP_3 receptor (Stricker, 1999). Normal oocyte fertilization requires sperm-specific phospholipase Cζ, which is targeted to membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) by its first EF-hand domain and catalytic X-Y domain, allowing cleavage of PIP_2 and release of IP_3 to act on its receptor (Nomikos et al., 2015). The calcium oscillations stimulated by the IP_3 receptor are maintained through ER calcium replenishment via SOCE (Carvacho et al., 2018). Not surprisingly, attenuation of STIM1 or ORAI1, which are necessary for SOCE, negatively affects porcine embryo fertilization and development (Lee et al., 2012; Wang et al., 2012). Other proteins important in cellular signaling and differentiation in embryonic development have also been shown to contain the calcium binding EF-hand motif, further indicating the importance of calcium metabolism in the embryo (Leclerc et al., 2012; Sasaki-Osugi et al., 2013). Finally, cell signaling throughout embryo development is mediated by calcium, including axis determination in the early stages (Webb and Miller, 2003).

2.4.1.1.3 *Lipid metabolism*

Together with the Golgi, the smooth ER represents the major site of lipid biosynthesis in the cell. Lipid synthesis is essential for the maintenance of mammalian cell membranes considering the bulk of their composition is comprised of the phospholipid phosphatidylcholine (Fagone and Jackowski, 2009). These cell membranes are essential to cell viability given they encompass and protect the cell from the extracellular environment, as well as create the organelles and cellular compartments necessary for its various functions (Fagone and Jackowski, 2009). Phosphatidylcholine is largely synthesized through the Kennedy pathway in the ER, which begins with phosphorylation of choline to phosphocholine by the enzyme choline kinase (CK) (Fagone and Jackowski, 2009). This is followed by the rate-limiting step in phospholipid synthesis, the formation of CDP-choline from phosphocholine and CTP by CTP:phosphocholine

cytidylyltransferase (CCT) (Jackowski and Fagone, 2005). The final reaction combining CDP-choline and diacylglycerol to form phosphatidylcholine is catalyzed by the choline phosphotransferase (CPT) enzyme located in the ER (Fagone and Jackowski, 2009). The biogenesis of membranes is essential to cell function, and as a result, decreases in phospholipid synthesis have been shown to cause cellular stress and death (van der Sanden et al., 2003). Furthermore, ER coping responses aimed at mitigating cellular stress lead to the upregulation of lipid synthesis (Sriburi et al., 2004). It is clear the role of the ER in maintaining membrane integrity through the synthesis of lipids is vital to cell function and survival. This vital role holds true in early embryonic development, with the rate of phospholipid synthesis increasing significantly after initial cleavage (Pratt, 1980). Furthermore, protection of membranes and lipids from oxidative damage by incubating with the enzyme paraoxonase-1 during bovine *in vitro* maturation leads to improved embryonic development (Rincon et al., 2016).

2.4.1.2 *The Many Faces of ER Stress*

It is obvious the numerous functions of the ER, including protein synthesis, calcium homeostasis and lipid synthesis, highlight its significant role in cellular metabolism and hence embryonic development. ER stress impedes the ability of the organelle to carry out its functions and can lead to cellular dysfunction and death (Groenendyk et al., 2013; Hebert and Molinari, 2007). There are many causes implicated in ER stress including heat stress (Eriksson et al., 2004), glucose deprivation (Eriksson et al., 2004), excess oxidation products (Eriksson et al., 2004), hypoxia (De Jaco et al., 2006), unbalanced calcium (Eriksson et al., 2004; Rizzuto et al., 1998), phospholipid depletion (Cox et al., 1997; van der Sanden et al., 2003), mutated proteins, viral infection and many other problems that lead to misfolded proteins (Groenendyk et al., 2013). The list of potential ER stressors is long, but they all fundamentally converge on one main principal: loss of energy and nutrient homeostasis (Dicks et al., 2015; Groenendyk et al., 2013). This fundamental perturbation can affect many cellular compartments, not just the ER. For example, ER stress has been implicated with nuclear stress, or DNA damage (Dicks et al., 2015). This is conceivable since defects in energy metabolism affect the energy demanding processes of DNA replication, DNA damage repair and synthesis of nucleotide building blocks (Vincent et al., 2008). Furthermore, reactive oxygen species, a well-known inducer of ER stress, can also cause DNA damage (Kasai, 1997). Cross talk between cellular compartments, however, is not one-sided, and more likely, it goes both ways. DNA damage can incur mutations in genes, which

can ultimately lead to aberrant transcripts and improperly folded proteins due to sequence anomalies, thereby leading to ER stress. With the important role of the ER in many cellular functions, it is clear there are many faces of ER stress, and DNA damage is included.

2.4.2 Consequences of Cellular Stress

Disrupted cellular homeostasis essentially has two possible outcomes: return to homeostasis, or programmed cell death if the insult is too severe. Unfortunately, a return to cellular homeostasis and cell survival does not equate to normal cell function. Cells may establish an altered metabolic state, whereby the cell has been able to cope with the cellular stress and survive, but to the detriment of the tissue or organism (Figure 2.2.1) (Dicks et al., 2015; Groenendyk et al., 2013). What dictates the path the cell will take is not fully understood, but it is likely dependent on the inciting cellular stress, the cell type, surrounding environment as well as the chronicity and severity of the insult (Oslowski and Urano, 2011; Rich et al., 2000; Rutkowski et al., 2006). The source of cellular stress can determine which coping response pathways are activated, and also the nature of their activation (Rutkowski et al., 2006). For example, different effector caspases are involved depending on the inciting stimulus of ER stress. Misfolded proteins tend to induce programmed cell death in cells via caspase-12 activation (Nakagawa et al., 2000), whereas ER stress-induced apoptosis caused by aberrant calcium metabolism is independent of this caspase (Nakano et al., 2006). The outcomes of these signaling pathways may also depend on the type of cell affected (Rich et al., 2000). Even within the same cell type, discrepancies in pathway activation could mean the difference between health and disease. This is exemplified in melanocytes, where mutations in the MAPK pathway at different levels can result in either ER stress coping response induction and tumour prevention (upstream H-Ras mutation), or induction of melanoma (downstream B-Raf mutation) (Denoyelle et al., 2006).

Chronicity and severity of cellular stress are also likely major factors dictating cell outcome. While similar pathways are activated in both cells that undergo apoptosis and those that survive, the determining switch between them is unclear. Interestingly, cells subjected to mild ER stress actually continue to propagate, whereas those inflicted with higher stress do not (Rutkowski et al., 2006), indicating the severity of the inciting stress impacts the outcome between cell survival and death. Furthermore, it is believed that the stability of cell death effectors, such as C/EBP-homologous protein (CHOP) and caspases, will dictate whether programmed cell death will

progress or not, and this is dependent on the chronicity of ER stress (Rutkowski et al., 2006). Under conditions of oxidative stress, the NF- κB pathway can induce both anti- and pro-apoptotic mediators (Schoonbroodt and Piette, 2000). When ER stress is prolonged, however, this pathway will preferentially favour programmed cell death (Xu et al., 2005). Furthermore, sustained activation of inositol requiring enzyme 1 α (IRE1α), a regulator in the UPR pathway, leads to degradation of the microRNAs miR-17, miR-34a, miR-96 and miR-125b, all which normally repress translation of caspase-2 mRNA (Upton et al., 2012). The chronic activation of IRE1α in this situation promotes cell death over return to homeostasis.

2.4.3 Stress Coping Mechanisms

2.4.3.1 *The Unfolded Protein Response, UPR*

The unfolded protein response is the most well studied coping mechanism initiated in response to ER stress. It has most often been studied in the context of an ER stress response to the accumulation of misfolded proteins, but it is clear it can be activated by other cellular stressors. The "master regulator" of the UPR is GRP78 (Hendershot, 2004), which is highly conserved from yeast to humans (Hebert and Molinari, 2007). GRP78 binds misfolded and unfolded proteins to prevent their aggregation, along with another chaperone protein, GRP94, which is conserved among vertebrates (Lee, 2001). Both proteins are upregulated in the face of ER stress caused by glucose starvation or energy deficiency (Scheuner et al., 2001). This can help the ER deal with stress and return the organelle to homeostasis. Furthermore, GRP78 may prevent apoptosis by complexing with caspase-7 and caspase-12 at the ER membrane, thereby preventing their activation of the programmed cell death pathway in the cytosol (Rao et al., 2002).

Redundancy in some functions between GRP78 and GRP94 exist, given heterozygous mutant GRP78 mice upregulate GRP94 to compensate (Luo et al., 2006). Nonetheless, GRP78 possesses unique functions since gene-inactivated mice embryos cannot develop past the blastocyst stage (Luo et al., 2006). An important function of GRP78 is its ability to initiate the UPR pathway (Hebert and Molinari, 2007). The UPR has three main objectives in response to misfolded proteins: transiently attenuate protein translation, upregulate transcription of chaperone proteins that can help refold the aberrant proteins, and finally, increase ER-associated degradation (ERAD) of terminally misfolded proteins (Groenendyk et al., 2013). To accomplish these three objectives, there are three separate arms of the UPR: the PKR-like ER kinase (PERK) pathway,

the IRE1α pathway, and the activating transcription factor 6 (ATF6) pathway. All pathways are activated by a conformational change in GRP78, which is normally bound to these regulators and inhibiting their activity (Groenendyk et al., 2013). Activation of the PERK pathway leads to PERK dimerization and phosphorylation of the eukaryotic initiation factor 2 α (eIF2α), rendering it inactive and thereby reducing protein translation (Groenendyk et al., 2013). Paradoxically, this cascade increases the translation of activated transcription factor 4 (ATF4) (Groenendyk et al., 2013) and CHOP (Oyadomari and Mori, 2004). ATF4, in turn, activates transcription of other factors, such as GADD34, which appears to escape phosphorylated eIF2α translation inhibition due to the preferential binding of ribosomes to a second upstream ORF (Lee et al., 2009). The protein translation inhibition is transient, however, considering that GADD34 associates with protein phosphatase 1, and stimulates dephosphorylation of eIF2α (Brush et al., 2003). In essence, the PERK pathway stimulates preferential translation of ER stress proteins to facilitate the coping ability of the cell early in its response, but also allows re-initiation of general protein synthesis needed for cell survival.

As a result of PERK activation, the preferentially translated CHOP can initiate responses to ER stress that may have different outcomes. For example, it can upregulate ER oxidoreductin 1 α (ERO1α) (Gross et al., 2006), which can be utilized by PDI, along with molecular oxygen, to generate energy needed to form disulfide bonds in the face of oxidative stress (Otsu et al., 2006). In contrast, ERO1α can also activate IP_3R on the ER membrane to translocate calcium to the mitochondria and stimulate cell death (Li et al., 2009). As previously explained, the variable outcomes possible from similar pathways likely depends on the context, chronicity and severity of ER stress.

Activation of the ATF6 pathway causes release from GRP78 and translocation of ATF6 to the Golgi, where it is processed by site 1 and site 2 proteases to release a DNA binding domain that acts like a transcription factor (Groenendyk et al., 2013). This transcription factor binds to ER stress elements on the DNA, upregulating transcription of chaperone protein genes, and proteins involved in ER-associated degradation (Groenendyk et al., 2013). One such protein is Derlin-3, which forms the retrotranslocon necessary to transport unfolded proteins from the ER to the cytosol for proteasomal degradation (Belmont et al., 2010). These responses can help the cell

refold proteins, prevent aggregation of unfolded proteins, as well as dispose of those that are critically misfolded. ATF6 can also increase transcription of an important mediator of the IRE1α pathway, X-box binding protein 1 (XBP1), highlighting cross talk that occurs among the arms of the UPR (Yoshida et al., 2001). ATF6 may also be involved in the hypoxia response, as there are binding sites for ATF6 around the start codon of hypoxia inducible factor 1 (HIF-1) (Groenendyk et al., 2013). Coordinated responses by the cell thus occur to help alleviate ER stress and regain homeostasis.

Activation of the IRE1α pathway results in IRE1α dimerization and stimulation of its RNAse activity through a conformational change (Ron and Hubbard, 2008), thereby cleaving the XBP1 mRNA to a spliced form, XBP1s, which acts as a transcription factor. This factor similarly binds ER stress elements (ERSE) on the DNA and increases transcription of chaperones and ERAD proteins (Groenendyk et al., 2013). It equally may participate in the hypoxia response given it has binding domains near the initiation site of HIF-1 (Groenendyk et al., 2013). XBP1s has also been shown to upregulate the rate-limiting enzyme in phosphatidylcholine synthesis, CCT, thereby increasing membrane biogenesis (Kim et al., 2015; Sriburi et al., 2007), which is vital for the cell to survive. Choline kinase (CK), another enzyme involved in membrane lipid synthesis, can also be upregulated via c-jun binding to the AP-1 promoter of the CK gene (Aoyama et al., 2007). This is relevant since IRE1α can activate TNF receptor-associated factor 2 (TRAF2), which subsequently activates apoptosis signaling kinase 1 (ASK1) and then c-Jun N-terminal kinase (JNK) (Sano and Reed, 2013), the kinase responsible for activating c-jun. This cascade can equally activate p38 MAPK (Sano and Reed, 2013). These survival signaling pathways can help the cell regain homeostasis, however, they can equally target the cell for programmed cell death (Szegezdi et al., 2006). JNK and p38 MAPK can both promote apoptosis by phosphorylating and activating the pro-apoptotic proteins Bax (Kim et al., 2006) and Bim (Lei and Davis, 2003; Puthalakath et al., 2007) and repressing the anti-apoptotic protein Bcl-2 (Deng et al., 2001). Interestingly, IRE1α is bound by c-Jun activation domain binding protein (JAB1) in yeast under conditions of mild ER stress (Oono et al., 2004). With severe ER stress, JAB1 releases IRE1α for sustained activation, highlighting how the UPR can switch from pro-survival to programmed cell death (Oono et al., 2004).

2.4.3.2 *The DNA Damage Response*

The DNA damage response is another strategy employed by the cell to mitigate a particular form of cellular stress: nuclear stress (Dicks et al., 2015). The DNA damage response essentially has three objectives to deal with DNA damage: cause temporary cell cycle arrest, inhibit local transcription, and relax the chromatin to facilitate DNA repair (Dicks et al., 2015). Cell cycle checkpoint control is imperative for the cell to prevent replication of damaged DNA at the G1/S stage, the segregation of damaged chromosomes at the G2/M stage, and the associated negative effects that result (Blackford and Jackson, 2017; Khanna and Jackson, 2001). This is evident in mouse embryos constructed using DNA-damaged sperm. These embryos exhibited delayed replication prior to the resumption of cell cleavage and progressive development. Embryos that did not delay replication, however, were unable to develop to the blastocyst stage (Gawecka et al., 2013). Local inhibition of transcription is also needed near sites of DNA damage, as this would result in erroneous transcripts and proteins. Ataxia telangiectasia mutated (ATM) protein, an important mediator of the DNA damage response, has been shown to prevent transcription by RNA polymerase II downstream of DNA double-stranded breaks (DSBs) (Shanbhag et al., 2010). This has similarly been shown to occur for another important mediator of the DNA damage response, DNA-dependent protein kinase (DNA-PK) (Pankotai et al., 2012). Finally, chromatin relaxation is needed to allow access of DNA repair proteins to the site of damage. These areas of DNA damage have been shown to accumulate enzymes involved in chromatin modification. In particular, they tend to be hypoacetylated, and exhibit higher levels of H3K9 and H3K27 trimethylation, all histone markers that reduce transcription (Adam and Polo, 2014).

Double-stranded breaks (DSBs) to the DNA represent the most dangerous type of DNA damage (Collins and Jones, 2016; Khanna and Jackson, 2001; Rich et al., 2000) since they can lead to large translocations and rearrangements if improperly repaired (Ferguson and Alt, 2001; Richardson and Jasin, 2000). The response to DSBs entails two major pathways, the homologous recombination (HR) pathway and the non-homologous end-joining (NHEJ) pathway. The former occurs largely during the replication phase of the cell cycle and G2, since there is an undamaged partner strand available as a template for repair (Mimitou and Symington, 2009). NHEJ repair occurs primarily during G0 and G1 phases, when a partner strand is not available for repair, thus leading to a more error-prone repair process (Mimitou and Symington, 2009).

During NHEJ, the DNA-PK complex recognizes and binds to the site of DSB (Blackford and Jackson, 2017). The DNA-PK complex is composed of two DNA-binding subunits, the 70kDa Ku70 and the 80kDa Ku80, as well as a catalytic subunit, DNA-PKcs, which exhibits similar activity to those of the phosphatidylinositol kinase related kinase (PIKK) family (Blackford and Jackson, 2017; Furgason and Bahassi el, 2013; Mimitou and Symington, 2009). The DNA-PK complex recruits other DNA repair proteins, such as the MRN complex, composed of Mre11, Rad50 and NSB1 proteins, which together help tether and process the DNA to facilitate repair (Khanna and Jackson, 2001; Rupnik et al., 2008). The tumor protein p53 binding protein 1 (53BP1) also facilitates the joining of the DNA strand ends for repair. It does so by binding damaged DNA and improving chromatin mobility, allowing the damaged ends to interact (Dimitrova et al., 2008). Furthermore, 53BP1 appears to promote NHEJ by preventing DNA resection needed for HR repair (Ceccaldi et al., 2016). DNA-PK also recruits and activates X-ray repair and cross-complementing protein 4 (XRCC4) and DNA ligase IV (Blackford and Jackson, 2017; Furgason and Bahassi el, 2013; Khanna and Jackson, 2001). XRCC4 and DNA ligase IV stimulate ligation of DNA strand ends during end-joining repair (Karran, 2000). The DNA-PK complex, XRCC4 and DNA ligase IV are all necessary for successful DNA damage repair via the NHEJ pathway (Critchlow and Jackson, 1998; Pannicke et al., 2004).

During the HR pathway, DSBs activate ATM and ataxia telangiectasia and Rad3-related (ATR) protein kinases, which are also members of the PIKK family (Blackford and Jackson, 2017; Khanna and Jackson, 2001). The functions of ATM and ATR overlap given that overexpression of ATR can compensate for ATM deficiency (Cliby et al., 1998). This is supported by the fact they both phosphorylate similar downstream substrates (Kim et al., 1999), albeit with differing kinetics and in response to differing types of DNA damage (Khanna and Jackson, 2001). Both proteins mediate DNA damage repair by inducing various signaling pathways that result in cell cycle arrest, DNA damage repair, and expression of cellular stress response genes (Blackford and Jackson, 2017; Khanna and Jackson, 2001). The cell cycle is arrested via phosphorylation of the cell cycle checkpoint control proteins p53, Chk1 and Chk2 (Banin et al., 1998; Gatei et al., 2003; Matsuoka et al., 2000). Chk1 and Chk2 in turn phosphorylate the phosphatase Cdc25, rendering it inactive and unable to dephosphorylate Cdc2, which is needed to allow progression past the G2/M phase (Donzelli and Draetta, 2003; Mailand et al., 2000). Phosphorylated p53 also

prevents cell cycle progression by inhibiting Cdk2 and Cdc2, albeit through different mechanisms (Dulic et al., 1994; Innocente et al., 1999). Furthermore, inhibition of p53 is relieved by phosphorylation of Mdm2 by ATM (Maya et al., 2001).

The DNA repair process is initiated by phosphorylation of histone H2AX on serine 139 (γH2AX) by ATM and ATR, after their recruitment to sites of DNA damage by the MRN complex and ATR interacting protein (ATRIP), respectively (Blackford and Jackson, 2017; Paull et al., 2000; Rogakou et al., 1998; Rupnik et al., 2008). Marking of DNA damage with γH2AX stimulates the co-localization of DNA repair proteins to the site of DSB, such as BRCA1, RAD50 and RAD51 (Paull et al., 2000). The accumulation of RAD51 at sites of DNA damage (Tashiro et al., 2000) coincides with accumulation of other DNA repair proteins, RAD52 and BRCA2 (Haber, 1999). RAD51 is needed for replication fork progression, both during repair of DSBs and also during normal DNA synthesis (Tashiro et al., 2000). RAD52 can bind DSBs and increase the activity of RAD51 (Haber, 1999). Phosphorylation of RAD51, which is dependent on ATM and the tyrosine kinase, c-Abl, is needed to facilitate its interaction with RAD52 and promote its repair abilities (Chen et al., 1999). The precise roles of BRCA1 and BRCA2 in the DNA damage response are not fully elucidated, but they appear to stabilize complexes of DNA repair protein foci, participate in checkpoint control as well as regulate transcription of DNA damage response proteins (Mullan et al., 2006; Welcsh et al., 2000). Specifically, BRCA1 plays an important bridging role between DNA damage sensor complexes and DNA repair effector proteins (Roy et al., 2011). There is also evidence suggesting a role for BRCA1 in promoting HR over NHEJ repair (Hustedt and Durocher, 2016), perhaps through competitive recruitment to γH2AX foci, which are equally phosphorylated by DNA-PK (Scully and Xie, 2013). BRCA2, on the other hand, acts solely during HR and promotes RAD51 mediated DNA repair (Roy et al., 2011). Nonetheless, the role of BRCA1 and BRCA2 is critical, given inactivation of both genes result in impaired DNA damage repair (Bhattacharyya et al., 2000; Sharan et al., 1997).

2.5 Cellular Stress and Development

2.5.1 UPR in the early embryo

Important mediators of the UPR are essential for early embryo development. GRP78 gene-inactivated mice cannot survive past the blastocyst stage, indicating the necessity for the “master

regulator" during embryogenesis (Luo et al., 2006). This is also true for GRP94, which is embryonic lethal and cannot be rescued, even with overexpression of GRP78 (Audouard et al., 2011). Furthermore, it has been shown that other components of the UPR, such as PERK, IRE1α, ATF4 and ATF6 are also expressed in mouse pre-implantation embryo development (Michalak and Gye, 2015). The necessity for these UPR regulators during embryogenesis is explained by the inherent presence of ER stress during development (Michalak and Gye, 2015). Spliced XBP1 has shown to be present in freshly flushed *in vivo* fertilized mouse embryos at varying stages of development, including 2-cell, 4-cell and morula embryos (Abraham et al., 2012). Another group, Zhang and colleagues, have equally shown the presence of increased nuclear XBP1s in *in vivo* fertilized embryos, specifically during the 2-, 4-, and 8-cell stage, as well as in the morula and blastocyst (Zhang et al., 2012a).

It is evident that embryos must contend with ER stress during normal *in vivo* development. As such, it is not difficult to fathom that once placed in the artificial conditions of *in vitro* culture (IVC), with variations in temperature and pH as well as mechanical and oxidative stress, the effects of ER stress are likely amplified (Michalak and Gye, 2015). In fact, spliced XBP1 is abundant in parthenogenetically-activated (PA) porcine embryos at the 4-cell, morula and blastocyst stages (Zhang et al., 2012b). Bovine embryos constructed through SCNT and IVF exhibit increased GRP78 expression compared to their *in vivo* derived counterparts (Canepa et al., 2014). Culture conditions have been shown to affect the development of pre-implantation embryos, and this is related to ER stress. For example, IVC of embryos using glucose as an energy source, as opposed to pyruvate and lactate, decreased blastocyst rate and total cell number (Karja et al., 2006). This effect was related to increased reactive oxygen species generation (Karja et al., 2006), a known inducer of ER stress. Accordingly, low oxygen tension during IVC has been shown to improve embryo development and quality (Karja et al., 2004). This is mediated by reduced hydrogen peroxide and DNA fragmentation, leading to better development of porcine embryos *in vitro* (Kitagawa et al., 2004).

While culture conditions can affect levels of ER stress and hence development, other inherent factors can also be involved. During fertilization for example, calcium oscillation is needed for resumption of meiosis and further embryonic development (Kline and Kline, 1992). Given the

importance of the ER in calcium homeostasis, these fluctuating levels are expected to challenge the ER. In addition, increased protein synthesis after cleavage, as well as greater need for lipids with each cell division, further tax the ER. Consequently, defects in the ability of the ER to regulate calcium, as well as protein or lipid synthesis during this critical time will likely contribute to early embryonic death.

2.5.2 DNA damage response in the early embryo

DNA damage is another cellular stressor that can affect pre-implantation development. In fact, deficiency in ATM, the major sensor for the HR pathway during DNA repair, results in increased DNA damage and impaired embryo development and quality (Bohrer, 2016). With the rapid cell divisions that occur during embryogenesis, ensuring genome integrity prior to cleavage is imperative for normal development (Jaroudi and SenGupta, 2007). Transcripts involved in DNA damage repair are present in both oocytes and blastocysts (Jaroudi et al., 2009; Menezo et al., 2007). In fact, it has been shown that transcripts involved in DNA damage repair are overrepresented in oocytes (Zeng et al., 2004). This supports the notion that the ability of the embryo to repair damaged DNA relies on maternal transcripts already present in the oocyte (Vinson and Hales, 2002). In fact, MMR deficiency phenotype results from genetic alterations originating from the maternal side, and not so from the paternal side (Larson et al., 2004). It is clear DNA damage reponse transcripts in the mature oocyte prepare the zygote with the necessary machinery needed to repair any DNA damage during the early stages of development (Zeng et al., 2004).

In vitro culture also appears to increase DNA damage, and hence ER stress. Actually, 30% of *in vitro* produced embryos have DNA damage (Sturmey et al., 2009). In porcine embryos, more DNA damage was shown to be present in *in vitro* produced embryos compared to those developed *in vivo* (Long et al., 1998). In addition, when compared to *in vivo* derived rhesus monkey embryos, *in vitro* cultured embryos had increased expression of DNA damage repair proteins ATR and RAD51 (Zheng et al., 2005). This study suggested that dysregulated expression of DNA damage response genes during *in vitro* culture likely impairs the ability of the embryo to deal with DNA damage (Zheng et al., 2005).

Poorly developing embryos have also been associated with increased DNA damage. This is supported by the fact that RAD51 is embryonic lethal in mice, indicating the importance of DNA damage repair in pre-implantation development (Lim and Hasty, 1996). In fact, disruption of RAD51 mRNA expression in porcine embryos through RNA interference resulted in increased DNA damage and arrested development prior to the blastocyst stage (Jin et al., 2019). In addition, microbeam-induced DNA damage in mouse embryos impedes development (Wang et al., 2013). Upregulation of HR pathway genes, TP53 and RAD52, have also been shown in poorly developing embryos (Henrique Barreta et al., 2012). Correspondingly, better quality porcine embryos produced by SCNT, IVF or PA all had decreased DNA damage, as evidenced by lower numbers of γH2AX foci (Bohrer et al., 2013; Bohrer et al., 2015). Interestingly, in UV-damaged cloned embryos, DNA damage and the necessary repair genes are decreased and blastocyst rate is increased, by culturing with a histone deacetylase inhibitor (Bohrer et al., 2014). This beneficial effect on development is likely mediated by improved access of DNA damage response proteins to the DNA for any necessary repair (Bastos et al., 2008).

2.6 Strategies to Improve Development

2.6.1 Inhibitors of cellular stress

It is evident that cellular stress can have a negative impact on cell homeostasis and survival, and hence can impede early embryonic development. While ER stress is present in early embryonic development, it has been more widely studied in the context of other diseases, such as diabetes, steatohepatitis and metabolic syndrome (Bailly-Maitre et al., 2010; Cho et al., 2014; Hebert and Molinari, 2007; Ozcan et al., 2009; Ozcan et al., 2004). Much research has investigated pharmacological inhibitors of ER stress to help relieve its detrimental effects in a variety of disease states. Many of these ER stress inhibitors have been loosely termed “chemical chaperones”, however, they do not directly interact with other proteins like molecular chaperones do. While the mechanism for many is not clear, they do relieve ER stress by affecting protein processing, and therefore the term “proteostasis promoters” has been proposed (Vega et al., 2016). In essence, these molecules prevent cellular stress and therefore will be referred to inhibitors of cellular stress in this manuscript.

The modified fatty acid 4-phenyl butyrate (PBA)(Vega et al., 2016) is one such molecule that has been shown to reduce ER stress by preventing the aggregation of misfolded proteins (de Almeida et al., 2007; Kubota et al., 2006; Uppala et al., 2017). It has also been shown to improve signs of type II diabetes mellitus (Ozcan et al., 2006), α-1-antitrypsin deficiency (Burrows et al., 2000) as well as reduce ER stress in steatohepatitis (Suzuki et al., 2019).

Tauroursodeoxycholic acid (TUDCA), a taurine-conjugated bile acid, is another inhibitor of cellular stress that has been widely studied. For example, palmitate-induced ER stress in rat pancreatic β-cells was relieved with TUDCA treatment, as evidenced by reduced GRP78, CHOP and ATF4 expression (Zhu et al., 2013). Relief of ER stress was also shown by TUDCA in liver cells subjected to misfolded proteins and UV radiation (Uppala et al., 2017). In fact, results from this study even suggested TUDCA to be more efficient at maintaining protein homeostasis than PBA (Uppala et al., 2017).

While some papers state TUDCA exhibits chaperone-like activity, it is unlikely it enters the cells that do not normally metabolize bile acids (Torchia et al., 2001). Indeed, it is known that conjugated bile acids activate ERK1/2 receptor tyrosine kinases and AKT intracellular pathways in a G-protein coupled receptor (GPCR)-dependent manner (Dent et al., 2005). Furthermore, inhibition of the G_{α}stimulatory G-protein subunit can abrogate the action of TUDCA (Vettorazzi et al., 2016). As such, it is likely that TUDCA acts through the only G-protein coupled bile acid receptor 1, GPBAR1, otherwise known as TGR5 (Duboc et al., 2014). In fact, TUDCA has recently been shown to require TGR5 to reduce inflammation in microglial cells (Yanguas-Casas et al., 2017), however, whether this is holds true in other tissues is unknown.

TGR5 has been shown to be present in various tissues throughout the body, including the ovaries, however, its function in this tissue is not clearly understood (Zwicker and Agellon, 2013). The presence of TGR5 in the ovary is interesting, considering that bile acids are primarily localized within the enterohepatic circulation pathway, including the liver, biliary system, intestinal tract, and portal vein. They are efficiently reabsorbed from the portal blood into hepatocytes by bile acid transporters and hence very little are present in systemic circulation (Halilbasic et al., 2013; Zwicker and Agellon, 2013). This small spillover into systemic

circulation accounts for bile acid concentrations of less than 5μM during periods of fasting to 3-7μM post-prandial (Zwicker and Agellon, 2013). Additionally, the amount of TUDCA, the taurine conjugated form of ursodeoxycholic acid (UDCA), present in systemic circulation is likely minute given bacterial deconjugation occurs during intestinal transit, prior to reabsorption (Zwicker and Agellon, 2013). Nonetheless, bile acids, including UDCA-derived ones, have been identified within human ovarian follicles, and at concentrations double that of serum (Nagy et al., 2015). While some have suggested the enzymes needed for bile acid synthesis are present in the ovary (Smith et al., 2009), whether actual synthesis is occurring is not well established. Furthermore, it is unlikely UDCA-derived bile acids can be formed in any mammalian tissue, since their synthesis requires bacterial enzymes (Lepercq et al., 2007; Zwicker and Agellon, 2013). Further investigation is necessary to determine how UDCA-derived bile acids concentrate in the ovarian follicles, as well as if TUDCA and UDCA have physiologic importance in TGR5 signaling. Perhaps physiologically they have no impact, and are simply replicating *in vitro* the actions of other resident ligands, namely steroids (Keitel et al., 2010), which are known to be present and are structurally similar to bile acids.

