DNA double-strand breaks in early developing embryos: occurrence, consequences and regulation of repair mechanisms

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ABSTRACT

DNA DSBs are the most important type of DNA damage because their defective repair can induce genome instability, deleterious mutations and cellular death. Studies, most using mice and humans, have shown that DNA DSBs is an important component affecting early embryo development and somatic cell reprogramming during the production of iPS cells. However, the occurrence and effects of DNA DSBs, and the mechanism involved in DNA DSBs repair have not been systematically investigated during early embryo development and cell reprogramming in embryos produced by somatic cell nuclear transfer (SCNT). Therefore, in the first manuscript of this thesis, we characterized the occurrence of DNA DSBs during early development of IVF and SCNT porcine embryos. We observed that: i) DNA DSBs occur in a high proportion of embryos; ii) fast-cleaving embryos have fewer DNA DSBs than slow-cleaving embryos; iii) the oocyte activation protocol can affect DNA integrity in SCNT embryos; and iv) better-quality blastocysts have fewer DNA DSBs. In the second manuscript of this thesis, we investigated the effect of HDACi treatment on DNA DSBs repair and development of SCNT embryos. Our findings revealed that: i) DNA DSBs in nuclear donor cells is an important component affecting development of SCNT embryos; and ii) HDACi treatment after nuclear transfer enhances DNA DSBs repair and development of SCNT embryos. In the third manuscript, we compared the occurrence of DSBs between early- and late-cleaving embryos. Our results showed that the presence of DNA DSBs affects the cleavage kinetics and embryo developmental capacity to the blastocyst stage. Finally, in the fourth manuscript of this thesis, we assessed the importance of the HR and the NHEJ pathways for DNA DSBs repair during early embryo development. Our findings revealed that: i) the HR is the main DNA DSB repair pathway during early embryo development; and ii) the HR pathway is mainly activated by the protein kinase ATM. Together, findings reported

in this thesis revealed that DNA DSBs have important detrimental consequences on early embryo development and somatic cell reprogramming in SCNT embryos, and that the HR pathway is mainly responsible for DNA DSBs repair in preimplantation embryos. These findings have implications on fertility, development and assisted reproductive technologies. For instance, discoveries from our studies can be used to test and optimize in vitro culture environments and conditions having less detrimental effects on genome integrity and consequently improve the developmental rate quality of embryos that are transferred to recipient females.

RÉSUMÉ

Les ruptures bicaténaires de l'ADN représentent la plus importante sorte de dommage à l'ADN car elles peuvent causer de l'instabilité génomique, des mutations délétères, et même la mort cellulaire si la réparation est défectueuse. Plusieurs études, dont la plupart sont effectuées chez la souris et l'humain, démontrent que les ruptures bicaténaires de l'ADN ont un effet important dans le développement embryonnaire précoce et la reprogrammation des cellules somatiques lors de la production des cellules iPS. Cependant, l'occurrence et l'effet des ruptures bicaténaires de l'ADN, et le mécanisme de réparation des ruptures bicaténaires de l'ADN, n'ont jamais été caractérisés systématiquement pendant le développement embryonnaire précoce, ni pendant la reprogrammation des embryons crées par le transfert nucléaire de cellules somatiques (TNCS). Alors, dans le premier manuscrit de cette thèse, nous avons caractérisé l'occurrence des ruptures bicaténaires de l'ADN dans le développement précoce des embryons porcins crées par FIV et TNCS. Nous avons observé que : i) les ruptures bicaténaires de l'ADN sont présentes dans une grande proportion des embryons ; ii) les embryons à clivage rapide ont moins de ruptures bicaténaires de l'ADN que les embryons à clivage lente; iii) le protocole d'activation des ovocytes peut affecter l'intégrité de l'ADN dans les embryons crées par TNCS ; et iv) les blastocystes de meilleure qualité ont moins de ruptures bicaténaires de l'ADN. Dans le deuxième manuscrit de cette thèse, nous avons investigué l'effet des iHDAC sur la réparation des ruptures bicaténaires de l'ADN et le développement des embryons créés par TNCS. Nos trouvailles révèlent que : i) les ruptures bicaténaires de l'ADN dans les cellules nucléaires transférées représentent un élément important dans le développement des embryons créés par TNCS ; et ii) traitement avec iHDAC après transfert nucléaire améliore la réparation des ruptures bicaténaires de l'ADN et le développement des embryons créés par TNCS. Dans le troisième manuscrit, nous avons comparé

l'occurrence des ruptures bicaténaires de l'ADN dans les embryons à clivage lente et les embryons à clivage rapide. Nos résultats démontrent que les ruptures bicaténaires de l'ADN affectent les cinétiques de clivage et la capacité de développement embryonnaire au stade de blastocyste. Finalement, dans le quatrième manuscrit de cette thèse, nous avons vérifié l'importance des systèmes de réparation par recombinaison homologue et par jonction d'extrémités non-homologues (NHEJ) dans la réparation des ruptures bicaténaires de l'ADN pendant le développement embryonnaire précoce. Nos trouvailles révèlent que : i) le système de réparation par recombinaison homologue est le système de réparation principal dans le développement embryonnaire précoce ; et ii) le système de réparation par recombinaison homologue est activé principalement par la protéine kinase ATM. Ensemble, les données rapportées dans cette thèse révèlent que les ruptures bicaténaires de l'ADN ont des effets nocifs importants dans le développement embryonnaire précoce et la reprogrammation des cellules somatiques dans les embryons créés par TNCS, et que le système de réparation par recombinaison homologue est principalement responsable de la réparation des ruptures bicaténaires de l'ADN dans les embryons préimplantatoires. Ces trouvailles ont des conséquences importantes pour la fertilité, le développement et les technologies de reproduction assistées. Par exemple, les découvertes de nos études peuvent être utilisées pour tester et optimiser l'environnement et les conditions de culture *in vitro* pour minimiser les effets nocifs à l'intégrité de l'ADN, et en conséquent, améliorer le taux de développement et la qualité des embryons transférés aux femelles.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was prepared in accordance with the McGill University thesis preparation guidelines in a manuscript-based format. It was written by Rodrigo C. Bohrer, with editorial help of Dr. Vilceu Bordignon. The chapters 1 and 2 consist of introduction and literature review, respectively. The section 2.1 of the literature review, entitled as "Cloning animals by nuclear transfer", was published as a chapter in the book "Biotechnology of Animal Reproduction". Bohrer R.C. and Bordignon V. (2016) Cloning Animals by Nuclear Transfer. In: MM Seneda, KC Silva-Santos, LSR Marinho editors. Biotechnology of Animal Reproduction. Nova Science Publishers, Inc. (NOVA), New York, Ch. 13, pp 293-313.

The chapter 4 of this thesis consists in the first manuscript, entitled "Phosphorylated histone H2A.x in porcine embryos produced by IVF and somatic cell nuclear transfer", that was published in the "Reproduction" journal. Rodrigo C. Bohrer was responsible for the conduction of most experiments, data analysis and manuscript writing. Dr. Limei Che was responsible for the conduction of some experiments and data collection. Dr. Paulo Bayard Dias Gonçalves, Dr. Raj Duggavathi and Dr. Vilceu Bordignon contributed during experiment design, data analysis and manuscript preparation.

The chapter 5 of this thesis consists in the second manuscript, entitled "Inhibition of histone deacetylases enhances DNA damage repair in SCNT embryos", that was published in the "Cell Cycle" journal. Rodrigo C. Bohrer was responsible for the conduction of experiments, data analysis and manuscript writing. Dr. Raj Duggavathi and Dr. Vilceu Bordignon contributed during experiment design, data analysis and manuscript preparation.

The chapter 6 of this thesis consists in the third manuscript, entitled “The Incidence of DNA Double-Strand Breaks Is Higher in Late-Cleaving and Less Developmentally Competent Porcine Embryos”, that was published in the “Biology of Reproduction” journal. Rodrigo C. Bohrer was responsible for the conduction of most experiments, data analysis and manuscript writing. Dr. Ana Rita S. Coutinho was responsible for the conduction of some experiments and data collection. Dr. Raj Duggavathi and Dr. Vilceu Bordignon contributed during experiment design, data analysis and manuscript preparation.

The chapter 7 of this thesis consists in the fourth manuscript, entitled “Double-strand DNA breaks are mainly repaired by the homologous recombination pathway in early developing embryos”, which is under preparation for submission to the “Development” journal. Rodrigo C. Bohrer was responsible for the conduction of most experiments, data analysis and manuscript writing. Naomi Dicks and Karina Gutierrez helped in the conduction of experiments involving oocyte collection, media preparation and embryo culture. Dr. Raj Duggavathi and Dr. Vilceu Bordignon contributed during experiment design, data analysis and manuscript preparation.

LIST OF ABBREVIATIONS

5-aza-dC – 5-aza-2'-deoxycytidine

6-DMAP – 6-dimethylaminopurine

53BP1 – tumor suppressor p53-binding protein 1

ACTB – actin beta

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia and Rad3 related

ATRIP – ATR-interacting protein

BASC – BRCA1-Associated Genome Surveillance Complex

BRCA1 – breast cancer type 1 susceptibility protein

BSA – bovine serum albumin

BSE – bovine spongiform encephalopathy

cDNA – complementary DNA

CHEK1 – checkpoint kinase 1

CHEK2 – checkpoint kinase 2

Chk1 – Serine/threonine-protein kinases 1

Chk2 – Serine/threonine-protein kinases 2

CJD – Creutzfeld-Jacob disease

COCs – cumulus-oocyte complexes

CRISPR – clustered regularly interspaced short palindromic repeats

CtBP – C-terminal binding protein

CtIP – C-terminal binding protein (CtBP)-interacting protein

D3 – day 3

D5 – day 5

D7 – day 7

dbcAMP – dibutyryl cyclic adenosine monophosphate

DMEM – dulbecco's modified eagle's medium

DNA – deoxyribonucleic acid

DNA-PK – DNA-dependent protein kinase

DSBs – double-strand breaks

eCG – equine chorionic gonadotropin

EGA – embryonic genome activation

EGF – epidermal growth factor

FBS – fetal bovine serum

FSH – follicle-stimulating hormone

Gcn5 – histone acetyltransferase KAT2A

H2AX139ph – phosphorylated histone H2A.x

H3K9 – histone H3 lysine 9

H3K14 – histone H3 lysine 14

H3K18 – histone H3 lysine 18

H3K23 – histone H3 lysine 23

H4K5 – histone H4 lysine 5

H4K8 – histone H4 lysine 8

H4K12 – histone H4 lysine 12

H4K16 – histone H4 lysine 16

H4K20me2 – histone H4 dimethylated in the lysine 20

HATs – histone acetyltransferases

hCG – human chorionic gonadotropin

HDAC – histone deacetylases

HDACi – histone deacetylase inhibitors

HR – homologous recombination

ICM – inner cell mass

IGF-I – insulin-like growth factor I

IgG – immunoglobulin G

ION – ionomycin

iPS cells – induced pluripotent stem cells

IVM – in vitro maturation

KU70 – X-ray repair cross-complementing protein 6;

KU80 – X-ray repair cross-complementing protein 5

LH – luteinizing hormone

LIG4 – ligase IV

MMSET– Multiple myeloma SET domain-containing protein

MPF – M-phase Promoting Factor

MRE11 – double-strand break repair protein MRE11A

MRE11A – MRE11 meiotic recombination 11 homolog A

mRNA – messenger ribonucleic acid

NHEJ – nonhomologous end-joining

NSD2 – Nuclear SET domain-containing protein 2

PAGE – polyacrylamide gel electrophoresis

PARP1 – poly (ADP-ribose) polymerase 1

PB – polar body

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PRKDC – protein kinase, DNA-activated, catalytic polypeptide

PVA – polyvinyl alcohol

PZM-3 – porcine zygote medium

RAD51 – DNA repair protein RAD51 homolog 1

RNA – ribonucleic acid

RNF168 – E3 ubiquitin-protein ligase RNF168

RNF8 – E3 ubiquitin-protein ligase RNF8

ROS – reactive oxygen species

RPA – replication protein A

SCNT – somatic cell nuclear transfer

SDS – sodium dodecyl sulfate

$Sr2^{+}$ – strontium chloride

SSB – single-strand breaks

ssDNA – single-stranded DNA

TALEN – transcription activator-like effector nucleases

TBS – tris-buffered saline

TP53BP1 – tumor protein p53 binding protein 1

TSA – Trichostatin A

UV – ultraviolet

VPA – Valproic acid

WHSC1 – Wolf-Hirschhorn syndrome candidate 1 protein

XRCC4 – X-ray repair complementing defective repair in Chinese hamster cells 4

XRCC5 – X-ray repair complementing defective repair in Chinese hamster cells 5

XRCC6 – X-ray repair complementing defective repair in Chinese hamster cells 6.

ZFN – zinc finger nucleases

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CHAPTER 1

1.1. INTRODUCTION

Studies have recently shown that the occurrence of DNA double-strand breaks (DSBs) is an important factor affecting early embryo development. Indeed, induction of DSBs in either male or female pronuclei reduced the cleavage rate and impaired blastocyst formation (Wang et al., 2013b). Moreover, embryo development is compromised after fertilization with sperm containing increased number of DNA damage (Simon et al., 2014). These findings indicate that preserving genome integrity is a critical component for early embryo development. Occurrence of DSBs during early embryo development also have consequences on post-implantation development, by reducing implantation rates and compromising pregnancy outcome in human (Simon et al., 2014). Despite of having been thoroughly investigated in somatic cells, the occurrence, consequences and regulation of mechanisms involved in DNA DSBs repair have not been sufficiently explored during early embryo development.

Studies in somatic cells have shown that DNA DSBs are the most important type of DNA damage because their defective repair can induce genome instability, deleterious mutations and cellular death (Rich et al., 2000, Khanna and Jackson, 2001, Peterson and Côté, 2004, Furgason and Bahassi el, 2013). DNA DSBs can be caused by endogenous factors such as by-products of cellular metabolism and collapsed DNA replication forks (Lindahl and Wood, 1999, De Bont and van Larebeke, 2004), and by exogenous factors including ultraviolet (UV) light, ionizing irradiation and chemical agents (Grigaravičius et al., 2009, Mu et al., 2011). There are two main pathways involved in DSBs repair, the homologous recombination (HR) and the nonhomologous end-joining (NHEJ) pathway (Karran, 2000, Stiff et al., 2004, Stiff et al., 2006).

Previous studies have shown that preservation of genome integrity is indispensable for somatic cell reprogramming and production of induced pluripotent stem cells (iPS) (Marion et al., 2009). Moreover, defective DNA DSBs repair by either HR or NHEJ pathways impair cell reprograming and iPS production (González et al., 2013, Molina-Estevez et al., 2013). Findings from these studies suggest that increased DNA DSBs incidence and this defective repair could also affect cell reprogramming and development of embryos produced by somatic cell nuclear transfer (SCNT).

Previous studies conducted in our lab have shown that treatment of SCNT embryos with histone deacetylase inhibitors (HDACi) improved cell reprogramming and cloning efficiency in pig (Martinez-Diaz et al., 2010). It is thought that HDACi treatment improves cell reprogramming after SCNT by increasing chromatin accessibility to reprogramming factors present in the oocyte cytoplasm (Iager et al., 2008, Zhao et al., 2010, Wang et al., 2011b). Better access to chromatin is also required for DNA repair, since it facilitates the activation and action of signaling and repair proteins at the sites of DNA DSBs (Lee et al., 2010, Sharma et al., 2010, Ogiwara et al., 2011). Therefore, we hypothesized that HDACi treatment would also facility DSBs repair and preserve genome integrity during cell reprogramming in SCNT embryos.

The main research goal in this thesis was to get a comprehensive view of the occurrence, consequences and mechanisms involved in DSBs repair during early embryo development and cell reprograming in SCNT embryos. For that, in the first study we characterized the occurrence of DNA DSBs during early embryo development. In the second study, we investigated the effect of HDACi treatment on DNA DSBs occurrence and repair during cell reprogramming in SCNT embryos. In the third study, we evaluated the effects of DNA DSBs on embryo cleavage kinetics and developmental capacity to reach the blastocyst stage. In the fourth study, we assessed the

importance and regulation of the HR and NHEJ pathways for DNA DSBs repair during early embryo development.

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and in vivo developmental competence of somatic cell nuclear transfer porcine embryos. Cell Reprogram., 12, 75-83. doi: 10.1089/cell.2009.0038.

CHAPTER 2

LITERATURE REVIEW

The first section of the Literature Review was published as chapter in the book entitled "Biotechnology of Animal Reproduction", chapter 13: "CLONING ANIMALS BY NUCLEAR TRANSFER" (Bohrer R.C. and Bordignon V., 2016).

2.1. CLONING ANIMALS BY NUCLEAR TRANSFER

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2.1.1. Abstract

Since the first adult animal, Dolly the sheep, was cloned from a somatic cell, more than 20 different species of animals have been cloned by somatic cell nuclear transfer (SCNT). Animal cloning has many applications including the replication of genetically superior farm animals, rescuing of endangered species, and the production of genetically modified animals for research. However, the efficiency of animal cloning by SCNT remains very low. This is likely due to insufficient nuclear reprogramming leading to altered gene expression during embryo and fetal development. However, recent attempts to improve nuclear reprogramming have significantly improved animal cloning efficiency, particularly in laboratory species. This indicates that more effective protocols for SCNT cloning may also be developed for large animal species, including

livestock. This chapter presents an overview of animal cloning history, applications, technical approaches, limitations and proposed alternatives for improving SCNT cloning efficiency.

Keywords: animal cloning, nuclear transfer, cell reprogramming, epigenetics, embryo development

2.1.2. Introduction

Somatic cell nuclear transfer (SCNT) is a technique that involves the transfer of a somatic cell nucleus to the cytoplasm of an enucleated oocyte. Factors present in the oocyte cytoplasm are able to reprogram differentiated cells back to an embryonic state, which allows creation of cloned animals. Thus, SCNT has many applications including production of high-quality farm animals or endangered animals, and creating genetically modified animals for used in research.

Following the production of the first adult cloned mammal, Dolly the sheep, from a somatic cell derived from the mammary gland, SCNT has been used to clone animals from various species, including domestic and wild animals. However, the developmental efficiency of SCNT embryos compared to embryos produced by other assisted reproductive technologies, including in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI), remains much lower. This is likely due to insufficient or aberrant epigenetic reprogramming of the transferred somatic nucleus. It has been commonly observed in a number of studies that embryos produced by SCNT have higher DNA and histone methylation patterns compared to control embryos. Moreover, hypoacetylation of different lysine residues has been reported in a number of publications. These changes result in abnormal gene regulation, which compromises pre- and post-implantation development of SCNT embryos. Different approaches have been tested attempting to increase

epigenetic reprograming in SCNT embryos. Treatment with epigenetic modulators, especially inhibitors of histone deacetylase enzymes (HDACi), has been shown to improve cell reprogramming and increase animal cloning efficiency from somatic cells in various species. The main objective of this chapter is to provide an overview on the SCNT technology used for animal cloning including historical aspects, applications, efficiency, problems and alternatives for improving animal cloning efficiency.

2.1.3. History of Animal Cloning by Nuclear Transfer

Nuclear transfer was first performed in the early 1950s in frogs, to transfer embryonic cell nuclei to enucleated oocytes with the objective of studying cellular differentiation (Briggs and King, 1952). Nuclear transfer technology was later adapted to mammals for the transfer of embryonic cell nuclei to enucleated oocytes, which are approximately 1,000 times smaller than frog' eggs. The first clones of mammals from embryonic cells were produced in the 1980s, including mice (Illmensee and Hoppe, 1981), rabbits (Stice and Robl, 1988), sheep (Willadsen, 1986), cattle (Prather et al., 1987) and pigs (Prather et al., 1989). Gurdon (1962) reported the production of cloned frogs after transferring somatic cell nuclei to enucleated eggs. This was the first study demonstrating that differentiated cells can be reprogrammed to a totipotent state. This study paved the way for other ground-breaking studies by Wilmut et al. (1997) and Takahashi and Yamanaka (2006). These studies have produced the first cloned mammal from a somatic cell of an adult animal, and induced somatic cells in culture to reprogram into pluripotent stem cells (iPS), respectively. For their significant contribution to the field of cellular reprograming, which has great promise for development of cell-based therapies, Gurdon and Yamanaka were awarded the Nobel Prize in Physiology or Medicine in 2012.