Much of the beneficial effects of TUDCA in the face of cellular stress appear to relate to a reduction in apoptosis. ER-stressed rat pancreatic acini treated with TUDCA demonstrated decreased GRP78, decreased phosphorylation of PERK and JNK, decreased CHOP expression, decreased caspase-3 activation and consequently, decreased apoptosis (Malo et al., 2010). In a rat model of liver ischemia and reperfusion, TUDCA also decreased inflammation, apoptosis and necrosis by reducing activation of IRE and PERK, caspase-12, JNK and CHOP (Ben Mosbah et al., 2010). It also inactivated glycogen synthase kinase-3 (GSK-3), which resulted in less cytochrome c release from mitochondria, decreased activation of caspase-9 and reduced mitochondrial damage (Ben Mosbah et al., 2010). Human hepatocyte lines treated with thapsigargin to induce calcium-mediated ER stress were also rescued after treatment with TUDCA (Xie et al., 2002). TUDCA reduced calcium efflux from the ER in response to thapsigargin, as well as decreased GRP78, caspase-12 activation, effector caspases and apoptosis (Xie et al., 2002). In addition, TUDCA has been shown to inhibit the association of the pro-apoptotic mediator Bax with mitochondria as well as cytochrome c release (Rodrigues et al., 2000). More specifically, TUDCA prevents apoptosis by reducing Bax-associated mitochondrial

membrane perturbation (Rodrigues et al., 2003). These anti-apoptotic effects are clearly seen in multiple studies where embryos treated with TUDCA show decreased pro-apoptotic transcripts and rates of cell death (Kim et al., 2012; Lin et al., 2016; Zhang et al., 2012b). The specific signaling pathways activated by TUDCA to reduce programmed cell death are varied and likely depend on the inciting stressors, the duration and severity of stress, as well as the cell type affected. Survival pathway activation plays a role, however, given protection from glycochenodeoxycholic acid-induced apoptosis in rat hepatocytes with TUDCA required activation of p38, ERK MAPK and PI3K pathways (Schoemaker et al., 2004). In addition, TUDCA has been shown to signal through the PKA pathway and its downstream target cAMP response element binding protein (CREB) (Vettorazzi et al., 2016; Wang et al., 2005).

2.6.2 Resolution of cellular stress improves development

As increased ER stress is present in the developing embryo, many researchers have studied the use of cellular stress inhibitors to mitigate these effects and improve development. TUDCA has been widely used in studies involving pre-implantation embryo development. Parthenogenetically-activated porcine embryos cultured with TUDCA exhibited increased blastocyst rates, as well as improved quality of these embryos, as evidenced by increased total cell number (Zhang et al., 2012b). The extent of their inner cell mass was also greater, as was their expression of the anti-apoptotic mediator, Bcl-2 (Zhang et al., 2012b). Similar results have been demonstrated in IVF porcine embryos (Kim et al., 2012), *in vivo* fertilized mouse embryos (Zhang et al., 2012a) and SCNT-derived porcine embryos treated with TUDCA (Lin et al., 2016). In all of these studies, TUDCA treatment increased the rate of blastocyst development, as well as total cell number (Kim et al., 2012; Lin et al., 2016; Zhang et al., 2012a). Remarkably, TUDCA has even been shown to improve development past the pre-implantation period, with improved implantation and live birth rates demonstrated in mice (Lin et al., 2015). Finally, there is evidence that human embryos collected from follicles with higher concentrations of UDCA-derived bile acids have better development, as evidenced by more cells at day three of development (Nagy et al., 2015). While this study was retrospective in nature, with some concerns including old frozen follicular samples as well as the inability to exclude their contamination with blood, the results are interesting and warrant further investigation (Nagy et al., 2015).

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CONNECTING STATEMENT 1

The literature review presented in Chapter 2 highlights how cellular stress negatively affects early embryo development and how stress coping responses, such as the UPR and the DNA damage response, determine not only the fate of the blastomere, but that of the embryo and fetus. Given the extensive functions of the ER, it is no wonder ER stress and the UPR have garnered much attention. Other coping mechanisms, however, do exist to deal with cellular stress originating in other cellular compartments, such as the heat shock response, the hypoxia response and the DNA damage response.

These coping mechanisms are likely intricately intertwined. While many different cellular insults can initiate different coping pathways, they ultimately affect ER function and so cannot be isolated in their response. In fact, the UPR has been shown to affect mediators of the hypoxia response (Groenendyk et al., 2013), while studies in human cells exposed to heat stress, oxidative stress and classic instigators of ER stress, show overlap of gene expression responses between the various types of insult (Murray et al., 2004). Furthermore, nuclear stress, in the form of DNA damage, can negatively affect ER function through the production of erroneous transcripts which result in the production of abnormal or misfolded proteins (Groenendyk et al., 2013). These findings support the notion that these stress coping mechanisms cannot be compartmentalized. This concept is further developed in the review article presented in Chapter 3, discussing the crosstalk between the UPR and the DNA damage response in the context of cancerous cells, which share similar biological behaviour to those of the early embryo (Ma et al., 2010). This review also identifies the response to DNA damage as a unique stress coping mechanism initiated in response to stress within the nuclear compartment. This response to nuclear stress is dedicated to chromatin and DNA damage repair and as such, is referred to as the genome damage response, or GDR.

CHAPTER 3

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Endoplasmic reticulum stress, genome damage, and cancer

Naomi Dicks[1], Karina Gutierrez[1], Marek Michalak[2], Vilceu Bordignon[1]* and
Luis B. Agellon[3]*

[1]Department of Animal Science, McGill University, Ste. Anne de Bellevue, QC, Canada
[2]Department of Biochemistry, University of Alberta, Edmonton, AB, Canada
[3]School of Dietetics and Human Nutrition, McGill University, Ste. Anne de Bellevue, QC, Canada

Running head: Coping responses to cellular stress

***Correspondence:**
Luis B. Agellon, School of Dietetics and Human Nutrition, Ste. Anne de Bellevue, QC, H9X 3V9 Canada. email: luis.agellon@mcgill.ca
Vilceu Bordignon, Department of Animal Sciences, Ste. Anne de Bellevue, QC, H9X 3V9, Canada. email: vilceu.bordignon@mcgill.ca

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3.1. Abstract

Endoplasmic reticulum (ER) stress has been linked to many diseases, including cancer. A large body of work has focused on the activation of the ER stress response in cancer cells to facilitate their survival and tumor growth, however, there are some studies suggesting that the ER stress response can also mitigate cancer progression. Despite these contradictions, it is clear that the ER stress response is closely associated with cancer biology. The ER stress response classically encompasses activation of three separate pathways, which are collectively categorized the unfolded protein response (UPR). The UPR has been extensively studied in various cancers and appears to confer a selective advantage to tumor cells to facilitate their enhanced growth and resistance to anti-cancer agents. It has also been shown that ER stress induces chromatin changes, which can also facilitate cell survival. Chromatin remodeling has been linked with many cancers through repression of tumor suppressor and apoptosis genes. Interplay between the classic UPR and genome damage repair mechanisms may have important implications in the transformation process of normal cells into cancer cells.

Keywords: carcinogenesis, cell death, chromatin damage, coping responses, DNA breaks, endoplasmic reticulum, unfolded proteins

3.2. Introduction

Cells in the body are continuously exposed to a dynamic environment dictated by the metabolic and nutritional status of the organism. Certain instances, such as exposure of the organism to nutrient excess or deprivation, extremes in temperatures, xenobiotics, and radiation, cause damage to cellular components and disruption of cellular processes. It has long been recognized that cells are adept at compensating for changes in their environment by altering certain cellular processes. The mobilization of such coping mechanisms is designed to maintain or recover proper function, overcome stressful conditions, and increase the chance for survival (Figure 3.9.1).

In the past several years, there has been increasing evidence linking ER stress with development of diseases, including certain types of cancers (Hebert and Molinari, 2007; Niederreiter et al., 2013; Wang and Kaufman, 2014; Wu et al., 2014; Yoshida, 2007). In the case of cancers,

especially non-inherited cancers that arise from genome damage, the cells capitalize on the ER stress response, which may be adaptive and advantageous at the cellular level, but deleterious to the organism. In this review, we discuss ER stress and genome damage in relation to cancer development. We provide observations supporting a link between different corrective strategies that cells adopt which may lead to malignancies.

The accumulation of unfolded and misfolded proteins disrupts ER homeostasis and leads to the activation of the classic coping mechanism termed the unfolded protein response (UPR) (Figure 3.9.1). The UPR is initiated by the molecular chaperone glucose-regulated protein 78 (GRP78). GRP78 not only binds to the misfolded and unfolded proteins, but also regulates the transmembrane ER stress sensors, namely protein kinase RNA like ER kinase (PERK), inositol-requiring protein 1α (IRE1α) and activating transcription factor 6 (ATF6) (Bertolotti et al., 2000; Shen et al., 2002; Yoshida et al., 2001).

Each ER stress sensor activates a separate arm of the UPR to facilitate immediate changes to a set of cellular functions designed to temporarily arrest general protein synthesis, and to produce active transcription factors that ultimately facilitate correct protein folding, degradation of proteins that cannot be properly processed, and regain of ER function. Under extreme conditions, these strategies may not be sufficient to alleviate the ER stress and thus require the removal of the malfunctioning cells. In such cases, cells undergo controlled cell death by activation of the apoptotic pathway. In some situations, certain adaptive strategies provide these cells with a selective growth advantage over other cells (Figure 3.9.1). This selective advantage could permit cells to survive and propagate even under chronic ER stress.

3.3. ER stress and Cancer

The high proliferative rates and inadequate vascularization of solid tumors culminate in a very unfavorable microenvironment. The low pH, low oxygen tension and low nutrient supply result in an accumulation of misfolded proteins and ER stress, which could signal cell death (Ma and Hendershot, 2004; Saito et al., 2009). Cancer cells, however, have developed a capacity to survive these extreme conditions, despite the presence of ER stress, through modulation of the

UPR response (Andruska et al., 2014; Kosakowska-Cholody et al., 2014; Pyrko et al., 2007; Wang et al., 2014a).

It has been observed that GRP78, a dominant regulator of the ER stress response, is increased in a variety of cancer types including breast, brain, lung, colon, prostate, skin and some other malignancies (Andruska et al., 2014; Lee et al., 2006; Lee et al., 2008; Pootrakul et al., 2006; Wang and Kaufman, 2014; Wang et al., 2005; Xing et al., 2006; Zhuang et al., 2009). This chaperone is associated with prolonged cell survival, mainly by preventing ER stress-induced apoptosis and thereby promoting cell malignancy, metastatic development and resistance to anti-cancer agents (Andruska et al., 2014; Lee, 2007; Pyrko et al., 2007; Visioli et al., 2014). High levels of GRP78 are also associated with rapid proliferation and malignancy of tumors (Andruska et al., 2014; Pyrko et al., 2007). In breast cancer cells that express estrogen receptor α [NR3A1], the estrogen-mediated increase in GRP78 abundance confers improved resistance to ER stress and cell proliferation, both of which can be decreased through siRNA mediated knockdown of estrogen receptor α (Andruska et al., 2014). Similarly, up-regulation of GRP78 has been shown to increase growth of a glioma cell line whereas its down-regulation inhibits tumor development (Pyrko et al., 2007). The reduction of GRP78 in glioblastoma cell lines and solid tumors treated with a chemotherapeutic agent increased the expression of CHOP and caspase 7, leading to cell apoptosis and inhibition of tumor formation (Kosakowska-Cholody et al., 2014; Pyrko et al., 2007). Moreover, the antitumor agent HKH40A, decreases GRP78 not only at the transcriptional level, but also at the protein level by directly binding GRP78 to facilitate its degradation (Kosakowska-Cholody et al., 2014). Based on these characteristics, GRP78 is considered as a biomarker of cancer progression (Lee, 2007).

The components of the UPR pathway have also been implicated in cancer (Lee, 2007; Manie et al., 2014; Wang and Kaufman, 2014; Wang et al., 2014a). Mutations in IRE1α have been found in some human malignancies (Greenman et al., 2007; Guichard et al., 2012). Under hypoxia, the effector of the IRE1α pathway, spliced XBP1 (XBP1s), is one of the factors involved in tumor growth and survival. It promotes cancer cell survival under low oxygen conditions by forming a transcriptional complex around hypoxia-inducible factor-1, a major gene regulator under hypoxic conditions (Chen et al., 2014). This transcription factor is also involved in human breast

tumorigenesis as well as in the progression of triple negative breast cancer (Chen et al., 2014; Fujimoto et al., 2003). Similarly, the PERK pathway can contribute to cell survival and growth through ATF4, a transcription factor that induces pro-survival genes (Blais et al., 2006; Ye et al., 2010). ATF4 is overexpressed in solid tumors and is essential for tumor cell survival in various mouse and human cancers whereas elimination of ATF4 in cancer cells induces apoptosis (Ye et al., 2010). PERK can also facilitate tumor growth by upregulating vascular endothelial growth factor (VEGF) and thereby inducing angiogenesis in tumors (Blais et al., 2006). Tumors derived from PERK-deficient mouse embryonic fibroblasts are considerably smaller compared to those derived from wildtype embryonic fibroblasts as a result of their impaired ability to stimulate angiogenesis (Blais et al., 2006).

Despite ample examples suggesting that the activation of the UPR is essential to cancer cell survival and tumor development, there are also indications that ER stress may provide protection against cancer (Itkonen et al., 2014; Niederreiter et al., 2013; Wang et al., 2014b). In particular, it has been shown that XBP1 is protective against intestinal tumorigenesis (Niederreiter et al., 2013). Prostatic cancer cells have been shown to produce high levels of UDP-N-acetylglucosamine pyrophosphorylase 1, which reduces ER stress in these cells and facilitates their growth (Itkonen et al., 2014). The flavonoid baicalein has also been shown to induce ER stress in hepatocellular cancer cells, resulting in increased apoptosis (Wang et al., 2014b). Interestingly, in this same study, increased IREα and eIF2α activation provided a survival advantage to theses cancerous cells. This finding highlights the paradoxical role of the UPR in cancer and our incomplete understanding of how signaling pathways may favour cell death or survival under different conditions (Clarke et al., 2014; Vandewynckel et al., 2013; Verfaillie et al., 2013). Whichever the outcome produced by ER stress, it is clearly apparent that the UPR plays a critical role in cancer biology.

3.4. Genome Damage and Cancer

Genome damage can be caused by a number of endogenous and exogenous genotoxic factors, including reactive oxygen species, altered cell metabolism, xenobiotics and radiation (Furgason and Bahassi el, 2013; Khanna and Jackson, 2001). These factors lead to DNA strand breaks, collapsed DNA replication forks, and damage to histones as well as other DNA-binding proteins

(Khanna and Jackson, 2001). In response to chromatin damage, cells can establish a genome damage response (GDR) to repair damage to both DNA and nuclear proteins, adapt to genome damage and reestablish nuclear function (Figure 3.9.1). Adaptation to genome damage can lead to cell survival but also chromatin alterations, which may have severe consequences for tissue function and physiology (Furgason and Bahassi el, 2013).

The GDR is orchestrated by several factors encompassing sensors, transducers and effectors proteins (Figure 3.9.2), which require post-translational modification and accumulation of proteins to assemble multiprotein foci at the sites of DNA lesions (Luijsterburg and van Attikum, 2011; Roos and Kaina, 2013; Soria et al., 2012). In general, activation of GDR involves temporary cell cycle arrest, local inhibition of transcription, and relaxation of chromatin to facilitate repairs. This process requires post-translational modification of proteins including the activation of the kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR), which phosphorylate transducer proteins at the damages sites, including the histone H2A.x (H2AX139ph) which anchors some important effector proteins required for damage repair and cell cycle arrest (Luijsterburg and van Attikum, 2011; Lukas et al., 2011; Roos and Kaina, 2013; Soria et al., 2012). ATM and ATR also activate the serine-threonine checkpoint effector kinases, Chk1 and Chk2, which regulate a number of proteins involved in transcription, cell cycle progression and apoptosis, including the tumor suppressor protein p53 and BRCA1 (Chen et al., 2005; Lee et al., 2000), and the cell cycle regulator proteins Cdc25 and Wee1 (Falck et al., 2002; Lee et al., 2001). Phosphorylation of transcription factors, notably such as E2F1, NR4A, ATF2 and Sp1, also facilitate DNA repair in a transcription-independent fashion, by direct interaction with damaged DNA, and subsequently the co-localization of other DNA repair proteins (Malewicz and Perlmann, 2014). In addition to post-translational modifications, genotoxic lesions and DNA damaging agents can also trigger nucleosomal remodeling via eviction of resident histones and reincorporation of new histones into the reassembled nucleosomes after damage repair (Dinant et al., 2013; Ikura et al., 2007; Luijsterburg and van Attikum, 2011; Polo, 2014).

Local transcriptional arrest associated with GDR may be transient or stable (Adam and Polo, 2014; Svejstrup, 2010). Linked to this arrest are numerous histone modifications, including

hypoacetylation of histone H4, increased histone H3K9me3 and H3K27me3, and decreased histone H3K4me3 (Adam and Polo, 2014; Seiler et al., 2011). Histone chaperones, which transfer histones to the nucleosomes, are required for transcriptional reinitiation following DNA damage (Adam and Polo, 2014; Hamiche and Shuaib, 2013). GDR also involves the participation of ATP-dependent chromatin remodeling complexes, including the switch/sucrose non-fermentable (SWI2/SNF2), imitation switch (ISWI), inositol requiring 80 (INO80) and chromodomain helicase DNA-binding protein, which mediate nucleosome sliding and histone displacement promoting access for DNA repair proteins (Luijsterburg and van Attikum, 2011; Polo, 2014; Soria et al., 2012).

Non-coding RNAs are also known to affect DNA repair and genome instability. Indeed, DNA damage responsive microRNAs have been shown to be misexpressed in cancer cells and to affect chemotherapy sensitivity (van Jaarsveld et al., 2014; Wan et al., 2014; Wang and Taniguchi, 2013). It has also been shown that downregulation of Dicer and Ago2, two essential components for microRNA-processing, reduced cell survival and checkpoint response after UV-induced DNA damage (Pothof et al., 2009). Moreover, the miR-18a was shown to downregulate ATM expression, reduce DNA damage repair, and sensitize breast cancer cells to γ-irradiation treatment (Song et al., 2011). Also, upregulation of the miR-24 decreases H2AX and renders cells more vulnerable to DNA damage induced by γ-irradiation and genotoxic drugs (Lal et al., 2009). Another microRNA, miR-155, was shown to reduce the levels of RAD51, a recombinase required to repair double strand breaks by DNA homologous recombination, and consequently decreased DNA repair and enhanced sensitivity to ionizing radiation in human breast cancer cells (Gasparini et al., 2014).

3.5. UPR and GDR Crosstalk

There is accumulating evidence suggesting that ER stress and genome damage responses are intertwined. Indeed, ER stress induced with tunicamycin treatment or glucose deprivation decreases genomic DNA damage repair by stimulating proteasomal degradation of Rad51 (Yamamori et al., 2013). On the other hand, down-regulation of PERK enhances DNA damage repair in irradiated cancer cells (Oommen and Prise, 2013). Interestingly, induction of ER stress recruits the histone acetyltransferase p300 to the GRP78 promoter and this correlates with

increased histone H4 acetylation and GRP78 gene expression (Baumeister et al., 2005). Increased GRP78 transcription is associated with the recruitment of arginine histone methyltransferase, PRMT1 (Baumeister et al., 2005). It was suggested that arginine methylation of MRE11 by PRMT1 regulates the activity of MRN complex, which is required for proper DNA damage checkpoint control (Boisvert et al., 2005). Therefore, it appears that increased GRP78 transcription from ER stress can also facilitate DNA damage repair. These contradictory effects further demonstrate our incomplete understanding of the stress signaling pathways and how they interact to determine cell fate. However, it also illustrates how ER stress can cause chromatin remodeling and affect the GDR pathway. If genome damage responses are impaired by alterations in the UPR, this can affect DNA integrity and subsequently increase risks of carcinogenesis.

Signaling from both ER stress and DNA damage also appear to result in similar chromatin remodeling changes to respond to cellular insults. Increased H3K14 acetylation as a consequence of ER stress has been observed, and this can activate the expression of other target ER stress response genes (Schram et al., 2013). Similarly, GDR results in increased H3K14ac, which promotes the binding of BRG1, an ATPase component of SWI2/SNF2 complex, to H2AXph139 at the sites of DNA damage enabling chromatin remodeling for DNA repair (Lee et al., 2010; Ogiwara et al., 2011). Phosphorylation of the histone H2Ax also enables recruitment of other chromatin remodeling complexes including INO80 and SWR1, and the histone acetyltransferase complex NuA4 to facilitate DNA repair (Bird et al., 2002; Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). Therefore, H3K14ac and H2AXph139 seem to be important in connecting ER stress and GDR.

Chromatin remodeling has also been shown to occur as a result of hypoxia and heat stress, two common causes of ER stress that also have effects on GDR (Groenendyk et al., 2013). Hypoxia-induced ER stress leads to global deacetylation and methylation of histones in the proximity of genes involved in the hypoxia-inducible factor-1 mediated response (Islam and Mendelson, 2006; Jung et al., 2005; Kasper et al., 2005). This facilitates transcription of the genes needed for an adaptive response to hypoxia (Bristow and Hill, 2008; Johnson and Barton, 2007). Yet, there is evidence confirming that hypoxia can lead to defective DNA repair, genomic instability,

and consequently, to cellular transformation (Bristow and Hill, 2008). In addition, it has been well documented that chromatin remodeling in response to heat stress results in increased transcription of heat shock proteins (Sullivan et al., 2001). These proteins have been shown to reduce accumulation of H2AXph139, decrease DNA damage repair, and increase radiation sensitivity and genome instability (Gabai et al., 2010; Laszlo and Fleischer, 2009).

While there is evidence demonstrating crosstalk between the UPR and GDR, it is not well understood at this time. Increased reactive oxygen species appears to be a common by-product of most cellular insults, ER stress and DNA damage included (Jiang et al., 2011). Oxidative stress can modulate multiple signaling pathways through activation of common transducers and transcription factors (Simmons et al., 2009).

3.6. Role of UPR and GDR in Carcinogenesis

Classically, the development of cancer is largely associated with inherited or acquired mutations of specific genes that regulate cell cycle, proliferation and apoptosis (Stratton et al., 2009; Vogelstein et al., 2013). However, similar effects can be seen with epigenetic changes, which, alone or associated with genetic mutations, can alter the expression of tumor suppressor genes (Baylin and Jones, 2011; Easwaran et al., 2014; Shen and Laird, 2013; You and Jones, 2012). There are many examples of chromatin changes that lead to cancers. Hypermethylation of the DNA repair gene BRCA1 has been associated with both breast and ovarian cancer (Birgisdottir et al., 2006; Esteller et al., 2000; Ibanez de Caceres et al., 2004). Aberrant promoter methylation of the Kelch-like-ECH-associated protein 1 gene, which codes for an adaptor protein involved in degradation of cell survival and anti-apoptosis gene products, has been linked to a poorer prognosis and increased carcinogenesis in breast cancer patients (Barbano et al., 2013). Hypermethylation of tumor suppressor genes has been observed in renal carcinomas and hematopoietic cancers (Herman et al., 1997; Herman et al., 1994; Schafer et al., 2010). Hypermethylation of the cell cycle regulation gene RB1 and cyclin-dependent kinase inhibitor genes, CDKN2B and CDKN2A, which are respectively associated with the ocular tumor, retinoblastoma (Greger et al., 1994) and various leukemias and lymphomas (Herman et al., 1997). Histone deacetylation has been associated with a more aggressive form of acute myeloid leukemia (AML) through it repressive effect on the tumor suppressor gene death-associated

protein kinase 1 (Shanmugam et al., 2012). AML has also been associated with changes is histone methylation patterns (Yamazaki et al., 2013). Finally, chromatin remodeling agents, including inhibitors of histone deacetylases, histone lysine demethylases, and DNA methyltransferases, have been tested for the treatment of various cancers (Azad et al., 2013; Bojang and Ramos, 2014; Schafer et al., 2010; Thinnes et al., 2014; Yang et al., 2013; Zhou et al., 2011).

Since both ER stress and GDR coping mechanisms affect chromatin remodeling and DNA repair, adaptations based on these mechanisms could lead to emergence of malignant cells with self-renewal properties due to both genomic and epigenomic alterations. For example, hypermethylation of promoter regions around ER stress response genes have been implicated in the development of alcohol-induced liver cancer (Han et al., 2013). GRP78-deficient mice fed large quantities of alcohol throughout their lives show high incidence of hepatic tumors, and correlate with hypermethylation of ATF6, which upregulates genes involved in ER-associated degradation to deal with the accumulation of misfolded proteins (Han et al., 2013) . Also, increased GRP78 stimulates the VEGF receptor 2 and subsequently VEGF-induced endothelial cell proliferation, which facilitates angiogenesis and tumor survival and growth (Dong et al., 2008; Katanasaka et al., 2010; Virrey et al., 2008). The apparent contradictory effect on neoplasticity as both inhibition and promotion of cancer progression, predicted by GRP78 abundance, suggests that the nature and context of coping response activation is an important determinant of the outcome.

Acetylation of H3K14 has also been implicated in cell survival and carcinogenesis, both with respect to the UPR and GDR. Increase in H3K14 acetylation in response to ER stress results in stimulation of transcription, promoting cell survival (Schram et al., 2013). Increased H3K14ac during GDR enhances access of BRG1 to the sites of DNA damage to promote chromatin remodeling required for DNA repair (Lee et al., 2010; Ogiwara et al., 2011). However, in addition to promoting DNA repair, BRG1 has been associated with cancer development. For example, BRG1 was shown to: impair the recruitment of BRCA1 to DNA-damage sites, which is important in DNA-damage repair and in the maintenance of genomic stability (Zhang et al., 2013); to activate the melanoma inhibitor of apoptosis gene (Saladi et al., 2013); and to support

oncogenic transcriptional program, including Myc (Shi et al., 2013), for the survival of leukemic cells (Buscarlet et al., 2014). Finally, chromatin changes in response to genotoxic conditions have been shown to alter the regulation of the Hedgehog-Gli signaling pathway, which has been implicated in genome instability and in several types of cancers (Carrillo et al., 2014; Malatesta et al., 2013; Mazza et al., 2013).

3.7. Summary

Coping mechanisms are designed to correct, minimize or overcome damage caused by harsh environments, and promote cell survival. The UPR pathway is mobilized in response to the accumulation of unfolded proteins and to ultimately regain ER homeostasis. Similarly, the GDR pathway operates in response to chromatin damage and to restore normal nuclear function. Some adaptive strategies allow cells to overcome defects in cellular function through metabolic adaptation and gain a survival advantage, such as in certain types of malignancies. A better understanding of the interplay between UPR and GDR pathways may provide new insights into the pathogenesis of cancers, which could give rise to more effective anti-cancer therapies.

3.8. Acknowledgements

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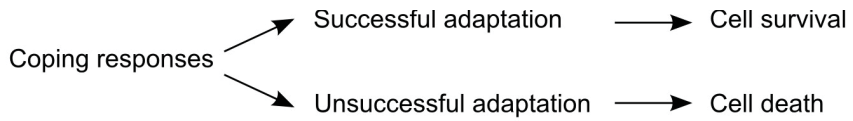
3.9. Figures



Unfolded Protein Response pathway
- elimination of unfolded and misfolded proteins
- recovery from ER stress

Genome Damage Response pathway
- chromatin repair
- restoration of normal nuclear function

Figure 3.9.1. Coping response mechanisms.

The unfolded protein response (UPR) pathway operates to restore correct folding of proteins and recovery of the ER from stress. The genome damage response (GDR) pathway enables repair of damaged DNA, histones and other DNA binding proteins and restores normal nuclear function.

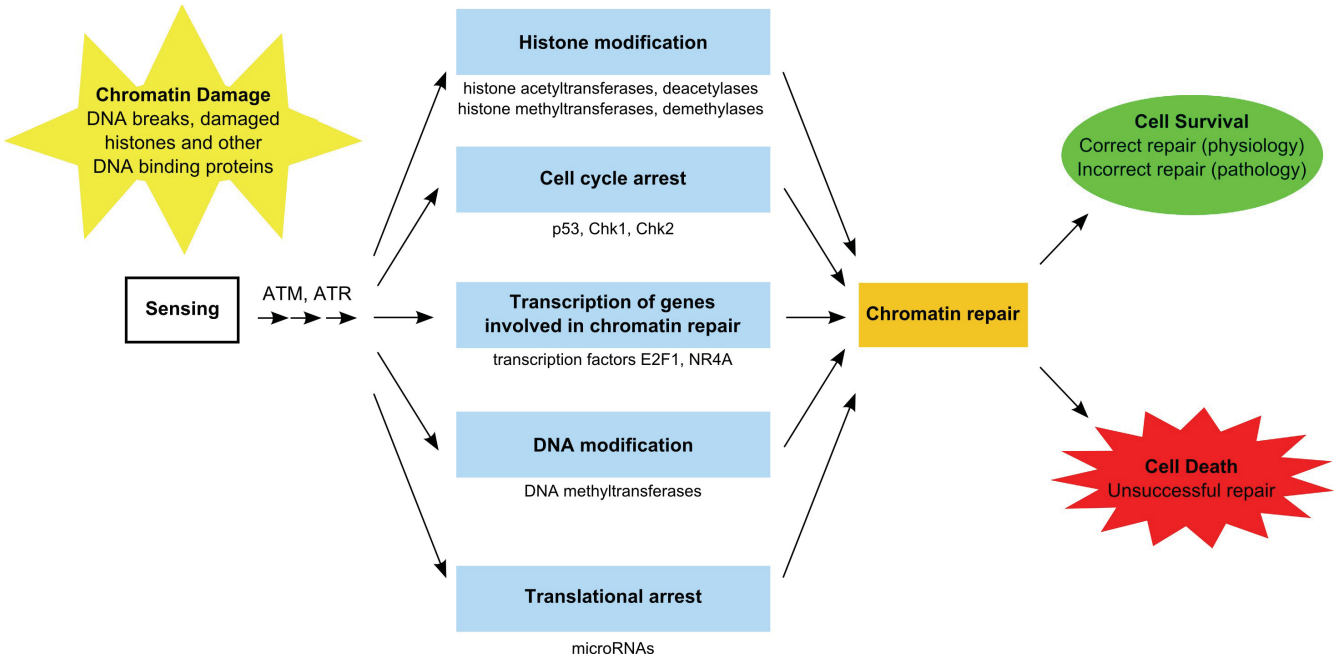


Figure 3.9.2. Functional components of the GDR pathway.