2.1.4. Cloning Applications

The production of the first adult cloned animal from a differentiated cell has boosted interest in the use of SCNT as a reproductive method for different animal species and applications.

2.1.4.1. Cloning to Rescue Endangered Species

The fact that differentiated cells can be reprogrammed to an embryonic state opened a new avenue for preservation of endangered species, for which normal breeding is not an option due to limited number of animals. A cow of the Enderby Island breed was the first endangered animal cloned by SCNT using host oocytes from other cattle breeds (Wells et al., 1998). The availability of host oocytes is one of the main limitations for the application of SCNT to rescue endangered species. One option would be interspecies SCNT cloning, which consists of using host oocytes from a different species than the nuclear donor cells. Given their availability and well developed condition for in vitro maturation, oocytes from domestic species would be the ideal option for interspecies SCNT cloning. The idea of interspecies SCNT cloning comes from experiments in the early days of SCNT showing that nuclei of mammal somatic cells could be reprogrammed by amphibian oocytes.

Interspecies SCNT cloning has been successful when donor cells and recipient oocytes are from closely related species. Examples include the cloning of gaur (Bos gaurus) using bovine oocytes (Bos taurus), mouflon (Ovis orientalis musimon) using domestic sheep oocytes (Ovis aries), the African wildcat (Felis silvestris lybica) using domestic cat oocytes (Felis catus), gray wolf (Canis lupus) using domestic dog oocytes (Canis lupus familiaris), sand cat (Felis margarita)

using domestic cat oocytes, and coyote (Canis latrans) using domestic dog oocytes (Lanza et al., 2000, Loi et al., 2001, Gomez et al., 2004, Kim et al., 2007, Aguilera and Gomez-Gonzalez, 2008, Srirattana et al., 2012, Hwang et al., 2013).

Interspecies SCNT cloning using far related species have not been successful and no live cloned animals have been produced so far, which is likely a consequence of molecular incompatibilities between the donor cell and the recipient oocyte. Indeed, SCNT embryos constructed with nuclear donor cells and host oocytes from far related species seem to progress normally through the first cell division, but show developmental abnormalities starting near the embryonic genome activation (EGA) period. Before EGA, which is mainly controlled by the recipient oocyte, DNA transcription and corresponding mRNA translation by the somatic nucleus has to be stopped. However, this process seems to be defective in interspecies SCNT embryos, which affects normal embryo development (Wang et al., 2011a). In addition, the efficiency of maternal mRNA degradation, which is important for normal embryo development after EGA, is reduced in embryos produced by interspecies SCNT compared to conventional SCNT embryos. Studies have also reported aberrant expression of pluripotency genes that are important for normal development (e.g., NANOG, OCT4) after EGA in interspecies SCNT embryos, which indicates abnormal nuclear reprogramming (Arat et al., 2003, Lagutina et al., 2010).

2.1.4.2. Cloning to Multiply Elite Farm Animals

A large number of farm animals have been cloned by SCNT over the last 2 decades. Although many studies have been conducted attempting to improve the efficiency of the technology, animal cloning by SCNT is still very inefficient compared to in vitro fertilization.

Indeed, the percentage of transferred embryos that develop to viable offspring has often ranged from 1 to 5% (Table 1). Despite the low efficiency, SCNT allows making copies of elite animals with desirable phenotypic traits, such as increased milk production, feed conversion, growth rate, total milk protein, and meat/carcass quality.

There has been some concern about safety of foods derived from cloned animals or their offspring. However, chemical and biological properties of products from cloned cattle were shown to be equivalent to products from control cattle. Also, the nutritional value of milk and meat was similar in cloned and control cattle (Takahashi and Ito, 2004, Tome et al., 2004). Based on those analyses, the US Food and Drug Administration (FDA) concluded that meat and milk from cloned cattle, swine, goats or their offspring are as safe as those from animals produced through conventional breeding. Nevertheless, regulation of animal cloning differs in various countries around the world. In Canada, the department of health (Health Canada) considers products derived from cloned animals as novel food. These products are subject to food and drug regulations and cannot enter the human food supply until a pre-market safety assessment has been conducted. In Europe, the European Parliament proposed a ban of any product derived from cloned animals or their descendants.

Table 2.1. SCNT embryo development and cloning efficiency in different animal species

Species	Cleavage	Blastocyst	Efficiency	References
Swine	70-82%	11-41%	0-5%	(Martinez-Diaz et al., 2010, Zhao et al., 2010, Kurome et al., 2013, Li et al., 2013)
Bovine	70-80%	12-40%	3.3-14%	(Cibelli et al., 1998, Yang et al., 2007, Sangalli et al., 2014)
Ovine	73-91%	9-54%	3-17%	(Wilmut et al., 1997, Alexander et al., 2006, Wen et al., 2014)
Caprine	35-84%	20-34%	1-7%	(Baguisi et al., 1999, Baldassarre et al., 2004, Liu et al., 2011, Feng et al., 2015)
Equine	60-100%	3-37%	0-3%	(Galli et al., 2007, Gambini et al., 2012, Choi et al., 2013)
Murine	49-90%	26-66%	1-4.9%	(Wakayama et al., 1998, Yang et al., 2007, Mallol et al., 2015)

2.1.4.3. Cloning to Produce Transgenic Animals

Pronuclear microinjection of DNA was the first technique developed to produce transgenic animals. However, with the exception of mice, the efficiency of this technique is low. In general, less than 10% of the animals produced by pronuclear microinjection express the transferred gene. Animal cloning by SCNT from transgenic cells has increased the efficiency to produce transgenic animals compared to pronuclear injection of DNA, particularly in domestic animal species. SCNT has a number of advantages that include the possibility of selecting nuclear donor cells having correct integration and expression of the transferred gene before use for cloning a transgenic

animal. It also allows the creation of cloned animals after gene targeting in cultured cells. Moreover, SCNT permits exclusive creation of transgenic animals of the desired sex, depending on the nuclear donor cell used for cloning. Finally, transgenic nuclear donor cells can be easily cryopreserved and stored for further creation of more transgenic animals as needed. Examples of transgenic animals that were created by SCNT from genetically manipulated cells include sheep expressing the human coagulation factor IX (Schnieke et al., 1997), transchromosomic cattle producing human polyclonal antibodies (Kuroiwa et al., 2002), cows producing milk with higher levels of casein (Brophy et al., 2003), cows resistant to intramammary Staphylococcus aureus infection (Wall et al., 2005), pigs with omega-3 fatty acid-enriched meat (Lai et al., 2006) and lambs with a deletion in the PrP gene, which encodes the Prion protein that is associated with scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeld-Jacob disease (CJD) in humans (Denning et al., 2001).

There are different techniques that can be used to induce gene manipulation in somatic cells cultured in vitro. Traditional techniques such as homologous recombination (HR) have low efficiency and require long periods of cell culture and selection prior to SCNT, which may reduce the efficiency of animal cloning from those cells. More recently, engineered meganucleases, including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems, have been developed for targeted genome editing and gene manipulation in eukaryotic cells with much higher efficiency than HR. These meganucleases have been used for gene manipulation in a variety of cell types and animals including the production of biallelic knockouts in large animal species (Hauschild et al., 2011, Carlson et al., 2012, Whitworth et al., 2014). The combination of SCNT

with gene editing technologies will further facilitate the production of genetically engineered animals.

2.1.4.4. Cloning to Produce Animals for Biomedical Research

Cloning by SCNT has been used to produce unique animal models for biomedical and biopharmaceutical purposes. Examples include the production of animals expressing recombinant human proteins in their milk, which can be purified and then used as therapeutics. The first recombinant protein approved for medical use in humans was the Antithrombin III (ATryn®) from GTC-Biotherapeutics, produced in the mammary gland of transgenic goats (Baguisi et al., 1999). Currently, other recombinant proteins produced in the milk of transgenic animals are in different phases of clinical trials.

SCNT has also been used to produce animal models for the study of human diseases. Traditionally, mice models have been produced by pronuclear injection or by embryonic stem cell technology. However, translational research using mice models has important limitations due to differences in physiology, metabolism, body size and short life span compared to humans. Since pronuclear injection is not effective and stem cell technology is not well developed, cloning by SCNT represents the method of choice for the production of large animal models for research. In comparison to other large animals, there has been growing interest in using pigs as animal models for the study of human diseases because of physiopathological, anatomical and metabolic similarities with humans. Examples of porcine models that have been created for the study of human diseases include Alzheimer's, cystic fibrosis, diabetes mellitus and cardiovascular diseases (Hao et al., 2006, Rogers et al., 2008, Kragh et al., 2009, Umeyama et al., 2009).

SCNT can also be used for the production of genetically engineered animals as a source of organs, tissues or cells for testing in cross-species transplantation or xenotransplantation. Pigs have been the animal of choice among other domestic species for studies in xenotransplantation because of similarities in organ size and physiology with humans, and their lower cost and broad availability. Genetically modified pigs with increased immunocompatibility have been produced by SCNT for research use in xenotransplantation (Phelps et al., 2003, Kuwaki et al., 2005). It is still not possible to overcome all the barriers that impede successful xenotransplantation but SCNT from genetically manipulated cells will further contribute to the progress of xenotransplantation.

2.1.5. The SCNT Technique

The success of animal cloning by SCNT depends on the proper coordination and execution of multiple technical steps, as schematically represented in the Figure 1. The main elements of the cloning process are nuclear donor cells, mature oocytes to be used as host cytoplasts, oocyte manipulation (enucleation, cell transfer, and fusion), oocyte activation (to induce embryo development), embryo culture, synchronization of recipient females and embryo transfer, monitoring of postimplantation development and recipient females' care, control of parturition and periparturition assistance of the cloned offspring. Although the general procedures of the nuclear transfer technology for animal cloning have not undergone important changes, several small adaptations and improvements that have been established and adopted over the years. As a consequence, most animal cloning laboratories have adopted their own protocol, which overall are only slightly different from each other. Although changes in animal cloning protocols have not dramatically affected efficiency, variations in steps of the cloning process seem to be fundamental when cloning different species. A description of the cloning process will be presented hereafter,

but detailed descriptions of protocols used in different species have been published elsewhere, including in a special issue of Cloning and Stem Cells in 2003 (http://www.liebertonline.com/toc/clo/5/4).

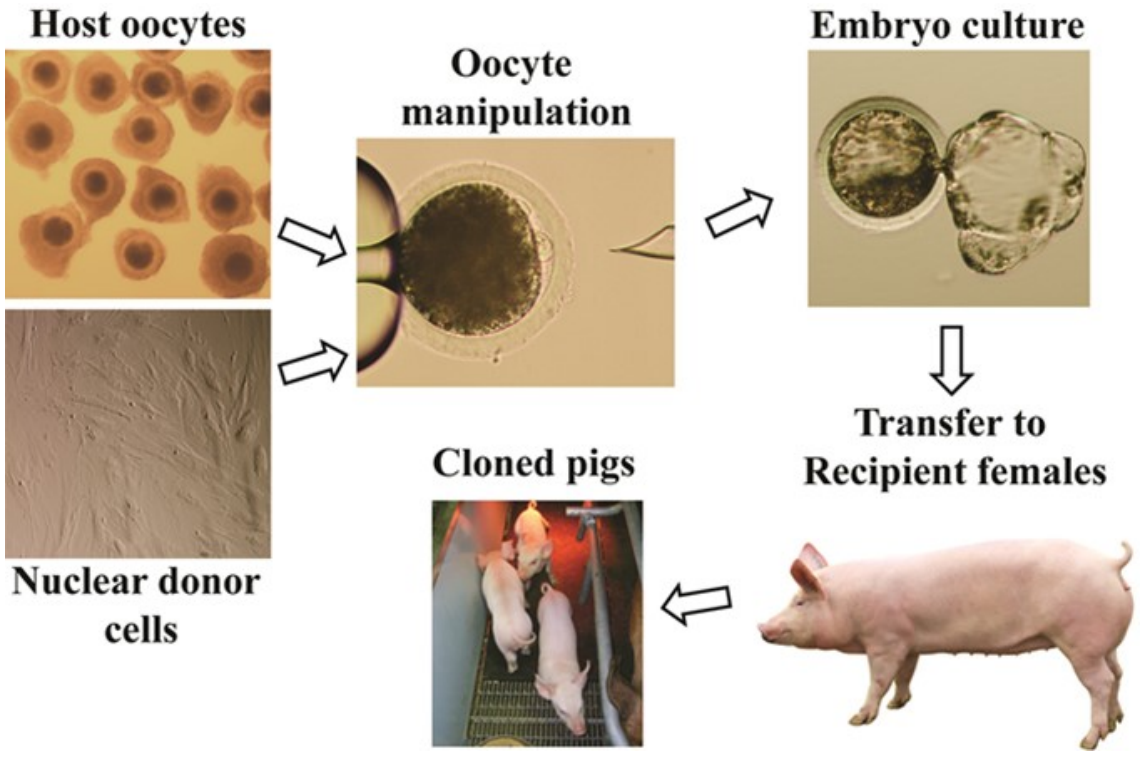

Figure 2.1. Representative images of the main steps for the production of cloned animals by SCNT. Nuclear donor cells are usually derived from biopsies taken from the animal to be cloned, in vitro cultured until reaching confluence and then stored frozen in liquid nitrogen. Host oocytes are normally collected from ovaries recovered in abattoirs and then in vitro matured. After maturation, the oocytes are enucleated using micropipettes connected to a micromanipulator and a nuclear donor cell is placed under the zona pellucida of the enucleated oocyte. The host cytoplasm and the nuclear donor cell are then fused using an electrofusion chamber. After fusion, the oocytes are activated and then cultured in vitro until embryo transfer. The SCNT embryos are

finally transferred to synchronized recipient females either to the oviduct or the uterus depending on the developmental stage at the time of embryo transfer. Pregnancy is confirmed and monitored by ultrasonography until parturition, which is normally induced and closely monitored for assistance if necessary.

2.1.5.1. Preparation of Host Oocytes for SCNT

Matured oocytes of good quality represent a major component for the success of animal cloning by SCNT. Factors present into the oocyte cytoplasm are responsible for reprogramming the transplanted nucleus and initiate embryo development. The main steps in the preparation of host oocytes for SCNT include maturation, micromanipulation (enucleation and cell transfer), oocyte-cell fusion, and activation.

2.1.5.1.1. Oocyte Maturation

During maturation, oocytes need to complete the first meiosis and progress from prophase I (immature) to metaphase II (mature) with an extruded polar body (Figure 2A). Communications via gap junctions permit the transfer of factors between the oocyte and the surrounding cumulus cells that are important for oocyte maturation and early embryo development (Macaulay et al., 2014). Host oocytes are usually matured in vivo when cloning laboratory animals and in vitro when cloning large domestic animals. Hormonal treatments are used to induce the growth and ovulation of a large number of follicles when using oocytes matured in vivo. The protocol used in mice consists in an intraperitoneal injection of 5-10UI of eCG (equine chorionic gonadotropin) to induce the recruitment and growth of follicles. Ovulation is then induced by injecting 5UI of hCG

(human chorionic gonadotropin) 48h later, and the matured oocytes are collected from the ampullae of the oviducts 12-15h after hCG injection. Before micromanipulation, oocytes are incubated in culture media (e.g., HEPES-CZB medium) containing 0.1-0.3 mg/ml of hyaluronidase to remove cumulus cells.

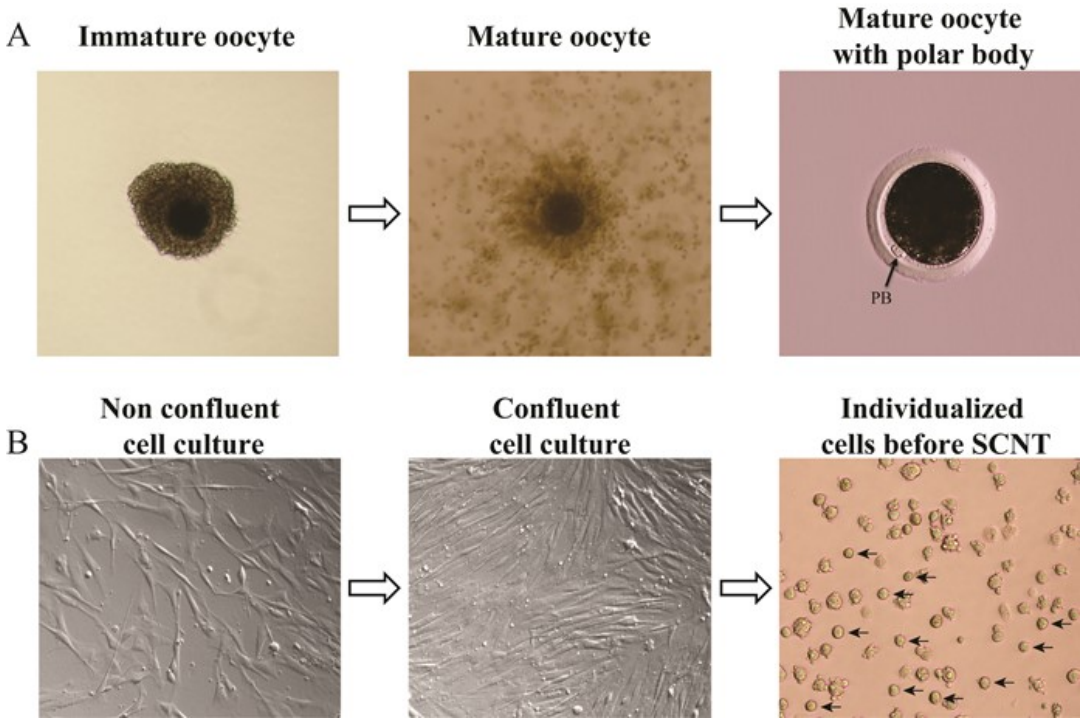

Figure 2.2. Preparation of host oocytes and nuclear donor cells. (A) Porcine oocyte before and after in vitro maturation showing cumulus cells expansion and presence of the first polar body (PB = polar body). (B) Non confluent cell culture, confluent cell culture and individualized cells after trypsinization to be used for SCNT. Arrows indicates cells normally used for SCNT.

Due to several reasons, which include broadly availability of ovaries from slaughtered animals, lower cost, well established protocols, and less welfare issues, in vitro oocyte maturation is more commonly used than in vivo maturation for cloning domestic animal species. Ovaries

collected from abattoirs are transported to the laboratory within a few hours after animal slaughtering in saline solution supplemented with antibiotics at 32-35℃. The size of the follicles from which the oocytes are harvested varies between species to obtain developmental competent oocyte, i.e., those that are better able to support early embryo development likely due to a more accomplished cytoplasmic maturation. For example, porcine and bovine follicles are generally used when their diameter is between 3 and 8 mm. After collection, the immature cumulus–oocyte complexes (COCs) are selected based on morphological aspects of both the oocyte and the surrounding cumulus cells. Groups of 25-30 oocytes are matured in 100-200 µl of maturation medium covered with mineral oil in a humidified atmosphere of 5% CO2 and 95% air at 38-39℃. The maturation medium usually consists of TCM199 supplemented with different compounds including: fetal bovine serum (FBS), bovine serum albumin (BSA), heat-inactivated estrus serum or follicular fluid; hormones (e.g., follicle-stimulating hormone (FSH), luteinizing hormone (LH), 17-β-estradiol); growth factors (e.g., epidermal growth factor (EGF), insulin-like growth factor I (IGF-I)); sodium pyruvate; and antibiotics (e.g., gentamicin, kanamycin, penicillin/streptomycin). The time of oocyte maturation in vitro varies according to the species but is in general 20-24 h for ruminants (e.g., cattle, sheep and goat), 24-36 h for equine, and 42-48h for swine. After maturation, the cumulus cells are removed by pipetting or vortexing the oocytes in a buffered medium containing 0.2-0.3 mg/ml of hyaluronidase. Matured oocytes are then selected based on their intact morphology and the presence of a polar body.

2.1.5.1.2. Preparation of Nuclear Donor Cells

SCNT cloning is normally performed using cultured cells established from biopsies taken from live animals. Studies in different species have confirmed that nuclear donor cells are one of the major factors affecting the success of SCNT cloning. Although many different cell types have been used for cloning animals of different species, there is no agreement regarding the most suitable cell type for SCNT cloning. Indeed, enormous inter- and intra-animal variations on animal cloning efficiency have been reported when using the same cell type. Therefore, skin fibroblasts have been preferentially used for cloning large animals, because they are easily accessible through skin biopsies taken from live animals. In the case of cloning for production of transgenic animals, the use of fetal cells (fetal fibroblasts) is preferable, given the fact that fetal cells have in general longer proliferative capacity in vitro compared to adult cells.