Sensing of damaged DNA, histones and other DNA-binding proteins results in activation of specific kinases (e.g., ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) proteins) (Luijsterburg and van Attikum, 2011; Lukas et al., 2011; Roos and Kaina, 2013; Soria et al., 2012). Access to damaged chromatin is facilitated by histone modification involving histone acetyltransferases/deacetylases and histone methyltransferase/demethylases as well as by DNA modification involving DNA methyltransferase (Adam and Polo, 2014; Luijsterburg and van Attikum, 2011; Polo, 2014; Seiler et al., 2011; Soria et al., 2012). Certain transcription factors (e.g., E2F1, NR4A) stimulate genes involved in chromatin repair (Malewicz and Perlmann, 2014). Translational arrest is facilitated by microRNAs (e.g., mIR-155, miR-18a) (Gasparini et al., 2014; Song et al., 2011). Cell cycle arrest (e.g., via p53, Chk1, Chk2) may be required in order to complete chromatin repair (Chen et al., 2005; Falck et al., 2002; Lee et al., 2001; Lee et al., 2000). Unsuccessful chromatin repair due to extensive damage commits the cell to die. Successful chromatin repair enables cells to survive and restore normal function. In certain cases, incorrect repair escapes quality control surveillance and leads to altered cell function, which may provide the cell with a survival advantage, but manifest as pathology at the organismal level.

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CHAPTER 4

SUMMARY

There are many instigators of cellular stress, including nuclear stress in the form of DNA damage, as well as numerous causes of ER stress. It is apparent that perturbations to energy and nutrient homeostasis result in cellular stress, which negatively impacts early embryonic development. Given the extensive functions of the ER, dysfunction of this organelle impacts other cellular compartments, and the same is true with nuclear stress. As such, coping mechanisms initiated in response to cellular stress likely exhibit significant crosstalk. The UPR and GDR pathways are activated in response to ER and nuclear stress, respectively, and they are potentially linked through various regulators and effectors in their signaling cascades. These coping responses play important roles in facilitating successful embryonic development. It has been shown that by reducing ER stress, developmental potential of embryos can be improved. In addition, more developmentally competent early-cleaving embryos have reduced DNA damage, indicating embryos with lower levels of nuclear stress also have a greater chance at normal development.

A better understanding of the factors contributing to differential developmental potential in early embryo development is essential to improve the efficiency of IVP. Efficient IVP translates into improved ART, which are needed to facilitate the production of high quality agricultural livestock, generate more efficient animal models for biomedical research, promote the conservation of endangered species, and to circumvent fertility problems faced by current human society.

While cellular stress has been shown to be present in developing embryos, it is not clear if ER stress affects cleavage kinetics and embryo developmental potential. Furthermore, treatment of IVC embryos with the cellular stress inhibitor, TUDCA, has been shown to improve embryo developmental competence and quality, however, the mechanism behind its action is unclear. Finally, it is unknown if TUDCA can relieve nuclear stress, and whether the UPR and GDR coping responses are linked in early embryo development. A more in depth understanding of the

types of cellular stress and coping responses that affect cleavage kinetics and developmental capacity is necessary. This information represents a first step towards the development of mitigation techniques to improve IVP. Furthermore, an understanding of the mechanism of action by which cellular stress inhibitors improve embryo development can facilitate new rescue treatments as well as help elucidate the inherent coping responses utilized by the early stage embryo. This doctoral thesis aims to address these questions through the following hypothesis and objectives:

Hypothesis

It is hypothesized that functional UPR and GDR coping responses necessary to combat cellular stress are essential for successful pre-implantation embryo development.

More specifically, it is hypothesized that:

1. Inherent cellular stress is present during early development and this negatively affects cleavage kinetics, development and quality of embryos.
2. Inhibition of cellular stress using TUDCA is mediated by the bile acid receptor TGR5 in early embryo development.
3. TUDCA activation of TGR5 signaling relieves nuclear stress and affects the downstream pathways of the UPR and GDR during early embryo development.

Objectives

To evaluate the general and specific hypotheses, three main objectives are proposed:

1. To evaluate the inherent effect of cellular stress on cleavage kinetics, development and quality in parthenogenetic embryos.
2. To evaluate whether TUDCA mediates its beneficial effect on early embryo development through the membrane-bound bile acid receptor, TGR5.
3. To evaluate whether TUDCA can alleviate nuclear stress through the TGR5 receptor and affect UPR and GDR responses.

CHAPTER 5

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Relief of endoplasmic reticulum stress enhances DNA damage repair and improves development of pre-implantation embryos

Naomi Dicks[a¶], Rodrigo C. Bohrer[a], Karina Gutierrez[a], Marek Michalak[c], Luis B. Agellon[b¶*], Vilceu Bordignon[a¶*]

[1]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[2]School of Human Nutrition, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[3]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

*Corresponding author
E-mail: vilceu.bordignon@mcgill.ca or luis.agellon@mcgill.ca

¶ These authors contributed equally to this work.

5.1 Abstract

Early-cleaving embryos are known to have better capacity to reach the blastocyst stage and produce better quality embryos compared to late-cleaving embryos. To investigate the significance of endoplasmic reticulum (ER) stress on early embryo cleavage kinetics and development, porcine embryos produced *in vitro* were separated into early- and late-cleaving groups and then cultured in the absence or presence of the ER stress inhibitor tauroursodeoxycholic acid (TUDCA). Developing embryos were collected at days 3 to 7 of culture for assessment of ER stress status, incidence of DNA double-strand breaks (DSBs), development and total cell number. In the absence of TUDCA treatment, late-cleaving embryos exhibited ER stress, higher incidence of DNA DSBs, as well as reductions in development to the blastocyst stage and total embryo cell numbers. Treatment of late-cleaving embryos with TUDCA mitigated these effects and markedly improved embryo quality and development. These results demonstrate the importance of stress coping responses in early developing embryos, and that reduction of ER stress is a potential means to improve embryo quality and developmental competence.

5.2 Introduction

Embryo death during early developmental stages is a leading component of infertility. In livestock, most naturally fertilized oocytes are unable to survive beyond the implantation stage(Diskin et al., 2011). It is also well documented that less than 50 % of embryos produced by *in vitro* fertilization (IVF) are able to establish pregnancy either in humans or livestock(Paramio and Izquierdo, 2014; Pontes et al., 2011; Sunderam et al., 2015). Such failures are attributable to many factors including gamete integrity and quality, genetic and genomic defects, and environmental factors affecting the embryo developmental milieu, such as the health, and hormonal and metabolic status of the zygote/embryo host(Leroy et al., 2015; Swain et al., 2016; Wrenzycki and Stinshoff, 2013).

During *in vitro* embryo production, distinct populations of embryos can be differentiated according to cleavage kinetics, which can predict developmental potential. Early-cleaving embryos begin to divide shortly after fertilization or activation, generally within the first 24 h in the porcine species, whereas late-cleaving embryos initiate their first cell division after 24

h(Isom et al., 2012; Lechniak et al., 2008). It has been observed in pigs and cattle that early-cleaving embryos have a greater ability to develop to the blastocyst stage and also have better quality, as assessed by total embryo cell numbers(Booth et al., 2007; Coutinho et al., 2011; Dang-Nguyen et al., 2010; Isom et al., 2012; Lonergan et al., 1999). This improved developmental capacity has been shown in many mammalian species, including mice, pigs, cattle, hamsters and even humans(Booth et al., 2007; Coutinho et al., 2011; Dang-Nguyen et al., 2010; Isom et al., 2012; Lee et al., 2015; Lonergan et al., 1999; McKiernan and Bavister, 1994; Sakkas et al., 1998). In livestock, it is possible to accurately predict successful development to the blastocyst stage by evaluating embryo cell cleavage kinetics(Booth et al., 2007). In humans, it has been shown that transfer of early-cleaving embryos produce higher pregnancy rates(Sakkas et al., 1998).

While it is now clear that cleavage kinetics represents an important parameter of embryo competence, the nature of these differences is not understood. Recently, Bohrer and colleagues showed that late-cleaving porcine embryos have increased DNA damage and this affects their quality and ability to reach the blastocyst stage(Bohrer et al., 2015). DNA damage is an inciting cause of ER stress, a state of cellular dysfunction that is associated with aberration of multiple cellular metabolic pathways(Dicks et al., 2015; Groenendyk et al., 2010). Cells normally mount stress coping strategies, such as the unfolded protein response(Groenendyk et al., 2013; Hebert and Molinari, 2007) and genome damage response pathways(Adam and Polo, 2014; Dicks et al., 2015; Khanna and Jackson, 2001), to regain homeostasis.

The ability to maintain cellular homeostasis throughout the execution of the metabolically active developmental program is paramount for ensuring the survival of the embryo. ER stress is of interest in development since important regulators of the ER stress response have been implicated in murine embryo survival or death(Audouard et al., 2011; Hao et al., 2009; Iwawaki et al., 2009; Luo et al., 2006; Michalak and Gye, 2015). Glucose-regulated protein 78kDa (GRP78) is known as the “master regulator” of the unfolded protein response given its ability to modulate the activation of the three main pathways involved in this coping mechanism(Groenendyk et al., 2013; Groenendyk et al., 2010). GRP78 has been shown to be essential for early embryo cell growth in mice(Luo et al., 2006). One of the main arms activated

by GRP78 is the inositol-requiring enzyme 1 (IRE1) pathway(Groenendyk et al., 2013; Groenendyk et al., 2010). This pathway has equally been implicated in murine embryo survival(Iwawaki et al., 2009). Dimerization and phosphorylation of IRE1 stimulates its endoribonuclease activity, which removes a 26-nucleotide fragment from the X-box binding protein 1 (XBP1) mRNA(Groenendyk et al., 2013; Groenendyk et al., 2010). This spliced form of XBP1 mRNA encodes the XBP1s form of the transcription factor that translocates to the nucleus to induce the expression of chaperones and other proteins important in the mitigation of ER stress(Groenendyk et al., 2013; Groenendyk et al., 2010). Functional XBP1s has been identified in murine and porcine developing embryos(Zhang et al., 2012a; Zhang et al., 2012b), indicating inherent ER stress during preimplantation development in these species. Interestingly, treatment with the ER stress inhibitor, TUDCA, improves development in these models(Zhang et al., 2012a; Zhang et al., 2012b). There is also evidence that XBP1s regulates genes involved in the genome damage response(Acosta-Alvear et al., 2007; Argemi et al., 2017) and its knockdown results in increased foci of DNA damage(Argemi et al., 2017).

While regulators of ER stress have been shown to be involved in development(Audouard et al., 2011; Hao et al., 2009; Iwawaki et al., 2009; Luo et al., 2006; Michalak and Gye, 2015), to date, there has been no investigation into the impact of ER stress on embryo cleavage kinetics, an important indicator of embryo competence. It has been clearly shown in mice and pigs that induction of ER stress in embryos impedes their development (Kim et al., 2012; Zhang et al., 2012a; Zhang et al., 2012b), however, inherent ER stress status has never been evaluated in early- and late-cleaving embryos. Further insight into the nature of late-cleaving embryos can shed light on the cause of their poor development, as well as provide a means to improve fertility. The objective of the present study was to assess inherent ER stress status and DNA damage in both early- and late-cleaving porcine embryos, and determine if these factors affect the developmental capacity and quality of embryos. We found that ER stress is a major determinant of developmental potential in late-cleaving porcine embryos, and that DNA damage is linked to ER stress.

5.3 Results

To assess the effects of resolving inherent ER stress on embryo development, both early- and late-cleaving embryos were treated with TUDCA, an ER stress inhibitor. Treatment of early-cleaving embryos with TUDCA did not result in a significant increase in the rate of development to the blastocyst stage (Fig 5.7.1A). However, the blastocyst rate of late-cleaving embryos increased 2.5 fold from 11.7% to 31.4% (Fig 5.7.1B). Interestingly, inhibition of ER stress increased development of late-cleaving embryos (31.4 %) to a rate similar to untreated early-cleaving embryos (30.2 %). These TUDCA-treated late-cleaving embryos, which normally develop poorly, had improved quality during both early development (Fig 5.7.1D) and at the blastocyst stage (Fig 5.7.1F and 5.7.1G), as indicated by an increase in mean total cell number by 32.1% (2.8 to 3.7, $p = 0.02$) and 43.0% (25.8 to 36.9, $p = 0.004$), respectively. Treatment of early-cleaving embryos did not improve quality in early development (Fig 5.7.1C), but did so at the blastocyst stage (Fig. 5.7.1E and 5.7.1G). Importantly, TUDCA treatment increased the number of cells in late-cleaving embryos to similar numbers of untreated early-cleaving embryos at both the developing (3.7 vs. 4.1) and blastocyst stages (36.9 vs. 38.7), respectively.

ER stress was evaluated in all embryos by measuring the mRNA abundance of XBP1s and GRP78. Indeed, both were increased in untreated day 5 late-cleaving embryos compared to their untreated counterparts (Fig 5.7.2B and 5.7.2F, respectively). This effect was not seen in the more developmentally competent day 5 early-cleaving embryos (Fig 5.7.2A and 5.7.2E, respectively), nor in any embryos of any group that had successfully attained the blastocyst stage (Fig 5.7.2C, 5.7.2D, 5.7.2G and 5.7.2H). Furthermore, evaluation of GRP78 protein via immunofluorescence showed a similar trend. Untreated late-cleaving developing embryos had a higher relative GRP78 fluorescence compared to their TUDCA-treated counterparts (Fig 5.7.3B and 5.7.E). While treatment with TUDCA caused a significant decrease in GRP78 fluorescence in late-cleaving developing embryos (Fig 5.7.3B and 5.7.E), this effect was not seen in more competent early-cleaving developing embryos (Fig 5.7.3A and E), nor in any embryos of any group that had successfully attained the blastocyst stage (Fig. 5.7.3C, D). Importantly, treatment with TUDCA decreased mRNA abundance of XBP1s in late-cleaving developing embryos to similar levels of early-cleaving developing embryos, treated or not with TUDCA.

Given DNA damage is an inducer of ER stress, it was also evaluated in both developing embryos and blastocysts. The incidence of DNA double strand breaks was determined by assessing the number of fluorescent foci of H2AX139ph. The mean number of H2AX foci per cell was not different in treated and non-treated early-cleaving developing embryos (Fig 5.7.4A). However, inhibition of ER stress by TUDCA treatment decreased the mean number of H2AX foci per cell in late cleaving embryos (Fig 5.7.4B). Interestingly, treatment of late-cleaving developing embryos with TUDCA reduced DNA damage to a level similar to that of untreated early-cleaving embryos. At the blastocyst stage, the mean percentage of cells with H2AX foci was decreased by TUDCA treatment in early-cleaving embryos (Fig 5.7.4D), but not in late-cleaving embryos (Fig 5.7.4E).

5.4 Discussion

Results of this study are based on experiments in parthenogenetically-activated porcine oocytes. This model was selected in place of the standard IVF produced embryos since it was essential to accurately measure the time until the embryo's first cleavage to carry out the experiments. Furthermore, abnormal oocyte penetration and polyspermy are common problems seen in porcine IVF, which can have detrimental effects on embryo cleavage and development(Meseguer et al., 2011). Finally, parthenogenetic embryo development is an established model for early embryo development(Fair et al., 2004; Kirkegaard et al., 2015; Mourot et al., 2006; Sugimura et al., 2012; Wong et al., 2010) and the blastocyst rate and quality of embryos produced using this method have been shown to be similar to IVF in pigs(Bastos et al., 2008; Brevini et al., 2002).

Distinction between early- and late-cleaving embryos is important since the former exhibits improved developmental competence. The findings of the present study demonstrate that ER stress affects embryo cleavage kinetics, and ultimately both the quality and the ability of embryos to develop to the blastocyst stage. Studies in humans and livestock using diverse embryo production methods, including *in vitro* fertilization, intra-cytoplasmic sperm injection, parthenogenetic activation and nuclear transfer, consistently show that late-cleaving embryos have reduced developmental competence(Booth et al., 2007; Coutinho et al., 2011; Edwards et al., 1984; Isom et al., 2012; Lonergan et al., 1999; Meseguer et al., 2011; Sakkas et al., 1998; Yadav et al., 1993). Thus, the kinetics of embryo cleavage has been used as a parameter for

embryo selection in assisted reproduction in both humans and livestock(Kirkegaard et al., 2015; Sugimura et al., 2012).

The precise causes of compromised development of late-cleaving embryos are not known. These may originate from endogenous sources such as inherent in the gametes, or exogenous sources such as the developmental milieu, of the gametes and embryos. Regardless, late- and early-cleaving embryos exhibit differences in metabolic profile(Lee et al., 2015), mRNA transcriptome(Fair et al., 2004; Mourot et al., 2006; Wong et al., 2010), mRNA processing(Brevini et al., 2002), and chromatin remodeling(Bastos et al., 2008). In this study, we found increased UPR activity, an ER stress coping response, as revealed by increased XBP1s and GRP78 mRNA abundance and GRP78 immunofluorescence, in untreated late-cleaving embryos. Remarkably, treatment with TUDCA(Vega et al., 2016) which has been shown to decrease ER stress in embryos and increase blastocyst rates(Kim et al., 2012; Lin et al., 2016; Zhang et al., 2012a; Zhang et al., 2012b), rescued development and quality of late-cleaving embryos. TUDCA treatment had no apparent negative impacts on early-cleaving embryos. These findings indicate that increased ER stress is a major determinant of reduced developmental potential of late-cleaving embryos, and that embryos at the earliest cell division stages are extremely sensitive to ER stress.

It is known that a variety of common factors are associated with both ER stress and DNA damage(Ciccia and Elledge, 2010; Groenendyk et al., 2013). There is also evidence indicating that similar signaling pathways are induced by either DNA damage or ER stress responses in somatic cells(Dicks et al., 2015). Recent studies showed that late-cleaving embryos have higher incidence of DNA DSBs than early-cleaving embryos(Bohrer et al., 2015; Bohrer et al., 2014) and treatment with inhibitors of histone deacetylase enzymes was shown to enhance DNA damage repair(Bohrer et al., 2014), as well as reduce ER stress(Song et al., 2014) in embryos produced by nuclear transfer. These studies suggest that DNA damage and ER stress may have common effects on embryo cleavage kinetics and development. In our study, higher incidence of DNA damage was detected in untreated late-cleaving developing embryos, as demonstrated by an increase in the mean number of DSBs per cell. These embryos appear to accumulate more DNA damage foci during early development, whereas those that successfully develop to the

blastocyst stage are no different from their TUDCA-treated counterparts. Interestingly, early-cleaving blastocysts treated with TUDCA had reduced DNA damage compared to their untreated counterparts, an effect not seen in the late-cleaving blastocysts. This improvement seems to have occurred after genome activation, which coincides with the observation that the DNA damage response is most active near the blastocyst stage(Vinson and Hales, 2002). The difference in TUDCA effect at this stage between early and late-cleaving blastocysts is unclear, but may be attributed to greater cellular differentiation, and hence increased activity of DNA damage repair, in the more developmentally advanced early-cleaving groups. Our findings indicate that relief of ER stress had the same positive effect on embryo genome integrity by either decreasing the incidence of DSBs or enhancing genome damage repair(Dicks et al., 2015). Importantly, our study provides clear evidence linking the genome damage coping response(Dicks et al., 2015) to ER stress coping response pathways in developing embryos.

In conclusion, we show here that increased ER stress and genome damage are present in developing late-cleaving embryos. Pharmacological relief of ER stress also decreased genome damage and improved embryo development. Therefore, reduction of ER stress is a potential means to rescue the developmental competence and quality of late-cleaving and poorly developing embryos. Our findings have significant implications in human and livestock fertility as well as for the refinement of assisted reproductive technologies.

5.5 Materials and Methods

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Animal Care

All animal work performed in this study was approved by the Animal Care and Use Committee of McGill in compliance with guidelines from the Canadian Council on Animal Care.

Oocyte retrieval and maturation

Oocytes were aspirated from ovaries of pre-pubertal gilts collected from a local abattoir (Olymel, S.E.C./L.P, Saint Esprit, Quebec, Canada). Cumulus oocyte complexes (COC) were collected from 3-6 mm follicles and those having at least three layers of cumulus cells and homogeneous cytoplasm were selected for maturation. Groups of 30 COCs were matured for 22 h in 100 µl of maturation medium consisting of TCM 199 (Life technologies, Burlington, ON, Canada), supplemented with 20% of porcine follicular fluid, 1mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor (EGF; Life technologies), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.5 µg/mL LH (SIOUX Biochemical Inc., Sioux Center, IA, United States), 0.5 µg/mL FSH (SIOUX Biochemical Inc.), and 20 µg/mL gentamicin (Life technologies). COCs were transferred to the same IVM medium, but without LH, FSH and dbcAMP, for an additional 20 to 22 h as previously described(Bohrer et al., 2015).

Oocyte activation

After maturation, COCs were denuded with 0.1% hyaluronidase (H3506) to facilitate selection of mature MII oocytes. Mature oocytes were activated for 5 min in 15 µM ionomycin (I0634) followed by 4 h in calcium-free porcine zygote medium (PZM-3) supplemented with 10 mM strontium chloride (255521), 7.5 µg/ml cytochalasin B (C6762) and 10 µg/ml cyclohexamide (C1988), as previously described(Che et al., 2007).

Embryo culture

After activation, embryos were cultured in PZM-3 medium supplemented with 3 mg/ml BSA (A6003). Groups of up 20-30 embryos were cultured in 60 µl droplets under mineral oil at 5 % CO_2, 95 % air and 38.5 °C. After 24-30 h, cleavage was assessed and early-cleaving embryos were separated from the rest of the group. After 48-54 h, cleavage was re-assessed and late-cleaving embryos were identified. The categorization of early and late-cleaving embryos according to these time points has been based on previous studies (Bohrer et al., 2015; Coutinho et al., 2011; Dang-Nguyen et al., 2010; Isom et al., 2012; Lechniak et al., 2008; Totey et al., 1996) . Any remaining uncleaved embryos were discarded at this stage. Early- and late-cleaving embryos were each divided into two equal groups, one that was subsequently cultured in PZM-3 supplemented with 50 µM TUDCA (Millipore, Billerica, MA, USA, 580549), and another

supplemented with an equal volume of vehicle (PBS) (Life Technologies, Carlsbad, CA, USA, 21600-010). The concentration of TUDCA was chosen based on a previous study optimized for porcine embryo culture(Zhang et al., 2012b). Embryos were maintained in culture and collected for analysis at day 3 and 5 (developing embryos), and at day 7 (blastocysts). Embryo quality was evaluated by determining the total cell number of developing and blastocyst stage embryos. qPCR analysis was performed on embryos collected at day 5, and blastocysts collected at day 7. Embryos were not evaluated for mRNA prior to 5 days of culture to ensure that zygotic genome activation had occurred. Embryos cultured beyond 5 days were supplemented with 10 % FBS (Life Technologies, 16170-078). All data from the study was collected from a minimum of 3 replicates.

Evaluation of blastocyst rate and cell number

Blastocyst rates were calculated at day 7 based on the number of cleaved embryos initially maintained in culture. Blastocysts were fixed in 4 % formaldehyde (HT501128) for 15 min and permeabilized with 1 % Triton X-100 (T8787) in PBS at 37 °C for 30 min. Fixed embryos were stained with DAPI (4,6-Diamidino-2-Phenylindole, Dilactate) (Life Technologies, D3571) for 15 min at room temperature, and then mounted on microscope slides in a drop of Mowiol (Polyvinylalcohol, 10852). Cell numbers were counted based on DAPI staining using a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY, USA).

Quantitative real-time PCR

RNA was extracted from embryos using the PicoPure™ RNA Isolation Kit (Life Technologies, KIT0202) and cDNA synthesized using Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, 11754050), according to manufacturer's recommendations for both procedures. Quantitative real-time PCR was carried out using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA, 185-5200) and the iQ™ SYBR® Green Supermix (Bio-Rad, 170-8880). Primers used are listed in Table 5.8.1. Thermocycler parameters were 5 min at 95 °C, 40 cycles of 15 s at 95 °C followed by 30 s at 58 °C, and finally 10 s at 95 °C and 5 s at 60 °C. Samples were run in duplicate and specificity of reaction products were verified by melting-curve analyses. Relative quantities of mRNA were calculated using the $\Delta\Delta C_T$ method(Schmittgen and Livak, 2008) with the reference 18S ribosomal RNA gene and H2A

gene used for normalization. Spliced XBP1 (XBP1s) mRNA abundance was compared to unspliced XBP1 mRNA abundance prior to normalization. All reactions used for quantification had efficiency between 90 -110%, $R^2 \geq 0.98$ and slope values from -3.6 to -3.1.

Immunofluorescence analysis

Immunofluorescence experiments were performed as previously described with minor alterations (Coutinho et al., 2011, Bohrer et al., 2015). Embryos were fixed and permeabilized as described above for evaluation of blastocyst cell number. Embryos were stored at 4 °C in 1 % Triton X-100 in PBS until immunofluorescence analyses could be performed on all replicates simultaneously, to reduce experimental variability in fluorescence staining. Data are from a minimum of 3 replicates with a minimum of 15 embryos per group. Fixed and permeabilized embryos were incubated for 90 min at room temperature in blocking solution, consisting of 3 % BSA (Roche, Basel, Switzerland, 10775835001) and 0.2 % Tween-20 (P1379) in PBS, and then overnight at 4 °C in the presence of the primary antibody. Primary antibodies used were rabbit polyclonal anti-GRP78 (Abcam, Ab191023) and mouse monoclonal anti-phospho-histone H2A.X (Ser139) (Millipore, 05-636) diluted at 1:100 and 1:400 in blocking solution, respectively. Negative control samples were incubated in blocking solution overnight in the absence of primary antibodies. Samples were then rinsed 3 times for 30 min each in blocking solution before incubation in the dark for 50 min in the presence of 1:1000 diluted secondary antibodies, which were either goat polyclonal anti-rabbit Alexa Fluor 488-conjugated IgG antibody (Life Technologies, A-11008) or goat polyclonal anti-mouse Cy3-conjugated IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, 115-165-146). Embryos were then rinsed in blocking solution for 30 min, incubated for 20 min in 10 μg/ml DAPI, and rinsed once more in blocking solution for 30 min before mounting on slides in Mowiol. Slides were analyzed using a Nikon Eclipse 80i microscope (Nikon Instruments Inc.) and images were captured using a Retiga 2000R monochrome digital camera (Qimaging, Surrey, BC, Canada) and the Simple PCI Imaging Software (Compix, Inc., Sewickly, PA, USA). Total GRP78 fluorescence was recorded, adjusted based on embryo cell number, and normalized against negative controls. H2AX139ph foci greater than $0.3\mu m^3$, which indicate sites of DNA damage repair and exclude areas of cell cycle regulation and mitosis(Grenier et al., 2012; McManus and Hendzel, 2005), were counted in individual nuclei and expressed as the average

number of positive foci per cell for developing embryos (collected at day 3 and 5), or as the average number of positive cells for blastocysts (collected at day 7). Cell numbers were counted based on DAPI staining for all embryos and blastocysts analyzed.

Statistical analyses

All statistical analyses were performed using the JMP 10 program (SAS, Cary, NC, USA). Continuous values satisfying a normal distribution were tested using the one-way ANOVA method and means compared using the Student's t-test. For data sets not normally distributed, continuous values were tested using the Kruskal-Wallis method and means compared using the Wilcoxon test. Significant differences were attributed for P-values < 0.05.

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5.7 Figures

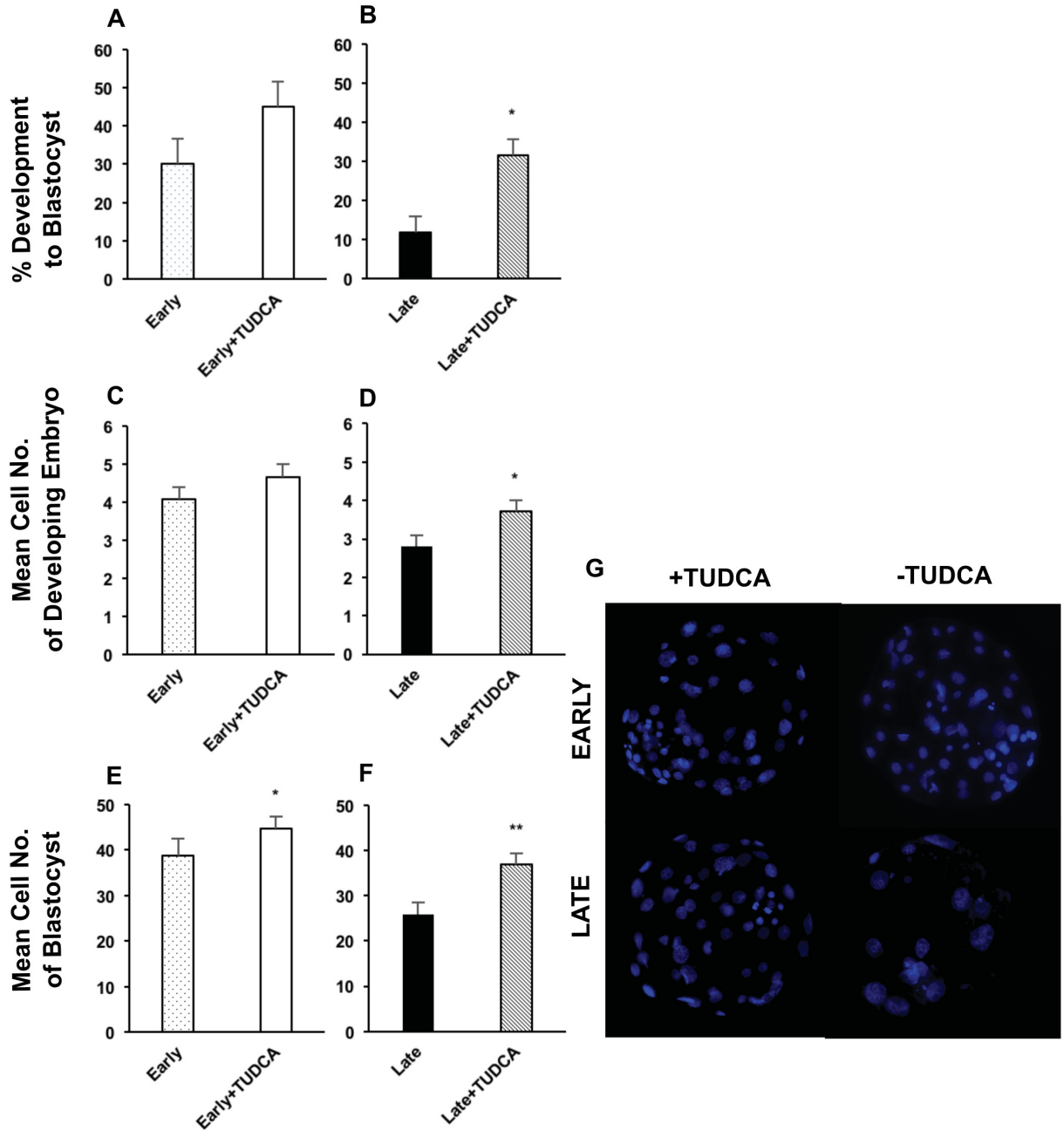

Figure 5.7.1. Culture with TUDCA Improves Embryo Quality and Blastocyst Rate

Evaluation of embryo quality, as indicated by mean total cell number, was performed in developing embryos and blastocysts cultured without or with TUDCA. Development to the blastocyst stage was also investigated. Percent development to the blastocyst stage (blastocyst rate) in each treatment group of early-cleaving embryos, n = 335 (A) and late-cleaving embryos, n = 585 (B). Mean total cell number for each treatment group of early-cleaving developing

embryos, n = 42 (C) and late-cleaving developing embryos, n = 42 (D). Mean total cell number for each treatment group of early-cleaving blastocysts, n = 89 (E) and late-cleaving blastocysts, n = 65 (F). Representative images of early-cleaving and late-cleaving blastocysts cultured without or with TUDCA (G). Images were captured at 20X magnification. Developing embryos are defined as those collected at day 3 and 5 of culture. Late = late-cleaving embryos cultured without TUDCA; Late + TUDCA = late-cleaving embryos cultured with TUDCA; Early = early-cleaving embryos cultured without TUDCA; Early + TUDCA = early-cleaving embryos cultured with TUDCA. Statistically significant differences among groups are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

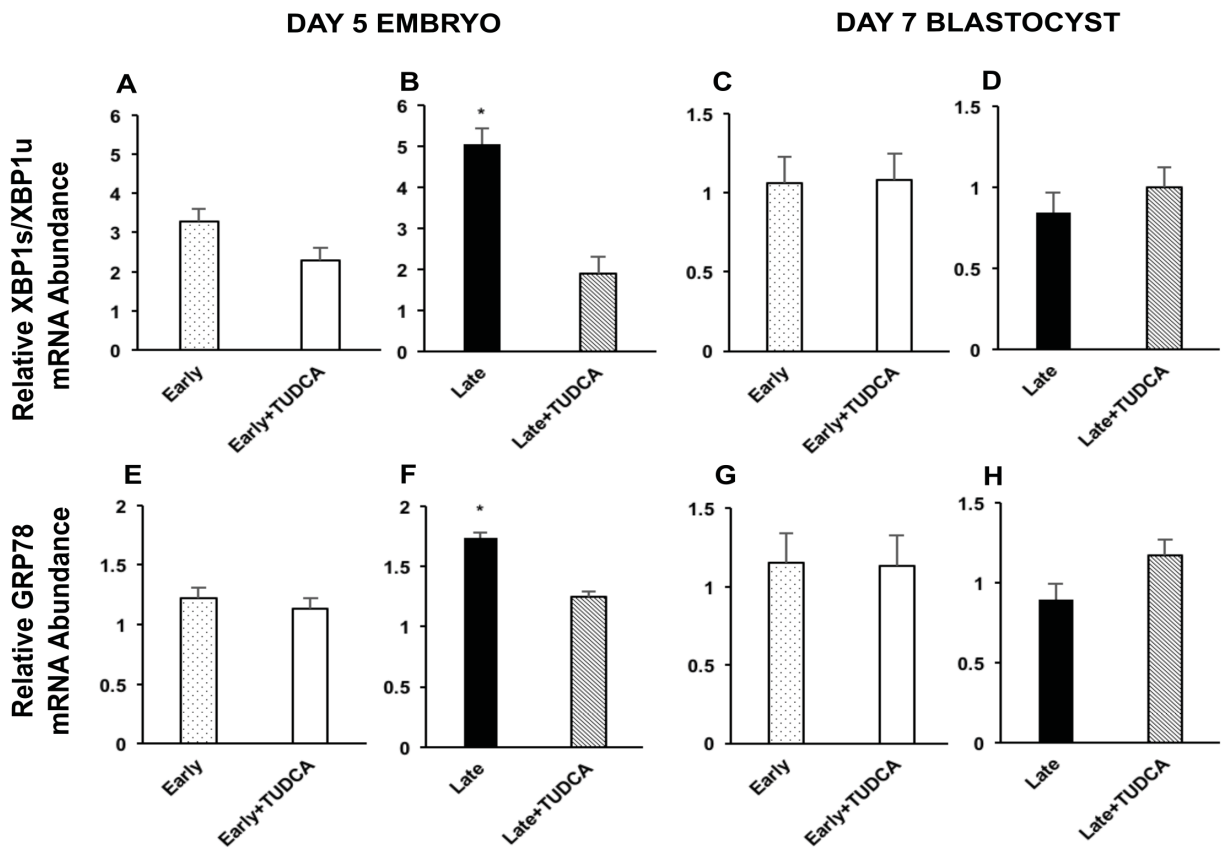

Figure 5.7.2. ER Stress in the Developing Embryo and Blastocyst: mRNA Abundance
Comparison of ER stress marker mRNA abundance in embryos to evaluate ER stress status. XBP1s/XBP1 mRNA abundance in the early-cleaving day 5 embryo, n = 6 (A) and late-cleaving day 5 embryo, n = 6 (B). XBP1s/XBP1 mRNA abundance in the early-cleaving blastocyst, n = 6 (C) and late-cleaving blastocyst, n = 6 (D). GRP78 mRNA abundance in the early-cleaving day 5 embryo, n = 6 (E) and late-cleaving day 5 embryo, n = 6 (F). GRP78 mRNA abundance in the early-cleaving blastocyst, n = 6 (G) and late-cleaving blastocyst, n = 6 (H). Each sample represents a pool of 15 day 5 embryos or 5 blastocysts. Day 3 embryos were not evaluated for mRNA since zygotic genome activation is not complete at this time point. Late = late-cleaving embryos cultured without TUDCA; Late + TUDCA = late-cleaving embryos cultured with TUDCA; Early = early-cleaving embryos cultured without TUDCA; Early + TUDCA = early-cleaving embryos cultured with TUDCA. Statistically significant differences among groups are indicated by * ($p < 0.05$).

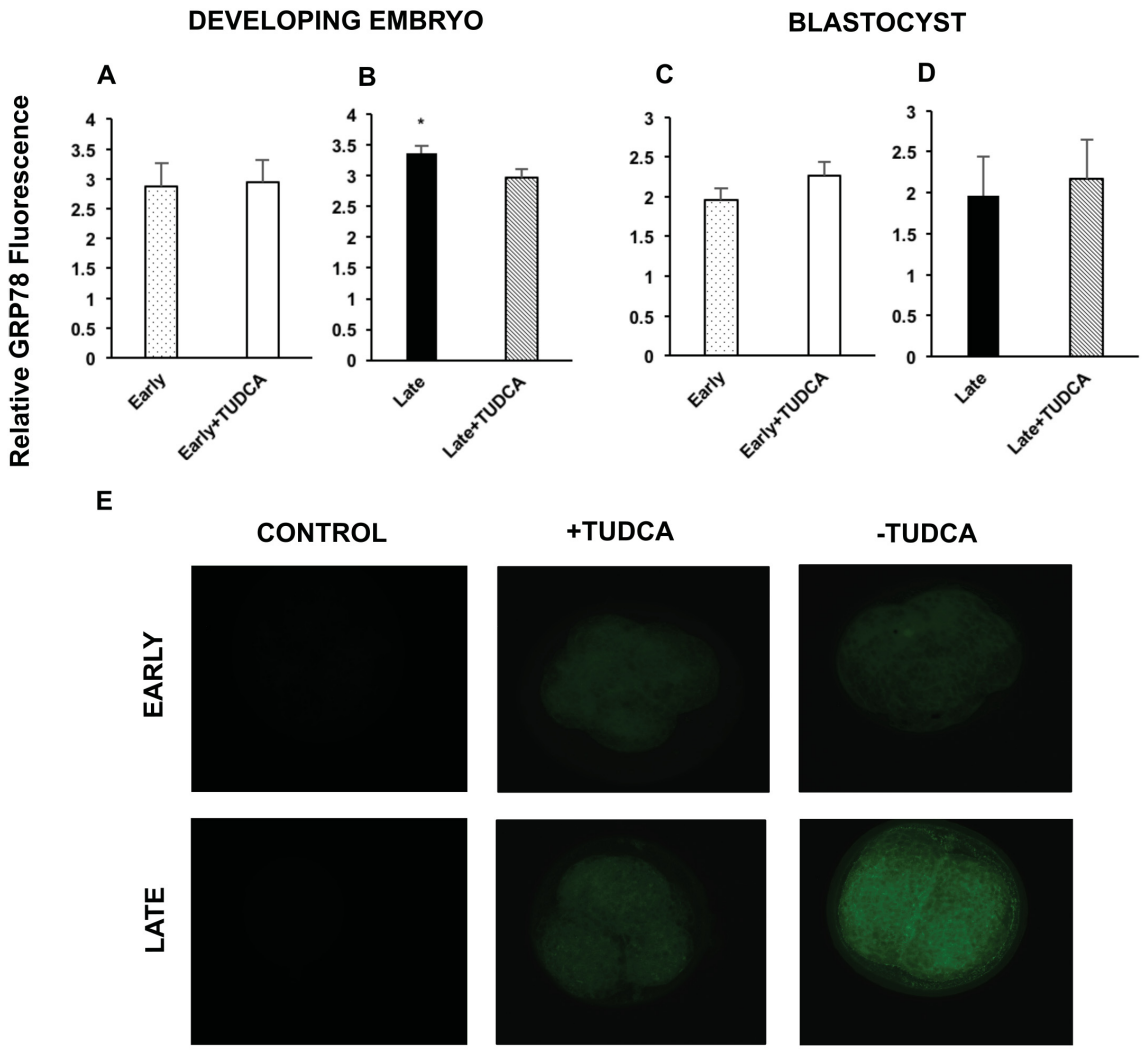

Figure 5.7.3. ER Stress in the Developing Embryo and Blastocyst: Immunofluorescence
Comparison of ER stress marker immunofluorescence in embryos to evaluate ER stress status. Mean relative GRP78 immunofluorescence in the early-cleaving developing embryo, n = 19 (A) and late-cleaving developing embryo, n = 13 (B). Mean relative GRP78 immunofluorescence in the early-cleaving blastocyst, n = 9 (C) and late-cleaving blastocyst, n = 6 (D). Representative images of GRP78 immunofluorescence for each treatment group in developing embryos (E). Images were captured at 20X magnification. Developing embryos are defined as those collected at day 3 and 5 of culture. Late = late-cleaving embryos cultured without TUDCA; Late + TUDCA = late-cleaving embryos cultured with TUDCA; Early = early-cleaving embryos cultured without TUDCA; Early + TUDCA = early-cleaving embryos cultured with TUDCA. Statistically significant differences among groups are indicated by * ($p < 0.05$).

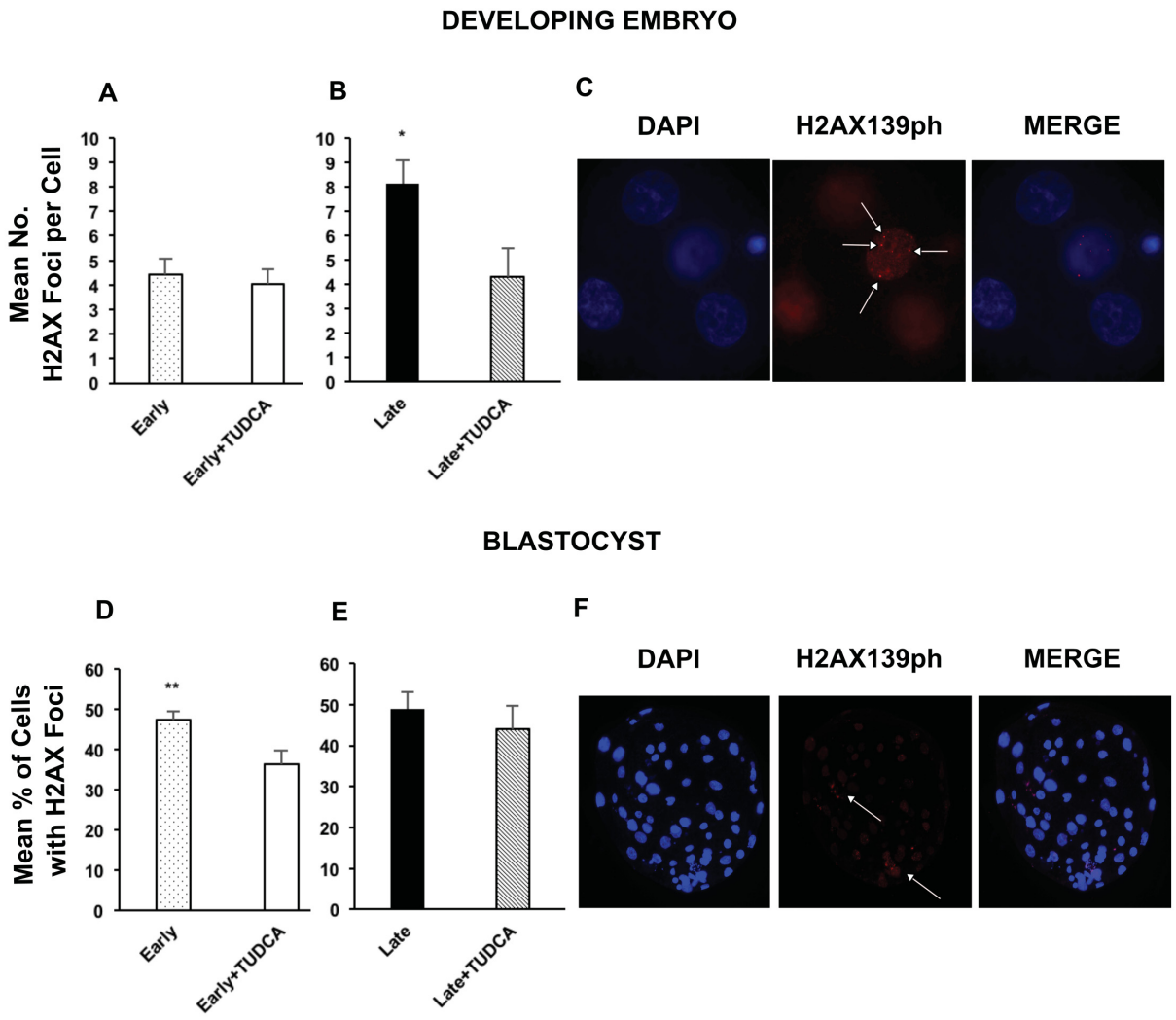

Figure 5.7.4. DNA Damage in the Developing Embryo and Blastocyst

Evaluation of H2AX139ph foci to assess DNA damage. Comparison of mean H2AX139ph foci per cell in early-cleaving developing embryos, n = 64 (A) and late-cleaving developing embryos, n = 55 (B). Comparison of proportion of cells positive for H2AX139ph foci in early-cleaving blastocysts, n = 52 (D) and late-cleaving blastocysts, n = 23 (E). Representative images of H2AX139ph immunofluorescence in developing embryos (C) and blastocysts (F), indicating nuclei (blue) and the H2AX139ph foci (red) used to assess DNA damage. Images were captured at 20X magnification. Developing embryos are defined as those collected at day 3 and 5 of culture. Late = late-cleaving embryos cultured without TUDCA; Late + TUDCA = late-cleaving embryos cultured with TUDCA; Early = early-cleaving embryos cultured without TUDCA; Early + TUDCA = early-cleaving embryos cultured with TUDCA. Statistically significant differences among groups are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

5.8 Tables

Table 5.8.1. Primers used for quantitative real-time PCR.

Gene	Forward Primer	Reverse Primer	Accession No.
18S	GACATCTAAGGGCATCACAGA	ACACGGACAGGATTGACAGA	NR_046261.1
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	XM_001927727.2
GRP78	AGGTGATCTGGTCCTGCTTG	GTCGCTCACCTTCATAGACCTT	XM_001927795.5
XBP1	GAGACCAAGGGGAATGGAGC	GCAGAGGTGCACGTAGTCTG	NM_001142836.1
XBP1s	CTGAGTCCGCAGCAGGTG	GGCTGGTAAGGAACTGGGTC	NM_001271738.1

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CONNECTING STATEMENT 2

In Chapter 5, TUDCA was shown to rescue developmentally-incompetent late-cleaving embryos by reducing ER stress and DNA damage. In fact, TUDCA was able to increase development to rates similar to that of developmentally-competent early-cleaving embryos. This remarkable improvement in developmental potential raised questions regarding the mechanism by which the bile acid was able to effect these changes. While it is known that TUDCA activates survival signaling pathways (Schoemaker et al., 2004) and reduces apoptosis (Kim et al., 2012; Lin et al., 2016; Rodrigues et al., 2003; Zhang et al., 2012), the upstream mechanism by which it exerts these effects is not clear. Evidence suggests TUDCA requires a receptor for its action, given bile acids are normally highly toxic intracellularly (Torchia et al., 2001), and its action relies on the presence of the G_{α} stimulatory protein (Vettorazzi et al., 2016). In fact, a recent study indicated TUDCA required the TGR5 receptor to reduce inflammation in rat microglial cells (Yanguas-Casas et al., 2017), however, it is not known if TGR5 is even present in early embryos. TGR5 mRNA expression has been shown in multiple tissues, including low expression in the ovary, however, its existence in oocytes or embryos has yet to be identified (Duboc et al., 2014).

In the second study, the necessity of the TGR5 receptor for the beneficial action of TUDCA on embryo development was evaluated. Firstly, to determine if a receptor was in fact needed, development of embryos was compared after intracellular injection of TUDCA or incorporation of TUDCA in the culture medium. Secondly, to evaluate the necessity of the TGR5 receptor, TGR5 protein abundance was reduced using DsiRNA under standard IVP conditions, and also in a model of glucose-induced ER stress. Embryo development, quality, as well as markers of ER stress, oxidative stress, and DNA damage repair were evaluated.

CHAPTER 6

Manuscript in preparation for submission to the "FASEB Journal".

The TGR5 receptor is needed for tauroursodeoxycholic acid-mediated reduction of ER and oxidative stress and improvement of early embryo development

Authors and Affiliations: Naomi Dicks[a], Karina Gutierrez[a], Luke Currin[a], Mariana Priotto de Macedo[a], Werner Glanzner[a], Marek Michalak[c], Luis B. Agellon[b*], Vilceu Bordignon[a*]

[a]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[b]School of Human Nutrition, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[c]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

***Corresponding Authors:**
Vilceu Bordignon, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9, vilceu.bordignon@mcgill.ca, Tel: +1(514) 398-7793, Fax: +1(514) 398-7990
Luis B. Agellon, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9, luis.agellon@mcgill.ca, Tel: +1(514) 398-7862

Short Title: TUDCA needs TGR5 in early embryo development

6.1 Abstract

Impaired glucose metabolism associated with metabolic syndrome is a known cause of infertility. In fact, hyperglycemia increases endoplasmic reticulum (ER) and oxidative stress and reduces the quality and development of embryos. The natural bile acid and proteostasis promoter tauroursodeoxycholic acid (TUDCA) reduces ER stress and rescues developmentally-incompetent late-cleaving embryos, however, the upstream mechanism by which it acts is not clear. This study aimed to investigate how TUDCA improves embryo development by: 1) determining if TUDCA acts intracellularly; and 2) determining if TUDCA requires the G-protein-coupled membrane-bound bile acid receptor, TGR5, for its mode of action. Development was compared between zygotes injected intracellularly with TUDCA or cultured in TUDCA-supplemented medium. TGR5 receptor abundance was reduced using DsiRNA under standard conditions and those of nutrient excess. Blastocyst rates and quality were assessed by total cell number and percentage of CC3-positive cells. Embryos were assessed for markers of ER stress, oxidative stress, apoptosis regulation, DNA damage repair and pluripotency using immunofluorescence and qPCR. Results indicate that TUDCA needs the TGR5 receptor to reduce ER and oxidative stress, and improve pre-implantation embryo development, but is toxic when introduced intracellularly. This data provides insight into potential therapeutic targets for the improvement of metabolic syndrome-associated infertility.

Key Words: GPBAR1, TUDCA, preimplantation development, UPR, porcine

6.2 Introduction

The use of assisted reproductive technologies (ART), including artificial insemination, *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP), has increased markedly in the past few decades due to impaired fertility (Stephen et al., 2016). Numerous factors are responsible for increasing rates of infertility, including the rising incidence of insulin resistance and metabolic syndrome (Aguilar et al., 2015; Apridonidze et al., 2005; Kasturi et al., 2008). Not surprisingly, diabetes mellitus has equally been associated with infertility issues in both men (Alves et al., 2013) and women (Livshits and Seidman, 2009). In fact, mice embryos cultured with high concentrations of glucose demonstrate impaired pre-implantation development (Fraser et al., 2007), while hyperglycemia-associated embryopathy has been well documented in humans

(Eriksson et al., 2003; Moley, 2001). Excess glucose induces both endoplasmic reticulum (ER) and oxidative stress (Ozcan et al., 2006; Poitout and Robertson, 2008; Wellen and Thompson, 2010), suggesting that increased cellular stress is a driving factor contributing to impaired embryo development and fertility. Indeed, oocytes of diabetic mice have shown increased levels of oxidative stress (Ou et al., 2012), while infants born to hyperglycemic mothers were shown to have increased oxidative and DNA damage (Durga et al., 2018). These data indicate that insulin resistance, excess glucose, and subsequent cellular stress negatively affect fertility and development.

Cellular stress may originate in various organelles, including the ER and the nucleus (Michalak and Agellon, 2018). ER stress and nuclear stress, in the form of DNA damage, have both been implicated in early embryo development (Dicks et al., 2017). ER stress is fundamentally the result of impaired energy and nutrient homeostasis (Dicks et al., 2015), and known inducers of ER stress, such as excess oxidation products (Yoon et al., 2014), heat (Sakatani et al., 2004), abnormal cell calcium level or distribution (Takahashi et al., 2009), and misfolded proteins (Zhang et al., 2012a; Zhang et al., 2012b), have been shown to reduce pre-implantation embryo development in multiple species. Evidently, ER stress plays an important role in embryo development, highlighting the need for appropriate coping mechanisms to mitigate this stress (Groenendyk et al., 2013; Michalak and Gye, 2015). The embryo cell or blastomere can respond to increased ER stress by activating the unfolded protein response (UPR) to either mitigate stress and re-establish homeostasis, or trigger apoptosis and embryo arrest if the insult cannot be resolved (Dicks et al., 2015). Although the UPR may be successful in re-establishing cellular homeostasis, this may result in either a normal adaptation of the cell or a pathological adaptation to ensure cell survival at the detriment to the organism (Dicks et al., 2015). The UPR involves three main pathways that can be activated in response to ER stress: the inositol-requiring enzyme 1-α (IRE1α) pathway, the protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathway, and the activating transcription factor 6 (ATF6) pathway (Groenendyk et al., 2013). These pathways are regulated by the glucose-regulated protein 78, or GRP78, which releases these inducers so that they may activate downstream effectors (Groenendyk et al., 2013). IRE1α dimerization and activation of its endoribonuclease activity results in cleavage of a 26-nucleotide intron from the X-box binding protein 1 (XBP1) mRNA, forming the active transcription factor,

spliced XBP1 (XBP1s). Cleaved ATF6 also acts as a transcription factor, while PERK induction promotes preferential translation of the activating transcription factor 4 (ATF4). Together, these transcription factors upregulate ER stress response genes and ER associated degradation to facilitate cell survival and a return to homeostasis.

The negative implications of increased cellular stress on embryo development have fueled considerable research into pharmacological compounds that are able to mitigate this stress. Interestingly, the natural bile acid and proteostasis promoter (Vega et al., 2016) tauroursodeoxycholic acid (TUDCA) has been shown to reduce ER stress (Vega et al., 2016) and improve early embryo development in numerous species, including the mouse, pig and cow (Kim et al., 2012; Lin et al., 2016; Song et al., 2011; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b). It has also been shown to improve bovine pre-implantation embryo development by relieving oxidative stress (Yoon et al., 2014). Recently, the developmental potential of poorly developing late-cleaving porcine embryos was rescued with TUDCA treatment, by reducing both ER stress and DNA damage (Dicks et al., 2017). Despite clear evidence TUDCA reduces cellular stress and improves embryo development, the upstream mechanism by which this occurs is not clear. While TUDCA is often referred to as a "chemical chaperone", it unlikely functions in this way given bile acids are highly toxic to cells when present intracellularly (Torchia et al., 2001). Given the toxic nature of bile acids, it is more likely TUDCA exerts its beneficial effects indirectly by interacting with a cell surface receptor, such as the strictly membrane-bound G-protein coupled bile acid receptor, Takeda-G-protein-receptor-5 (TGR5) (Schaap et al., 2014). .

The purpose of this study was to determine how TUDCA reduces cellular stress and improves early embryo development. Firstly, comparison of development between porcine embryos injected intracellularly with TUDCA with those cultured in TUDCA-supplemented medium was performed. Data indicated TUDCA was toxic intracellularly, suggesting an indirect mechanism of action. To determine if TUDCA mediated its beneficial effect on development through activation of a signaling pathway, the necessity of the TGR5 receptor was assessed using DsiRNA in standard *in vitro* culture and under conditions of glucose-induced ER stress. Results demonstrated that the TGR5 receptor is needed for TUDCA to reduce ER and oxidative stress

and improve pre-implantation embryo development. These findings elucidate for the first time the upstream mechanism of action of TUDCA in early embryo development and provide insight into potential therapeutic targets to mitigate metabolic-syndrome associated infertility.

6.3 Materials and Methods

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from MilliporeSigma (Burlington, MA, USA).

Oocyte collection and maturation

Ovaries of pre-pubertal gilts were collected from a local abattoir (Olymel, S.E.C./L.P, Saint Esprit, QC, Canada) and 3-6 mm follicles were aspirated to retrieve oocytes. Cumulus oocyte complexes (COC) with a minimum of three layers of cumulus cells and homogeneous cytoplasm were collected for maturation. Groups of 30-35 COCs were matured for 22 h in 100 µl of *in vitro* maturation medium 1 (IVM1) consisting of TCM 199 (ThermoFisher Scientific, Waltham, MA, USA), supplemented with 20% porcine follicular fluid, 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP D-0260), 0.1 mg/mL cysteine (6852), 10 ng/mL epidermal growth factor (EGF; ThermoFisher Scientific, PHG0311), 0.91 mM sodium pyruvate (P-4562), 3.05 mM D-glucose (G-6152), 0.5 µg/mL LH (SIOUX Biochemical Inc., Sioux Center, IA, USA, 725), 0.5 µg/mL FSH (SIOUX Biochemical Inc., 715), and 20 µg/mL gentamicin (G-1272). Subsequently, COCs were transferred to IVM2 medium, consisting of IVM1 without LH, FSH and dbcAMP, for an additional 20-22 h, as previously described (Bohrer et al., 2015).

Parthenogenetic activation

After 42-44 h of maturation, COCs were denuded with 0. 1 % hyaluronidase (H3506) and mature MII oocytes were selected. Mature oocytes were activated for 5 min in 15 µM ionomycin (I0634) followed by 4 h in calcium-free porcine zygote medium (PZM-3) supplemented with 10 mM strontium chloride (255521), 7.5 µg/ml cytochalasin B (C6762) and 10 µg/ml cyclohexamide (C1988), as previously reported (Che et al., 2007).

In vitro fertilization

Mature oocytes were selected and *in vitro* fertilized as previously described (Bohrer et al., 2013), with minor modifications. Prior to *in vitro* fertilization (IVF), oocytes were rinsed in porcine Tris-Buffered media (TBM) supplemented with 30 mg/ml bovine serum albumin (BSA) (A6003), 2 mM caffeine (C-07500) and 20 µg/mL gentamicin (G-1272), herein referred to as TBM-Fert. After rinsing, oocytes were placed in groups of 80-120 in 500 µl wells of TBM-Fert. Boar semen was assessed for sperm progressive motility and then washed in the TBM-Fert devoid of caffeine, to prevent capacitation. Washed semen was re-suspended in TBM-Fert (with caffeine), and an appropriate volume was added to the wells of mature oocytes to obtain approximately 200,000 motile sperm/ml. After 5 h of co-incubation, oocytes were removed from the fertilization media and rinsed in TBM-Fert to remove any sperm from the zona pellucida prior to embryo culture.

Embryo injection

After activation or IVF, embryos were injected or not, depending on the experimental group. Embryos were manipulated in HEPES-buffered TCM-199 supplemented with 2mg/ml BSA (A6003) and 20 µg/mL gentamicin (G-1272) using an inverted microscope (Nikon, Tokyo, Japan) and a micromanipulator system (Narishige International, Long Island, NY, USA). The volume injected into individual embryos was regulated using a FemtoJet 4i programmable microinjector (Eppendorff, Hamburg, Germany). If any further manipulation was required after injection, embryos were recovered in *in vitro* culture medium (IVC) for a minimum of 30 min.

Embryo culture

After activation or IVF, as well as embryo injection, embryos were rinsed and then cultured in IVC medium, consisting of PZM-3 supplemented with 3 mg/ml BSA (A6003) and 5mM hypotaurine (H1384). Depending on the experiment, this culture medium was supplemented with 50 µM TUDCA (580549) and/or 7 mM glucose (G-6152). The concentration of TUDCA was selected based on previous optimization studies performed by our laboratory (Appendix 9.1) and other groups (Zhang et al., 2012b). The concentration of glucose was selected based on a previously published early embryo development diabetic model (Fraser et al., 2007) as well as dose experiments performed by our laboratory (Appendix 9.1). Groups of 20-30 embryos were

cultured in 60 µl droplets under mineral oil at 5 % CO_2, 95 % air and 38.5 °C. After 48 h, cleavage was assessed and any uncleaved embryos were discarded. Embryos cultured beyond 5 days were supplemented with 10 % FBS (ThermoFisher Scientific, 16170-078).

Validation of TGR5 knockdown

Dicer substrate siRNA (DsiRNA) was designed to target the TGR5 mRNA sequence using Integrated DNA Technologies' (IDT) Custom Dicer-Substrate siRNA Design Tool (Coralville, IA, USA) (https://www.idtdna.com/site/order/designtool/index/DSIRNA_CUSTOM). Specificity of the TGR5 DsiRNA was confirmed by using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA) prior to being synthesized by IDT (Coralville, IA, USA). A scrambled DsiRNA designed by IDT was used as a negative control. Sense and antisense sequences of the DsiRNA are indicated: si-TGR5 (sense – CCAAAGCAGCGUGGACCUUGACUTG, antisense – ACGGUUUCGUCGCACCUGGAACUGAAC) and scrambled, si-CTRL (sense – CGUUAAUCGCGUAUAAUACGCGUAT, antisense – AUACGCGUAUUAUACGCGAUUAACGAC). Porcine neonatal fibroblasts and porcine embryos were used to validate the knockdown of TGR5 expression using si-TGR5. Porcine fibroblasts were transfected with 1nM and 10nM of si-TGR5 or si-CTRL using the Neon® Transfection System (ThermoFisher Scientific). Transfected fibroblasts were cultured in standard Dulbecco Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (D8900) supplemented with 10 % FBS (ThermoFisher Scientific, 16170-078) and 1 % penicillin-streptomycin (P-4333) for 48 h and then collected for mRNA and protein extraction. Abundance of TGR5 mRNA and protein were assessed using qPCR and immunoblotting, respectively. Porcine parthenogenetically-activated porcine embryos were injected with ~10 pl of 2 0µM si-TGR5 or si-CTRL and cultured in IVC for 60 h prior to collection of cleaved embryos. Abundance of TGR5 mRNA was assessed by qPCR and TGR5 protein was assessed by immunofluorescence microscopy. Data for these experiments were collected from a minimum of 3 replicates.

Validation of glucose -induced ER stress model

Excess glucose was used to perturb nutrient and energy homeostasis and thereby induce ER stress (Chen et al., 2018). Glucose was also selected since it is well known to impede fertility (Apridonidze et al., 2005; Kasturi et al., 2008) and has been shown to reduce early embryo development and quality (Fraser et al., 2007). Parthenogenetically-activated embryos were cultured in either standard IVC (Control), IVC + 7 mM glucose to cause ER stress (Glucose), and IVC + 7 mM glucose and 50 μM TUDCA (Glucose + TUDCA). Embryos were assessed at Day 7 for blastocyst development.