The procedures used to establish primary cell cultures may vary according to the cell type. In the case of skin fibroblasts, a sterile skin biopsy of 1-2 cm is collected after shaving and disinfecting the biopsy area with 1% iodine solution followed by 70% ethanol. The biopsy is then transported to the laboratory on ice in a buffered medium containing antibiotics (e.g., Penicillin/Streptomycin) and an antimycotic agent (e.g., Fungizone). In the lab, the biopsied sample is rinsed several times and the external layer (epidermis) is removed with a sterile scalpel blade. The remaining tissue is cut into small pieces (1-2 mm) and then placed in culture. Alternatively, the tissue pieces can be digested with collagenase (2-5 mg/ml) to disperse the cells before culture. When using fetal cells, the fetal tissues are minced using a blade and then digested using Trypsin-EDTA (2.5-0.2 mg/ml) solution. Cell culture is normally performed in Dulbecco's Modified Eagle's medium/F12 medium (DMEM/F12) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.5-1% antibiotics (10000 U/ml penicillin and 10 mg/ml streptomycin). Cells

can be maintained in culture for several passages or frozen and stored in liquid nitrogen. Although a few studies have proposed that the time in culture does not seem to negatively affect cloning efficiency, most of the cloned animals have been produced using cell lines that were maintained in culture for less than six passages.

Cell cycle synchronization between the host cytoplast and the nuclear donor cell is indispensable for the success of animal cloning (Campbell et al., 1996, Wilmut et al., 1997, Zakhartchenko et al., 1999). Cell cycle incompatibilities can lead to compromised chromatin integrity and aneuploidy. The main factor responsible for these conditions is the M-phase Promoting Factor (MPF), a protein kinase with high activity in M-II stage oocytes, which causes nuclear membrane degradation and chromatin condensation after SCNT. Detrimental consequences of cell cycle incompatibilities are more commonly observed when interphase cells are transferred to M-II cytoplasts, since MPF activity can lead to premature chromatin condensation resulting in DNA damage and lower embryo development. To prevent cell cycle incompatibilities after SCNT, different strategies have been tested. A simple procedure consists in culturing the cells until reaching full confluence, which results in a majority of cells synchronized at the G0/G1 phase. Serum depletion or starvation, which consists in reducing the normal 10% FBS concentration to 0.5%, is another alternative to enrich the population of cells synchronized at the G0 phase, which reduces the risk of cell cycle incompatibly with M-II cytoplasts. In preparation for nuclear transfer, nuclear donor cells are detached from the culture dish and individualized by treatment with 0.25% of Trypsin-EDTA (Figure 2B).

2.1.5.1.3. Oocyte Enucleation and Reconstruction

The main steps required for oocyte reconstruction are enucleation, cell transfer and cell membrane fusion of the nuclear donor cell with the host cytoplast (Figure 3A-D). There are many technical variations for completing these steps, which include the use of micromanipulator versus "handmade methods" without micromanipulators; presence versus absence (removal) of the zona pellucida; mechanical versus chemical enucleation; fluorescence-assisted versus blind enucleation; and nuclear donor cell injection versus cell fusion. In our laboratory, SCNT has been performed using micromanipulators for mechanical removal of the oocyte chromatin without using fluoresce and without removing the zona pellucida, followed by injecting a nuclear donor cell in the perivitelline space, and then electrofusion of cell and oocyte membranes. For these procedures, matured oocytes are incubated for 1-2 h in Hepes-buffered TCM199 medium containing 0.4 μg/ml demecolcine and 0.05 M sucrose. This treatment results in a protrusion in the ooplasm membrane resembling a polar body, which contains the metaphase chromosomes. Oocyte enucleation is performed in Hepes-buffered TCM199 medium supplemented with 2.5-10 μg/ml cytochalasin B, an inhibitor of cytoskeleton assembly, to prevent oocyte lysis. The protruded pseudo polar-body along with a small portion of the surrounding oocyte cytoplasm is removed using a micropipette connected to a micromanipulator. A single somatic cell is then placed between the perivitelline space (between ooplasm membrane and the zona pellucida), in the space left by the enucleation procedure. The oocyte and nuclear donor cell are then fused by applying one or more electrical pulses using electroporation equipment (Figure 3D). The cell-oocyte couplets to be fused are placed one by one or in small groups (5-10) between the electrodes of the fusion chamber, which is attached to the electroporator. Fusion conditions can vary according to different factors including the cell type, species, electroporation equipment, and cell fusion medium. Electrofusion

normally comprises one alternating current (AC) pulse to induce alignment of the cell-oocyte couplets in a parallel position in relation to the fusion chamber electrodes, and one or more direct current (DC) pulses to induce cell membrane poration and fusion. Typically, the AC pulse range is 5-6 V at 600-1000 kHz for 5-10 μs, and the DC pulses are 1-3.5 kV/cm for 30-250 μs. A low-conductivity solution is used when applying the electrical pulses to prevent any damage to the cell-oocyte couplets. A commonly used fusion solution consists of 0.28-0.3 M mannitol, 0.1 mM magnesium sulfate, 0.05 mM calcium chloride and 0.05–0.1% BSA, with pH adjusted to 7.2–7.4. After applying the electrical pulses, cell-oocyte couplets are cultured in TCM199 medium supplemented with 3mg/ml BSA and 20 μg/ml gentamicin for 1 h before oocyte activation.

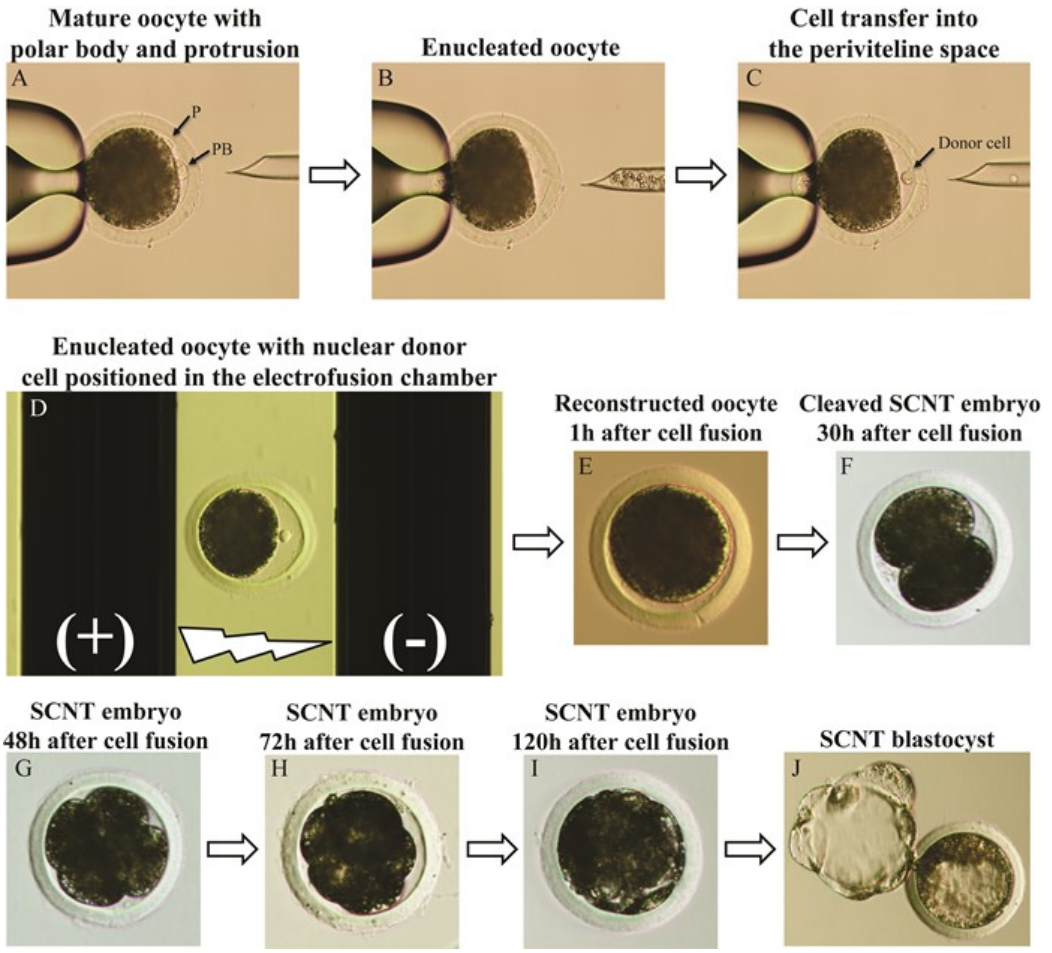

Figure 2.3. SCNT embryo construction and culture. (A) Oocyte with a polar body (PB) and pseudo-polar body (P) indicating the location of the chromatin before enucleation. (B) Enucleated oocyte after aspiration of the PB and P. (C) Nuclear donor cell in the perivitelline space of the enucleated oocyte. (D) Host oocyte and donor cell couplet in the electrofusion chamber for the application of the cell fusion pulse. (E-J) Porcine SCNT embryos at different stages of in vitro development after cell fusion and activation.

2.1.5.1.4. Oocyte Activation and Embryo Culture

Oocyte activation is another decisive step for the success of animal cloning. Abnormal activation can lead to cell cycle incompatibilities and affect normal embryo development (Campbell, 1999). Matured oocytes at the M-II stage have high MPF activity. MPF is a kinase protein formed of two sub-units, a regulatory (cyclin B) and a catalytic (cyclin-dependent kinase p34cdc2). High MPF activity causes chromatin condensation, meiotic spindle stabilization, and meiotic arrest of oocytes at the M-II stage. During normal fertilization, the penetrating sperm induces degradation of MPF activity by promoting repeated rises of calcium ion levels in the oocyte cytoplasm, which results in degradation of cyclin B, exit from M-II arrest and initiation of embryo development. Therefore, protocols used for oocyte activation are based on raising calcium levels in the oocytes to induce MPF degradation in association with inhibitors of MPF activity. Induction of intracellular calcium can be performed using physical (e.g., electrical pulse) or chemical agents (e.g., ionomycin, strontium chloride, ethanol). Although a single increase in intracellular calcium levels is normally used to induce oocyte activation after nuclear transfer, it is known that MPF degradation after fertilization is gradually induced by several calcium oscillations promoted by the sperm. It has been shown that strontium chloride (Sr2+) is able to induce calcium oscillations and activation of oocytes of different species, including mice, rats, cattle and sheep. Our group has established an oocyte activation protocol for porcine oocyte based on a combination of ionomycin (ION) and $Sr2^{+}$ (Che et al., 2007), which has been successfully applied to cloned pigs by SCNT (Martinez-Diaz et al., 2010).

Treatment with inhibitors of protein phosphorylation (e.g., 6-dimethylaminopurine (6-DMAP), roscovitine, butyrolactone I) or protein synthesis (cycloheximide) for a few hours (2–5 h) after an initial stimulus is applied to increase intracellular calcium represents another alternative

to increase activation efficiency. In addition, cytochalasin B can also be added during this period to preserve the normal ploidy of the reconstructed oocyte by preventing the possible extrusion of a polar body. Common protocols used for oocyte activation when cloning livestock species are:

1. Ionomycin + 6-DMAP: 5–15 μM ionomycin for 4-15 min., followed by 2 mM 6-DMAP for 2-5 h.

2. Ionomycin + cycloheximide + cytochalasin B: 5-15 μM ionomycin for 4-15 min., followed by 10 μg/ml cycloheximide and 7.5 μg/ml cytochalasin B for 3-5 h.

3. Ionomycin + cycloheximide + cytochalasin B + strontium chloride: 15 μM ionomycin for 5 min., followed by 10 μg/ml cycloheximide, 7.5 μg/ml cytochalasin B and 10 mM strontium chloride for 4 h.

4. Electrical pulse + 6-DMAP: 1-3 DC pulses of 100-320 V/mm for 10-50 μs each followed by 2mM 6-DMAP for 2-5h.

5. Electrical pulse + cycloheximide + cytochalasin B: 1-3 DC pulses of 100-320 V/mm for 10-50 μs, followed by 10 μg/ml cycloheximide and 7.5 μg/ml cytochalasin B for 3-5h.

After oocyte activation, embryos are washed in culture medium and then cultured in vitro up to the blastocyst stage before they are transferred to synchronized recipient females. Embryo culture conditions vary according to the species. For instance, swine embryos are often cultured in PZM-3 medium supplemented with 3 mg/ml BSA (fatty acid free) in a humidified atmosphere with 5% CO2 and 95% air at 38.5 oC. After 5 days of culture, the media can be supplemented with 10% fetal bovine serum (FBS), which will increase the average cell number of SCNT porcine

embryos. Bovine SCNT embryos are normally cultured in Synthetic Oviductal Fluid (SOF) supplemented with 10% of FBS in a humidified atmosphere with 5% CO2, 5% O2 and 90% N2. In most domestic species, first cell cleavage occurs between 24 and 48 h after oocyte activation and embryos reach the blastocyst stage after 6-7 days of in vitro culture (Figure 3F and J).

2.1.6. Efficiency of SCNT Cloning

To date, animals of more than 20 different species have been cloned by SCNT using cells collected from different tissues. Although pre-implantation development of SCNT embryos, including cleavage and blastocyst rates, is similar to embryos produced by IVF, pregnancy and birth rates are lower in SCNT compared to IVF embryos (Table 1). Thus, cloning efficiency, as determined by the number of embryos that need to be transferred to recipient females to produce a live born cloned animal, remains much lower when compared to embryos produced by fertilization.

2.1.7. Problems of SCNT Cloning

A variety of developmental problems from early embryonic to post-natal stages have been reported with SCNT embryos of different species. For normal development and creation of a cloned animal, the nuclear donor cell needs to regain a totipotent state to be able to recapitulate all the stages of embryo, foetus and fetal membranes formation. As part of this process, epigenetic marks acquired during cellular differentiation need to be erased after SCNT and then re-established during development of the cloned embryos to ensure proper cell differentiation and metabolism. This requires major chromatin remodeling, which is largely dependent on changes in DNA

methylation and histone modifications. However, aberrant epigenetic reprogramming, including DNA and histone hypermethylation, and histone hypoacetylation, has been described in embryos produced by SCNT (Dean et al., 2001, Santos et al., 2003, Wee et al., 2006).

The differentiation state of the nuclear donor is another important factor affecting animal cloning efficiency. Indeed, negative correlations have been observed between cloning efficiency and the differentiation state of the nuclear donor cell in different species including cattle (Heyman et al., 2002) and mice (Blelloch et al., 2006). This supports the idea that epigenetic reprogramming is more difficult to attain when using more differentiated cells for SCNT. Abnormal or insufficient epigenetic reprogramming leads to improper gene regulation during development of SCNT embryos. It has been proposed that during somatic cell reprogramming more than 8000 genes expressed in the nuclear donor cell need to be silenced and the same number of genes activated in the SCNT embryos to support normal development (Niemann et al., 2008). However, aberrant expression of genes regulating embryo-fetal and fetal membranes development has been detected in embryos and fetal tissues derived by SCNT (Wrenzycki et al., 2001, Wrenzycki et al., 2002, Li et al., 2005).

Faulty gene regulation is likely the cause of the high rates of embryo-fetal deaths (50-100%) often observed when SCNT embryos are transferred to recipient females. This has been correlated with fetal membrane abnormalities, such as rudimentary placental development, reduced number and altered anatomy of caruncles and cotyledons, and hydrallantois observed in SCNT gestations of cattle and other species (Hill et al., 2000, Heyman et al., 2002, Palmieri et al., 2008). In addition to gestational problems, a number of conditions including respiratory problems, hyper or hypothermia, enlarged umbilical cord, contracted flexor tendons, septicemia, and persistent urachus have commonly been observed in newborn cloned animals. These and other

conditions result in higher rates of early postnatal death (Panarace et al., 2007). Despite of the higher incidence of pre- and post-natal developmental problems, a proportion of SCNT embryos develop normally and result in cloned animals that grow, reproduce and produce like naturally conceived animals. This confirms that, at least in a small proportion of cases, proper nuclear reprogramming is attained in embryos produced by SCNT. Better understanding the complex mechanisms required for cell reprogramming has been the focus of many recent researches and will likely reveal new routes to improve SCNT cloning efficiency.

2.1.8. Alternatives to Improve SCNT Cloning Efficiency

Since insufficient epigenetic reprogramming is thought to be the main constraint for improving SCNT cloning efficiency, strategies being tested to enhance cell reprogramming include the treatment of nuclear donor cells and/or reconstructed oocytes with epigenetic modulators. For instance, treatment of SCNT embryos with histone deacetylase inhibitors (HDACi), such as Trichostatin A (TSA), Valproic acid (VPA) and Scriptaid, was shown to facilitate nuclear reprogramming, increase embryo development and improve animal cloning efficiency (Maalouf et al., 2009, Zhao et al., 2010, Wang et al., 2011b, Xu et al., 2012). HDACi treatment, which increases acetylation of lysine residues in the tails of the histones H3 and H4, is thought to make the chromatin of the donor cell more accessible to reprogramming factors present in the cytoplasm of the reconstructed embryo. Changes in chromatin accessibility are important for various cellular processes including DNA replication, transcription and repair (Tamburini and Tyler, 2005, Clayton et al., 2006). We have recently observed that HDACi treatment facilitates DNA damage repair and preservation of genome integrity during cell reprogramming in SCNT embryos (Bohrer et al., 2014).

Treatment of nuclear donor cells prior to SCNT with cytoplasm extract from oocytes is another tested alternative to improve cell reprogramming and development of SCNT embryos. For instance, exposure of nuclear donor cells to cytoplasm extract of Xenopus eggs has reduced DNA methylation and increased development of SCNT embryos (Yang et al., 2012). In addition, cytoplasm extract from oocytes of mammals was shown to reduce histone methylation, facilitates epigenetic reprogramming in reconstructed embryos, and improve embryo development and SCNT cloning efficiency (Bui et al., 2008, Bui et al., 2012).

Treatment of nuclear donor cells prior to nuclear transfer with inhibitors of DNA methylation, including 5-aza-2'-deoxycytidine (5-aza-dC), was also shown to facilitate cell reprogramming (Wang et al., 2011c). In addition, improved embryo development and animal cloning efficiency has been observed when nuclear donor cells were treated with both 5-aza-dC and HDACi before nuclear transfer (Diao et al., 2013). Among the different epigenetic marks that need to be reprogrammed, it has recently been proposed that the trimethylation of the lysine 9 in the histone H3 (H3K9me3) represents a major barrier for somatic cell reprogramming. Indeed, by reducing the levels of H3K9me3 in nuclear donor cells a dramatic improvement in SCNT efficiency was observed (Matoba et al., 2014).

Abnormal epigenetic reprogramming during SCNT cloning also results in downregulation of many genes on the X chromosome as a consequence of higher expression of the Xist gene. Xist, a noncoding RNA that participates in the X chromosome inactivation in females, was found to be ectopically expressed from the active X chromosome in both male and female cloned mice. Surprisingly, a remarkable increase in cloning efficiency was observed when donor cells containing Xist-deficient X chromosome were used for SCNT (Inoue et al., 2010). In addition, Xist knockdown by microinjection of small interfering RNA into oocytes before SCNT can rescue

the impaired development of cloned embryos (Matoba et al., 2011). Xist is also aberrantly expressed in bovine and pig SCNT embryos, and is implicated in prenatal death in these species (Xue et al., 2002, Jiang et al., 2008). Treatment of pig cloned embryos with HDACi (Scriptaid) and DNMT (RG108) increased cloning efficiency by restoring the reprogramming of Xist to similar pattern observed in IVF embryos (Xu et al., 2013).