Evaluation of cleavage, blastocyst rate and cell number

Cleavage rates were calculated after 48 h of culture using the number of embryos having completed their first cell division over the total number of embryos placed in culture on Day 1. Blastocyst rates were calculated on Day 7 based on the number of cleaved embryos maintained in culture after 48 h. Since embryo cleavage was greatly reduced by direct injection of TUDCA, blastocyst rates were calculated at Day 7 based on the total number of embryos placed in culture on Day 1. No embryos were collected prior to Day 7 in these experiments evaluating blastocyst development. Cell number was evaluated in Day 5 and Day 7 embryos using DAPI (4,6-Diamidino-2-Phenylindole, Dilactate) (ThermoFisher Scientific, D3571) staining at the same time as processing for immunofluorescence, as described below.

Quantitative real-time PCR

RNA was extracted from embryos using the PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, KIT0202) and cDNA synthesized using Superscript® VILO™ cDNA Synthesis Kit (ThermoFisher Scientific, 11754050), according to manufacturer recommendations for both procedures. A minimum of 15 embryos per replicate were collected for Day 5 analysis, while a minimum of 5 blastocysts per replicate were collected for Day 7 analysis. RNA from porcine neonatal fibroblasts was extracted using TRIzol™ Reagent (ThermoFisher Scientific, 15596026) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA 170-8890), both according to manufacturer recommendations. Quantitative real-time PCR was carried out using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, 185-5200) and the Advanced qPCR Mastermix (Wisent Bio Products, Montreal, QC, Canada 800-435-UL). Primers used are listed in Table 6.9.1. Thermocycler parameters were 5 min at 95 °C, 40 cycles

of 15 s at 95 °C followed by 30 s at the optimal annealing temperature (Table 6.9.1), and finally 10s at 95 °C and 5 s at 60 °C. Samples were run in duplicate and melt-curve analyses were used to verify the specificity of reaction products. Relative quantities of mRNA were calculated using the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008) or the Pfaffl method, if efficiencies of reactions differed by more than 10% (Pfaffl, 2001). Multiple reference genes were used for normalization including the 18S ribosomal RNA gene, H2A gene and ß-actin gene. Spliced XBP1 (XBP1s) mRNA abundance was expressed as a ratio to unspliced XBP1 mRNA abundance (XBP1s/u). All reactions used for quantification had efficiency between 90 -110 %, $R^2 \geq 0.98$ and slope values from -3.6 to -3.1.

Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described (Bohrer et al., 2013) with minor modifications. At collection, embryos were fixed in 10 % formalin (HT501128) for 15 min and then permeabilized with 1 % Triton X-100 (T8787) in PBS at for 30min at 37 °C. Embryos were stored at 4 °C until immunofluorescence analyses were performed. To reduce experimental variability in fluorescence staining, embryos from all replicates in a group were assessed simultaneously. Fixed and permeabilized embryos were incubated three times for 30 min at room temperature in blocking solution, consisting of 3 % BSA (Roche, Basel, Switzerland, 10775835001) and 0.2 % Tween-20 (P1379) in PBS. Embryos were then incubated overnight at 4 °C in the presence or absence (control) of the primary antibody. Primary antibodies used were rabbit polyclonal anti-cleaved caspase 3 (CC3) (Cell Signaling, 9661S), rabbit polyclonal anti-TGR5 (Abcam, Cambridge, UK, 72531), rabbit polyclonal anti-GRP78 (Abcam, Ab191023), and mouse monoclonal anti-phospho-histone H2A.X (Ser139) (05-636), diluted at 1:400, 1:500, 1:1000 and 1:400 in blocking solution, respectively. The following morning, samples were rinsed three times for 30 min in blocking solution and then incubated in the dark for 50 min in the presence of secondary antibodies, diluted 1:1000. Secondary antibodies used were goat polyclonal anti-rabbit Alexa Fluor 488-conjugated IgG antibody (ThermoFisher Scientific, A-11008) or goat polyclonal anti-mouse Cy3-conjugated IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, 115-165-146). Embryos were rinsed once again: once in blocking solution for 30 min, then 20 min in 10 μg/ml DAPI in blocking solution, and finally once more in blocking solution for 30 min, before mounting on

slides in Mowiol (Polyvinylalcohol, 10852). A Nikon Eclipse 80i microscope (Nikon Instruments Inc.) equipped with a Retiga 2000R monochrome digital camera (Qimaging, Surrey, BC, Canada) as well as the Simple PCI Imaging Software (Compix, Inc., Sewickly, PA, USA) were used to capture and analyze embryo images. Cells were considered apoptotic if they were CC3 immunofluorescence positive. Total TGR5 and GRP78 fluorescence was recorded, adjusted based on embryo cell number, and normalized against negative controls. Foci of phosphorylated H2AX at serine 139 (γH2AX) greater than $0.3\mu m^3$, indicating sites of DNA damage repair (McManus et al., 2005; Grenier et al., 2012), were counted in individual nuclei and expressed as the average number of positive foci per cell for developing embryos (collected at Day 5), or as the average number of positive cells for blastocysts (collected at Day 7). All embryos analyzed were evaluated for total cell number based on DAPI staining.

Immunoblot analysis

Transfected porcine neonatal fibroblasts were collected after 48 h of culture, rinsed twice in cold PBS and re-suspended in Laemmli buffer (Bio-Rad, 1610737) containing 5 % ß-mercaptoethanol (Bio-Rad, 1610710), and protease and phosphatase inhibitors (G Biosciences, St. Louis, MO, USA, 786-331, 786-450). Protein samples were vortexed, pipetted, sonicated and boiled at 95 ºC for 5 min before being resolved on a polyacrylamide gel (7.5 %) and then transferred to a nitrocellulose membrane. Membranes were blocked in 5 % BSA (Roche, Basel, Switzerland, 10775835001) in Tris-buffered saline with 0.1 % Tween (MP Biomedicals, Santa Ana, CA, USA, 0210317091) (TBS-T) for 2 h before incubation overnight at 4 °C in their appropriate primary antibodies. Primary antibodies used were rabbit polyclonal anti-TGR5 (Abcam, 72531) and rabbit polyclonal anti-ß-actin (Abcam, ab8227) at dilutions of 1:5000 and 1:10,000 in TBS-T, respectively. The following morning, membranes were rinsed 5 times in TBS-T for 7 min prior to incubation in secondary antibody, goat anti-rabbit IgG (Abcam, ab6721), at a 1:10,000 dilution for 1 h. Membranes were rinsed again 5 times in TBS-T for 7 min. Protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad, 710-5060) and a Chemidoc Analyzer (Bio-Rad 170-8265).

Statistical analyses

All statistical analyses were performed using the JMP 13 program (SAS, Cary, NC, USA). Continuous values satisfying a normal distribution were tested using the one-way ANOVA method and means compared using the Tukey test for multiple comparisons, or the Student's t-test for pair-wise comparisons. For data sets not normally distributed, continuous values were tested using the Kruskal-Wallis method and means compared using the Dunn's test for multiple comparisons, or Wilcoxon's test for pair-wise comparisons. Significant differences were attributed for P-values < 0.05.

6.4 Results

Intracellular injection of TUDCA impairs embryo development

To determine if the action of TUDCA was mediated intracellularly or via an extracellular membrane receptor, activated embryos were separated into 4 groups: cultured in standard IVC (Control), cultured in IVC supplemented with 50 μM TUDCA (Cultured with TUDCA), injected with ~10 pl of sterile water (Injected with Water) and cultured in IVC, or injected with ~10 pl of 2.5 mM TUDCA (Injected with TUDCA) and cultured in IVC. The volume of TUDCA injected was calculated to obtain a final concentration of 50 μM based on the approximate volume of an early zygote ($\approx 4/3\pi r^3$, or 5.24×10^{-7} cm^3, where r = 50 μm). Embryos injected with either water or TUDCA had significantly decreased cleavage rates at 43.2 % and 27.1 %, respectively, compared to 64.2 % and 68.6 % for the Control and TUDCA-treated groups ($p < 0.001$) (Fig.6.8.1A). Given the significant effect on cleavage, blastocysts rates were calculated based on the total number of embryos placed in culture to ensure inclusion of any embryos not making it to the cleavage stage. As expected, the TUDCA-treated group had a significantly increased rate of blastocyst development compared to the Control group (33.8 % vs. 25.3 %, $p < 0.05$) (Fig.6.8.1B). Embryos injected with water had a similar rate of development compared to Control (21.0 % vs 25.3 %, $p > 0.05$), whereas those injected with TUDCA had a significantly reduced number of blastocysts, at only 11.7% ($p < 0.01$) (Fig.6.8.1B). The quality of blastocysts was assessed by evaluating total cell number (Fig.1C) and mean percentage of apoptotic cells (Fig.6.8.1D) via cleaved caspase-3 (CC3) immunofluorescence. No significant differences were seen among any of the groups (Fig.6.8.1 C,D). These results demonstrate that the beneficial effect of TUDCA on embryo development was achieved indirectly via modulation of a cellular signaling pathway rather than by direct interaction with intracellular components.

TUDCA needs TGR5 to improve early embryo development

To evaluate if the TGR5 receptor is involved in mediating the action of TUDCA, DsiRNA targeting the TGR5 mRNA sequence was used to inhibit TGR5 gene expression. Firstly, efficacy of the designed TGR5 DsiRNA was validated by transfecting porcine neonatal fibroblast cells with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) at 1.0 nM and 10 nM concentrations (Fig.6.8.2 A,B,C). At the 1.0 nM concentration, the mean relative mRNA abundance of TGR5 was significantly reduced by 49.3 % ($p < 0.05$) (Fig.2 A), however no difference in protein abundance was seen (Fig.6.8.2 B). At the 10 nM concentration, however, si-TGR5 significantly decreased TGR5 protein abundance by 38.1 % compared to si-CTRL ($p < 0.01$) (Fig.6.8.2 C,D). Testing of TGR5 DsiRNA was also performed in parthenogenetically-activated porcine embryos by injecting either si-CTRL or si-TGR5 into zygotes after activation. Both TGR5 mRNA and protein abundance were significantly reduced in the si-TGR5 porcine embryos, by 50 % ($p < 0.01$) (Fig.6.8.2 E) and 29.5 % ($p < 0.01$) (Fig.6.8.2 F,G), respectively.

Secondly, to determine if the TGR5 receptor was involved in mediating the beneficial effect of TUDCA on development, activated or IVF embryos were injected with ~10 pl of 20 μM si-TGR5 or si-CTRL, and then divided into 4 different culture groups: si-TGR5 in standard IVC (si-TGR5), si-CTRL in standard IVC (si-CTRL), si-TGR5 in IVC supplemented with 50 μM TUDCA (si-TGR5 + TUDCA) and si-CTRL in IVC supplemented with 50 μM TUDCA (si-CTRL + TUDCA). Embryos injected with either si-CTRL or si-TGR5 and cultured in the absence or presence of TUDCA showed no effect on cleavage, whether they were parthenogenetically activated or *in vitro* fertilized (Fig.6.8.3 A,C). In addition, TGR5 was not essential for embryo development given there was no difference in blastocyst development between si-CTRL and si-TGR5, when cultured in standard IVC alone (Fig.6.8.3 B,D). When evaluating si-CTRL and si-TGR5 in the presence of TUDCA, however, a significant reduction in blastocyst rate based on cleavage was seen in both parthenotes and IVF embryos (Fig.6.8.3 B,D). In parthenotes, the si-CTRL + TUDCA group had the highest blastocyst rate at 42.7 %, whereas the si-TGR5 + TUDCA group was significantly lower than this at 26.1 % ($p < 0.05$) (Fig.6.8.3 B). Similarly, in IVF embryos, the blastocyst rate in the si-CTRL + TUDCA group was 20.3%, whereas it was significantly reduced in the si-TGR5 + TUDCA group to 10.1 % ($p < 0.05$)

(Fig.6.8.3 D). With TGR5 attenuation, the rate of embryo development plummets, despite the presence of TUDCA. These results indicated that TUDCA needs the TGR5 receptor to facilitate embryo development to the blastocyst stage.

As indicators of embryo quality, mean total cell number and mean percentage of CC3-positive, or apoptotic, cells were evaluated, however, no significant differences were detected between the groups (Appendix 9.2). To further investigate embryo quality between the si-CTRL + TUDCA and si-TGR5 + TUDCA groups, embryos were assessed for mean total cell number (Supplemental Fig.6.10.1 A,D), ER stress via mean GRP78 fluorescence (Supplemental Fig.6.10.1 B,E) and DNA damage via γH2AX immunofluorescence (Supplemental Fig.6.10.1 C,F), in both developing Day 5 embryos and Day 7 blastocysts, respectively. No significant differences were seen at any of the time points. These results were surprising, given the significant effect seen on development, and suggested a possible dilution of the TUDCA effect in this unstressed model.

TUDCA needs TGR5 to rescue development in the face of glucose-induced ER stress

In the model of glucose-induced ER stress, rate of blastocyst development dramatically decreased when embryos were cultured with glucose, falling from 64.6 % in the Control group to 6.1 % ($p < 0.001$) (Fig.6.8.4 A). TUDCA was able to rescue development of these embryos by increasing the blastocyst rate back up to 28.1 % ($p < 0.05$) (Fig.6.8.4 A), validating its beneficial effect on development in this model of glucose-induced ER stress.

To determine if TUDCA needs the TGR5 receptor to improve development in the face of glucose-induced ER stress, TGR5 gene expression was knocked down using si-TGR5 as previously described. Activated embryos were cultured in IVC supplemented with 7 mM glucose and 50 μM TUDCA after being injected with either ~10 pl of 20 μM si-TGR5 (si-TGR5 + Glucose + TUDCA) or si-CTRL (si-CTRL + Glucose + TUDCA). Embryos that had the action of TUDCA attenuated had a 2.7-fold decrease in the rate of blastocyst development in the face of glucose-induced ER stress (4.3 % vs. 11.25 %, $p < 0.05$) (Fig.6.8.4 B,C). Similar to the unstressed system, no difference in mean blastocyst total cell number or percentage of CC3-positive cells was identified (Fig.6.8.4 D,E, respectively). These results demonstrated that

TUDCA needs TGR5 to rescue development to the blastocyst stage when exposed to conditions of glucose-induced ER stress.

TUDCA needs TGR5 to relieve cellular stress and regulate cell survival

To further evaluate the effect of TUDCA under conditions of glucose-induced ER stress, embryo quality was assessed in the developing stages. Attenuating the action of TUDCA in the Day 5 developing embryo did not alter mean total cell number (Fig.6.8.5 A), but significantly increased X-linked inhibitor of apoptosis (XIAP) mRNA abundance (2.18 vs. 1.16 fold increase, $p < 0.05$) (Fig.6.8.5 C). The increased abundance of XIAP mRNA, which is consistent with upregulated survival signaling during UPR activity (Brown et al., 2016; Hu et al., 2004), coincided with a significant increase in ER stress based on a 3-fold increase in mean GRP78 fluorescence (6.53 vs. 2.03 fold increase, $p < 0.05$) (Fig.6.8.5 B) and a 2-fold increase in mean relative XBP1s/u mRNA abundance (1.16 vs. 0.50 fold increase, $p < 0.01$) (Fig.6.8.5 C). In addition, TUDCA failed to reduce oxidative stress when TGR5 was knocked down, as shown by a 2-fold increase in mean relative mRNA abundance of SOD1 (2.12 vs. 1.00 fold increase, $p < 0.05$) (Fig.6.8.5 C). DNA damage repair was also evaluated by mean relative mRNA abundance of genes involved with the homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways (Fig.6.8.5 D), however, no significant differences were noted. These results demonstrated that TUDCA needs the TGR5 receptor to reduce ER and oxidative stress in the developing embryo, explaining its ability to facilitate development to the blastocyst stage. Furthermore, attenuation of TGR5 gene expression to block the action of TUDCA increased the mRNA abundance of the cell survival gene, XIAP. This finding suggested sustained activation of the UPR, which indicates an unsuccessful attempt to return to homeostasis (Groenendyk et al., 2013) and a greater risk of apoptosis and embryo arrest (Dicks et al., 2015).

Attenuating TUDCA action in the face of glucose-induced ER stress alters mRNA abundance of genes controlling pluripotency

The increase in XIAP mRNA abundance in si-TGR5 embryos suggested impaired developmental potential despite survival of embryos to the blastocyst stage. Therefore, indicators of normal blastocyst development, including the trophectoderm marker, CDX2, and pluripotency factors, NANOG, OCT4 and SOX2, were evaluated in both developing embryos (Fig.6.8.6 A-D) and

blastocysts (Fig.6.8.6 E-H). Remarkably, mRNA abundance of pluripotency factors were significantly reduced in embryos with attenuation of TUDCA action (Fig.6.8.6 C,F,G). In developing Day 5 embryos, the mean relative mRNA abundance of the OCT4 gene was reduced by 30.8 % (1.46 vs. 2.11 fold increase, $p < 0.05$) (Fig.6.8.6 C), whereas in the blastocyst, it was reduced by 37.3 % (0.74 vs. 1.18 fold increase, $p < 0.05$) (Fig.6.8.6 G). In addition, mean relative mRNA abundance of the NANOG gene was also significantly decreased in the blastocyst (0.68 vs. 2.81 fold increase, $p < 0.05$) (Fig.6.8.6 F). These changes in pluripotency gene mRNA abundance could have important implications for embryo viability.

6.5 Discussion

Results presented in Chapter 5 showed TUDCA can rescue developmentally-incompetent late-cleaving embryos by reducing ER stress and DNA damage, however, the mechanism and mode of action by which TUDCA was acting was not clear. While it has been suggested that TUDCA acts as a "chemical chaperone", this study demonstrated that it is in fact toxic when injected intracellularly. This was not surprising, given bile acids are normally transported intracellularly only in specialized cells, such as hepatocytes and enterocytes, which possess appropriate bile acid transporters and bile acid binding proteins (Halilbasic et al., 2013; Torchia et al., 2001; Zwicker and Agellon, 2013). These results provided evidence that the mechanism of action by which TUDCA exerts its effects is indirect, through a receptor-mediated signaling pathway. Naturally, the only exclusively membrane-bound bile acid receptor (Duboc et al., 2014), TGR5, represented a prime candidate. In fact, TGR5 is a G-protein-coupled receptor and data suggests that TUDCA may act through TGR5 given inhibition of the G-stimulatory protein abrogates the activation of cell survival signaling pathways (Vettorazzi et al., 2016). Indeed, TUDCA was recently shown to act through TGR5 to reduce inflammation in microglial cells (Yanguas-Casas et al., 2017), however, expression of the TGR5 gene within embryos and its ability to be activated by TUDCA has never been proven. To investigate the role of TGR5 in the mechanism of action by which TUDCA improves development, it was first identified in the early stage embryo using qPCR and immunofluorescence, and then targeted for manipulation using RNA interference to ensure specificity of inhibition. Results demonstrated for the first time that TGR5 is needed for TUDCA to improve early embryo development. With TGR5 attenuation, the efficacy of TUDCA was lost as it could no longer activate its mode of action. Rates of blastocyst

development were no longer rescued, while ER and oxidative stress were no longer relieved in a model of nutrient excess. Furthermore, attenuating the action of TUDCA caused an increase in XIAP mRNA abundance, an expected response with UPR activation (Brown et al., 2016; Hu et al., 2004), but with potential deleterious effects as embryo development progresses if homeostasis is never attained, or if a pathological adaptation occurs (Dicks et al., 2015). In fact, si-TGR5 embryos showed deficient mRNA abundance of genes that control pluripotency, with a reduction occurring in both OCT4 and NANOG. While these results could indicate increased differentiation of cells to the trophectoderm lineage (Liu et al., 2015), this was not corroborated by increased CDX2 mRNA abundance. Alternatively, the heightened cellular stress in these embryos could be delaying differentiation of embryonic cells, or more seriously, a defect in pluripotency could be at play. Regardless, either cause for the altered mRNA abundance of genes controlling pluripotency can negatively impact the viability of the developing embryo and fetus.

The elucidation of the upstream mechanism of action of TUDCA is significant, as it opens the door to further investigation into its mode of action and potential therapeutic options that exploit the signaling pathways activated by TGR5. While TUDCA treatment improved embryo production *in vitro*, it is unlikely to be acting on the developing embryo in a physiological setting. Most conjugated bile acids such as TUDCA are de-conjugated during intestinal transit prior to reabsorption by the portal system and delivery to the liver (Zwicker and Agellon, 2013). Furthermore, hepatocytes efficiently absorb bile acids from portal blood, such that only very low concentrations reach the systemic circulation (Zwicker and Agellon, 2013). Moreover, TUDCA is a minor component of the total bile acid species found in enterohepatic circulation (Lepercq et al., 2007; Zwicker and Agellon, 2013). At best, unconjugated bile acids may be physiologic ligands for the TGR5 receptor, albeit in minute amounts. One report has confirmed the unconjugated form of TUDCA, or UDCA, in human follicular fluid at double the concentration of serum, however, the authors could not rule out contamination of samples with peripheral blood (Nagy et al., 2015). Nevertheless, as the developing embryo is not normally exposed to bile acids, it is possible other unknown ligands for TGR5 are involved, such as the locally more abundant and structurally-related steroid hormones.

An additional finding in this study was that the TGR5 receptor is not essential for early embryonic development. Si-TGR5 embryos developed similarly to si-CTRL embryos when no other treatment was applied. Despite the incomplete inhibition of TGR5, these data are supported by studies showing mice with global TGR5 deficiency are born live without apparent developmental or morphological defects and adults have normal fertility (Maruyama et al., 2006; Vassileva et al., 2006). Nonetheless, the attenuation of TGR5 gene expression in this study was sufficient to suppress the efficacy of TUDCA treatment in alleviating ER stress, as demonstrated by an increase in UPR status.

Taken together, these results demonstrate that the TGR5 receptor is needed for TUDCA to reduce ER and oxidative stress and improve pre-implantation embryo development. Furthermore, this study highlights the positive effects of TGR5 activation on embryo survival under conditions of glucose-induced ER stress. These findings identify an upstream therapeutic target which can potentially be exploited to mitigate infertility associated with metabolic syndrome.

6.6 Acknowledgements

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6.7 Author Contributions

V.B. and L.B.A. designed the experiment. M.M. provided expertise to support the project. N.D., K.G., L.C., M.P.M., and W.G. carried out the experiments. N.D. performed the statistical analyses. N.D., V.B. and L.B.A. wrote and edited the manuscript.

6.8 Figures

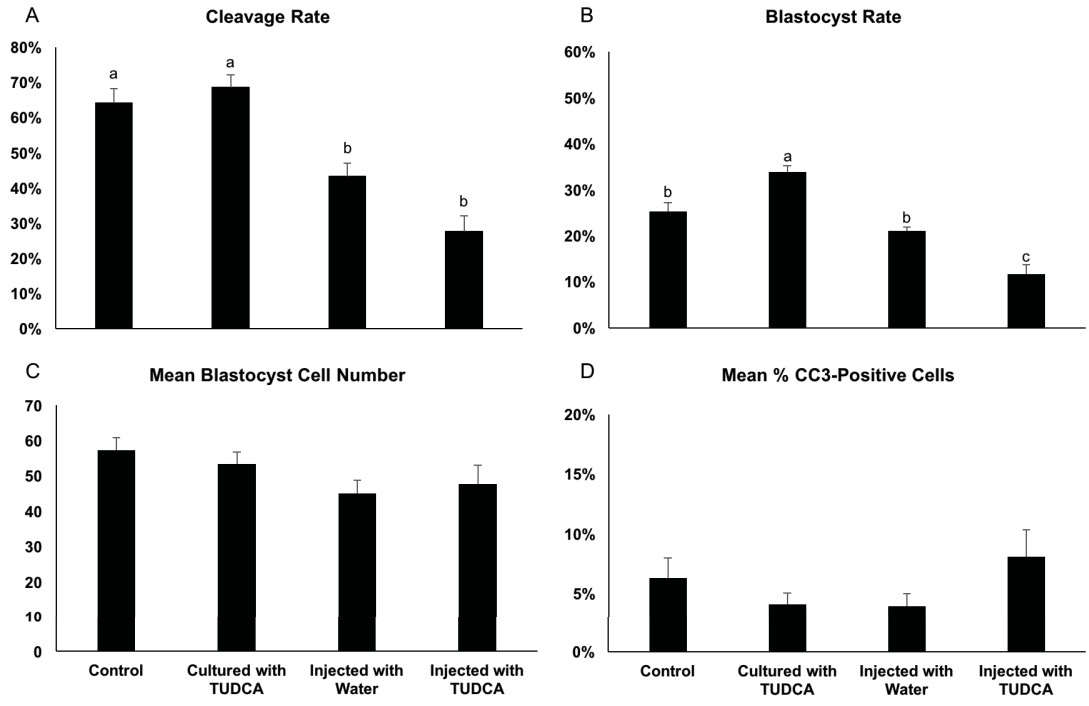


Figure. 6.8.1. Intracellular injection of TUDCA impairs embryo development

Parthenogenetically-activated (PA) porcine embryos were cultured in standard IVC media alone (Control), media supplemented with 50µM TUDCA (Cultured with TUDCA), injected with water, or injected with TUDCA to a final concentration of 50µM. Cleavage rate was assessed after 48h (A). At Day 7 of culture, blastocyst rate based on the total number of embryos (B) was recorded and embryos were collected for analysis. Mean blastocyst total cell number (C) and mean percentage of apoptotic cells in blastocysts (D), assessed by CC3 immunofluorescence, are shown. Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

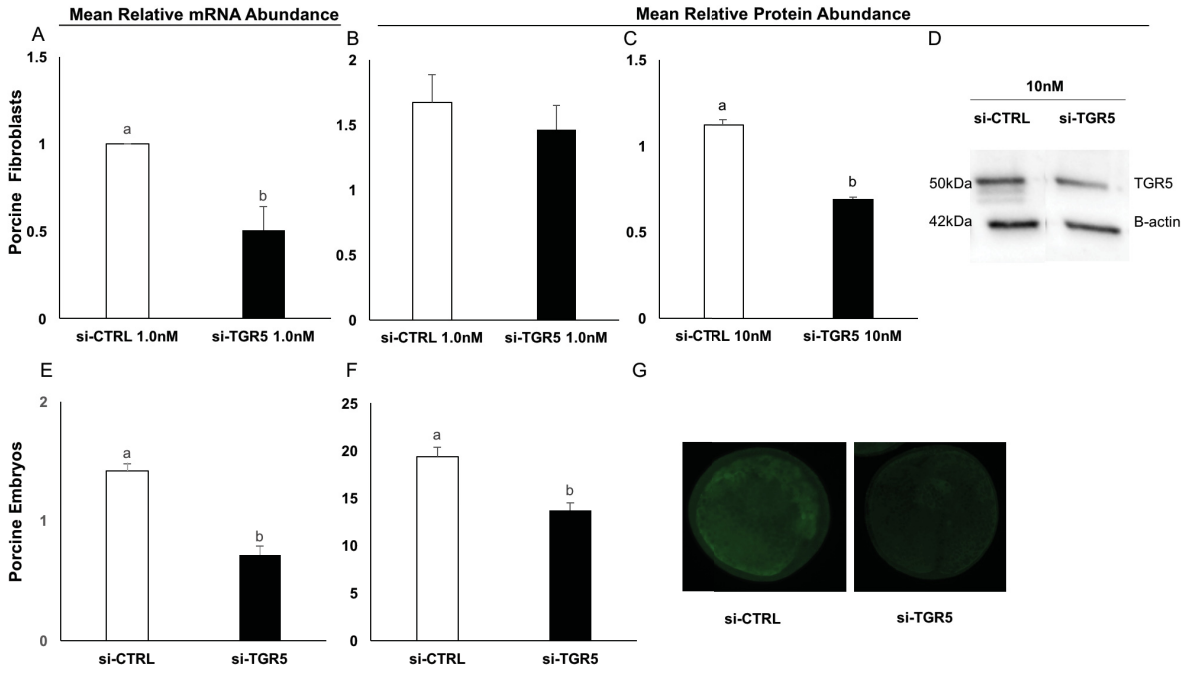


Figure 6.8.2. TGR5 DsiRNA reduces TGR5 receptor mRNA and protein abundance

DsiRNA was used to reduce expression of the TGR5 receptor gene. Mean relative TGR5 mRNA abundance (A) and mean relative TGR5 protein abundance (B,C) in porcine neonatal fibroblasts 48h after transfection with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) at 1nM (A,B) and 10nM (C). Representative TGR5 immunoblot of porcine neonatal fibroblasts transfected with si-CTRL or si-TGR5 at 10nM (D). Mean relative TGR5 mRNA abundance (E) and mean relative TGR5 protein abundance based on immunofluorescence (F) of porcine PA embryos 60h after injection with either si-CTRL or si-TGR5. Representative image of TGR5 immunofluorescence in embryos injected with si-CTRL or si-TGR5 (G). Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

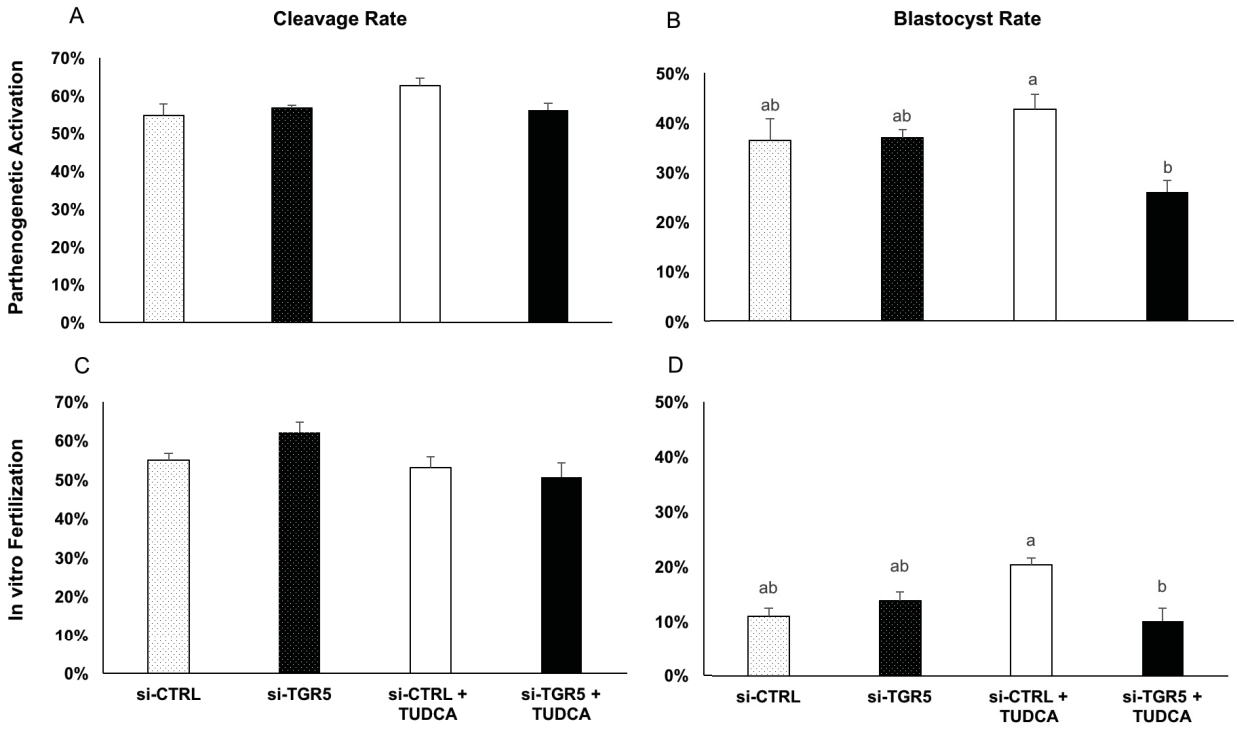


Figure. 6.8.3. TUDCA needs TGR5 to improve early embryo development

Parthenogenetically activated (PA) and in vitro fertilized (IVF) porcine embryos were injected with si-CTRL or si-TGR5 and cultured with or without 50μM TUDCA. Cleavage rate (A, C) was recorded after 48h in PA and IVF embryos, respectively. After 7 days of culture, blastocyst rate based on cleavage was assessed in PA (B) and IVF (D) embryos. Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

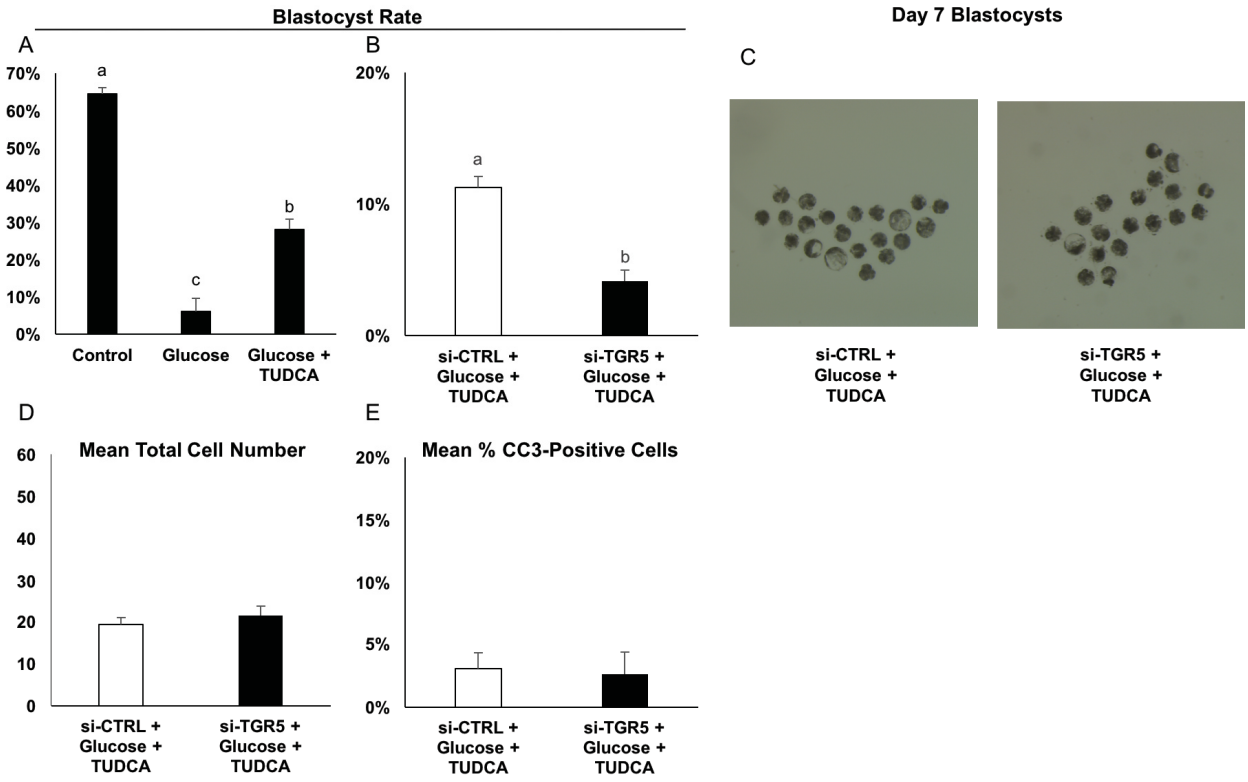


Figure 6.8.4. TUDCA needs TGR5 to rescue development in the face of glucose-induced ER stress

A model of glucose-induced oxidative stress is shown with PA embryos cultured in standard IVC media (Control), IVC with 7mM glucose (Glucose), and IVC with 7mM glucose and 50μM TUDCA (Glucose + TUDCA). Blastocyst rates based on cleavage at Day 7 of culture are indicated (A). To evaluate the necessity of the TGR5 receptor in this model, PA embryos injected with si-CTRL or si-TGR5 after activation were cultured with 7mM glucose and 50μM TUDCA. Blastocyst rates based on cleavage (B) as well as representative images of blastocyst development at Day 7 (C) are indicated. Blastocysts were collected at Day 7 for analysis of mean blastocyst total cell number (D), mean percentage of apoptotic cells (E), assessed by CC3 immunofluorescence. Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

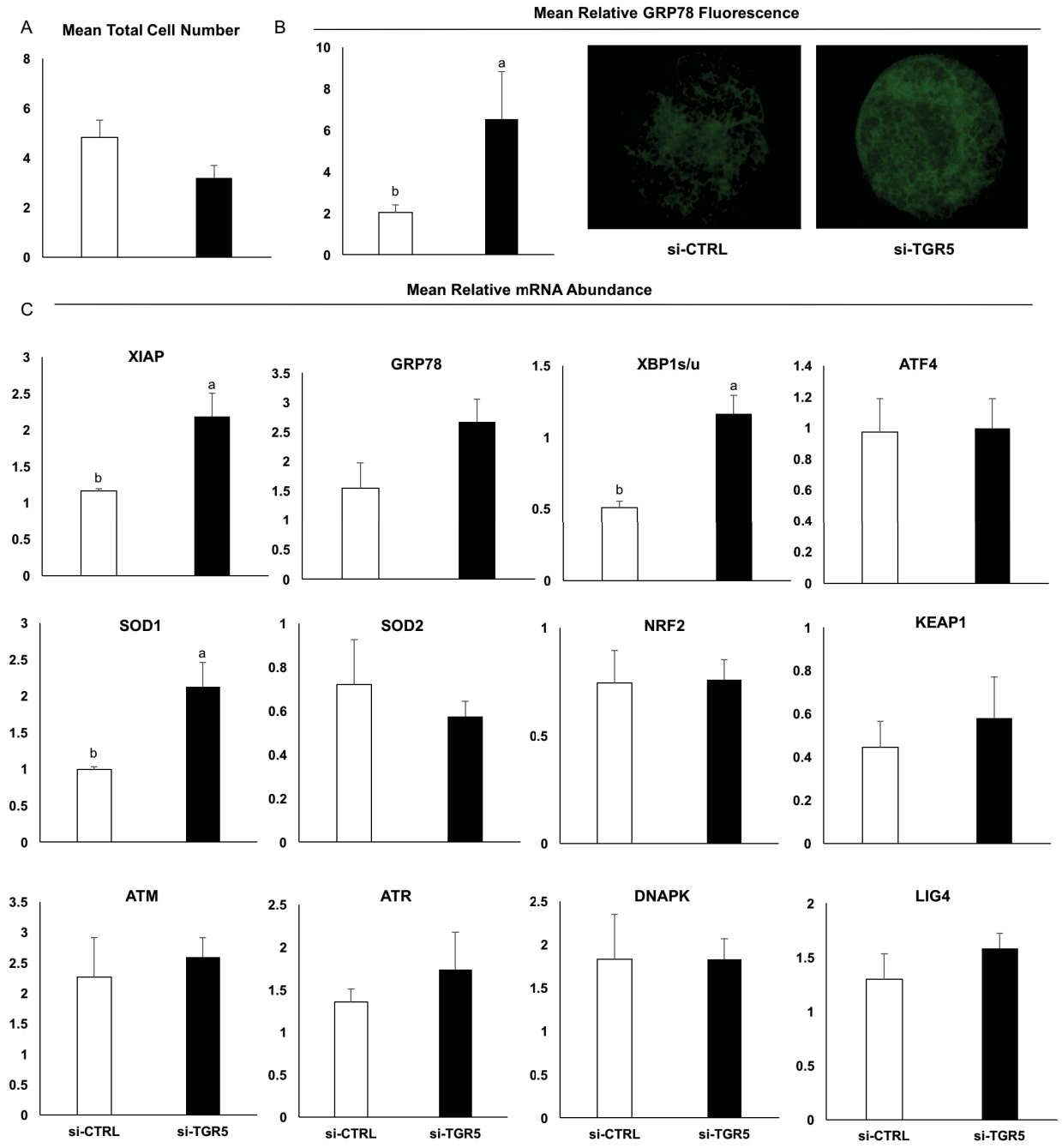


Figure 6.8.5. TUDCA needs TGR5 to relieve cellular stress and regulate cell survival

PA embryos were injected with si-CTRL or si-TGR5 after activation and cultured with 7mM glucose and 50μM TUDCA. Developing Day 5 embryos were collected for analysis. Mean total cell number of embryos (A) and mean relative mRNA expression of the regulator of apoptosis, XIAP (C) are shown. ER stress was evaluated by mean relative GRP78 fluorescence (B) and mean relative mRNA abundance of GRP78, XBP1 and ATF4 (C). XBP1 was expressed as the

ratio of spliced to unspliced XBP1 (XBP1s/u) (C). Oxidative stress was evaluated by mean relative mRNA abundance of SOD1, SOD2, NRF2, and KEAP1 (C). DNA damage repair was evaluated by mean relative mRNA abundance of ATM, ATR, DNAPK and LIG4 (C). Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

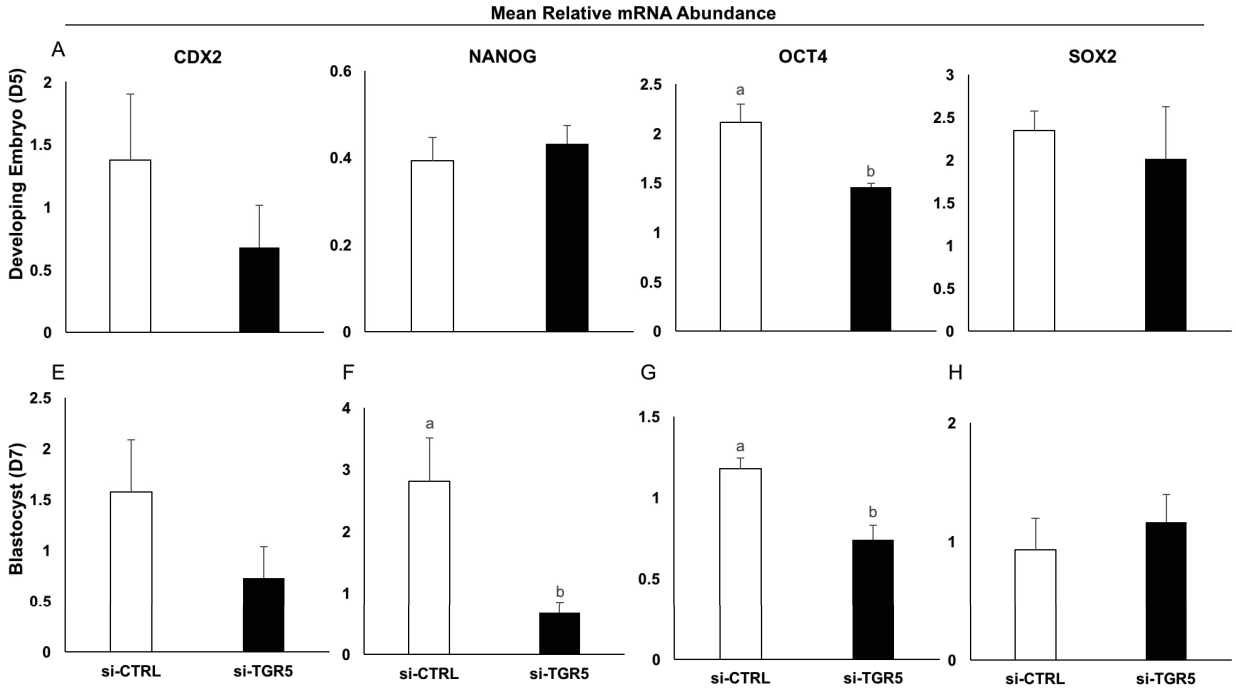


Figure 6.8.6. Attenuating TUDCA action in the face of glucose-induced ER stress alters mRNA abundance of genes controlling pluripotency

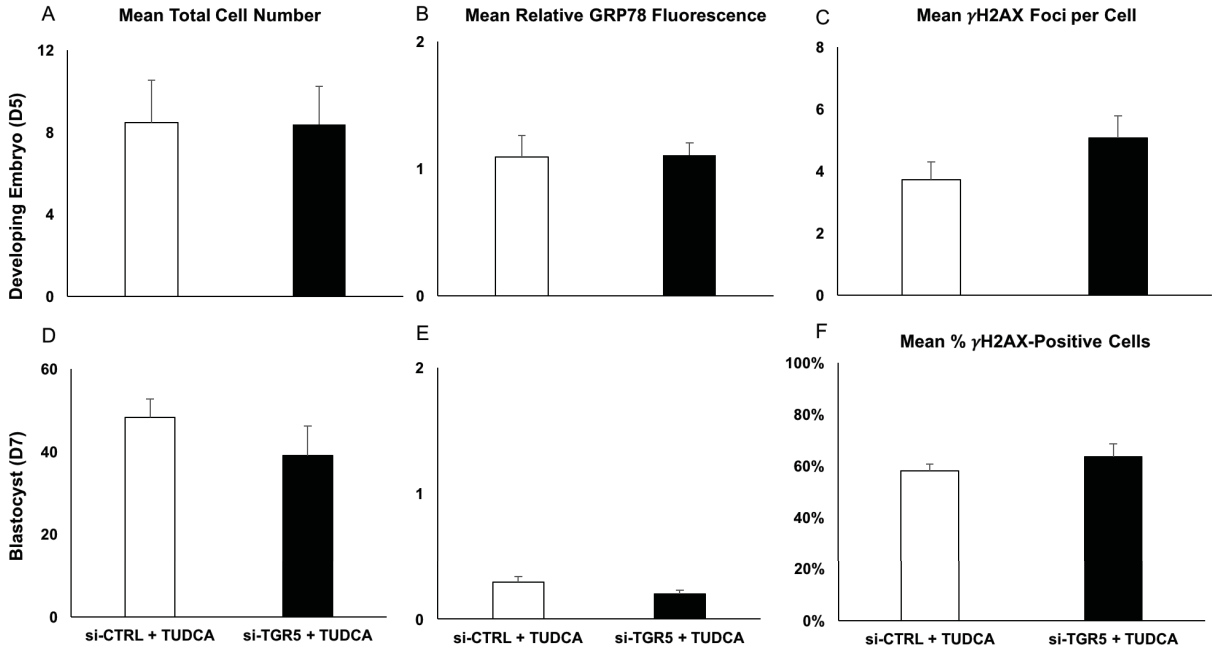
PA embryos were injected with si-CTRL or si-TGR5 after activation and cultured with 7mM glucose and 50µM TUDCA. Developing Day 5 (D5) embryos as well as Day 7 (D7) blastocysts were collected for analysis. Mean relative mRNA abundance of the trophectoderm marker (CDX2) and pluripotency markers (NANOG, OCT4, SOX2) are shown for both developing D5 embryos (A) and D7 blastocysts (B). Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

6.9 Tables

Table 6.9.1. Primers used for quantitative real-time PCR

Gene	Forward Primer	Reverse Primer	Accession No.	Anneal. T (ºC)
18S	GACATCTAAGGGCATCACAGA	ACACGGACAGGATTGACAGA	NR_046261.1	60
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	XM_001927727.2	60
ACTB	GCAGATGTGGATCAGCAAGC	GAATGCAACTAACAGTCCGCC	XM_021086047.1	60
TGR5	GTGGACCTTGACTTGAACTAGAG	TCAGAGAGGTTTGGTAGGGT	XM_013984487.2	72
GRP78	AGGTGATCTGGTCCTGCTTG	GTCGCTCACCTTCATAGACCTT	XM_001927795.5	64
XBP1u	GAGACCAAGGGGAATGGAGC	GCAGAGGTGCACGTAGTCTG	NM_001142836.1	64
XBP1s	CTGAGTCCGCAGCAGGTG	GGCTGGTAAGGAACTGGGTC	NM_001271738.1	71
ATF4	ACAGCAAGGAGGATGCTTTC	TGGCATGGTTTCCAGGTCAT	NM_001123078.1	66
SOD1	AAGGCCGTGTGTGTGCTGAA	GATCACCTTCAGCCAGTCCTTT	NM_001190422.1	60
SOD2	GGCCTACGTGAACAACCTGA	TGATTGATGTGGCCTCCACC	NM_214127.2	66
NRF2	AGCCCAGTCTTCATTGCTCC	CGTGCTAGTCTCAGCAAGGT	XM_013984303.2	60
KEAP1	ACGTGGAGACAGAAACGTGG	GTGTCCGTGTCTGGGTCATA	NM_001114671.1	60
ATM	CCGGTGTTTTGGGAGAGTGT	CTTCCGACCAAACTCAGCGT	NM_001123080.1	60
ATR	TGAGCTCCAGTGTTGGCATC	GCCAGTTCTCAGTGTGGTCA	XM_021069571.1	60
DNAPK	ATTCTTTGTCGGGAGCAGCA	CCTAGCTGTGTGGCACATGA	XM_021089419.1	60
LIG4	AGCTAGACGGCGAACGTATG	CCTTCCTGTGGGGAAACTCC	XM_003131089.5	60
MFN2	TGGAGTCAACGCAATCAGCA	AGGGACATCGCGTTTTTGGA	XM_021095349.1	58
XIAP	AAGCCCAGTGAAGACCCTTG	TTCAGCACTTCTCACCAGAGAC	NM_001097436.1	60
CDX2	TCGCTACATCACCATTCGGAG	TTCGTCCTGCGGTTCTGAAA	NM_001278769.1	60
NANOG	CAACGACAGATTTCAGAGGCAGA	GGTTCAGGATGTTGGAAAGTTCTTG	NM_001129971.1	60
OCT4	GCCAAGCTCCTAAAGCAGAAG	GCCAAGCTCCTAAAGCAGAAG	NM_001113060.1	60
SOX2	AACCAGAAGAACAGCCCAGAC	CTCCGACAAAAGTTTCCACTCG	NM_001123197.1	60

6.10 Supplemental Figure



Supplemental Figure 6.10.1. Attenuating TUDCA action does not affect the quality of unstressed developing embryos

Comparison of quality, ER stress and DNA damage in PA porcine embryos injected with si-CTRL or si-TGR5 after activation and cultured with 50μM TUDCA. Mean total cell number (A,D) and mean relative GRP78 fluorescence (B,E) of developing embryos (Day 5) and blastocysts (Day 7) are shown, respectively. DNA damage was assessed by evaluation of the mean number of γH2AX foci per cell in developing embryos (C) and the mean percentage of γH2AX positive cells in blastocysts (F). Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

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CONNECTING STATEMENT 3

Based on the results in Chapter 6, TUDCA needs the TGR5 receptor to reduce ER and oxidative stress and improve early embryo development. Interestingly, no effect on DNA damage repair was noted when the action of TUDCA was attenuated via TGR5 inhibition in a model of glucose-induced ER stress. Given the suggested link between the UPR and GDR (Dicks et al., 2015) and the negative effect oxidative stress has on genome stability (Kitagawa et al., 2004), an increase in DNA damage was expected. To further investigate if TUDCA and TGR5 signaling affect DNA damage and the GDR, a more direct model of DNA damage was utilized. In the third study, parthenogenetically-activated and *in vitro* fertilized porcine embryos were subjected to UV radiation with or without TUDCA in the culture medium, and development, ER stress and DNA damage were compared. In addition, the necessity of the TGR5 receptor for the action of TUDCA in the face of nuclear stress was assessed. Embryos were evaluated for markers of DNA damage, ER stress, as well as UPR and GDR activity.

CHAPTER 7

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Tauroursodeoxycholic acid acts via TGR5 receptor to facilitate DNA damage repair and improve early embryo development

Authors and Affiliations: Naomi Dicks[a], Karina Gutierrez[a], Luke Currin[a], Mariana Priotto de Macedo[a], Werner Glanzner[a], Marek Michalak[c], Luis B. Agellon[b*], Vilceu Bordignon[a*]

[a]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[b]School of Human Nutrition, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada
[c]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

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7.1 Abstract

DNA damage associated with assisted reproductive technologies (ART) is an important factor affecting gamete fertility and embryo development. The DNA damage incurred during *in vitro* embryo production (IVP), in addition to the inability to reliably select genetically stable gametes prior to ART, highlight the importance of efficient genome damage responses (GDR). Activation of the TGR5 receptor by tauroursodeoxycholic acid (TUDCA) has been shown to reduce ER stress in embryos, however its effect on the activation of GDR to facilitate DNA damage repair has not been examined. This study aimed to investigate the effect of TUDCA on DNA damage repair and embryo development. Firstly, in a porcine model of UV-induced nuclear stress, TUDCA reduced DNA damage and ER stress in developing embryos, as measured by γH2AX and GRP78 immunofluorescence, respectively. TUDCA was equally able to rescue early embryo development. No difference in total cell number, DNA damage, or percentage of apoptotic cells, measured by CC3 immunofluorescence, was noted in embryos that reached the blastocyst stage. Secondly, disruption of TGR5 signaling with DsiRNA abrogated the beneficial effects of TUDCA on development, resulting in increased DNA damage and ER stress in the developing embryo, as well as a reduction in the rate of blastocyst development. qPCR analysis revealed activation of the GDR, through increased mRNA abundance of DNAPK, 53BP1 and LIG4, as well as the ER stress response, or UPR, through increased spliced XBP1 and XIAP mRNA abundance. Results from this study demonstrated that TUDCA treatment activates TGR5 signaling to reduce DNA damage and improve embryo development after UV exposure.

Keywords: TUDCA, GPBAR1, genome damage, UPR, preimplantation embryo

7.2 Introduction

Assisted reproductive technologies (ART) represent important methods applied to mitigate various causes of impaired fertility. While these techniques have resulted in the birth of millions of children worldwide (Adamson et al., 2013), challenges and concerns associated with their use still remain. DNA damage during ART can be associated with severe consequences, such as birth defects or congenital diseases (Lewis and Aitken, 2005; Sakkas et al., 1998). Unfortunately, despite current advances, there is no reliable selection criteria for gametes with minimal DNA damage (Lewis and Aitken, 2005; Swain and Pool, 2008). While inherent differences in gamete

DNA damage or repair capability are important, stress during the IVP process can also increase the risk of genomic instability (Dicks et al., 2017). Endoplasmic reticulum (ER) stress is inherently present during the production of embryos *in vitro* (Zhang et al., 2012a), and it has also been linked to increased DNA damage (Dicks et al., 2017). In addition, oxidative stress, a known inducer of DNA damage, is also present under standard *in vitro* culture conditions (Kawahito et al., 2009). Unfortunately, even if development occurs in the ideal *in vivo* environment, the embryo will encounter DNA damage during normal cellular processes, including replication and energy metabolism (Kawahito et al., 2009; Takahashi, 2012; Torgovnick and Schumacher, 2015). As a result, the only assurance that DNA damage will not interfere with embryo viability is the presence of an efficient and effective genome damage response (GDR).

Double-stranded breaks (DSBs), caused by numerous factors including ionizing radiation, ultraviolet light, or endogenously produced reactive oxygen species (ROS), represent the most dangerous type of DNA damage, as they can lead to large deletions or translocations (Khanna and Jackson, 2001; Torgovnick and Schumacher, 2015). The GDR elicited in the face of DSBs include two main pathways: the homologous recombination (HR) pathway, and the non-homologous end-joining pathway (NHEJ) (Blackford and Jackson, 2017; Khanna and Jackson, 2001). The HR pathway is often considered a more efficient process given it uses a sister chromatid as a template to ensure precise repair of the defect (Torgovnick and Schumacher, 2015). As the primary pathway involved in GDR during early embryo development (Bohrer et al., 2018), it also represents a more physiological repair mechanism in this context. Conversely, the NHEJ pathway is considered more error-prone since it does not utilize a template for repair (Mimitou and Symington, 2009). In fact, inhibition of HR has been used as a tool to promote NHEJ and increase mutation rates during genome editing in embryos (Gutierrez, 2019). Important inducers of the DNA damage response during HR include ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) proteins, while DNA protein kinase (DNA-PK) coordinates the response during NHEJ (Blackford and Jackson, 2017; Khanna and Jackson, 2001). These kinases phosphorylate the histone H2AX at serine 139 (γH2AX), marking the site of DNA damage, and activate downstream effectors to inhibit local transcription, halt cell cycle progression, and recruit other proteins to effect DNA repair (Blackford and Jackson, 2017; Khanna and Jackson, 2001). Important effectors of DNA repair

include RAD51 during HR and both X-ray repair and cross-complementing protein 4 (XRCC4) and DNA ligase IV (LIG4) during NHEJ (Blackford and Jackson, 2017; Furgason and Bahassi el, 2013; Karran, 2000; Khanna and Jackson, 2001).

DNA damage has recently been linked to ER stress in poorly developing embryos (Dicks et al., 2017). In addition, given oxidative stress can induce both DNA damage (Kawahito et al., 2009) and ER stress (Yoon et al., 2014), it is likely the GDR and ER stress responses share similar pathways. One ER stress coping mechanism, commonly referred to as the unfolded protein response (UPR), is regulated by glucose regulated protein 78 (GRP78) which activates three separate transducers: the protein kinase RNA-like kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and the activating transcription factor 6 (ATF6) (Groenendyk et al., 2013; Michalak and Gye, 2015). All three arms of the UPR work to reduce stress on the ER by transiently reducing translation of proteins, as well as upregulating transcription of genes involved in ER-associated degradation as well as the ER stress response (Groenendyk et al., 2013; Michalak and Gye, 2015).

Interestingly, numerous studies have found the natural bile acid tauroursodeoxycholic acid (TUDCA), a proteostasis promoter (Vega et al., 2016), can reduce ER stress and improve embryo development in multiple species (Dicks et al., 2017; Kim et al., 2012; Lin et al., 2016; Song et al., 2011; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b), however, very little attention has been paid to its effect on the GDR. Recently, a study showed pre-treatment of liver cancer cells with high concentrations of TUDCA could reduce apoptosis after UV exposure (Uppala et al., 2017). In addition, TUDCA treatment was able to reduce DNA damage in poorly-developing slow cleaving porcine embryos (Chapter 5). These data provide evidence that TUDCA not only affects the UPR, but the GDR as well. In fact, TUDCA has recently been shown to necessitate the G-protein-coupled bile acid receptor, TGR5 (Chapter 6), suggesting TGR5-activated pathways may be involved.

The purpose of this study was to investigate the effect of TUDCA on DNA damage, the GDR, and early embryo development. Firstly, a DNA damage model was used to determine the effects of TUDCA on embryo development and the incidence of DNA damage. Secondly, the

mechanism of action underlying the effect of TUDCA was investigated by disrupting TGR5 signaling using DsiRNA. Results indicated for the first time that TUDCA can reduce DNA damage and rescue development in embryos exposed to UV radiation. Furthermore, this study showed that activation of TGR5 pathways facilitate physiologic GDR and cell survival responses necessary for normal embryo development. These findings provide insight into upstream signaling pathways involved in regulating DNA damage repair and provide potential therapeutic targets for the improvement of genome stability, fertility and embryo development.

7.3 Results

TUDCA treatment rescues embryo development after UV radiation

It was demonstrated previously that TUDCA can improve early embryo development in various species, however, its particular effects on DNA damage are less understood. To evaluate if TUDCA plays a role in the genome damage response (GDR), parthenogenetically-activated (PA) and *in vitro* fertilized (IVF) embryos were subjected to UV radiation and cultured in the absence or presence of 50 μM TUDCA. Rate of cleavage was not significantly affected in parthenotes or IVF embryos of any group (Fig.7.7.1 A, B). When subjected to 10 s of UV radiation, however, the rate of blastocyst development decreased by 2.6-fold and 4.4-fold compared to controls, in PA and IVF embryos respectively (18.1 % vs. 47.7 %, $p < 0.05$; 4.1 % vs. 18.3 %, $p < 0.05$) (Fig.7.7.1 C, D). Interestingly, in both PA and IVF embryos, TUDCA treatment rescued the development of UV-treated embryos such that the blastocyst rate was comparable to that of the control (30.0 % vs. 47.7 %, $p > 0.10$; 13.6 % vs. 18.3 %, $p > 0.10$) (Fig.7.7.1 C, D), indicating that TUDCA treatment prevented lethality due to DNA damage induced by UV radiation.

To further investigate the effects of TUDCA treatment on the GDR and UPR status, the quality of blastocysts that ultimately formed after UV radiation exposure was evaluated. PA and IVF blastocysts were collected at Day 7 for immunofluorescence staining. Mean blastocyst total cell number was not significantly different among any of the groups regardless of the means of embryo activation (Fig.7.7.1 E, F), nor was the percentage of cleaved caspase-3 (CC3)-positive cells, measured by immunofluorescence (Fig.7.7.1 I, J). Similarly, treatment with TUDCA did not significantly affect the mean percentage of DNA damaged cells, measured by γH2AX immunofluorescence, in either PA or IVF blastocysts compared to their untreated counterparts,

or the controls (Fig. 7.7.1 G, H). These results indicated that while TUDCA can rescue embryo development after UV radiation exposure, embryos that ultimately developed into blastocysts did not differ in their level of DNA damage. Given blastocysts represent a selected population, these results were not surprising. These data also indicated that the effects of TUDCA on the GDR and UPR after UV exposure are most important early in development.

TUDCA reduces DNA damage and ER stress in UV-treated developing embryos

To determine if the beneficial effect of TUDCA on development after UV stress was due to activation of stress coping response strategies, PA embryos were evaluated at Day 3 and Day 5 of development for GDR and UPR status. No difference in mean total cell number (Fig.7.7.2 A, B) was noted compared to untreated control embryos at either of the time points. Treatment with TUDCA, however, significantly reduced DNA damage in UV-exposed embryos compared to their untreated counterparts, at both Day 3 and Day 5 of culture (Fig.7.7.2 C, D, respectively). The mean number of γH2AX foci per cell decreased 5-fold at Day 3 (1.0 vs. 5.1; $p < 0.05$) (Fig.7.7.2 C), and 14-fold at Day 5 (0.2 vs. 2.7; $p < 0.05$) (Fig.7.7.2 D), in UV-exposed embryos treated with TUDCA compared to those that were not. Interestingly, ER stress, measured by mean GRP78 fluorescence, was also significantly reduced in the TUDCA-treated UV-exposed embryos at Day 3 (4.4 vs. 5.8; $p < 0.05$) (Fig.7.7.2 E), but not at Day 5 (0.6 vs. 0.9; $p > 0.10$) (Fig.7.7.2 F) of culture. These results indicated that TUDCA treatment significantly reduces DNA damage and ER stress in developing embryos after exposure to UV radiation, allowing more embryos to develop to the blastocyst stage. In addition, these findings suggested that the GDR and UPR are linked in their response to cellular stress caused by UV radiation.