2.1.9. Conclusion

To date, healthy cloned animals have been produced by SCNT in more than 20 different species, including farm, laboratory and wild animals. Indeed, the possibility to reprogram a somatic genome back to an embryonic state has many applications including replication of high-quality farm animals, rescue of endangered species, creation of genetically engineered animals, and production of unique animal models for research. However, SCNT cloning applications are limited by the low efficiency due to developmental problems. The low efficiency of SCNT cloning is likely due to defective epigenetic reprogramming leading to abnormal gene regulation and expression. However, recent evidence indicates that cloning efficiency can be significantly improved using epigenetic modulators. These treatments, along with new discoveries on cells dedifferentiation and reprogramming will likely allow the establishment of improved protocols with higher efficacy to create healthy copies of desired animals for different purposes. We believe that further understanding cell reprograming and epigenetics will allow establishment of improved protocols for SCNT cloning, which may increase the efficiency of this technology to similar levels currently obtained with other assisted reproductive technologies such as IVF and ICSI.

2.2. DNA DOUBLE-STRAND BREAKS AND REPAIR

2.2.1. Causes of DNA Double-Strand Breaks

DNA damage occurs in most cell types and it can be induced by endogenous or exogenous factors. Endogenous factors include reactive oxygen species (ROS) and alkylating agents produced as by-products of cellular metabolism, collapsed DNA replication forks and oxidative destruction of deoxyribose residues (Lindahl and Wood, 1999, Lindahl, 2000). Exogenous factors such as ultraviolet (UV) light, ionizing irradiation and chemical agents can also induce DNA damage. For example, frequent exposure to solar UV light can induce DNA damage by the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Ravanat et al., 2001). Endogenous and exogenous factors can induce the formation of both single-strand breaks (SSB) and double-strand breaks (DSBs) (Rich et al., 2000). DNA DSBs are the most important form of DNA damage because their defective repair can induce genome instability, deleterious mutations, and cellular death (Rich et al., 2000, Khanna and Jackson, 2001, Peterson and Côté, 2004, Furgason and Bahassi el, 2013). Therefore, the presence of mechanisms for DNA DSBs repair is important to maintain genome integrity and cellular viability. DNA DSBs are repaired by the homologous recombination (HR) or the nonhomologous end-joining (NHEJ) pathways (Karran, 2000, Stiff et al., 2004, Stiff et al., 2006).

2.2.2. Phosphorylated Histone H2A.x

Histone H2A.x is encoded by the gene H2AFX and is a member of the histone H2A family that also includes the H2A1, H2A2 and H2AZ. DNA is wrapped around the nucleosome, which is an octamer formed by two from each of the histones H2A, H2B, H3 and H4 families, and they are

linked by the histone H1. H2A.x is very abundant in different cell lines and tissues, with 2-25% of the total H2A family (West and Bonner, 1980). In addition to participating in DNA organization and packaging, the histone H2A.x is indispensable for genome integrity maintenance by participating in DSBs repair. The importance of H2A.x in the maintenance of genome integrity is evidenced by knockout mice (H2A.x-/-) exhibiting chromosomal instability, DNA repair defects with impaired recruitment of DNA repair proteins to the sites of DSBs (Celeste et al., 2002). After DNA DSB formation, H2A.x is immediately activated by phosphorylation of the C-terminal serine 139 (ser139) forming the H2AX139ph (also known as γH2AX). This phosphorylation is mediated by one or more members of PI3K-like kinase family, which includes ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PK) (Rogakou et al., 1998, Stiff et al., 2004, Stiff et al., 2006). The phosphorylation of H2A.x involves large chromatin domains around the sites of DSBs, forming a nuclear focus that can be visualized by immunostaining (Rogakou et al., 1999). Studies have shown that most H2AX139ph foci larger than 0.3 μm^3 are co-localized with DSB repair proteins (Paull et al., 2000b, McManus and Hendzel, 2005). H2AX139ph formation opens the chromatin and facilitate the access of proteins involved in DNA-damage signaling and repair at DSB sites (Lee et al., 2010). Moreover, H2AX139ph serves as a platform for accumulation of DNA repair proteins, from both HR (e.g., RAD51, RAD52, MRE11 and BRCA1) and NHEJ (e.g., 53BP1, KU80, DNA ligase IV, XRCC4) pathways, around the DSBs sites (Paull et al., 2000b, McManus and Hendzel, 2005). Therefore, the presence of H2AX139ph foci larger than 0.3 μm^3 in the nuclei of cells is considered a good marker of DNA DSBs.

2.2.3. DNA Repair by Homologous Recombination (HR)

In somatic cells the DNA DSBs are repaired by HR pathway during the synthesis (S) and growth phase 2 (G2) of the cell cycle, which is when the homologous sister chromatid is present. The HR pathway uses the sister chromatid as a template for information exchange and error free DNA repair. During DSBs repair by HR pathway, the MRN (Mre11–Rad50–Nbs1) complex recognizes the DSBs and recruit the protein ataxia telangiectasia mutated (ATM) to the damaged sites (Lee and Paull, 2005). ATM is then phosphorylated at residue S1981 in its FAT domain, resulting in dissociation of ATM heterodimer and activation (Bakkenist and Kastan, 2004). Activated ATM immediately stimulates the phosphorylation of H2A.x to form the H2AX139ph around the sites of DSBs, which induces chromatin modifications to facilitate the binding of proteins involved in DNA repair (Burma et al., 2001). The mediator of DNA damage checkpoint protein-1 (MDC1) binds to the H2AX139ph at the sites of DSBs and promotes further accumulation of active ATM and MRN complex to the damaged chromatin (Lukas et al., 2004, Stucki et al., 2005). MRN complex associates with the C-terminal binding protein (CtBP)-interacting protein (CtIP) and stimulates the 5'-3' DSBs end resection to generate 3' single-stranded DNA (ssDNA) needed for strand exchange (Sartori et al., 2007). Studies suggest that the protein BRCA1 interacts with CtIP and is also involved in DSB end resection (Chen et al., 2008). In addition, BRCA1 is a RING finger protein that promotes ubiquitination of proteins at DSB sites and facilitates DNA damage response (Lorick et al., 1999, Morris and Solomon, 2004, Polanowska et al., 2006). Interestingly, it has been shown that BRCA1 also associates with a group of proteins including ATM and the MRN complex to form the BASC (BRCA1-Associated Genome Surveillance Complex) complex that is a sensor of DNA damage and, therefore, is required for genome integrity maintenance (Wang et al., 2000).

During the DSB repair by the HR pathway, the protein ataxia telangiectasia and Rad3 related (ATR) is activated in response to a variety of DNA lesions, including DSBs, base adducts, crosslinks and replication stress, which induce the formation of ssDNA (Zou and Elledge, 2003, Fanning et al., 2006). The recruitment of ATR to the DSB sites depends on ATR-interacting protein (ATRIP) and on replication protein A (RPA)-bound ssDNA (Falck et al., 2005). The activation of ATR also leads to the H2AX139ph formation around the DSBs sites (Stiff et al., 2006). During DNA-damage response, ATR and ATM also activate the Serine/threonine-protein kinases 1 (Chk1) and 2 (Chk2), respectively, that regulate the cell cycle progression allowing enough time for DNA repair before DNA replication (Falck et al., 2001, Donzelli and Draetta, 2003). During DSB repair, the protein RAD52 will replace the protein RPA present in the ssDNA tails with RAD51 (McIlwraith and West, 2008). BRCA2 stimulates the binding of RAD51 into the RPA-ssDNA complex and, therefore, it is also important for DNA DSBs repair by HR pathway (Jensen et al., 2010). RAD51 will then promote homology recognition and initiates strand exchange with intact homologous DNA regions (Baumann et al., 1996), which will serve as a template for proper DNA repair (Kass and Jasin, 2010), and the DNA polymerase η promotes DNA synthesis (McIlwraith et al., 2005).

2.2.4. DNA Repair by Non-homologous End Joining (NHEJ)

Despite DNA repair by NHEJ pathway can occur throughout the cell cycle, it is more active during G1 when the sister chromatid is absent (Takata et al., 1998, Delacôte and Lopez, 2008). As DNA repair by NHEJ pathway is not guided by a template DNA strand, it is error-prone and partial loss of genetic information can occur at DSB sites (Lieber, 2008). During DNA repair by NHEJ pathway, the heterodimer protein ku70/80 senses and binds to the double-stranded DNA ends and

rapidly recruits DNA-PK to the damaged sites (Hammarsten and Chu, 1998, Mari et al., 2006), which will form a multiprotein complex to stabilize and align the two DNA ends to be repaired (Gottlieb and Jackson, 1993, DeFazio et al., 2002). Activation of DNA-PK induces the H2AX139ph formation, which promotes chromatin modification around the DSB sites to facilitate the access of signaling and repair proteins (Stiff et al., 2004). The activation of DNA-PK also induces the recruitment of the endonuclease Artemis that processes the 3' and 5' single-stranded overhangs at DNA ends to find the complementary nucleotide stretches (Ma et al., 2002). Subsequently, the DNA polymerases μ and λ are recruited to fill the gaps remaining on both strands before joining (Capp et al., 2006, Andrade et al., 2009). The final step of repair is the joining of DNA ends by the DNA ligase IV/XRCC4 complex (Critchlow and Jackson, 1998, Koch et al., 2004).

In general, the DNA repair pathway is chosen based on the cell cycle phase. The HR is more active during S and G2, whereas the NHEJ pathway is more active during G1 (Delacôte and Lopez, 2008). However, the proteins 53BP1 and BRCA1 play important roles in the pathway choice. The protein 53BP1 binds to the DNA DSB ends, which is most prominent in G1 phase, and inhibits the HR pathway by blocking DNA resection. In contrast, the BRCA1 binds to the DSB ends during S and G2 phases and exclude the 53BP1 from the damaged sites to promote end resection and stimulate the HR pathway (Yun and Hiom, 2009, Bunting et al., 2010, Chapman et al., 2012). Therefore, it seems to be always a competition between HR and NHEJ pathways for DNA DSBs repair.

2.2.5. Chromatin Remodeling During DNA-Damage Repair

During DNA DSBs repair the compacted chromatin needs to be opened to facilitate the access of signaling and repair factors that will recognize and promote DNA repair, and subsequently the chromatin returns to their original configuration. Studies have shown that some chromatin remodeling processes involved in DNA repair are energy dependent. A study using live cells showed decondensation of chromatin fibers by 30-40% around the sites of DNA DSBs, in an energy-dependent manner, immediately after DSBs induction (Kruhlak et al., 2006). Indeed, the poly (ADP-ribose) polymerase 1 (PARP1) participates in the ATP-dependent chromatin expansion around the sites of DSBs (Smeenk et al., 2013). Moreover, studies have shown that the chromatin-remodeling complexes INO80 and SWI/SNF also use energy to alter the chromatin configuration. INO80 is required for the binding of RPA to DNA DSBs ends and appropriate DNA repair by the HR pathway (Gospodinov et al., 2011). In the other hand, SWI/SNF is required to promote H2A.x phosphorylation to form H2AX139ph around the DNA DSBs sites (Lee et al., 2010) and, therefore, is required for DNA-damage response.

Histone acetylation is a post-translational modification highly involved in the process of chromatin remodeling during DNA-damage repair. Acetylation of lysine residues of histone N-terminal tails weakens their interaction with DNA and, therefore, opens the chromatin. The acetylation of various lysine residues of the histone H3, including H3K9, H3K14, H3K18 and H3K23 by the acetyltransferase Gcn5 is required for the interaction of SWI/SNF with the H2A.x in the sites of DNA DSBs (Lee et al., 2010). Moreover, deletion of Gcn5 results in loss of cellular viability after DNA DSBs induction (Tamburini and Tyler, 2005). Histone acetylation is also important for the recruitment of repair proteins to the sites of DNA DSBs. Studies have shown that the acetylation of H3K18, H4K5, H4K8, H4K12 and H4K16 by the histone acetyltransferases CBP

and p300 is required for the recruitment of proteins ku70 and ku80 to the DNA DSBs ends and DNA repair by NHEJ pathway (Ogiwara et al., 2011). Acetylation of H4K16 by the acetyltransferase MOF is also critical for DNA-damage response and DNA DSBs repair in mammals. Depletion of MOF delays the H2AX139ph formation and impairs the recruitment of RAD51 and 53BP1 to the sites of DNA DSBs (Sharma et al., 2010).

The chromatin remodeling process during DNA DSBs repair is tightly controlled and, therefore, factors involved in promoting chromatin acetylation and deacetylation around the sites of DNA DSBs are important. Studies have shown that the acetylation level of H3 and H4 changes during DNA-damage repair and that the histone deacetylases (HDAC) Rpd3, Sir2, and Hst1 are recruited to the sites of DNA DSBs (Tamburini and Tyler, 2005). Studies have also shown that the HDAC1 and HDAC2 are recruited to sites of DNA DSBs to promote deacetylation of H3K56 and H4K16, and their depletion results in impaired DNA DSBs repair (Miller et al., 2010).

Ubiquitination of H2A/H2A.x is another post-translational modification important for DNA repair. Studies have shown that the phosphorylation of MDC1 induced by ATM triggers the recruitment of RNF8, a RING-finger ubiquitin ligase, to the sites of DNA DSBs. Subsequently, the RNF168, another RING ubiquitin ligase, binds to the RNF8-ubiquitinated histones (Mailand et al., 2007). It has been shown that the recruitment of RNF8/RNF168 to the sites of DSBs facilitates the binding of repair proteins, including 53BP1 and BRCA1 (Stewart et al., Doil et al., 2009). The protein 53BP1 interacts with the sites of DNA DSBs by binding to the histone H3 methylated on lysine 79 (H3K79 methyl) (Huyen et al., 2004) and the histone H4 methylated on lysine 20 (H4K20 methyl) (Pei et al., 2011). Despite the fact that the level of H3K79 methyl does not change with the induction of DNA DSBs, the histone methyltransferase MMSET, also known

as NSD2 or WHSC1, increases the methylation of H4K20 at the sites of DNA DSBs. These findings highlight the importance of chromatin remodeling during DNA-damage response.

2.2.6. Genome Integrity Maintenance During Embryo Development

Studies have shown that DNA DSBs affect early embryo development in various species (Simon et al., 2014, Bohrer et al., 2015, Juan et al., 2015). Indeed, increased DNA damage in spermatozoids before fertilization results in embryo development failure (Morris et al., 2002, Gawecka et al., 2013). Moreover, experimentally induced DNA DSBs in early embryos reduces the cleavage rate and impairs development to the blastocyst stage (Wang et al., 2013b). These studies indicated that DNA-damage signaling and repair mechanisms are active during early embryo development to prevent the multiplication of DNA-damaged cells. Indeed, oocytes and early embryos are prepared to respond to genome instability by having transcripts for a large number of genes involved in DNA-damage signaling and repair (Jaroudi et al., 2009). Moreover, imbalance of key factors involved in DNA DSBs repair affects genome integrity maintenance. In this sense, inhibition of RAD51 increase the percentage of damaged DNA in mouse and bovine oocytes (Kujjo et al., 2012). Interestingly, microinjection of RAD51 protein into DNA-damaged oocytes improved DNA repair, reduced apoptosis and increased embryo development (Perez et al., 2006).

Induction of DNA DSBs in embryos from different species, including mice, rat and bovine, has been used to better understand the mechanisms of DNA repair and its consequences during embryo development (Grenier et al., 2012, Henrique Barreta et al., 2012, Gawecka et al., 2013). The phosphorylation of ATM in response to DNA DSBs in the nuclei of mouse embryos indicates

that the HR pathway is activated during DNA-damage response in early developing embryos (Mu et al., 2011, Wang et al., 2013a). However, if the NHEJ pathway is active and its relative importance for genome integrity maintenance during early embryo development has not been determined. Interestingly, studies have shown that early developing embryos are able to activate the checkpoint proteins Check1 and 2 in response to DNA DSBs to stop the cell cycle and prevent the multiplication of DNA-damaged cells (Wang et al., 2013a, Wang et al., 2013b).

Studies have shown that early-cleaving embryos produce higher blastocyst rates (Yadav et al., 1993, Sakkas et al., 1998, Lonergan et al., 1999, Booth et al., 2007, Coutinho et al., 2011, Isom et al., 2012). In addition, early-cleaving embryos have higher capacity to stablish pregnancies (Edwards et al., 1984, Meseguer et al., 2011, Sugimura et al., 2012). Although early-cleaving embryos have better capacity to sustain development, the cellular and molecular features involved in this process are not known. Interestingly, studies have shown that late-cleaving bovine embryos have higher intracellular levels of ROS and higher fluorescence intensity for the histone H2AX139ph in the nuclei (Bain et al., 2013). Even though the quantification of diffuse H2AX139ph is not a good marker of DNA DSBs (Paull et al., 2000b, McManus and Hendzel, 2005, Ziegler-Birling et al., 2009), it has been shown that increased intracellular levels of ROS profoundly affect genome integrity in different cell types (Chen et al., 1995, Lu and Finkel, 2008, Rai et al., 2009). Therefore, it suggests that embryo cleavage kinetics can be affected by DNA damage. Interestingly, embryos produced in vivo have lower levels of ROS than embryos produced in vitro (Goto et al., 1993). Moreover, reduction of intracellular ROS increased development of in vitro produced bovine embryos and reduced the fluorescence intensity for H2AX139ph (Betts et al., 2014). These findings suggest that by-products of cellular metabolism can affect the genome integrity and developmental competence of in vitro produced embryos.

Studies have shown that only the H2AX139ph foci larger than 0.3µm^3 indicates the sites of DNA DSBs (Paull et al., 2000b, McManus and Hendzel, 2005). H2AX139ph was also shown to be an important marker for DNA DSBs during different stages of embryo development (Henrique Barreta et al., 2012, Bohrer et al., 2013). However, in mice H2AX139ph seems to be absent in 1- and 2-cell stage embryos (Yukawa et al., 2007). This suggests that there are differences in the mechanism of DNA-damage signaling and repair between species. In summary, there is limited information regarding the occurrence of DNA DSBs during in vitro and in vivo embryo development, the effect of assisted reproductive technologies on genome integrity, and the mechanisms of DNA repair during embryo development. Therefore, additional studies are required to investigate the occurrence and effects of DNA DSBs on embryo development, as well as the mechanisms involved in DNA DSBs repair in early developing embryos.

2.3. HISTONE DEACETYLASE INHIBITORS (HDACi)

2.3.1. Types of HDACi

Histone acetylation is a post-translational modification that is regulated by histone acetyltransferases (HATs) and by histone deacetylases (HDACs). HATs mediate the acetylation of lysine residues of histones that will reduce the binding strength between DNA and histones and, therefore, increase DNA accessibility to enzymes and transcription factors. HDACs remove the acetyl group from histones and consequently increase the interaction between histones and the negatively charged DNA. These physiological modifications are indispensable during development and cell differentiation by regulating cellular processes that include transcription (Clayton et al., 2006) and DNA repair (Tamburini and Tyler, 2005). There are 18 different HDACs divided in four classes. The classes I, II, and IV are zinc-dependent amidohydrolases, and the class III requires NAD+ for deacetylation reaction. The HDACs 1, 2, 3 and 8 belong to class I, HDACs 4, 5, 7, 9 belong to class IIa, HDACs 6 and 10 belong to class IIb and the HDAC 11 belongs to class IV (Groselj et al., 2013).

Increase in histone acetylation can also be induced by numerous pharmacological inhibitors of histone deacetylases (HDACi). They are classified according to their structure and their HDAC class inhibitory specificity. The hydroxamic acids, which include TSA, Scriptaid, Vorinostat, H6CAHA and Abexinostat, are active against class I and II HDACs. The short-chain fatty acids, which include Sodium Butyrate, Pivanex and Valproic acid, inhibit the class I and IIa HDACs. The cyclic peptides, such as romidepsin, inhibit mainly class I HDACs, but can also inhibit class II HDACs at higher concentrations. The benzamides, which include entinostat and mocetinostat, are active against class I HDACs (Spiegel et al., 2012, Groselj et al., 2013).

Scriptaid or 6-(1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide is a potent HDACi that induces >100-fold increase in histone acetylation. In cultured cells, Scriptaid has maximal transcriptional activity at 2–2.5 μg/ml (6–8 μM) with minimal cellular toxicity. In contrast, the HDACi TSA has the optimal transcriptional activity at 0.32 μg/ml (1 μM) but with a higher cytotoxic effect. The concentration of TSA with minimal toxic effect is 0.1 μg/ml, but at this concentration TSA is much less efficient than Scriptaid to inhibit histone deacetylation (Su et al., 2000). Therefore, in our laboratory we have used Scriptaid as HDACi to improve cellular reprogramming during the production of cloned embryos by SCNT.

2.3.2. Effect of HDACi Treatment in SCNT Embryos

Studies have described aberrant epigenetic reprogramming in blastocysts produced by SCNT, which include histone hypoacetylation (Wee et al., 2006) and hypermethylation (Santos et al., 2003), and higher patterns of DNA methylation (Dean et al., 2001). Incomplete epigenetic reprogramming of SCNT embryos results in low cloning efficiency, with less than 5% of embryos developing to term and producing a healthy newborn animal in most of cases. Treatment of SCNT embryos with either TSA or Scriptaid results in higher blastocyst rates and increased cloning efficiency in various species (Van Thuan et al., 2009, Zhao et al., 2010, Wang et al., 2011b, Chen et al., 2013). TSA and Scriptaid treatment facilitate donor cell reprogramming during SCNT. For instance, it has been shown that Scriptaid treatment increases the acetylation levels of H3K9, H4K5 and H4K8 (Zhao et al., 2010, Wang et al., 2011b, Chen et al., 2013) and that TSA treatment increases the acetylation levels of H3K14 and H4K5 (Iager et al., 2008, Martinez-Diaz et al., 2010, Chen et al., 2013). Moreover, Scriptaid and TSA treatment reduces the methylation levels of H3K9me2 and the global DNA methylation levels, respectively, in SCNT embryos (Wee et al.,

2007, Wang et al., 2011b). In addition, it was suggested that HDACi treatment improves normal gene expression in SCNT embryos (Li et al., 2008, Shao et al., 2009).

Findings from the studies listed above indicate that HDACi treatment increases chromatin accessibility to reprogramming factors present inside the recipient oocyte and, therefore, increasing cellular reprogramming in SCNT embryos. Interestingly, increasing chromatin accessibility around the sites of DNA damage is also important for DNA DSBs repair (Rossetto et al., 2010). Preservation of genome integrity is indispensable for somatic cell reprogramming during the production of iPS cells (Marion et al., 2009, González et al., 2013, Molina-Estevez et al., 2013). Therefore, these findings suggest that HDACi treatment may have a positive effect on DNA DSBs repair during somatic cell reprogramming in SCNT embryos.

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CHAPTER 3

HYPOTHESIS AND OBJECTIVES

3.1. HYPOTHESIS

The occurrence of DNA DSBs is an important component affecting embryo cleavage kinetics, development and quality, and cell reprogramming in SCNT embryos.

3.2. OBJECTIVES

Objective 1: Characterize the occurrence of DNA DSBs during in vitro development of IVF and SCNT embryos.

Objective 2: Evaluate the effect of HDACi treatment on DNA DSBs repair in SCNT embryos.

Objective 3: Determine the effect of DNA DSBs on the developmental kinetics of in vitro produced embryos.

Objective 4: Study the importance of the HR and the NHEJ pathways for DNA DSBs repair during early embryo development.

CONNECTING STATEMENT 1

Studies have shown that DNA DSBs affects early embryo development in different species (Henrique Barreta et al., 2012, Wang et al., 2013b, Simon et al., 2014). Moreover, the presence of signaling and repair proteins has been characterized during early embryo development especially in mouse and rat embryos (Barton et al., 2007, Yukawa et al., 2007, Derijck et al., 2008, Grenier et al., 2012). Interestingly, findings from those studies suggest differences between species in the DNA damage response and the presence of DNA signaling and repair proteins. To our knowledge, the occurrence of DSBs in porcine embryos was not investigated in previous studies. Therefore, in the first manuscript of this thesis (Chapter 4), we characterized the presence of DNA DSBs during early development of porcine embryos produced by IVF and SCNT.

Oocyte activation is an important step for the success of embryo production by SCNT. Abnormal activation can lead to cell cycle incompatibilities between the nuclear donor cell and the host cytoplast, which can compromise embryo development (Fulka and Fulka, 2007). Abnormal cell cycle compatibilities are thought to affect chromatin replication, integrity, and segregation in SCNT embryos (Campbell, 1999). We have previously shown that porcine oocytes activated with Ionomycin and Strontium chloride have superior development of SCNT embryos compared with oocytes activated just with Ionomycin (Che et al., 2007). Therefore, in the first manuscript we also evaluated the effect of different oocyte activation protocols on the occurrence of DNA DSBs in SCNT embryos.

CHAPTER 4

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Phosphorylated histone H2A.x in porcine embryos produced by IVF and SCNT

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Short title: H2AX139ph in porcine embryos

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4.1. Abstract

Phosphorylated histone H2A.x (H2AX139ph) is a key factor for the repair of DNA double-strand breaks (DSBs) and the presence of H2AX139ph foci indicates the sites of DSBs. In this study we have characterized the presence of H2AX139ph during *in vitro* development of swine embryos produced by IVF and SCNT. Pronuclear stage embryos produced by IVF had in average 9.29 H2AX139ph foci per pronucleus. The number of H2AX139ph foci was higher in 2-cell than 4-cell stage embryos fixed at 48 h post-fertilization. The percentage of H2AX139ph positive nuclei was higher in SCNT embryos that were activated with ionomycin alone (ION) than in those activated with ION and strontium chloride (ION+Sr^{2+}). A negative correlation was found between the percentage of H2AX139ph positive cells and total embryo cell number in day-7 blastocysts produced by IVF or SCNT. Based on the detection of H2AX139ph foci, findings of this study indicate that: DSBs occur in a high proportion of swine embryos produced by either IVF or SCNT; fast cleaving embryos have less DSBs than slow cleaving embryos; the oocyte activation protocol can affect DNA integrity in SCNT embryos; and better quality blastocysts have less DSBs. We propose that the presence of H2AX139ph foci can be a useful marker of embryo quality.

Keywords**:** H2AX139ph, DNA double-strand breaks, IVF, SCNT, swine embryos.

4.2. Introduction

Embryo technologies such as *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) have been applied to produce embryos in a number of species. Because swine are physiologically similar to humans, there is growing interest in the use of these technologies to

create special animal models for the study of physiopathological processes and xenotransplantation (Kues and Niemann, 2004, Kuwaki et al., 2005, Aigner et al., 2010). However, development of IVF and SCNT swine embryos is influenced by different factors including oocyte quality, polyspermy, abnormal cell cycle interactions, deficient epigenetic reprogramming and altered gene expression (Abeydeera et al., 2000, Bortvin et al., 2003, Lucifero et al., 2006, Niemann et al., 2008, Nascimento et al., 2010, Whitworth and Prather, 2010, Yoshioka, 2011).

Oocyte activation is an important step for the success of SCNT. Abnormal activation can lead to cell cycle incompatibilities between the nuclear donor cell and the host cytoplast, which can compromise embryo development (Campbell, 1999, Fulka and Fulka, 2007). Intra-oocyte calcium oscillations induced by the sperm trigger oocyte activation during normal fertilization (Fissore et al., 1992). Strontium chloride (Sr^{2+}) was shown to induce calcium oscillations in oocytes and it has, consequently, been used for oocyte activation in different species such as mice, rats, bovine and ovine (Kline and Kline, 1992, Kishikawa et al., 1999, Méo et al., 2004, Tomashov-Matar et al., 2005, Zhang et al., 2005, Choi et al., 2012). We have previously shown that porcine oocyte activation using a combination of ionomycin (ION) and Sr^{2+} results in superior development of SCNT embryos compared to oocyte activation using ION alone (Che et al., 2007).

There is limited information currently available about the occurrence and consequences of DNA damage during embryo development, as well as about the potential effects of the technologies used for embryo production in the occurrence of DNA damage. In somatic cells, the histone H2A.x was shown to be indispensable for the preservation of genome integrity because it participates in the process of DNA double-strand breaks repair. The H2A.x is a member of the H2A histone family including H2A1, H2A2 and H2AZ, which help to package and organize DNA into chromatin (West and Bonner, 1980). The histone H2A.x is immediately activated by

phosphorylation of the C-terminal serine residue 139 (Ser139) at the nascent sites of DSBs. This phosphorylation is mediated by one or more members of the PI3K-like kinase family, which includes ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Stiff et al., 2004, Takahashi and Ohnishi, 2005, Stiff et al., 2006). In approximately 30 minutes of DSBs formation, H2AX139ph accumulates around the break sites creating foci where proteins involved in signaling and repair of DSBs begin to accumulate (Rogakou et al., 1999, Bonner et al., 2008). It has been demonstrated that only large H2AX139ph foci, i.e. those bigger than 0.30 μm^3, indicate the sites of DSBs because they are tightly co-localized with DNA-damage signaling and repair proteins (Paull et al., 2000a, McManus and Hendzel, 2005).

The presence of large H2AX139ph foci has been extensively used to evaluate the occurrence of DSBs in somatic cells (Bonner et al., 2008, Kinner et al., 2008, Jucha et al., 2010). There are also few studies reporting the presence of H2AX139ph foci in mouse and rat embryos (Forand et al., 2004, Luo et al., 2006, Adiga et al., 2007, Ziegler-Birling et al., 2009, Wossidlo et al., 2010, Grenier et al., 2012). Interestingly, DNA damage was shown to impair cellular reprogramming and reduce the efficiency to produce induced pluripotent stem (iPS) cells (Marion et al., 2009). This suggests that genome integrity might be an important component affecting cell reprogramming and development of SCNT embryos. The objectives of this study were to: a) investigate the occurrence of large H2AX139ph foci as indicators of DSBs during *in vitro* development of swine embryos produced by IVF and SCNT; b) evaluate the effect of the protocol used for oocyte activation on the occurrence of H2AX139ph foci in SCNT embryos; and c) assess whether the presence of H2AX139ph foci is correlated with development and embryo cell number at cleavage and blastocyst stages.

4.3. Materials and Methods

4.3.1. Chemicals

Unless otherwise indicated, all chemical reagents were purchased from Sigma Chemical Company (Sigma–Aldrich, Oakville, ON, Canada).

4.3.2. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were obtained from a local abattoir (Olymel S.E.C./L.P.) and transported to the laboratory in sterile 0.9% NaCl at 30 to 35°C. Follicles from 3 to 6 mm in diameter were aspirated using an 18-gauge needle attached to a 10 mL disposable syringe. Only cumulus-oocyte complexes (COCs) surrounded by a minimum of three cummulus cell layers and having an evenly granulated cytoplasm were selected for *in vitro* maturation (IVM). Groups of 20 COCs were cultured in 0.1 mL of maturation medium under mineral oil in a humidified atmosphere of 5% CO2 and 95% air at 38.5°C. Maturation medium consisted of TCM 199 (Life technologies, Burlington, ON, Canada), supplemented with 20% of porcine follicular fluid, 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., IA, USA), 0.5 µg/mL FSH (SIOUX Biochemical Inc.), and 20 µg/mL gentamicin (Life technologies). After 22 to 24 h of maturation, oocytes were transferred to the same IVM medium, but without LH and FSH, for an additional 20 to 22 h under the same conditions. Cumulus cells were removed by vortex in TCM 199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% of hyaluronidase and then oocytes were used for IVF or SCNT.

4.3.3. In vitro fertilization (IVF)

Matured oocytes were washed three times with IVF medium (113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 0.1% BSA) (Abeydeera and Day, 1997). Groups of 20 oocytes were then placed in 90 µl drops of IVF medium covered with mineral oil. Sperm was prepared from fresh semen samples of a proven fertile boar. Sperms were washed twice in IVF medium by centrifugation (800 rpm) for 3 minutes and the sperm pellet was resuspended with IVF medium. Oocytes in each drop were fertilized by adding 10 µl of the sperm preparation with concentration adjusted to 10,000 spermatozoa per oocyte. Oocytes were co-incubated with the sperm for 6 h and then collected, washed twice to remove unattached sperm cells and then cultured in PZM-3 medium (Yoshioka et al., 2002).

4.3.4. Somatic cell nuclear transfer (SCNT)

Cumulus-free oocytes selected with a polar body were cultured in TCM199 supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 60 minutes. This treatment resulted in a protrusion in the ooplasmic membrane that contained the metaphase chromosomes (Yin et al., 2002). Oocytes were then transferred to Tyrode's lactate-pyruvate-HEPES medium (TLP-HEPES: 5 mM glucose, 113 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 2 mM $NaHCO_3$, 20 mM lactate, 10 mM HEPES and 0.3% polyvinyl alcohol, pH 7.4) supplemented with 7.5 mg/mL CB for 5 to 10 min and enucleated by removing the protruded oocyte chromatin together with the first polar body. A fetal fibroblast cell derived from a confluent culture was transferred into the perivitelline space of each enucleated oocyte and electrically fused using a single DC pulse of 1.6 kV/cm for 70 µs. Electrofusion was performed into a 0.28 M mannitol solution supplemented with

50 μM $CaCl_2$, 100 μM $MgSO_4$ and 0.1% PVA. Reconstructed oocytes were cultured in TCM199 medium supplemented with 3 mg/mL BSA for 1 h before activation.

4.3.5. Oocyte activation

Reconstructed oocytes were activated with the following protocols (Che et al., 2007):

a) Ionomycin (ION): oocytes were exposed for 5 minutes to 15 μM ION in TCM 199, and then transferred to PZM-3 medium supplemented with cytochalasin B (CB; 7.5 μg/ml) and cycloheximide (CHX; 10 μg/ml) for 4 hours.

b) Ionomycin + Strontium chloride (ION+Sr^{2+}): oocytes were exposed to 15 μM ION for 5 minutes, then transferred to 10 mM Sr^{2+} in PZM-3 (Ca^{2+}-free) + 7.5 μg/ml of CB and 10 μg/ml of CHX for 4 hours. After activation, oocytes were washed and cultured in PZM-3.

4.3.6. Embryo culture

After IVF or SCNT oocytes were washed three times in PZM-3 medium supplemented with 3 mg/mL BSA (fatty acid-free) and cultured in a humidified atmosphere of 5% CO2 and 95% air at 38.5 ºC. The cleavage rate was determined 48 h after IVF or activation. Cleaved embryos were transferred to a fresh drop of PZM-3 medium and on day 5 of culture 10% fetal bovine serum (FBS) was added and embryos were maintained in culture until day 7.

4.3.7. Western Blot

In vitro produced porcine embryos (65 blastocysts) and porcine fetal fibroblasts were lysed in Laemmli buffer (Bio-Rad, Mississauga, Ontario, Canada) containing phosphatases and proteases inhibitor cocktail (G-Biosciences, St. Louis, MO, United States). Total lysate was resolved and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada). After blocking with 5% BSA in Tris-buffered saline with 0.1% tween (TBST), membranes were incubated overnight with anti-phospho-H2A.x (Ser139) mouse monoclonal antibody (dilution 1:1000; Millipore, Billerica, MA, United States) or with anti-beta actin rabbit polyclonal primary antibody (1:5000; Abcam Inc. Toronto, ON, Canada). Subsequently, membranes were incubated for 1 h with peroxidase-conjugated anti-mouse (1:3000) or anti-rabbit (1:10000) secondary antibody (Cell Signaling, Boston, MA, United States). Membranes were incubated for 2 minutes with Immun-Star WesternC Chemiluminescent Kit and images were captured using the ChemiDoc MP System (Bio-Rad, Mississauga, Ontario, Canada).

4.3.8. Immunofluorescence Staining

Samples of IVF and SCNT embryos at 1-cell, 2-4 cell, and day-7 blastocysts were fixed for 15-20 minutes in 4% paraformaldehyde and permeabilized in 1% Triton X-100 in phosphate-buffered saline (PBS) during 30 minutes at 37°C. Samples were incubated for 1 h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then maintained overnight in the presence of anti-phospho-H2A.x (ser 139) mouse monoclonal primary antibody (Millipore, Billerica, MA, United States) diluted (1:1000) in blocking solution. Embryos were then washed three times for 20 min each in blocking solution and incubated for 1 h at room temperature

(24-26°C) in the presence of 1:1000 diluted anti-mouse IgG Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Baltimore, PA). After this period, DNA was stained by exposing the samples to 10 µg/ml of 4',6-diamidino-2-phenylindole (DAPI; Life technologies) in blocking solution for 20 minutes. Samples were then washed three times (20 min each) in blocking solution and mounted on microscope slides using a drop of Mowiol. Slides were kept in a dark box and examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan). Images were captured at 200x, 400x or 600x magnification using a Retiga 2000R monochromo digital camera (Qimaging, BC, Canada) and the SimplePCI Imaging Software (Compix, Inc., Sewickley, PA). Nuclei were individually evaluated and H2AX139ph foci bigger than 0.30 μm^3, which indicates the sites of DNA DSBs (McManus and Hendzel, 2005, Grenier et al., 2012), were counted. The number of H2AX139ph foci was determined in IVF embryos at pronuclear stage (at 10, 15 and 20 h post-fertilization - hpf), SCNT embryos at 20 h post-nuclear transfer (hpnt), and in both IVF and SCNT cleaved embryos at 48 h (2- and 4-cells) and d-7 blastocysts. Results are presented as average number of H2AX139ph foci per nuclei or the proportion of nuclei with <10 foci, ≥10 foci and total positives.

4.3.9. Statistical analysis

All analyses were performed using the JMP software (SAS Institute, Inc., Cary, NC). Continuous data were analyzed by ANOVA and the means were compared by Student`s *t*-test or Tukey–Kramer HSD test for single or multiple comparisons, respectively. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. The correlations were done by simple linear regression analyzes, and the nominal data were analyzed by chi-square test. Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

4.4. Results

4.4.1. Presence of H2AX139ph on in vitro fertilized embryos

The specificity of H2AX139ph antibody was confirmed by western blot using porcine fetal fibroblasts and porcine embryos at day 7 of in vitro development. A unique protein band of the expected molecular weight (17 KDa) was detected in samples from both fibroblast cells and blastocysts (Fig. 1A).

The presence of H2AX139ph foci was first determined by immunofluorescence in IVF embryos at different stages of in vitro development. H2AX139ph was detected in pronuclei with an average of 9.29 foci per pronucleus (Fig. 1B). The average number of foci was numerically higher but not statistically significant in zygotes fixed at 15 h and 20 h compared to 10 hpf (P=0.096; Fig. 1C). H2AX139ph foci were found in most of zygotes regardless the time when they were fixed after fertilization (Fig. 1D). Based on the average number of foci counted per pronucleus (approximately 10), we decided to classify H2AX139ph positive nuclei as below and above 10 foci. The proportion of pronuclei with <10 foci compared to ≥10 foci was not statistically different in zygotes fixed at 10, 15 and 20 hpf (Fig. 1D).

Cleaved embryos fixed at 48 hpf were grouped in 2- (slow cleaving) and 4-cell (fast cleaving) stage. The mean number of H2AX139ph foci per nucleus (Fig. 2A) was higher (P=0.0119) in embryos with 2 cells (16.71 foci) than those with 4 cells (6.09 foci; Fig. 2B). H2AX139ph foci were detected in most of the nuclei of both 2- and 4-cell stage embryos (Fig. 2C). The proportion of nuclei presenting more than 10 foci was also higher (P=0.0432) in embryos with 2 cells (46.42%) than those with 4 cells (21.87%; Fig. 2C). Embryos at 4-cell had a higher proportion of nuclei with <10 foci than ≥10 foci (Fig. 2C).

In d-7 blastocysts produced by IVF (Fig. 3A), H2AX139ph was detected in 49.85% of the nuclei, whereas 36.69% of the positive cells had <10 foci and 13.16% had ≥10 foci ($P<0.0001$; Fig. 3B). Interestingly, there was a negative correlation ($r^2=0.6834$; $p<0.0001$) between the percentage of cells with H2AX139ph foci and the total number of cells per blastocyst (Fig. 3C).

4.4.2. Presence of H2AX139ph in SCNT embryos

This experiment evaluated the presence of H2AX139ph foci in SCNT embryos produced with different activation protocols (ION vs. ION+Sr^{2+}). In 1-cell stage embryos fixed at 20 hpnt, the proportion of nuclei presenting H2AX139ph foci, as well as the proportion of nuclei with <10 foci or ≥10 foci was not statistically different between groups activated with ION and ION+Sr^{2+} (Fig. 4).