TGR5-knockdown abolishes the ability of TUDCA to rescue of UV-treated embryos

The results of experiments discussed in Chapter 6 indicated the TGR5 receptor is needed for TUDCA to reduce ER and oxidative stress and improve early embryo development. To determine if TGR5 signaling is also required for the therapeutic effect of TUDCA on the GDR, the TGR5 receptor was knocked down using TGR5 DsiRNA (si-TGR5) prior to UV radiation exposure. Injection with scrambled control DsiRNA (si-CTRL) served as the control. Interestingly, the rate of cleavage was mildly increased from 62.6 % to 66.0 % in UV-exposed embryos after DsiRNA mediated inhibition of TGR5 ($p < 0.05$) (Fig.7.7.3 A). As expected,

however, the rate of blastocyst development was reduced by 42.2 % in UV-exposed si-TGR5 embryos treated with TUDCA, indicating that TGR5 is involved in the mechanism of action for DNA damage repair (17.3 % vs. 29.9 %; $p < 0.05$) (Fig.7.7.3 B, C). Quality of the blastocysts was assessed by mean total cell number (Fig.7.7.3 D), mean percentage of γH2AX-positive cells (Fig.7.7.3 E), and ER stress, by mean relative GRP78 fluorescence (Fig.7.7.3 F). No significant differences were seen in any of the parameters assessed (Fig.7.7.3 D-F). In addition, no significant difference in mean percentage of CC3-positive cells was seen (Appendix 9.3). These development results replicated those obtained from the UV-exposed groups treated with or without TUDCA (Fig.7.7.1), indicating that TGR5 signaling is necessary for TUDCA to improve early embryo development in the face of nuclear stress.

Altered GDR occurs in UV-exposed embryos when TGR5 activation is attenuated

While no effect on DNA damage was seen at the blastocyst stage in UV-exposed si-TGR5 embryos treated with TUDCA (Fig.7.7.3 G, H), previous results (Fig.7.7.2 C, D) indicated that any changes related to the GDR were only detectable in earlier stages of development. To assess this, si-CTRL and si-TGR5 embryos exposed to UV irradiation and treated with TUDCA were collected at Day 5 of culture for immunofluorescence and qPCR analysis. As expected, inhibition of TGR5 as a means to block the action of TUDCA after UV radiation caused a significant increase in DNA damage in developing embryos, with the mean γH2AX foci per cell doubling from 3.4 to 7.6 ($p < 0.05$) (Fig.7.7.4 A). These results suggested that embryos needed an intact TGR5 signaling pathway to activate the GDR and minimize DNA damage in response to UV radiation. Mean relative mRNA abundance of genes involved in DNA repair pathways were also significantly altered (Fig.7.7.4 B). Interestingly, mean relative abundance of mRNAs involved in HR were unchanged, while those involved in the error-prone NHEJ repair were significantly increased in UV-exposed embryos that had disrupted TUDCA/TGR5 signaling (Fig.7.7.4 B). mRNA abundance of DNAPK (1.23 vs. 0.54; $p < 0.05$) and 53BP1 (1.21 vs. 0.54; $p<0.05$) more than doubled (Fig.7.7.4 B), whereas the mRNA abundance of LIG4 nearly tripled (1.10 vs. 0.40; $p < 0.05$) (Fig.7.7.4 B). UV-exposed embryos with impaired TGR5 signaling showed elevated NHEJ gene expression, coinciding with their increased level of DNA damage foci (Fig.7.7.4 A). These results demonstrated that TGR5 signaling is important to facilitate physiological and not pathological GDR in response to nuclear stress.

Sustained UPR and altered regulation of apoptosis occurs in UV-exposed embryos when TGR5 activation is attenuated

While data from this study demonstrated aberrant GDR in the face of UV radiation when TGR5 signaling was disrupted, effects on the UPR warranted investigation. Similarly, UV-exposed si-CTRL and si-TGR5 embryos treated with TUDCA were collected at Day 5 of culture to evaluate UPR activity as an indicator of ER stress. Developing embryos that had disrupted TUDCA-TGR5 signaling after UV radiation showed a significant increase in mean GRP78 fluorescence, from 0.99 to 1.97 ($p < 0.001$) (Fig.7.7.5 B), compared to controls. These embryos demonstrated activation of the IRE1α arm of the UPR given a significant increase in mean relative mRNA abundance of XBP1s/u, compared to controls (0.74 vs. 0.43; $p < 0.01$) (Fig.7.7.5 C). ATF4, a downstream effector of the PERK pathway, nor its target, C/EBP-homologous protein (CHOP), showed no significant alteration in mRNA abundance (Fig.7.7.5 C). Similarly, mRNA abundance of ATF6 was not significantly affected (Fig.7.7.5 C). Since a normal response to sustained ER stress and UPR activation is controlled cell death, evaluation of total cell number and apoptosis was performed (Groenendyk et al., 2013). Interestingly, while there was no difference in mean total cell number (Fig.7.7.5 A), nor mRNA abundance of the mitochondrial function and pro-apoptotic gene, MFN2 (Fig.7.7.5 C), si-TGR5 embryos showed a marked increase in expression of the X-linked inhibitor of apoptosis (XIAP) gene (Fig.7.7.5 C), which was shown previously to be upregulated early in the response to UPR activation (Brown et al., 2016; Hu et al., 2004). In fact, mean relative mRNA abundance of XIAP nearly doubled in UV-exposed si-TGR5 embryos (1.41 vs. 0.82; $p<0.05$) (Fig.7.7.5 C). Together, these data suggested UV-exposed si-TGR5 embryos with increased DNA damage (Fig.7.7.4 A) and increased ER stress (Fig.7.7.5 B) exhibit sustained UPR and altered regulation of apoptosis to promote their survival.

7.4 Discussion

This study demonstrated for the first time that TUDCA treatment activates the GDR through TGR5 signaling to reduce DNA damage and rescue embryo development after UV exposure. While previous work alluded to cross-talk between TUDCA and the GDR (Dicks et al., 2017; Uppala et al., 2017), the current findings provide evidence of reduced DNA damage in the face

of genotoxic UV stress. In addition, these results confirm that the GDR and UPR are coordinately activated by TUDCA via the TGR5 signaling pathway.

Disrupted TUDCA/TGR5 signaling has a significant impact on the GDR. During early embryo development, it has been shown that embryos primarily utilize the HR pathway in response to DSBs caused by UV radiation, with DsiRNA targeting ATM, but not DNAPK, significantly reducing blastocyst rate and quality (Bohrer et al., 2018). Given the greater fidelity and efficiency of the HR pathway (Blackford and Jackson, 2017; Khanna and Jackson, 2001), it is not surprising embryos would preferentially employ this method to prevent replication of damaged DNA and subsequent deleterious effects. Remarkably, when TGR5 activation by TUDCA was disrupted in this study, not only was DNA damage increased in the face of UV stress, but mediators of the NHEJ pathway were preferentially upregulated. Expression of DNAPK, 53BP1 and LIG4 genes were increased, whereas no significant difference was seen in mRNA abundance of ATM, ATR or RAD51. These data suggest the GDR is altered in embryos with impaired TGR5 activation. Considering there was no difference seen in cell number and, in fact, increased XIAP mRNA abundance to prevent cell death, these changes can be interpreted as a successful adaptation from the perspective of the embryo cell, or blastomere (Dicks et al., 2015). Although stressed and damaged, these blastomeres were surviving. Alternatively, at the organismal level, upregulation of NHEJ over HR in the face of nuclear stress could represent a pathological adaptation (Dicks et al., 2015), raising concerns of impaired genome integrity and abnormal embryo development (Bohrer et al., 2018). This concern is amplified, considering these DNA-damaged and ER-stressed blastomeres have sustained UPR activation and upregulated expression of XIAP, promoting their survival despite their poor quality. Continued survival of these blastomeres in the face of DNA damage could have significant deleterious consequences in the embryo, fetus and neonate.

The impact of TGR5 pathway activation on the GDR also provides further evidence that the UPR and GDR are linked. It is well established that TUDCA, which activates TGR5 (Chapter 6), reduces the expression of the UPR-induced transcription factor, XBP1s (Lin et al., 2016; Yoon et al., 2014; Zhang et al., 2012a), however, it has also been shown in yeast that sustained XBP1s activity can increase NHEJ repair (Tao et al., 2011). Based on this, chronically elevated XBP1s

abundance in UV-exposed embryos might explain the switch from HR to NHEJ DNA repair. Interestingly, in this yeast model, the NHEJ pathway is promoted through histone H4 deacetylation by Rpd3, a histone deacetylase (HDAC) which resembles the mammalian HDAC1 (Tao et al., 2011). Similarly, hyperacetylation of histone H4, Ku proteins and poly ADP-ribose polymerase 1 (PARP1) in human leukemia cells results in impaired NHEJ repair (Robert et al., 2016). More specifically, acetylated PARP1 preferentially binds sites of DNA damage, blocking access of NHEJ repair proteins (Robert et al., 2016). Together these studies demonstrate that reduced acetylation of both histones and non-histone proteins can facilitate NHEJ (Robert et al., 2016; Tao et al., 2011). This is interesting since the *in vivo* induction of ER stress in rats resulted in the upregulation of HDACs (Yao et al., 2013), suggesting that ER stress causes hypoacetylation. Furthermore, TUDCA has been shown to reduce HDAC1 and increase H3K9 acetylation in nuclear donor cells for somatic cell nuclear transfer, indicating relief of ER stress increases acetylation (Zhang et al., 2018). Taken together, these results suggest disrupted TGR5 signaling increases XBP1s-mediated hypoacetylation of histones and proteins, leading to activation of NHEJ repair. While these studies illustrate how TGR5 pathway activation may affect chromatin remodeling and impact both the UPR and GDR, further investigation is needed to understand these complex interactions, which are undoubtedly cell context and cell-type specific (Ogiwara et al., 2011; Robert et al., 2016; Tao et al., 2011; Yaneva et al., 2005; Yao et al., 2013; Zhang et al., 2018).

In conclusion, this study demonstrated for the first time that TGR5 activation by TUDCA improves embryo development by reducing DNA damage and ER stress after UV radiation exposure. In addition, TGR5 signaling facilitates physiological GDR and cell survival regulation necessary to promote normal embryonic development. Together these data demonstrate coordinated activation of UPR and GDR through TGR5, underscoring its potential as a therapeutic target for improved genome stability, gamete fertility and embryo development.

7.5 Materials and Methods

Reagents

All chemicals and reagents used in this study were purchased from MilliporeSigma (Burlington, MA, USA). Sources for all other materials are indicated within the text.

Oocyte retrieval and in vitro maturation

Ovaries from pre-pubertal gilts were collected from a local slaughterhouse (Olymel, S.E.C./L.P., Saint Esprit, Quebec, Canada). Follicles of 3-6 mm in diameter were aspirated from the ovaries to retrieve cumulus oocyte complexes (COCs). COCs were collected for maturation, as previously described (Dicks et al., 2017) and cultured for 22 h in *in vitro* maturation medium 1 (IVM1) consisting of TCM 199 (ThermoFisher Scientific, Waltham, MA, USA), 20 % porcine follicular fluid, 1mM dibutyryl cyclic adenosine monophosphate (dbcAMP D-0260), 0.1 mg/mL cysteine (6852), 10 ng/mL epidermal growth factor (EGF; ThermoFisher Scientific, PHG0311), 0.91 mM sodium pyruvate (P-4562), 3.05 mM D-glucose (G-6152), 0.5 µg/mL LH (SIOUX Biochemical Inc., Sioux Center, IA, United States, 725), 0.5 µg/mL FSH (SIOUX Biochemical Inc., 715), and 20 µg/mL gentamicin (G-1272). COCs were rinsed and matured for an additional 20-22h in IVM2, consisting of IVM1 devoid of LH, FSH and dbcAMP.

Embryo activation

Matured oocytes were denuded with 0.1 % hyaluronidase (H3506) and activated parthenogenetically (PA), or fertilized *in vitro* (IVF). Parthenogenetic activation was performed as previously described (Dicks et al., 2017) using 15 µM ionomycin (I0634) for 5 min followed by 4 h in calcium-free porcine zygote medium (PZM-3) supplemented with 10mM strontium chloride (255521), 7.5 µg/ml cytochalasin B (C6762) and 10 µg/ml cyclohexamide (C1988). For IVF, matured oocytes were rinsed in porcine TBM-Fert, consisting of Tris-buffered media supplemented with 30 mg/ml bovine serum albumin (BSA) (A6003), 2 mM caffeine (C-07500) and 20 µg/mL gentamicin (G-1272). Porcine semen was prepared by washing in TBM-Fert devoid of caffeine and then re-suspending in regular TBM-Fert, immediately prior to co-incubation with the mature oocytes. Oocytes were incubated in 500 µl wells of TBM-Fert with approximately 100,000 motile sperm for a total of 5 h.

UV treatment

After parthenogenetic activation or IVF, embryos were thoroughly rinsed in porcine *in vitro* culture medium (IVC) prior to UV light exposure. Embryos were placed in 2 ml of IVC medium in a 35 mm cell culture dish (Corning, Tewksbury, MA, USA) and exposed inside a biological

safety cabinet (1300 Series Class II, Thermo Fischer Scientific, Waltham, MD, USA) to 10 s of UV light, based on previous UV dose-response results (Bohrer et al., 2018).

TGR5 knockdown

For experiments requiring reduction of TGR5 receptor protein, embryos were injected with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) (Table 7.8.1), designed using Integrated DNA Technologies' (IDT) Custom Dicer-Substrate siRNA Design Tool (Coralville, IA, USA) (https://www.idtdna.com/site/order/designtool/index/DSIRNA_CUSTOM) and previously validated to significantly reduce both TGR5 mRNA and protein abundance, as well as to have no effect on development when knocked down alone under standard culture conditions (Chapter 6). Injections were performed as previously described (Chapter 6) using an inverted microscope (Nikon, Tokyo, Japan) and a micromanipulator system (Narishige International, Long Island, NY, USA). Approximately 10 pl of 20 uM DsiRNA was injected into each embryo using the FemtoJet 4i programmable microinjector (Eppendorff, Hamburg, Germany). Embryos were permitted a minimum of 30minutes of recovery in IVC after injection, prior to UV exposure.

Embryo culture

After parthenogenetic activation/IVF and other treatments (microinjection, UV light exposure), embryos were rinsed and then cultured in IVC, consisting of porcine zygote medium supplemented with 3 mg/ml BSA (A6003) and 5 mM hypotaurine (H1384) (PZM-3). IVC was supplemented with or without 50 µM TUDCA (580549), depending on the experiment and treatment group. The concentration of TUDCA has previously been optimized for use in porcine embryo culture (Zhang et al., 2012b). Groups of 20-30 embryos were cultured in 60 µl droplets under mineral oil at 5 % CO_2, 95 % air and 38.5 °C. Cleavage rates were calculated after 48 h of culture and any uncleaved embryos were discarded. Culture medium was supplemented with 10 % FBS (ThermoFisher Scientific, 16170-078) after 5 days. Blastocyst rates were recorded on Day 7 based on the total number of cleaved embryos.

Evaluation of TUDCA effect on DNA-damaged embryos after UV exposure

To determine if TUDCA has a significant effect on the GDR, embryos were subjected to UV treatment to cause DNA damage and were subsequently cultured in the absence or presence of 50 μM TUDCA. Embryos not exposed to UV light and cultured in standard IVC served as controls. This experiment was replicated in both PA and IVF embryos. Cleavage and blastocyst rates were recorded, while the mean percentage of both γH2AX-positive and apoptotic cells were determined in Day 7 blastocysts. In addition, developing embryos at Day 3 and Day 5 of development were evaluated for mean total cell number, mean γH2AX foci per cell and mean GRP78 fluorescence. Data was collected from a minimum of 3 independent experiments.

Evaluation of disrupted TUDCA-TGR5 signaling on the GDR, UPR and embryo development

To determine if TGR5 was necessary for the effect of TUDCA on development in the face of nuclear stress, the TGR5 receptor was targeted using DsiRNA as previously performed (Chapter 6). PA embryos were injected with either scrambled control DsiRNA (si-CTRL), or TGR5 DsiRNA (si-TGR5) to reduce the abundance of TGR5 receptor protein. A minimum of 30 min was given for embryos to recover prior to UV light exposure to induce DNA damage, followed by culture in IVC supplemented with 50 μM TUDCA. Cleavage and blastocyst rates were recorded. Blastocysts collected at Day 7 were analyzed for total cell number, apoptosis, DNA damage and ER stress. After confirming TGR5-knockdown interfered with the action of TUDCA and replicated development results from UV-exposed embryos cultured with or without TUDCA, further evaluation of the GDR and UPR was performed. Day 5 developing embryos were assessed for markers of DNA-damage repair, the UPR and apoptosis. Furthermore, both Day 5 and Day 7 embryos were analyzed for normal development by assessing the expression of trophectoderm and inner cell mass genes.

Quantitative real-time PCR

The PicoPure™ RNA Isolation Kit (ThermoFisher Scientific, KIT0202) and the Superscript® VILO™ cDNA Synthesis Kit (ThermoFisher Scientific, 11754050) were used according to manufacturer recommendations to extract embryo mRNA and synthesize cDNA. Quantitative real-time PCR reactions containing sample cDNA, appropriate primers (Table 7.8.2) and the Advanced qPCR Mastermix (Wisent Bio Products, Montreal, Quebec, Canada 800-435-UL) were run using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, 185-5200).

Thermocycler parameters were 5 min at 95 °C, 40 cycles of 15 s at 95 °C followed by 30 s at the optimal annealing temperature (Table 7.8.2), and finally 10 s at 95 °C and 5 s at 60 °C. Duplicate reactions were performed and specificity of reaction products confirmed by melt-curve analyses. The $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008) was used to calculate relative quantities of mRNA, with multiple reference genes used for normalization, including the18S ribosomal RNA gene, H2A gene and ß-actin gene. The efficiency of qPCR reactions was between 90 -110 %, R^2 ≥0.98 and slope values ranged from -3.6 to -3.1.

Immunofluorescence

Immunofluorescence analysis was performed as previously described (Dicks et al., 2017; Bohrer et al., 2017). Briefly, embryos were fixed in 10 % formalin (HT501128) and then permeabilized with 1 % Triton X-100 (T8787) in PBS at 37 °C. Embryos were placed in blocking solution containing 3 % BSA (Roche, Basel, Switzerland, 10775835001) and 0.2 % Tween-20 (P1379) in PBS for 90 min, transferring to fresh solution every 30 min. Primary antibody incubation overnight at 4 °C was performed using mouse monoclonal anti-phospho-histone H2A.X (Ser139) (05-636) for evaluation of DNA damage (1:400), rabbit polyclonal anti-cleaved caspase 3 (CC3) (Cell Signaling, 9661S) for evaluation of apoptosis (1:400), and rabbit polyclonal anti-GRP78 (Abcam, Ab191023) for evaluation of ER stress (1:500). Embryos were then rinsed for another 90 min in blocking solution, transferring to fresh solution every 30 min. Incubation with secondary antibody was performed for 50 min in the dark, using goat polyclonal anti-mouse Cy3-conjugated IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA, 115-165-146) and goat polyclonal anti-rabbit Alexa Fluor 488-conjugated IgG antibody (ThermoFisher Scientific, A-11008) (1:1000). Prior to mounting on slides with Mowiol (Polyvinylalcohol, 10852), embryos were rinsed once more for 80 min: 30 min in blocking solution, 20 min in 10 μg/ml DAPI (4,6-Diamidino-2-Phenylindole, Dilactate) (ThermoFisher Scientific, D3571) to stain nuclei, and a final 30 min in blocking solution. Slides were analyzed as previously described (Dicks et al., 2017) using a Nikon Eclipse 80i microscope (Nikon Instruments Inc.), Retiga 2000R monochrome digital camera (Qimaging, Surrey, BC, Canada) and the Simple PCI Imaging Software (Compix, Inc., Sewickly, PA, USA). Only γH2AX foci indicating sites of DNA damage repair (greater than 0.3 μm^3) (Grenier et al., 2012; McManus and Hendzel, 2005) were counted and expressed as mean γH2AX foci per cell in developing

embryos (Day 3, Day 5), or mean percentage of γH2AX-positive cells (Day 7). Apoptosis in Day 7 blastocysts was expressed as the mean percentage of CC3-positive cells. GRP78 fluorescence was adjusted to cell number and normalized based on background negative control fluorescence.

Statistical analyses

The JMP 13 program (SAS, Cary, NC, USA) was used for all statistical analyses. The one-way ANOVA was used for all normally distributed data, with the Tukey post-hoc test for multiple comparisons and the Student's t-test for pair-wise comparisons. The Kruskal-Wallis method was used for all data not normally distributed, with comparison of means performed using Dunn's test (multiple comparisons) or Wilcoxon's test (pair-wise comparisons). Differences among groups were considered significant if p-values were < 0.05.

7.6 Acknowledgements

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7.7 Figures

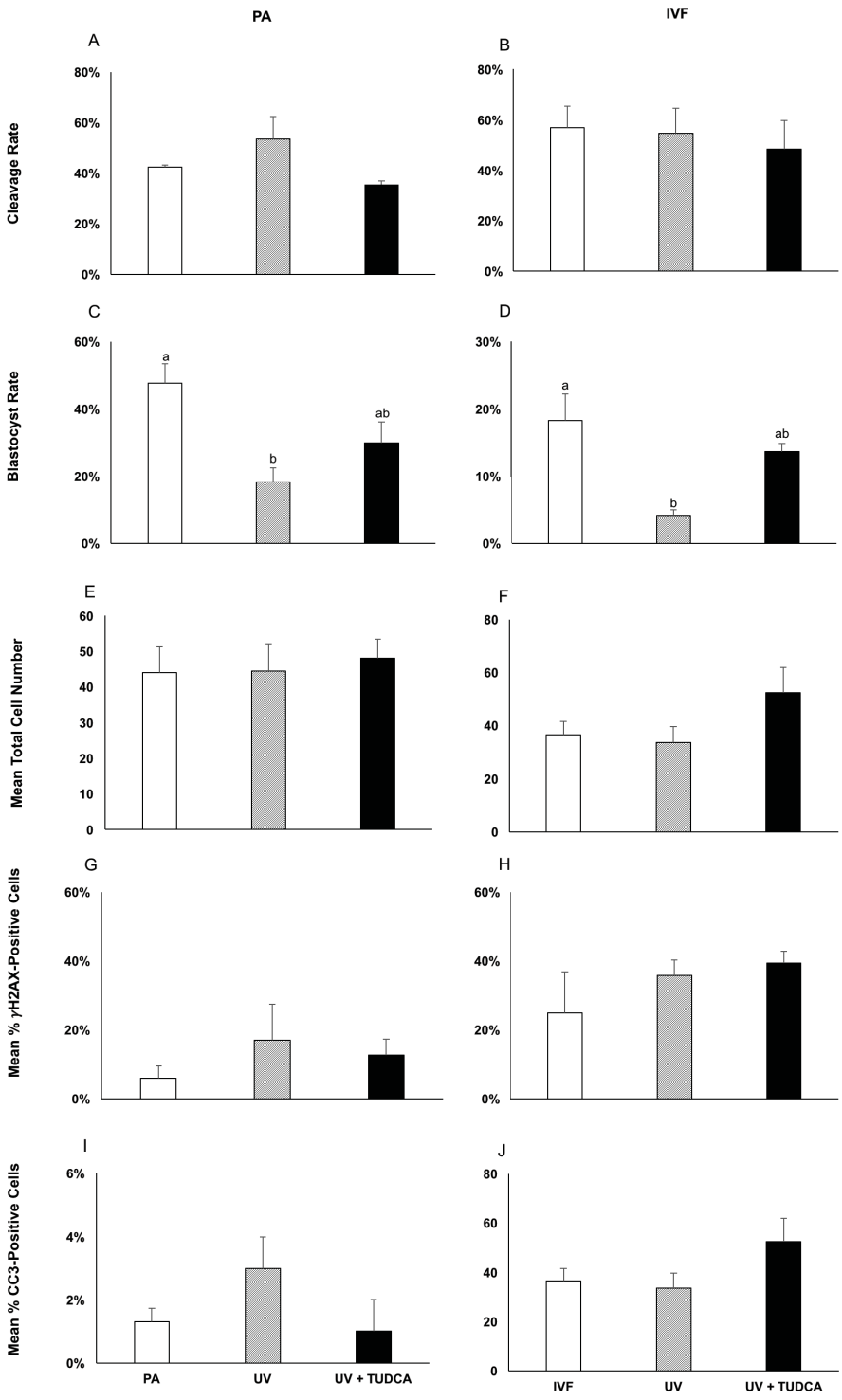

Figure 7.7.1. TUDCA treatment rescues embryo development after UV radiation

Embryo development was assessed in a DNA damage model of both parthenogenetically-activated (PA) and *in vitro fertilized* (IVF) porcine embryos. After activation or IVF, embryos were subjected to 0 or 10s of UV radiation and cultured with or without 50μM TUDCA. Cleavage after 48 h of culture (A,B) and blastocyst rates at Day 7 (C,D) are indicated for PA and IVF, respectively. Quality of blastocysts was also evaluated by mean total cell number (E,F), mean percentage of DNA-damaged cells, based on γH2AX immunofluorescence (G, H) and mean percentage of apoptotic cells, based on CC3 immunofluorescence (I, J), for PA and IVF, respectively. Data were collected from a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

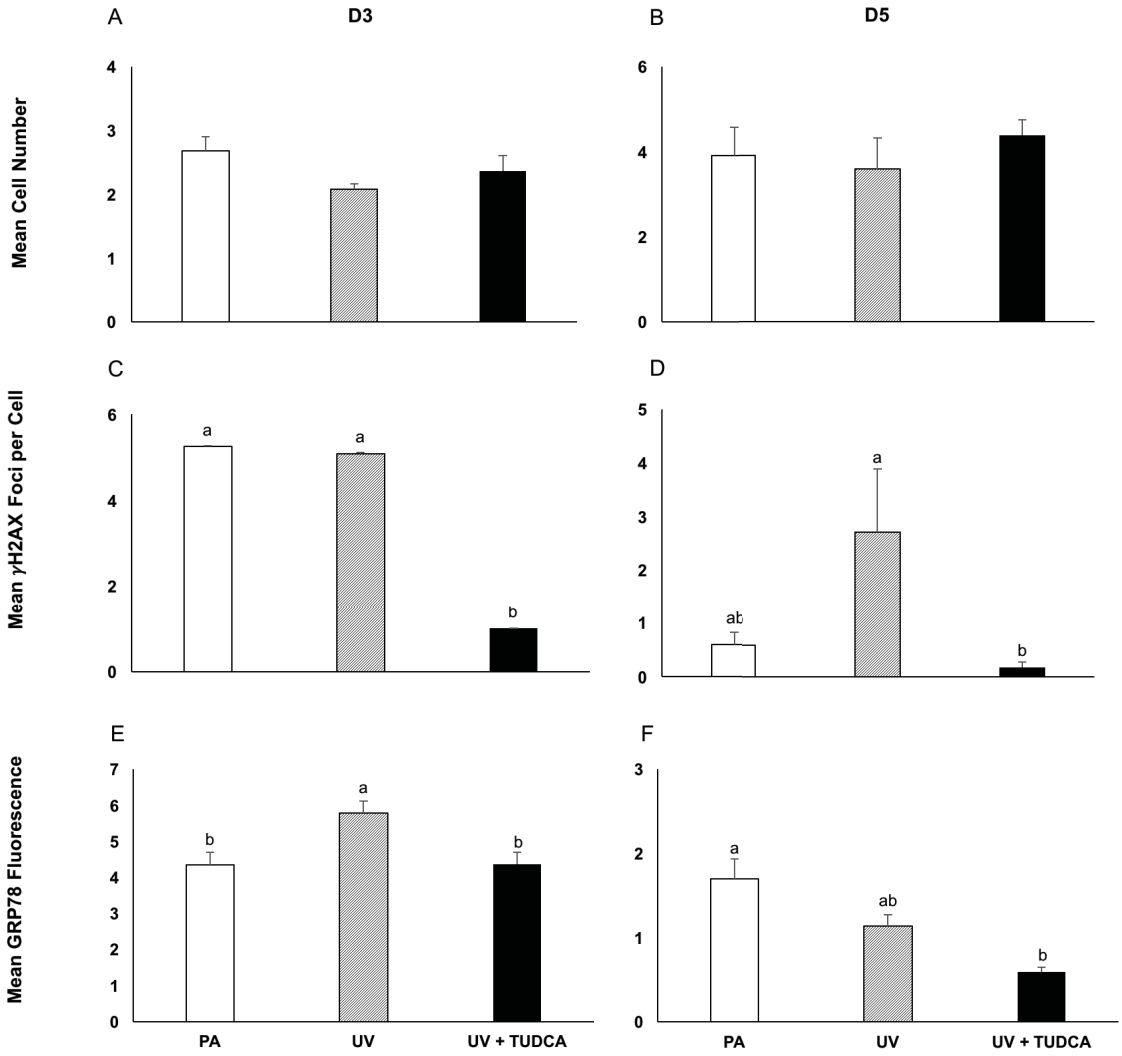

Figure 7.7.2. TUDCA reduces DNA damage and ER stress in UV-treated developing embryos

Evaluation of developing embryo quality in a DNA damage model of parthenogenetically-activated (PA) porcine embryos was performed. After activation, embryos were subjected to 0 or 10s of UV radiation and cultured with or without 50µM TUDCA. Embryos were collected at Day 3 (D3) and Day 5 (D5) of development. Mean total cell number of developing embryos at D3 and D5 are shown (A,B, respectively). DNA damage, evaluated by mean *γ*H2AX foci per cell (C,D), as well as ER stress, evaluated by mean GRP78 fluorescence (E,F) are indicated for both D3 and D5 embryos, respectively. Data were collected from a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

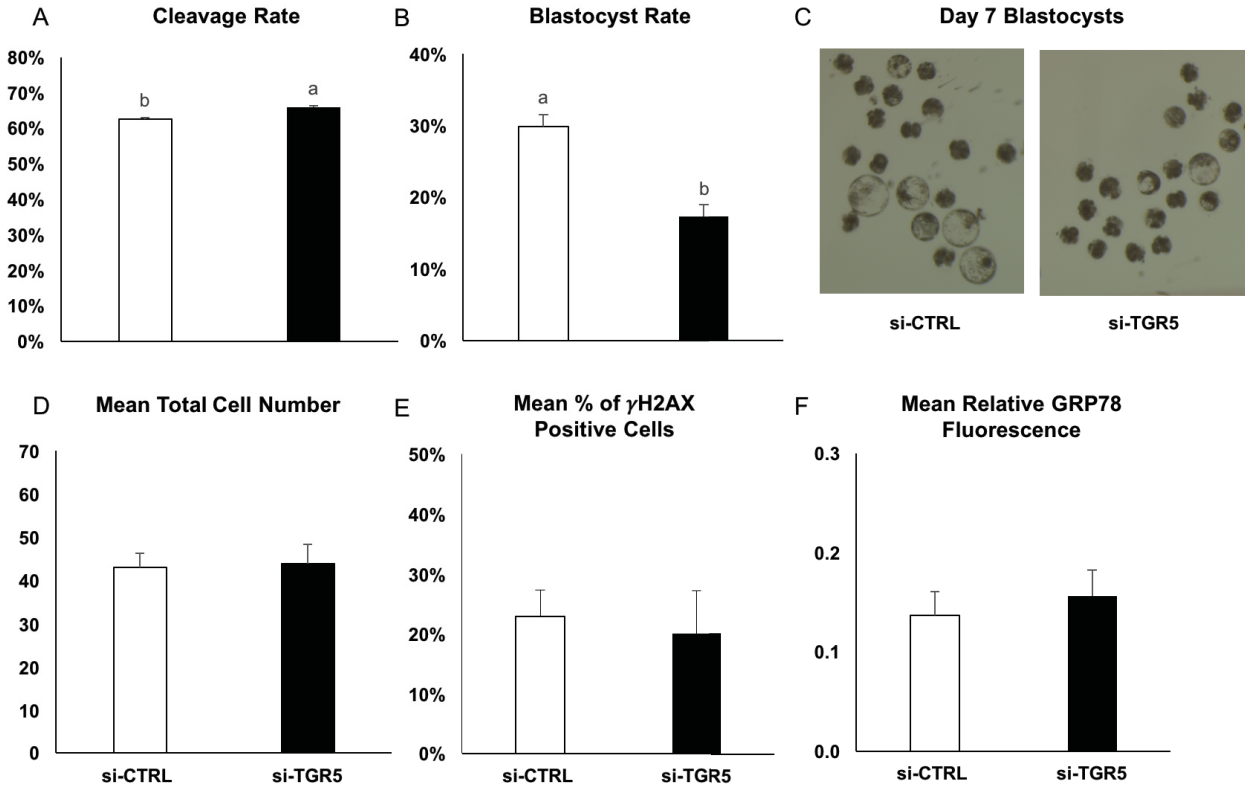

Figure 7.7.3. TGR5-knockdown abolishes the ability of TUDCA to rescue of UV-treated embryos

The necessity of TGR5 signaling for the ability of TUDCA to improve embryo development after DNA damage was investigated. PA embryos, injected with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) after activation, were subjected to 10s UV radiation and cultured with 50μM TUDCA. Cleavage was assessed 48 h after activation (A). After 7 days in culture, rate of blastocyst development was recorded (B) and blastocysts were analyzed for mean total cell number (D). DNA damage, assessed by mean percentage of γH2AX positive cells (E), and ER stress, assessed by mean relative GRP78 fluorescence (F) are indicated. All data were collected from a minimum of three replicates. Panel (C) shows representative images of blastocyst development at Day 7 of culture in both groups. Significant differences are indicated by different lower case letters ($P<0.05$).