In cleaved embryos fixed at 48 hpnt, the presence of H2AX139ph foci was affected by both the number of cells (2 cells vs. 4 cells) and the activation protocol (Fig. 5). The proportion of nuclei with <10 foci was higher in 4-cells (38.89%) compared to 2-cells (10.53%) stage embryos that were activated with ION. There was no statistical difference between 2- and 4-cell embryos in the group activated with ION+Sr^{2+} (Fig. 5A). The proportion of nuclei with ≥10 foci was higher in 2-cell (42.11% and 35.29%) than 4-cell (11.11% and 8%) stage embryos that were activated with either ION or ION+Sr^{2+}, respectively (Fig. 5B). Within the same cell stage, the activation protocol had a significant effect on the proportion of nuclei presenting H2AX139ph foci. The proportion of nuclei with <10 foci (38.89% vs. 16%; Fig. 5A) and total H2AX139ph positive nuclei (50% vs. 24%; Fig. 5C) were significantly higher in 4-cell stage embryos activated with ION compared to ION+Sr^{2+}.

In blastocysts produced by SCNT, the percentage of nuclei with <10 H2AX139ph foci (27.27% vs. 12.07%) and total H2AX139ph positive nuclei (43.75% and 20.75%) were significantly higher in SCNT blastocysts produced with ION compared to ION+Sr^{2+} activation protocol (Fig. 6A). There was a negative correlation (r^2=0.6284; $p<0.0001$) between the percentage of cells with H2AX139ph foci and the total number of cells in SCNT blastocysts (Fig. 6B).

4.5. Discussion

The phosphorylation of histone H2A.x is essential to promote DNA double-strand break repair by acting as a platform for accumulation and retention of important proteins involved in signaling and repair mechanisms (Kinner et al., 2008). Despite its importance in DSBs repair, H2AX139ph has not been thoroughly investigated during early embryo development. Few reports have shown the presence of H2AX139ph during embryo development in mice (Luo et al., 2006, Adiga et al., 2007, Pacchierotti et al., 2011, Xiao et al., 2012) and rats (Barton et al., 2007, Grenier et al., 2012), but whether the occurrence of H2AX139ph is correlated with embryo development and quality has not been investigated. Moreover, there were no studies conducted to investigate whether the technologies used for in vitro embryo production affect the occurrence of H2AX139ph. Therefore, this study used porcine embryos to investigate the occurrence of H2AX139ph during in vitro development of embryos produced by IVF and SCNT. Based on the presence of H2AX139ph foci as an indicator of DSBs, the main findings of this study were: a) DSBs are detected during all stages of in vitro embryo development; b) fast developing embryos at 48 h (4-cells) have less DSBs than slow developing embryos (2-cells); c) the oocyte activation protocol affects the occurrence of DSBs in embryos produced by SCNT; and d) better quality blastocysts with higher cell number have less DSBs than low quality blastocysts.

It has been shown that H2AX139ph foci larger than 0.30 μm^3 indicate the sites of DNA DSBs and co-localize with DNA-repair proteins including Rad51, BRCA1, 53BP1, NBS1, Mre11, Ku80, XRCC4, and DNA ligase IV (Paull et al., 2000a, McManus and Hendzel, 2005). In this study, we observed the presence of large H2AX139ph foci in high proportion of nuclei in swine embryos evaluated at day 1 (pronuclear stage), day 2 (2- and 4-cell stage) and day 7 (blastocyst stage) of in vitro culture. This suggests that swine embryos might rely on H2AX139ph to promote DNA-damage repair during all stages of development from zygote to the blastocyst stage. Although H2AX139ph foci were also reported in zygotes and cleaved embryos in rats (Barton et al., 2007, Grenier et al., 2012), there are likely differences among species since H2AX139ph foci were detected in cleaved embryos but not in zygotes of mice (2007, Yukawa et al., 2007).

Our study revealed that cultured swine embryos have high incidence of DSBs, which can likely compromise cell cycle progression, and embryo development and quality. Supporting this hypothesis are our findings showing that at 48 h of development the number of H2AX139ph foci was higher in 2-cell than in 4-cell embryos. Moreover, we have observed higher proportion of nuclei with ≥10 foci in 2-cell compared to 4-cell stage embryos. It has been shown that fast-cleaving swine embryos produce more and better quality blastocysts than slow-cleaving embryos (Dang-Nguyen et al., 2010, Coutinho et al., 2011). Similarly, fast developing porcine embryos produced by SCNT have higher potential to support embryo development than slow developing embryos (Isom et al., 2012). Therefore, based on current observations, it appears that higher incidence of DSBs delays early-embryo cleavages and compromises later embryo development and quality.

Our findings using blastocysts, both produced by IVF and SCNT, revealed a negative correlation between the percentage of cells presenting H2AX139ph foci and the total number of

cells per embryo. This suggests that the occurrence of DSBs is an important determinant of blastocyst quality. It has been shown that preimplantation stage embryos have the capacity to respond to DSBs by activating genes that control DNA damage repair mechanisms (Zheng et al., 2005, Henrique Barreta et al., 2012). DNA-damage response involves cell cycle coordination and arrest at G1/S or G2/M checkpoints to allow DNA-damage repair before DNA replication and cell division (Aquilina et al., 1999). This gives more time for the affected cells to repair DNA damage (Finkielstein et al., 2001), but in some cases can lead to activation of apoptotic pathways (Zhivotovsky and Kroemer, 2004, Cook et al., 2009). Our observation that embryos with higher proportion of H2AX139ph positive cells have lower cell number suggests that the checkpoint pathways are also induced in porcine embryos to repair/preserve genome integrity. Overall findings of this study were very similar in embryos produced by IVF and SCNT, which further supports an important effect of DSBs during in vitro embryo development.

Our study provides evidence that the occurrence of DSBs may represent an important component impairing development and efficiency of embryo production by SCNT. In somatic cells, DNA damage was shown to reduce the efficiency in the production of induced pluripotent stem cells (Marion et al., 2009). Moreover, H2AX139ph was shown to be important in maintaining self-renewal of mouse embryonic and induced pluripotent stem cells (Turinetto et al., 2012). Thus, in addition to affecting embryo development, one can speculate that DNA damage and/or presence of H2AX139ph may also play a role in cell reprogramming in SCNT embryos. However, this requires further investigation.

Previous studies have shown that oocyte activated with ION+Sr^{2+} result in superior development of SCNT swine embryos compared to those activated with ION alone (Che et al., 2007). One possibility is that Sr^{2+} treatment promotes a more physiological response in the oocyte,

and consequently reduces detrimental effects of cell cycle compatibilities between the transferred nucleus and the host cytoplasm. Abnormal cell cycle compatibilities are thought to affect chromatin replication, integrity and segregation in SCNT embryos (Campbell, 1999). In the present study, we investigated whether Sr^{2+} treatment affected the occurrence of DSBs in SCNT embryos. Interestingly, Sr^{2+} reduced the percentage of nuclei with less than 10 H2AX139ph foci and the total number of positive nuclei in 4-cell stage embryos. Moreover, the proportion of nuclei with H2AX139ph foci at the blastocyst stage was also reduced in the group activated with ION+Sr^{2+}. These observations demonstrate that better rates of embryo production from oocytes activated by Sr^{2+} can be a consequence of a reduction in the occurrence of DSBs in the developing embryo.

In summary, this study has for the first time characterized the presence of H2AX139ph during in vitro development of swine embryos produced by IVF and SCNT. Based on the presence of H2AX139ph foci, we confirmed that DSBs occur during all stages of culture from zygotes to blastocysts. We have also observed that fast developing embryos have less DSBs than slow developing embryos, as well as the existence of a negative correlation between the number of cells with DSBs and total number of cells in blastocyst-stage embryos. Moreover, we provide evidence that the activation protocol influences the occurrence of DSBs in cleaved stage embryo produced by SCNT. Together, these observations suggest that the presence of H2AX139ph foci is a good indicator of embryo quality, which could be used as a marker for developing improved protocols for in vitro embryo production.

4.6. Acknowledgments

The authors are thankful to Olymel S.E.C./L.P. for the donation of porcine ovaries. This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada. R.C.B. was supported by a scholarship from the Science Without Borders Program of the Brazilian Council of Scientific and Technological Development (CNPq).

4.7. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

4.8. Figures

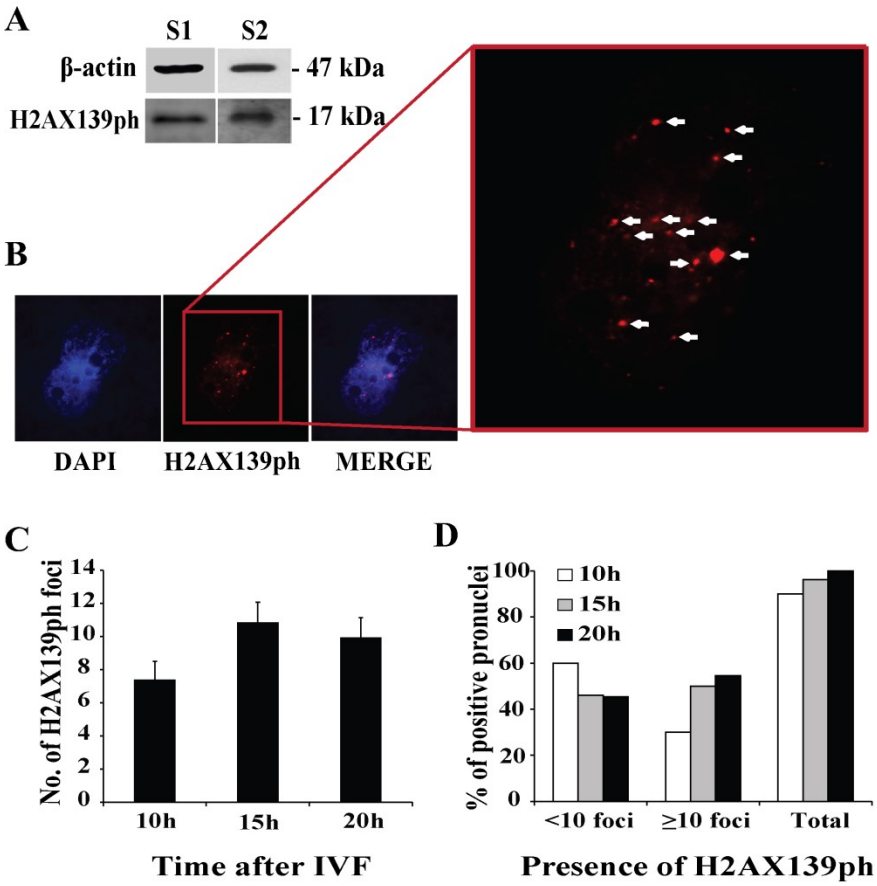

Figure 4.8.1. A) Immunoblots showing protein bands for H2AX139ph and β-actin in porcine fetal fibroblasts (S1) and day 7 porcine embryos (S2). B) Representative fluorescent images showing the presence of H2AX139ph foci (red) in the pronuclei (blue) of a porcine zygote fixed at 20 hour after IVF. Arrows indicate H2AX139ph foci bigger than 0.30 μm^3. C) Mean number of H2AX139ph foci in pronuclei of porcine zygotes fixed at 10, 15 and 20 h after IVF. D) Percentage of zygotes with H2AX139ph foci (<10 foci; ≥10 foci; total positives) at 10, 15 and 20 h after IVF. Data in C and D were not statistically different (P>0.05).

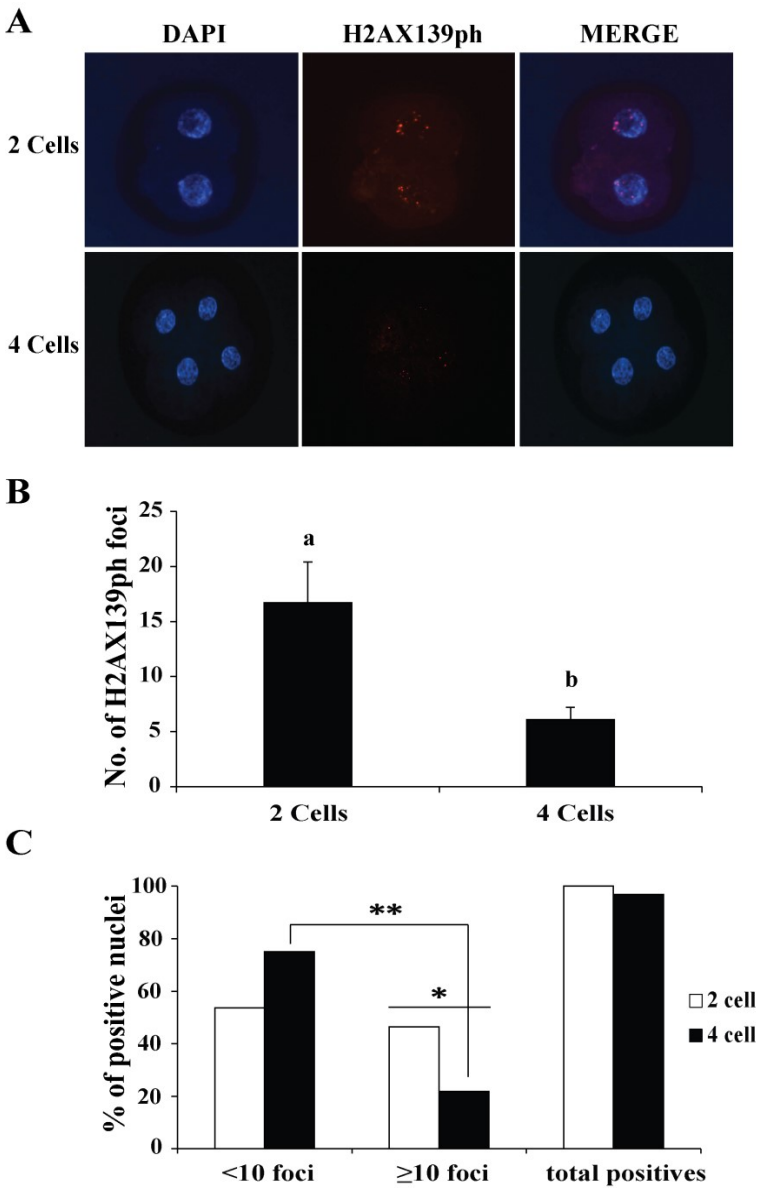

Figure 4.8.2. Presence of H2AX139ph foci in cleaved embryos fixed at 48 h after IVF. (A) Representative fluorescent images showing the presence of H2AX139ph foci in 2- and 4-cell embryos. (B) Mean number of H2AX139ph foci in 2- and 4-cell stage embryos (a vs. b, P=0.0119). (C) Percentage of nuclei with H2AX139ph foci (<10 foci; ≥10 foci; total positives) from the total number of evaluated nuclei. * P=0.0432; ** P=0.0041.

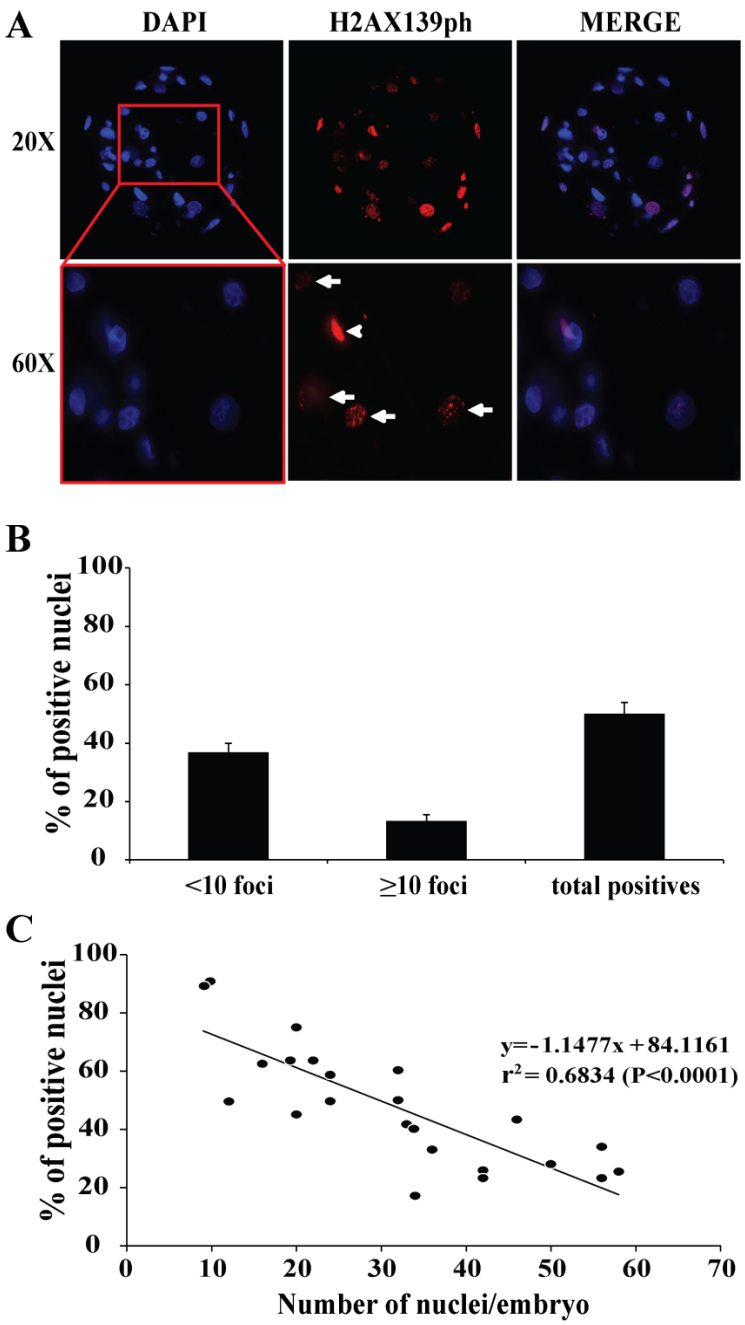

Figure 4.8.3. Presence of H2AX139ph in blastocysts at d-7 after IVF. (A) Representative fluorescent image of a day-7 blastocyst produced by IVF. Cell nuclei are stained in blue and H2AX139ph foci in red. Arrows indicate the presence of H2AX139ph foci in nuclei (≥10 foci and <10 foci) and arrowhead indicates a nucleus in M phase with diffuse H2AX139ph staining but absence of H2AX139ph foci. (B) Percentage of nuclei with H2AX139ph foci (<10 foci; ≥10 foci;

total positives) from the total number of evaluated nuclei. Different letters indicate statistical difference ($P<0.05$) between groups. (C) Correlation between the percentage of H2AX139ph positive nuclei and total number of nuclei in d-7 blastocysts ($P<0.0001$).

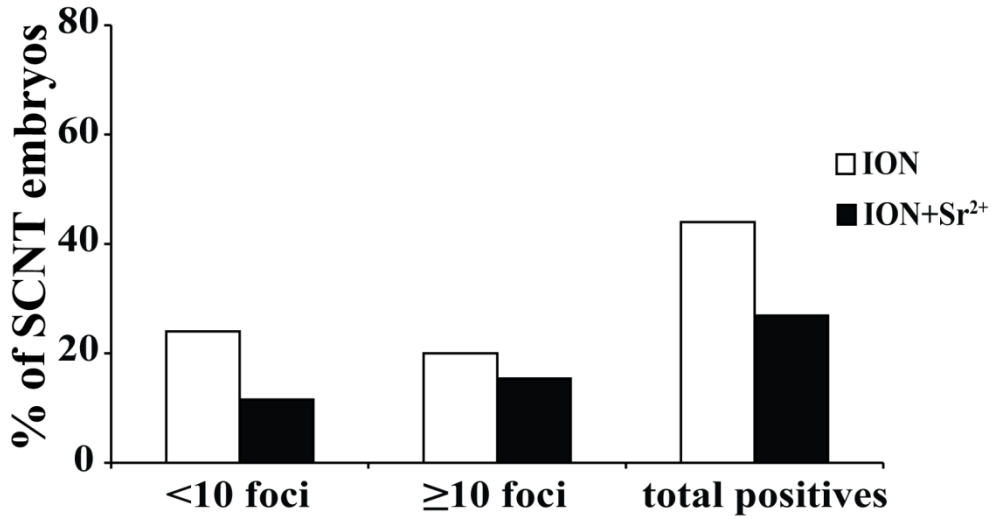

Figure 4.8.4. Presence H2AX139ph in 1-cell SCNT embryos fixed at 20 h after cell fusion. Bars represent the proportion of H2AX139ph positive embryos presenting <10 foci, ≥10 foci and total positives from the total number of evaluated nuclei. Open and black bars represent embryos activated with ION and ION+Sr^{2+}, respectively. Differences between ION and ION+Sr^{2+} groups were not statistically significant ($P>0.05$).

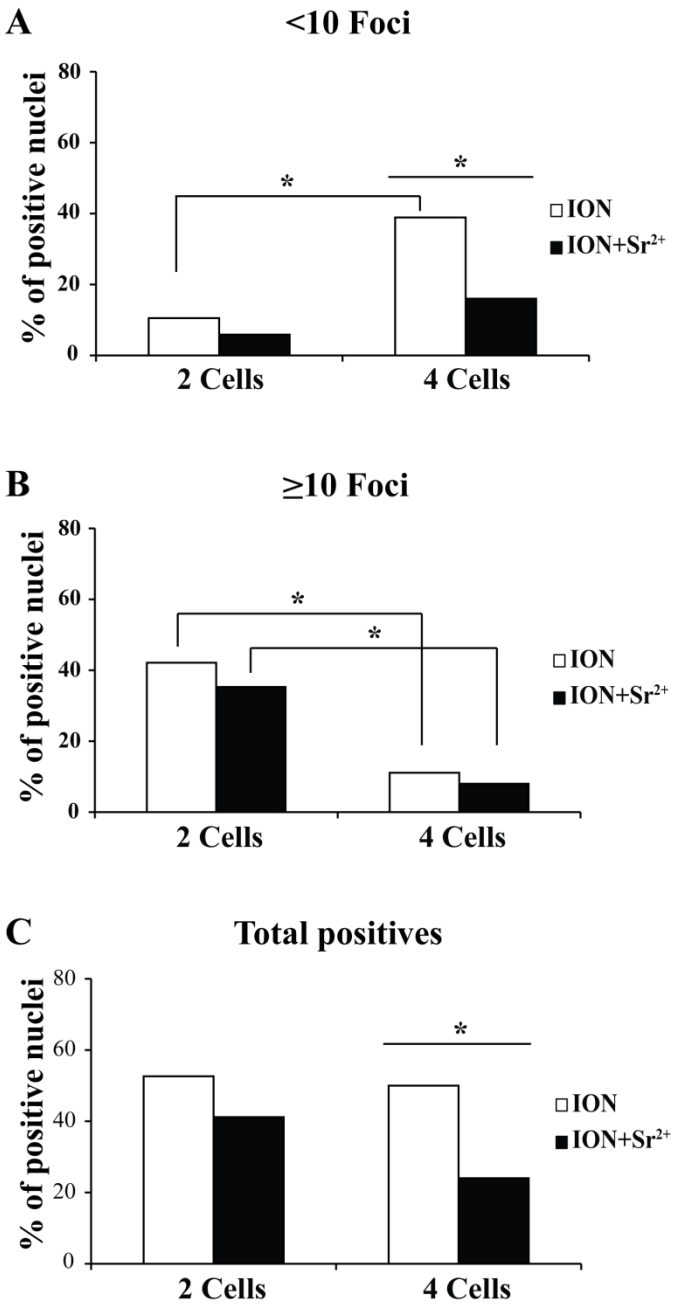

Figure 4.8.5. Effect of the activation protocol on the occurrence of H2AX139ph in SCNT embryos fixed at 48 h after reconstruction. Percentage of H2AX139ph positive nuclei with <10 foci (A), ≥10 foci (B) and total positives (C) from the total number of evaluated nuclei in 2- and 4-cell stage embryos. Open and black bars represent embryos activated with ION and ION+Sr^{2+}, respectively. The asterisk indicates significant difference ($P<0.05$) between groups.

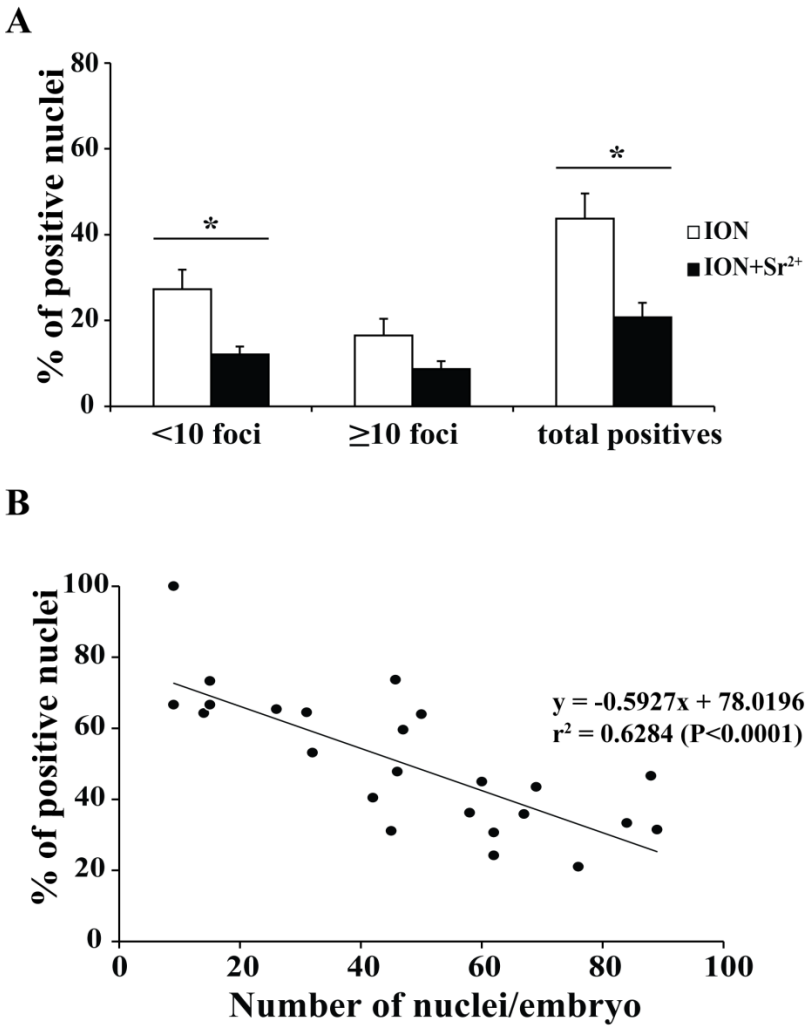

Figure 4.8.6. Presence of H2AX139ph foci and correlation between embryo cell number and presence of H2AX139ph in SCNT blastocysts produced with different activation protocols. (A) Percentage of nuclei with H2AX139ph foci (<10 foci, ≥10 foci and total positives) from the total number of evaluated nuclei in SCNT blastocysts fixed at day-7. Open and black bars represent embryos activated with ION and ION+Sr^{2+}, respectively. The asterisk indicates statistical difference ($P<0.05$) between ION and ION+Sr^{2+} groups. (B) Correlation between the percentage of H2AX139ph positive nuclei and total number of nuclei in d-7 SCNT blastocysts ($P<0.0001$).

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CONNECTING STATEMENT 2

Studies have shown that treatment of SCNT embryos with HDACi improves embryo development to blastocyst stage and cloning efficiency in various species (Van Thuan et al., 2009, Zhao et al., 2010, Wang et al., 2011b, Chen et al., 2013). It has also been shown that HDACi treatment facilitates donor cell reprogramming during SCNT by increasing chromatin accessibility to reprogramming factors present inside the oocyte. Indeed, previous studies from our group have shown that HDACi treatment increases the acetylation of the histone H3K14 (Martinez-Diaz et al., 2010). Interestingly, it was shown that increased chromatin accessibility is also important for DNA DSBs repair (Rossetto et al., 2010). Moreover, genome integrity preservation is indispensable for somatic cell reprogramming during the production of iPS cells (Marion et al., 2009, González et al., 2013, Molina-Estevez et al., 2013). Those findings suggest that HDACi treatment may facilitate DNA DSBs repair during cell reprogramming in SCNT embryos. Therefore, in the second manuscript of this thesis (Chapter 5) we investigated the effect of DNA DSBs on development of SCNT embryos and assessed the effect of HDACi treatment on DNA DSBs repair and development of SCNT embryos.

CHAPTER 5

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Inhibition of histone deacetylases enhances DNA-damage repair in SCNT embryos

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5.1. Abstract

Recent studies have shown that DNA damage affects embryo development and also somatic cell reprogramming into induced pluripotent stem (iPS) cells. It has been also shown that treatment with histone deacetylase inhibitors (HDACi) improves development of embryos produced by somatic cell nuclear transfer (SCNT) and enhances somatic cell reprogramming. There is evidence that increasing histone acetylation at the sites of DNA double-strand breaks (DSBs) is critical for DNA-damage repair. Therefore, we hypothesized that HDACi treatment enhances cell programming and embryo development by facilitating DNA-damage repair. To test this hypothesis we first established a DNA-damage model wherein exposure of nuclear donor cells to ultraviolet (UV) light prior to nuclear transfer reduced the development of SCNT embryos proportional to the length of UV exposure. Detection of phosphorylated histone H2A.x (H2AX139ph) foci confirmed that exposure of nuclear donor cells to UV light for 10 s was sufficient to increase DSBs in SCNT embryos. Treatment with HDACi during embryo culture increased development and reduced DSBs in SCNT embryo produced from UV treated cells. Transcript abundance of genes involved in either the homologous recombination (HR) or nonhomologous end-joining (NHEJ) pathways for DSBs repair was reduced by HDACi treatment in developing embryos at day 5 after SCNT. Interestingly, expression of HR and NHEJ genes was similar between HDACi-treated and control SCNT embryos that developed to the blastocyst stage. This suggested that the increased number of embryos that could achieve the blastocyst stage in response to HDACi treatment have repaired DNA damage. These results demonstrate that DNA-damage in nuclear donor cells is an important component affecting development of SCNT embryos, and that HDACi treatment after nuclear transfer enhances DSBs repair and development of SCNT embryos.

5.2. Introduction

Cell reprogramming, dedifferentiation and transdifferentiation are at the forefront of cell biology research. A better understanding of the mechanisms affecting these phenomena is required to fully realize the promise of cell-based therapies. The complete reprogramming of a somatic cell after it transfer into an enucleated oocyte resulting in the birth of the first live cloned animal represented a major breakthrough in cell reprogramming research (Wilmut et al., 1997). Indeed, the confirmation that differentiated somatic cells could be reprogrammed to a pluripotent state paved the way for the production of induced pluripotent stem cells (iPS) by transduction of a small number of transcription factors (Takahashi and Yamanaka, 2006). Cell reprogramming after somatic cell nuclear transfer (SCNT) relies on reprogramming factors present in the oocyte cytoplasm, which induce chromatin remodeling and epigenetic modifications to restore the pluripotent state (Bui et al., 2008). Many studies have investigated chromatin remodeling and epigenetic reprogramming after SCNT and provided substantial evidence that the low efficiency in the production of live animals by SCNT cloning is mainly a consequence of insufficient nuclear reprogramming (Dean et al., 2001, Wee et al., 2006, Wang et al., 2007, Fulka et al., 2008, Niemann et al., 2008). Nevertheless, the effect of genome integrity and DNA-damage repair on somatic cell reprogramming towards pluripotency has not been thoroughly investigated.

It has been shown that maintenance of genome integrity is critical for production of induced pluripotent stem (iPS) cells in humans and mice (Marion et al., 2009, González et al., 2013, Molina-Estevez et al., 2013), suggesting that genome integrity may also affect somatic cell reprogramming and development of SCNT embryos. DNA double-stranded breaks (DSBs) are recognized as the most biologically significant genotoxic lesions (Khanna and Jackson, 2001). DBS induces a rapid cellular response, which involves post-translational modification and

accumulation of proteins involved in DNA repair mechanisms thereby resulting in the formation of multiprotein foci. In mammals, two molecular pathways, homologous recombination (HR) and nonhomologous end-joining (NHEJ), are involved in DSB repair (Haber, 2000, Karran, 2000). In response to DNA damage, the protein kinases ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), and DNA-PK (DNA-dependent protein kinase) phosphorylate the histone H2A.x (H2AX139ph) at the sites of DSB formation (Burma et al., 2001, Stiff et al., 2004). This phosphorylation involves large chromatin domains forming nuclear foci that are easily detected by immunostaining (Rogakou et al., 1999). The phosphorylation of the histone H2A.X is critical for DSB repair because it anchors some important initiator proteins required for both HR (e.g., RAD51, MRE11, BRCA1) and NHEJ (e.g., 53BP1, KU80, DNA ligase IV, XRCC4) pathways, which can be colocalized with H2AX139ph at the sites of DSBs (Paull et al., 2000, McManus and Hendzel, 2005). The proteins RAD51 and 53BP1 are key factors for DNA-damage repair. RAD51 acts in the HR pathway by binding to single-stranded DNA (ssDNA) coated by the replication protein A (RPA) and promoting the invasion of the single-stranded end into the duplex DNA during homologous pair (Baumann et al., 1996, West, 2003). 53BP1 acts in NHEJ pathway by binding to dimethylated lysine 20 in histone H4 (H4K20me2) in the sites of DSBs and increasing the mobility of damaged chromatin to facilitate the joining of broken DNA ends (Botuyan et al., 2006, Dimitrova et al., 2008).

Exposure to UV light has been used to study DNA-damage signaling and repair mechanisms in somatic cells (Scarpato et al., 2013), oocytes (Carroll and Marangos, 2013) and zygotes (Mu et al., 2011). UV radiation induces DNA damage primarily by the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Ravanat et al., 2001). During DNA replication, single-stranded DNA gaps are generated by stalling of replication

forks at UV-damaged sites (Cortez, 2005). If not repaired, the stalled fork gaps can result in the formation of DSBs (Peak and Peak, 1990, Petermann and Helleday, 2010). UV radiation activates ATR and ATM kinases in ATR-dependent manner (Stiff et al., 2006), which will phosphorylate the histone H2A.x in a dose dependent manner (Staszewski et al., 2008). H2AX139ph has been co-localized with DNA-damage repair proteins, including RAD51 and 53BP1, at the sites of DSBs induced by UV exposure (de Feraudy et al., 2010, Oh et al., 2011).

Inhibitors of histone deacetylases (HDACi) have been used to improve cell reprogramming in embryos produced by SCNT in various species (Maalouf et al., 2009, Zhao et al., 2010, Wang et al., 2011). Porcine SCNT embryos treated with HDACi have increased acetylation of different lysine residues including H3K14 (Martinez-Diaz et al., 2010), H4K5 (Iager et al., 2008) and H4K8 (Zhao et al., 2010). Moreover, treatment with HDACi was shown to reduce DNA methylation and expression of DNA methyltransferases in donor cells and in SCNT embryos (Wee et al., 2007, Li et al., 2008). Indeed, there is evidence that HDACi treatment improves normal gene expression in developing SCNT embryos (Li et al., 2008, Shao et al., 2009). Scriptaid is a novel HDACi with low toxicity and enhanced transcriptional activity (Su et al., 2000). Scriptaid treatment of SCNT embryos increased development and live animal production in various species including swine (Van Thuan et al., 2009, Zhao et al., 2009, Wang et al., 2011, Chen et al., 2013). While effects of HDACi on epigenetic reprogramming have been comprehensively investigated, it potential effects on DNA-damage repair and maintenance of genome integrity during cell reprogramming in SCNT embryos has not been studied.

A higher incidence of DSBs has been detected in late-cleaving and lower quality embryos produced by SCNT or IVF (Bohrer et al., 2013). In somatic cells, histone modifications play important roles in DSB repair. For instance, the acetylation of histone H4K16 is critical for DNA

damage response and DSB repair by HR and NHEJ pathways (Sharma et al., 2010). In addition, acetylation of histone H4K5 and H4K12 at the sites of DSBs facilitates the recruitment of the RAD51 protein (Yang et al., 2013). These studies suggest that increasing histone acetylation by HDACi treatment may have a positive effect on DNA-damage repair during cell reprogramming in SCNT embryos. Therefore, the objectives of this study were: 1) to evaluate the effect of DNA damage on development of SCNT embryos; and 2) to assess the effect of HDACi treatment on DNA-damage repair and development in SCNT embryos.

5.3. Results

5.3.1. Exposure of donor cells to UV prior to nuclear transfer affects development of SCNT embryos

First, we evaluated the effect of UV light exposure (10, 30 or 60 s) of nuclear donor cells prior to nuclear transfer on the development of SCNT embryos. Exposure of nuclear donor cells to UV light had detrimental effects on SCNT embryo cleavage and development to the blastocyst stage (Fig. 1). The cleavage rate was similar between embryos reconstructed with control cells and UV light treated cells for 10 s, but it was approximately 50% lower when donor cells were exposed to UV light for 60 s (Fig. 1A).

Although a significant proportion of embryos that were reconstructed with cells in all UV treatments could develop beyond the first cleavage stage, development to the blastocyst stage at day 7 was dramatically reduced by UV exposure (Fig 1B). Indeed, development to blastocyst was completely blocked by 60 s of UV exposure, while blastocyst rate was approximately 65% reduced by 10 s treatment compared to control cells. Based on these results, we chose 10 s as the UV

treatment for subsequent studies because it did not completely suppress development of SCNT embryos.

5.3.2. UV exposure up-regulated DNA-damage response proteins in donor cells and in SCNT embryos

We then evaluated the effect of 10 s UV exposure on the abundance of DNA-damage sensor (H2AX139ph) and repair (RAD51 and 53BP1) proteins in nuclear donor cells and SCNT embryos. UV treatment increased the abundance of these three proteins 24 h later in nuclear donor cells (Fig. 2). Similarly, the abundance of these proteins in nuclear foci was significantly higher in 1-cell (20 h after SCNT) and cleaved (48 h after SCNT) SCNT embryos derived from the UV-treated compared to those derived from control cells (Figs. 3 and 4). There was a clear co-localization between the DNA-damage sensor and repair proteins in both 1-cell and cleaved SCNT embryos. These results confirmed the increased incidence of DNA damage in embryos produced from the UV-treated cells.

5.3.3. HDACi treatment increased development and reduced DNA-damage response proteins in SCNT embryos

The effect of HDACi treatment was evaluated in SCNT embryos produced from control and UV-exposed cells. Cleavage rates were similar between HDCAi treated and control embryos reconstructed either with control or UV-treated donor cells (Fig. 5A). Interestingly, compared to non-treated embryos, HDACi treatment resulted in a 4.5-fold increase (4.1% vs. 18.3%) in development to the blastocyst stage of embryos reconstructed using UV-exposed cells (Fig. 5B).

HDACi treatment also reduced the number of foci containing H2AX139ph, RAD51 and 53BP1 proteins in cleaved embryos produced using UV-treated cells (Fig. 6).

5.3.4. HDACi treatment reduced the expression of DNA-damage response genes in SCNT embryos

To further explore the effect of HDACi treatment on DBS repair, transcript abundance of DNA-damage response genes that participate in the HR and NHEJ repair pathways was evaluated in day-5 and -7 SCNT embryos. Day-5 embryos produced from UV-treated cells had higher mRNA levels of genes involved in HR (*RAD51*, *BRCA1*, *RPA1*, *MRE11A*; Fig. 7A) and NHEJ (*PRKDC*, *TP53BP1*, *XRCC6*, *XRCC5*, *LIG4*, *XRCC4*, Fig. 7B) than embryos derived from control cells. Interestingly, HDACi treatment reduced the overall expression of all assessed genes in embryos reconstructed with UV-treated donor cells to levels that were equivalent ($P > 0.05$) to those observed in embryos derived from control cells (Fig. 7A and B). Moreover, comparison between HDACi-treated and control embryos derived from UV-treated donor cells revealed significant reduction in the mRNA levels of *PRKDC* and *XRCC6* (Fig. 7B).

In contrast to findings in day-5 embryos, there was no difference in the levels of mRNA expression for genes involved in the two DNA-damage repair pathways in day-7 SCNT embryos (Fig. 8). Interestingly, SCNT embryos derived from UV-treated donor cells that successfully develop to the blastocyst stage have similar expression levels of DNA damage response genes compared to embryos derived from control donor cells. There was also no effect of HDACi treatment on gene expression at the blastocyst stage, either in embryos derived from UV-treated

or control donor cells. These results suggest that SCNT embryos that developed to the blastocyst stage had completed DNA damage repair by day-7.