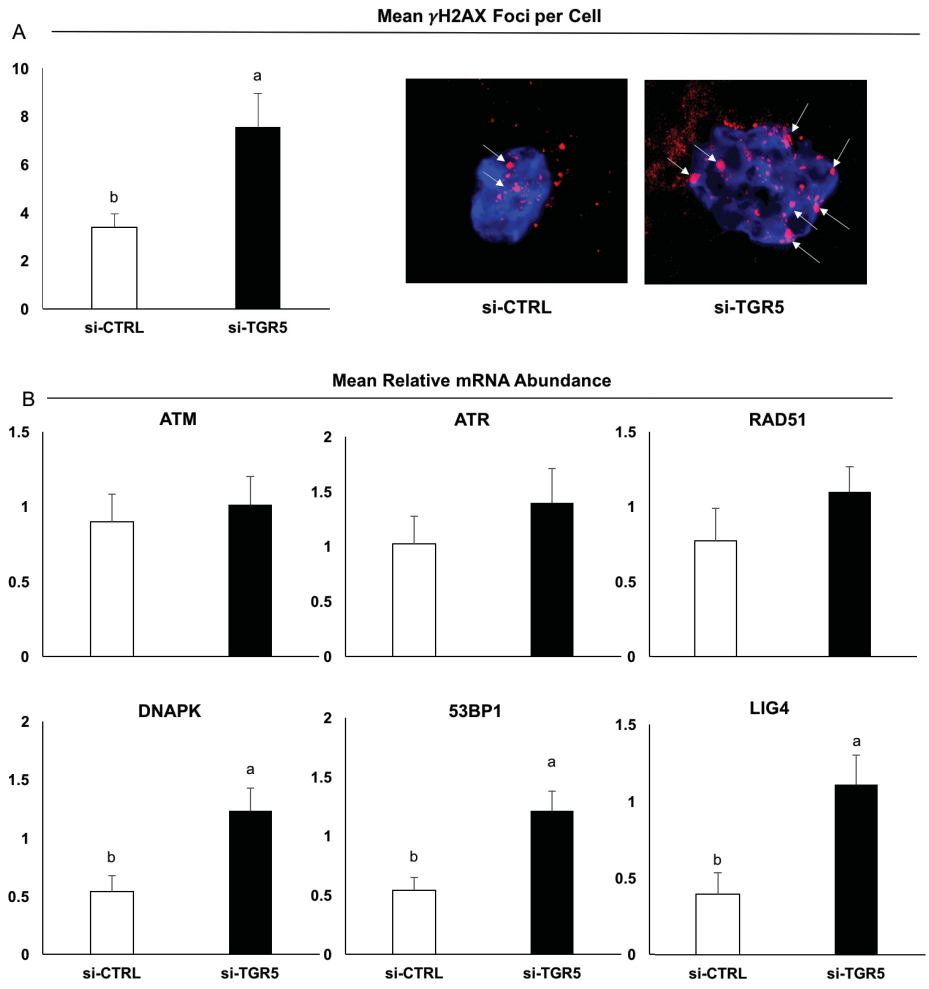

Figure 7.7.4. Altered GDR occurs in UV-exposed embryos when TGR5 activation is attenuated

The effects of TUDCA/TGR5 signaling attenuation on the GDR was assessed. PA embryos, injected with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) after activation, were subjected to 10s UV radiation and cultured with 50µM TUDCA. Developing embryos were collected at Day 5 of culture. DNA damage was assessed by γH2AX immunofluorescence and expressed as mean γH2AX foci per cell (A). Representative images of embryos are shown (A). Blue fluorescence indicates the nucleus; Red fluorescence indicates sites of phosphorylated H2AX (γH2AX); White arrows indicate γH2AX foci greater than 0.3µm^3 (sites of DNA damage). mRNA abundance of DNA damage repair genes involved with homologous recombination (ATM, ATR, RAD51) and non-homologous end-joining repair (DNAPK, 53BP1, LIG4) are shown (B). All data were collected from a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

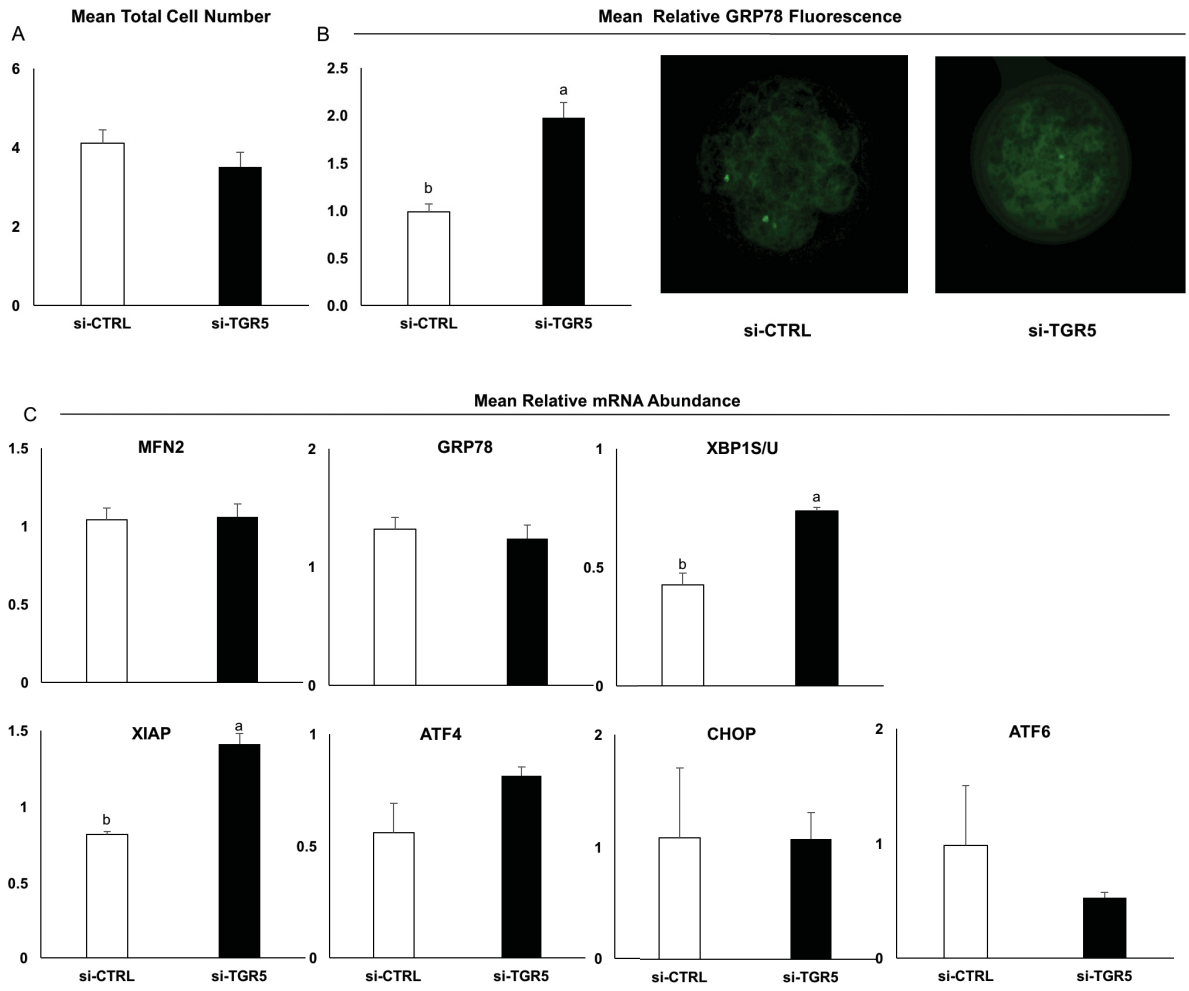

Figure 7.7.5. Sustained UPR and altered regulation of apoptosis occurs in UV-exposed embryos when TGR5 activation is attenuated

The effects of TUDCA/TGR5 signaling attenuation on the UPR as well as regulators of apoptosis were assessed. PA embryos, injected with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) after activation, were subjected to 10s UV radiation and cultured with 50µM TUDCA. Developing embryos were collected at Day 5 of culture. Mean total cell number of embryos (A) as well as mean relative mRNA abundance of genes involved with the regulation of apoptosis (MFN2, XIAP) are shown (C). ER stress was evaluated by mean relative GRP78 fluorescence (B) as well as mean mRNA abundance of UPR genes (GRP78, XBP1, ATF4, CHOP, ATF6) (C). Representative images of GRP78 fluorescence are indicated (B). XBP1 mRNA abundance was expressed as a ratio of spliced to unspliced XBP1 (XBP1S/U) (B). All data were collected from a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).

7.8 Tables

Table 7.8.1. DsiRNA sense and antisense sequences

DsiRNA	Sense	Antisense
si-TGR5	CCAAAGCAGCGUGGACCUUGACUTG	ACGGUUUCGUCGCACCUGGAACUGAAC
si-CTRL	CGUUAAUCGCGUAUAAUACGCGUAT	AUACGCGUAUUAUACGCGAUUAACGAC

Table 7.8.2. Quantitative real-time PCR primer sequences

Gene	Forward Primer	Reverse Primer	Annealing Temp. (ºC)	Accession No.
18S	GACATCTAAGGGCATCACAGA	ACACGGACAGGATTGACAGA	60	NR_046261.1
53BP1	GCAGATGGACCCTACTGGAA	GGCTTTCAGGCTGAGAATCTT	60	XM_001925938.4
ACTB	GCAGATGTGGATCAGCAAGC	GAATGCAACTAACAGTCCGCC	60	XM_021086047.1
ATF4	ACAGCAAGGAGGATGCTTTC	TGGCATGGTTTCCAGGTCAT	66	NM_001123078.1
ATM	CCGGTGTTTTGGGAGAGTGT	CTTCCGACCAAACTCAGCGT	60	NM_001123080.1
ATR	TGAGCTCCAGTGTTGGCATC	GCCAGTTCTCAGTGTGGTCA	60	XM_021069571.1
DNAPK	ATTCTTTGTCGGGAGCAGCA	CCTAGCTGTGTGGCACATGA	60	XM_021089419.1
GRP78	AGGTGATCTGGTCCTGCTTG	GTCGCTCACCTTCATAGACCTT	64	XM_001927795.5
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	60	XM_001927727.2
KEAP1	ACGTGGAGACAGAAACGTGG	GTGTCCGTGTCTGGGTCATA	60	NM_001114671.1
LIG4	AGCTAGACGGCGAACGTATG	CCTTCCTGTGGGGAAACTCC	60	XM_003131089.5
MFN2	TGGAGTCAACGCAATCAGCA	AGGGACATCGCGTTTTTGGA	58	XM_021095349.1
NRF2	AGCCCAGTCTTCATTGCTCC	CGTGCTAGTCTCAGCAAGGT	60	XM_013984303.2
RAD51	CTTCGGTGGAAGAGGAGAGC	CGGTGTGGAATCCAGCTTCT	60	NM_001123181.1
SOD1	AAGGCCGTGTGTGTGCTGAA	GATCACCTTCAGCCAGTCCTTT	60	NM_001190422.1
SOD2	GGCCTACGTGAACAACCTGA	TGATTGATGTGGCCTCCACC	66	NM_214127.2
TGR5	GTGGACCTTGACTTGAACTAGAG	TCAGAGAGGTTTGGTAGGGT	72	XM_013984487.2
XBP1s	CTGAGTCCGCAGCAGGTG	GGCTGGTAAGGAACTGGGTC	71	NM_001271738.1
XBP1u	GAGACCAAGGGGAATGGAGC	GCAGAGGTGCACGTAGTCTG	64	NM_001142836.1
XIAP	AAGCCCAGTGAAGACCCTTG	TTCAGCACTTCTCACCAGAGAC	60	NM_001097436.1

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The purpose of this thesis was to investigate cellular stress and coping responses in early embryos and how this affects their preimplantation development. The porcine model was selected for study given the readily available oocytes for experimentation as well as its similarities with human early embryo developmental kinetics and nutrient metabolism (Humpherson et al., 2005; Prather et al., 2009; Vodicka et al., 2005; Wolf et al., 2014; Bradley and Swann, 2019). Evidence explored in the literature review highlighted the negative implications of increased ER (Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b) and nuclear stress (Bohrer et al., 2015) on early embryos and how mitigation of cellular stress can improve, not only preimplantation development (Kim et al., 2012; Lin et al., 2016; Song et al., 2011; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b), but rates of pregnancy and live births (Lin et al., 2015). In particular, the beneficial effects of the natural bile acid and ER stress inhibitor, TUDCA, on preimplantation embryo development were clearly identified (Kim et al., 2012; Lin et al., 2016; Song et al., 2011; Yoon et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b), however, the mechanism of action by which it exerted these effects was not clear. Understanding how cellular stress affects early embryo development, as well as how TUDCA activates its mode of action and modulates stress coping mechanisms, represented a first step towards improving the efficiency of IVP.

In the first study (Chapter 5), investigation of how ER stress may impact cleavage kinetics was evaluated. It has been previously well documented that late-cleaving embryos have a much lower developmental potential compared to those that cleave early (Booth et al., 2007; Coutinho et al., 2011; Isom et al., 2012; Lonergan et al., 1999; Lundin et al., 2001; Sakkas et al., 1998; van Soom et al., 1997). More recently, it has been shown that these late-cleaving embryos also have increased DNA damage (Bohrer et al., 2015). Interestingly, results from this first study showed that these embryos not only have increased DNA damage, but they also have increased ER stress. Furthermore, by relieving ER stress with TUDCA, DNA damage was also reduced and embryo development improved to levels comparable to the more competent early-cleaving

embryos, suggesting a link between the UPR and GDR. Together, these findings indicated that ER stress is a dominant factor determining cleavage kinetics and the developmental potential of embryos. In other words, not all embryos are created equal and one important difference is ER stress. This has important implications for all the methods involved during IVP and even other ARTs. Optimization of techniques for gamete selection, *in vitro* maturation, and *in vitro* culture to minimize ER stress, or maximize the ability of the embryo to mitigate ER stress, has now been identified as an important factor to improve the efficiency of viable embryo production.

Given TUDCA was able to rescue developmentally incompetent late-cleaving embryos, investigation into its mechanism of action was performed. In this second manuscript (Chapter 6), TUDCA was shown to dramatically reduce development to the blastocyst stage when injected intracellularly, while incorporation of TUDCA in the culture medium increased development, as expected. These findings indicated TUDCA improved embryo development, not directly within the cell, but via an indirect mechanism of action. The G-protein-coupled TGR5 receptor is the only exclusively membrane-bound bile acid receptor (Duboc et al., 2014) and TUDCA has previously been shown to require the G_{α} stimulatory protein for its action (Vettorazzi et al., 2016). As a result, the TGR5 receptor was targeted for manipulation using DsiRNA. While TGR5 mRNA has been identified in many tissues, including the ovary (Duboc et al., 2014), this study showed for the first time that both TGR5 mRNA and protein are present in cleaved porcine embryos. Furthermore, this study showed that the TGR5 receptor was needed for TUDCA to improve development to the blastocyst stage in both IVF and PA porcine embryos. In a model of glucose-induced ER stress, this improvement in development was shown to be mediated by a decrease in ER and oxidative stress in developing embryos. Interestingly, attenuating TGR5 signaling in the face of glucose-induced ER stress altered the mRNA abundance of genes controlling pluripotency, more specifically, NANOG and OCT4. While these changes could be attributed to delayed development, it raised concerns of impaired pluripotency, which may have significant deleterious effects in the embryo and fetus. Together, these data provide insight into a potential therapeutic target to improve preimplantation embryo development. While TUDCA may not be a physiological ligand of TGR5 in the embryo (Zwicker and Agellon, 2013), further exploration of possible resident ligands, namely structurally-similar steroids, could improve our

understanding of the optimal environment for early embryo development as well as provide practical and bioavailable therapies to mitigate infertility.

While the TGR5 receptor was needed for TUDCA to reduce ER and oxidative stress, there was no effect seen on DNA damage repair. The link between the UPR and GDR implied in the first manuscript (Chapter 5), suggested a decrease in DNA damage would occur, however, this was not seen. Given DNA damage is a further downstream effect of glucose-induced ER stress, it was possible this model was not sensitive enough to evaluate GDR.

To further investigate the link between TGR5 signaling and the GDR, evaluation of the effects of TUDCA in a model of DNA damage was performed. In this third manuscript (Chapter 7), TUDCA was able to rescue development of embryos to the blastocyst stage after exposure to UV radiation, both in IVF and PA porcine embryos. Evaluation of developing embryos demonstrated that this improvement in development coincided with a decrease in DNA damage and ER stress. While TUDCA has previously been shown to reduce ER stress in the face of UV radiation in liver cells (Uppala et al., 2017), it has never been shown to ameliorate DNA damage before. Together, these findings provided further evidence of a link between the UPR and GDR (Dicks et al., 2015). In addition, the TGR5 receptor was shown to be needed for the beneficial effect of TUDCA on these UV irradiated embryos. In fact, sustained activation of the UPR and altered GDR was shown in these UV-exposed embryos that had disrupted TGR5 signaling. These data demonstrated coordinated activation of both the UPR and GDR through the TGR5 pathway. Remarkably, impaired TGR5 signaling in the face of DNA damage resulted in the upregulation of genes involved in NHEJ repair, an error-prone DNA repair pathway (Mimitou and Symington, 2009). This was intriguing given embryos rely primarily on the more precise HR repair pathway during early embryo development (Bohrer et al., 2018). Altered GDR responses as a result of disrupted TGR5 signaling can have important implications on genome stability and viability in developing embryos. Further investigation and targeted activation of TGR5 pathways represent a potential means to promote physiological over pathological DNA damage repair and improved embryo development.

While the manuscripts in Chapter 6 and 7 clearly identified that the mechanism of TUDCA action occurs through stimulation of the TGR5 receptor, its mode of action remains unclear. The intricacies of this mode of action are key to understanding the link between the UPR and GDR. Given the physical nature of the cellular reticular network (Agellon and Michalak, 2017), it seems evident that responses to stress in the ER would exhibit crosstalk with the nuclear membrane and nucleus. In addition to this physical connection, other links between these coping responses exist. For example, nutrient and energy deficiencies that activate the UPR have also been shown to affect the GDR (Bristow and Hill, 2008; Yamamori et al., 2013). Furthermore, regulation of apoptosis through GADD proteins, such as GADD153 (CHOP) and GADD34, which were originally identified for their expression after UV exposure (Hollander et al., 1997), are also involved in both stress coping responses (Jeon et al., 2016; Marciniak et al., 2004; Szegezdi et al., 2006). Another link between the UPR and GDR is through chromatin remodeling. Similar changes in histone acetylation and methylation that occur during the UPR and the GDR were reviewed in Chapter 3 in the context of cancerous cells, which behave similarly to those of the developing embryo (Ma et al., 2010). In addition, activation of cellular stress signaling proteins such as c-Abl and the JNK MAPK have been implicated in both the GDR and UPR (Ito et al., 2001; Kharbanda et al., 1998; Meltser et al., 2011; Urano et al., 2000). Interestingly, TUDCA has also been shown to activate other MAPK, including p38 and ERK (Schoemaker et al., 2004). Other signaling pathways have equally been implicated in the mode of action of TUDCA, such as the PI3K (Schoemaker et al., 2004), PKA (Vettorazzi et al., 2016; Wang et al., 2005) and PKB/AKT pathways (Dent et al., 2005). Notably, these pathways all interact with the PI3K-PKB pathway (Cosentino et al., 2007; McGuire et al., 2013; Wang et al., 2011). While data from this thesis (Chapter 7) demonstrated TUDCA affects the GDR, which is orchestrated by members of the PIKK family (Blackford and Jackson, 2017), the PIKK, DNA-PK, has previously been shown to phosphorylate and activate PKB to promote survival after DNA damage induced by ionizing radiation (Bozulic et al., 2008). Together these data suggest activation of TGR5 by TUDCA may ultimately activate the PI3K-PKB pathway, however, this remains to be proven. While Chapter 7 of this thesis provides evidence of coordinated activation of the UPR and GDR through the TGR5 pathway, understanding the mode of action by which TUDCA functions will reveal the precise mechanisms that link these two coping responses.

In conclusion, data from this thesis revealed that: 1) ER stress is a determining factor differentiating developmentally-incompetent late-cleaving embryos; 2) TUDCA-treated late-cleaving embryos can develop at similar rates as the developmentally-competent early-cleaving embryos; 3) TGR5 receptor is present in early porcine embryos; 4) TGR5 receptor is needed for TUDCA to reduce ER stress, oxidative stress, DNA damage and improve embryo development; 5) TUDCA reduces both nuclear and ER stress in early embryos after exposure to UV radiation; 6) Disrupted TGR5 signaling in the face of cellular stress results in sustained UPR and altered GDR; and 7) The UPR and GDR are coordinately activated through TGR5 pathways. Together, these results advance our understanding of cellular stress and coping responses in early embryo development, which is necessary to improve IVP and mitigate infertility.

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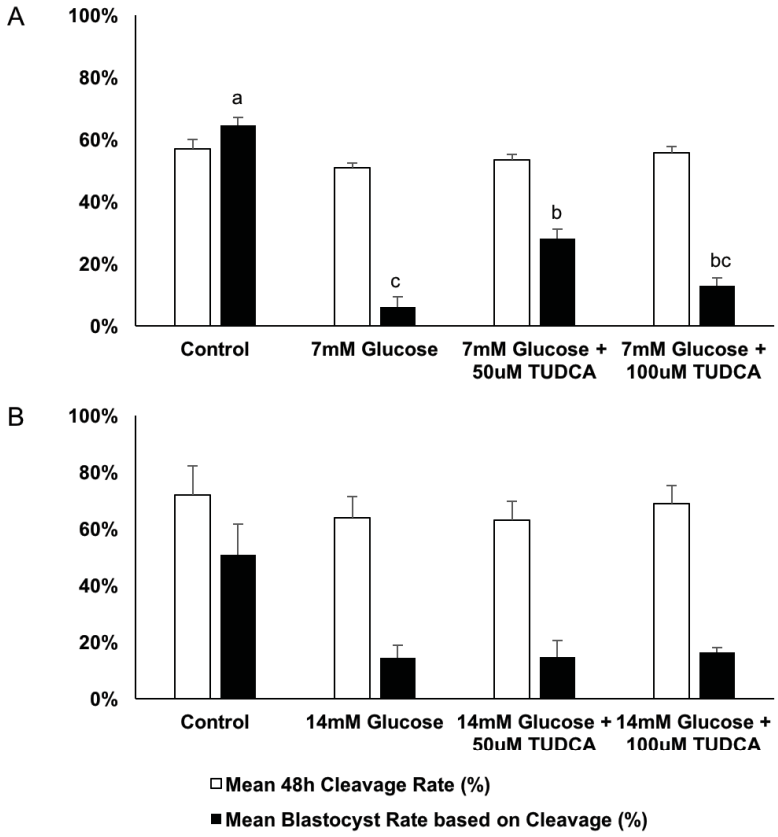
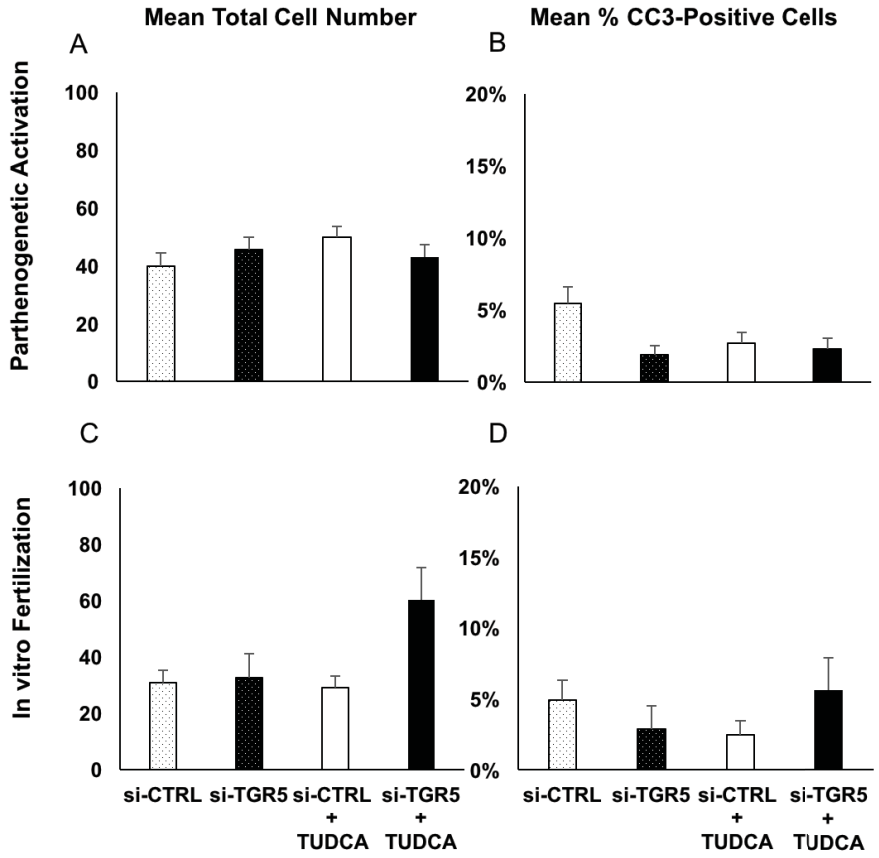
CHAPTER 9

APPENDICES



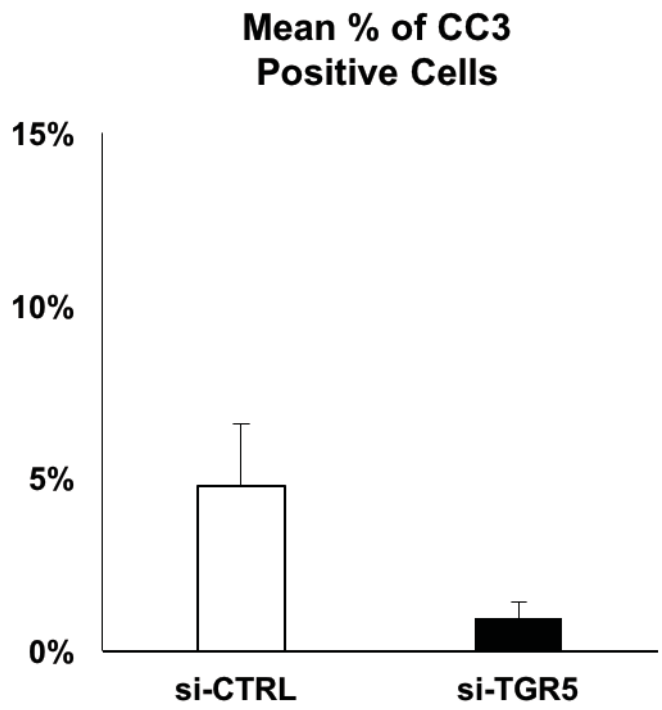
Appendix 9.1. Optimal glucose and TUDCA concentrations in an embryo model of stress and recovery

Optimal glucose and TUDCA concentrations were evaluated in a model of stress and recovery in porcine embryos. PA embryos were cultured in 7mM (A) or 14mM glucose (B) and the rescue effects on development of 50**µ**M and 100**µ**M TUDCA were compared. PA embryos cultured in standard IVC served as the control. Rate of embryo cleavage (open bars) after 48h, and rate of blastocyst development based on cleavage (black bars) are indicated.Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).



Appendix 9.2. Neither TUDCA or TGR5 affect total cell number or percentage of apoptotic cells in blastocysts under standard culture conditions

Parthenogenetically activated (PA) and *in vitro* fertilized (IVF) porcine embryos were injected with si-CTRL or si-TGR5 and cultured with or without 50μM TUDCA. After 7 days of culture, blastocysts were collected. Total cell number (A,C) and percentage of apoptotic cells based on CC3 immunofluorescence (B,D) were assessed in PA and IVF blastocysts, respectively. Data represent a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).



Appendix 9.3. Mean percentage of CC3-positive cells in TUDCA-treated si-CTRL and si-TGR5 blastocysts after UV exposure

The necessity of TGR5 signaling for TUDCA to improve embryo development after DNA damage was investigated. PA embryos, injected with either scrambled control DsiRNA (si-CTRL) or TGR5 DsiRNA (si-TGR5) after activation, were subjected to 10s UV radiation and cultured with 50μM TUDCA. Apoptosis was assessed by mean percentage of CC3-positive cells using immunofluorescence staining. All data were collected from a minimum of three replicates. Significant differences are indicated by different lower case letters ($P<0.05$).