5.4. Discussion

Findings in this study provide strong evidence that genome integrity is an important component affecting cell reprogramming in embryos produced by somatic cell nuclear transfer. More interestingly, we found that treatment with HDACi promoted DNA-damage repair during cell reprogramming and improved development of embryos produced by SCNT. These findings suggest that chromatin remodeling during cell reprogramming is required to promote DNA-damage repair in SCNT embryos. It also reveals that the positive role of HDACi treatment, which has been shown to increase the development of embryos produced by SCNT (Zhao et al., 2009, Martinez-Diaz et al., 2010, Zhao et al., 2010, Wang et al., 2011), is at least in part due to its role in promoting DSB repair.

In order to assess the impact of DNA integrity on cell reprogramming in embryos produced by SCNT, we first used nuclear donor cells that were exposed to UV light. As expected, but previously not confirmed, increasing exposure of nuclear donor cells to UV light progressively reduced embryo development after SCNT. The increased expression of H2AX139ph, RAD51 and 53BP1 proteins confirmed that UV treatment induced DSBs in nuclear donor cells. It is well documented that H2AX139ph accumulates around the sites of DSBs (Rogakou et al., 1999) and it plays an indispensable role in the recruitment of repair proteins, including RAD51 and 53BP1, which are key proteins for DNA-damage repair by HR and NHEJ pathways, respectively (West, 2003, Botuyan et al., 2006, Dimitrova et al., 2008).

Based on the number and co-localization of immunofluorescent foci for H2AX139ph, RAD51 and 53BP1 proteins, we have confirmed that SCNT embryos produced from UV treated cells have higher prevalence of DSBs compared to embryos produced from control cells. The dramatic reduction in development to the blastocyst stage of embryos produced from UV treated cells indicates that preserving genome integrity is critical for somatic cell reprogramming and the generation of SCNT blastocysts. These findings are in line with previous studies showing that the preservation of genome integrity is a crucial condition for somatic cell reprogramming and production of iPS cells in human and mouse (Marion et al., 2009, González et al., 2013, Molina-Estevez et al., 2013). In addition, there is also evidence that DNA damage affects cell cycle progression and compromises development of normal fertilized mouse embryos (Gawecka et al., 2013, Wang et al., 2013). Our results using SCNT embryos provided further evidence that both cell reprogramming and early embryo development are severely affected when the genome integrity is disrupted.

Chromatin remodelling and epigenetic changes, including histone modifications, are important parameters for cell reprogramming and normal development of SCNT embryos (Dean et al., 2001, Santos et al., 2003, Wee et al., 2006, Niemann et al., 2008). In this context, treatment with HDACi has been used to improve donor cell reprogramming and to increase the developmental capacity of SCNT embryos and the efficiency of animal cloning from somatic cells in various species including swine (Maalouf et al., 2009, Zhao et al., 2010, Wang et al., 2011, Chen et al., 2013). Although the mechanisms by which HDACi promote cell reprogramming and development have not been completely characterized, it has been shown that HDACi treatment increases the acetylation levels of multiple lysine residues on histones, including H3K14 (Martinez-Diaz et al., 2010), H4K5 (Iager et al., 2008) and H4K8 (Zhao et al., 2010), in SCNT

embryos. It is known from studies in somatic cells that acetylation of lysine residues, including H4K5, H4K12 and H4K16, by different histone acetyltransferases (HATs) is critical for DNA-damage repair (Tamburini and Tyler, 2005, Sharma et al., 2010, Tang et al., 2013, Yang et al., 2013). It was also shown that the HAT Gcn5 can interact with H2AX139ph in the sites of DSBs and then acetylate various lyse residues on the histone H3, including H3K9, H3K14, H3K18 and H3K23 (Lee et al., 2010a). Based on those discoveries we have hypothesized that increasing histone acetylation by HDACi treatment would facilitate DNA-damage repair during cell reprogramming in SCNT embryos. Indeed, we have found that HDACi treatment greatly increased development to the blastocyst stage of SCNT embryos produced from UV treated cells. Moreover, HDACi treatment consistently reduced the number of fluorescent foci for H2AX139ph, RAD51 and 53BP1 in cleaved SCNT embryos produced from UV treated cells. These findings indicate that HDACi treatment can facilitate DNA-damage repair in SCNT embryos. Although HDACi may affect other DNA functions, including transcription and replication (Wanczyk et al., 2011), its positive effect on DSB repair seems to be an important route by which HDACi treatment improves SCNT embryo development.

In this study, we have not evaluated the effect of HDACi on DNA-damage repair in fibroblast cells before nuclear transfer. Although similar effects of HDACi would be expected to occur in cultured cells, there is evidence from other studies that HDACi treatment may reduce DNA-damage repair in cultured human fibroblasts cells (Lee et al., 2010b, Purrucker et al., 2010). It is therefore possible that the positive effect of HDACi treatment observed after SCNT depend upon interactions with chromatin remodeling and/or DNA-damage repair factors present in the host oocyte cytoplasm, which does not occur when cultured cells are only exposed to HDACi without nuclear transfer. Duration of exposure represents another potential reason affecting cell

response to HDACi treatment. Indeed, HDAC inhibitors have been used as anticancer agents because prolonged exposure to these compounds, alone or in association with other drugs, affects cell proliferation and induce cell death in different types of cells in culture (Munshi et al., 2005, Marks and Xu, 2009, Di Micco et al., 2011, Lee et al., 2011, Diyabalanage et al., 2013). In the current study, SCNT embryos were exposed to HDACi for a period of 15h, which might be sufficient to promote chromatin remodeling without affecting cell viability as observed in cells treated for longer periods. In line with this are previous studies confirming that longer exposure to HDACi can also reduce development of SCNT embryos (Kishigami et al., 2006, Akagi et al., 2011).

In order to further evaluate the effect of HDACi treatment on DSB repair in SCNT embryos, we assessed the expression of several genes involved in either the HR or the NHEJ DNA-repair pathways in SCNT embryos at day-5 and -7 of development. Higher abundance of transcripts involved in the two DNA-damage repair pathways was detected in day 5 SCNT embryos derived from UV treated cells, which confirms that both repair pathways were regulated in SCNT embryos. This is in line with previous studies whereas genes involved in the HR and NHEJ pathways were induced by UV treatment in fertilized bovine embryos produced in vitro (Henrique Barreta et al., 2012). In this study, we observed an overall tendency for a decreasing expression of genes involved in the HR and NHEJ repair pathways in day 5 SCNT embryos that were treated with HDACi. Importantly, HDACi treatment significantly reduced the mRNA abundance for *XRCC6* and *PRKDC* genes in embryos produced from UV treated cells. These genes encode, respectively, for the KU70 and DNA-PK proteins, which are key factors for DSBs repair in the NHEJ pathway (Cary et al., 1997, Lieber, 2008). The lower expression of DNA-repair genes indicate that day 5 SCNT embryos treated with HDACi have less DSBs. In contrast to day-5

embryos, we observed that none of the assessed genes were differentially expressed in day-7 SCNT blastocysts, even in those derived from UV treated donor cells. These results suggest that only the embryos that have repaired DNA-damage were able to develop to the blastocyst stage. The fact that HDACi could rescue the development of higher proportion of SCNT embryos derived from UV treated cells and that important genes involved in either HR or NHEJ repair pathways were normally expressed in the developing blastocysts further indicate that HDACi treatment have promoted DSBs repair during embryo development.

In conclusion, based on a model of UV-induced DNA damage in cultured somatic cells followed by SCNT into enucleated oocytes, we have shown that genome integrity in nuclear donor cells has critical consequences on the development of SCNT embryos. Moreover, we have shown that HDACi exposure can rescue development of SCNT embryos derived from DNA-damaged donor cells by improving DNA-damage repair during early embryo development. Based on these findings, we propose that chromatin remodeling during cell reprogramming after SCNT is not only important for the resetting of the epigenetic program but also to promote DNA-damage repair and preserve the genome integrity for normal embryo development. In this context, increasing histone acetylation by treatment with HDACi seems to be a promising alternative.

5.5. Materials and Methods

5.5.1. Chemicals

Unless otherwise indicated, chemical and reagents were purchased from Sigma Chemical Company (Sigma–Aldrich, Oakville, ON, Canada).

5.5.2. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were obtained from a local abattoir (Olymel S.E.C./L.P., Saint-Esprit, QC, Canada) and transported to the laboratory in 0.9% NaCl at 32 to 35°C. Follicles ranging from 3 to 6 mm in diameter were aspirated using an 18-gauge needle. Only cumulus-oocyte complexes (COCs) surrounded by a minimum of three cumulus cell layers and having an evenly granulated cytoplasm were selected for *in vitro* maturation (IVM). Groups of 30 COCs were cultured in 0.1 mL of maturation medium under mineral oil in a humidified atmosphere of 5% CO2 and 95% air at 38.5°C. Maturation medium consisted 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). After 22 to 24 h of maturation, oocytes were transferred to the same IVM medium, but without LH, FSH and dbcAMP, for an additional 20 to 22 h under the same conditions. Cumulus cells were removed by pipetting in TCM 199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% of hyaluronidase and then oocytes were used for SCNT.

5.5.3. Nuclear donor cell culture and UV light exposure

Porcine fetal fibroblast cells were *in vitro* cultured in cell culture media (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM) supplemented with 10% fetal

bovine serum (FBS; Life Technologies) and 1% antibiotics (Penicillin 10,000U/ml and Streptomycin 10,000 µg/ml; Life Technologies) at 38.5ºC in 5% CO_2 and 95% air until confluence. Cells were then trypsinized (0.25% trypsin-EDTA; Life Technologies), washed and resuspended in cell culture media. To induce DNA-damage, trypsinized cells were placed in 35 mm culture dishes (Corning, Tewksbury MA, United States) containing 1 ml of cell culture media and exposed to UV light for 0 (control group), 10, 30 and 60 s in a type B2 biological safety cabinet (Thermo Scientific™ 1300 Series Class II). Immediately after UV light exposure, cells were used for SCNT. To analyse DNA-damage and repair proteins in donor cells, confluent fetal fibroblasts were exposed to UV light for 10 s, using the same conditions indicated above, and then *in vitro* cultured for 24 h before protein extraction and western blot analysis.

5.5.4. Somatic cell nuclear transfer (SCNT)

Cumulus-free oocytes selected with a polar body were cultured in TCM199 supplemented with 0.4 ∝g/mL demecolcine and 0.05 M sucrose for 60 minutes. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes.(Yin et al., 2002) Oocytes were then transferred to TCM 199 HEPES-buffered medium supplemented with 2 mg/ml of BSA (fatty acid free), 20 ∝g/ml gentamicin and 7.5 mg/mL cytochalasin B for 5 to 10 minutes and enucleated by removing the protruded oocyte chromatin together with the first polar body. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte and electrically fused using a single DC pulse of 1.6 kV/cm for 70 µs. Electrofusion was performed into a 0.28 M mannitol solution supplemented with 50 ∝M $CaCl_2$, 100 ∝M $MgSO_4$ and 0.1% PVA. Reconstructed oocytes were cultured in TCM199 medium supplemented with 3 mg/mL BSA for

1 h before activation. For activation, oocytes were exposed to ionomycin (15 μM) for 5 minutes, then transferred to 10 mM of Strontium chloride in porcine zygote medium (PZM-3) without Ca^{2+} supplemented with cytochalasin B (7.5 μg/ml) and cycloheximide (10 μg/ml) for 4 h. Reconstructed oocytes were treated (HDACi group) or not (Control group) with Scriptaid (500 μM) for 15 h starting after ionomycin treatment.

5.5.5. Embryo culture

After SCNT oocytes were washed in PZM-3 medium supplemented with 3 mg/mL BSA (fatty acid-free) and cultured in a humidified atmosphere of 5% CO2 and 95% air at 38.5 ºC. The cleavage rate was determined 48 h after activation. Culture medium was supplemented with 10% FBS on day 5 and blastocyst rate was evaluated on day 7 of embryo culture.

5.5.6. Western Blot

Porcine fetal fibroblasts were lysed in Laemmli buffer (Bio-Rad, Mississauga, ON, Canada) containing phosphatases and proteases inhibitor cocktail (G-Biosciences, St. Louis, MO, United States). Total lysate was size fractionated by SDS-PAGE 5% to 12.5% gel and electroblotted onto nitrocellulose membranes (Bio-Rad). After blocking with 5% BSA or skim milk powder in Tris-buffered saline (TBS) + 0.1% of Tween, pH 7.6, membranes were incubated overnight with primary antibodies (H2AX139ph (05-636; Millipore, Billerica, MA, United States) 1:1000; 53BP1 (4937; Cell Signaling, Boston, MA, United States) 1:1000; RAD51 (sc-8349; Santa Cruz Biotechnology, Dallas, TX, United States) 1:1000; Beta-actin (ab8227; Abcam Inc. Toronto, ON, Canada) 1:5000 diluted in blocking solution. Subsequently, membranes were incubated for 1

h with peroxidase-conjugated secondary antibody anti-mouse (7076; Cell Signaling) diluted 1:3000 or anti-rabbit (ab6721; Abcam) diluted 1:10.000 in blocking solution. Membranes were incubated for 3 minutes with Immun-Star WesternC Chemiluminescent Kit and images were captured using the ChemiDoc MP System (Bio-Rad). Images were analyzed and bands were quantified using the Image Lab 3.0 software (Bio-Rad).

5.5.7. Immunofluorescence Staining

SCNT embryos at 20h and 48h after oocyte activation were fixed for 15-20 minutes in 4% paraformaldehyde and permeabilized in 1% Triton X-100 in phosphate-buffered saline (PBS) during 30 minutes at 37°C. Samples were incubated for 1 h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then incubated overnight with primary antibodies (H2AX139ph, 1:1000; 53BP1, 1:300; RAD51, 1:500) diluted in blocking solution. Embryos were incubated for 1 h at room temperature in the presence of secondary antibodies anti-mouse IgG Cy3-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) diluted 1:1000 or anti-rabbit IgG Alexa Fluor 488 (Life technologies) diluted 1:1000. DNA was stained by exposing the samples to 10 ∝g/ml of 4',6-diamidino-2-phenylindole (DAPI; Life technologies) in blocking solution for 20 minutes. Samples were then mounted on microscope slides using a drop of Mowiol. Slides were kept in a dark box and examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan). Images were captured at 600x magnification using a Retiga 2000R monochromo digital camera (Qimaging, Surrey, BC, Canada). The number and size of H2AX139ph foci were evaluated in each nuclei using the SimplePCI Imaging Software (Compix, Inc., Sewickley, PA, United States) and foci lager than 0.3 μm^3 were

counted as a site of DSBs.(Bohrer et al., 2013) All RAD51 and 53BP1 foci colocalized with H2AX139ph foci were also counted.

5.5.8. RNA extraction and qRT-PCR

Total RNA was extracted from groups of 20 (day 5) and 10 (day 7) SCNT embryos using the PicoPure RNA Isolation Kit (Life Technologies) according to the manufacturer's instruction. RNA was treated with DNase I (Qiagen, Mississauga, ON, Canada) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR reaction was carried out using a CFX384 Real-Time Detection System (Bio-Rad), iQ SYBR Green Supermix (Bio-Rad), 400nM of primers and 2 µl of cDNA. Primers (Table 1) were designed using Primer-Blast and specificity was confirmed using BLAST (NCBI, Bethesda MD, United States). Common thermal cycling parameters (5 minutes at 95ºC and 40 cycles of 15 seconds at 95ºC and 30 seconds at 60ºC) were used to amplify each transcript and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene and expression was normalized to the abundance of the housekeeping gene ACTB.(Park et al., 2011) All reactions used for quantification had efficiency between 90 -110%, $R^2 \geq 0.98$ and slope values from -3.6 to -3.1.

5.5.9. Statistical analysis

All analyses were performed using the JMP Software (SAS Institute, Inc., Cary, NC, United States). Continuous data were analyzed using ANOVA, and the means were compared

using Student's t-test or Tukey–Kramer Honestly Significant Difference (HSD) test for single or multiple comparisons, respectively. All experiments were repeated at least three times and data are expressed as mean ± s.e.m.. Differences were considered statistically significant if $P<0.05$.

5.6. Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

5.7. Funding

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5.8. Acknowledgements

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5.9. Figures

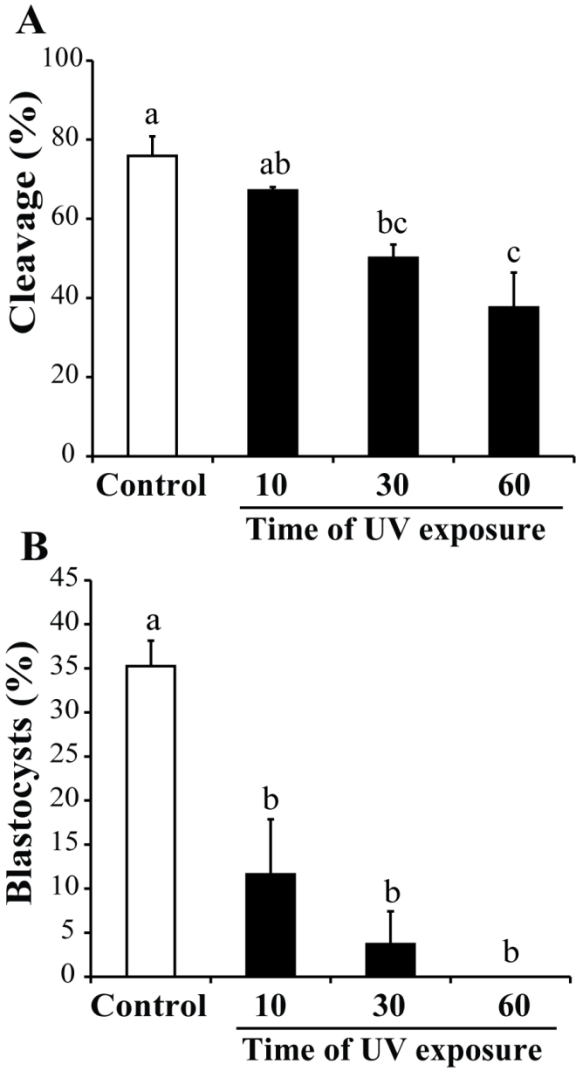

Figure 5.9.1. Development of SCNT embryos reconstructed with donor cells exposed to UV light for different lengths of time. Cleavage (A) and blastocyst (B) rates of SCNT embryos reconstructed with donor cells that were unexposed (Control) or exposed to UV light for 10, 30 and 60 seconds. Data are from three replicates with a minimum of 25 embryos in each group. Different letters indicate significant difference between groups ($p<0.05$).

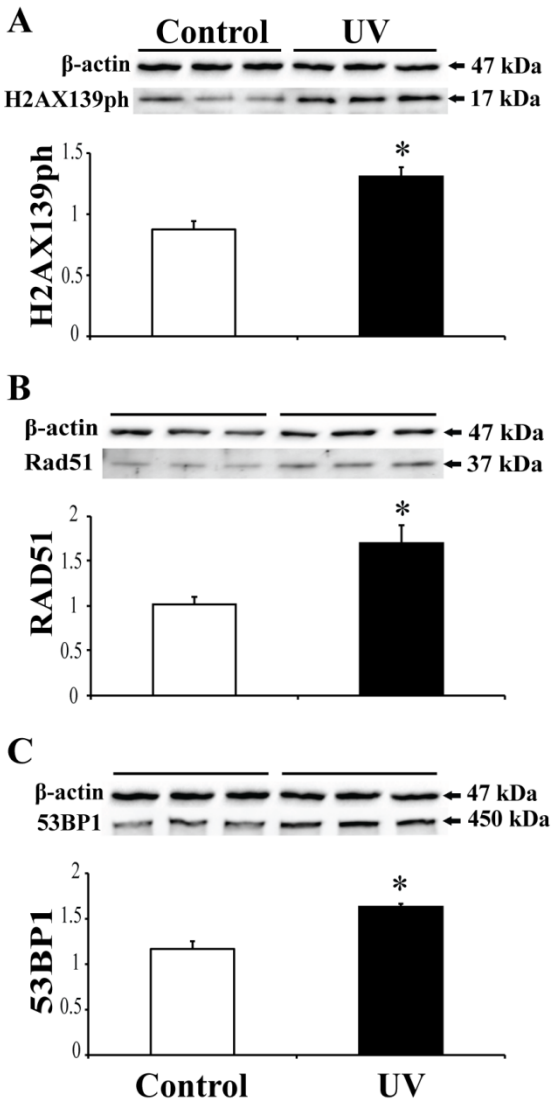

Figure 5.9.2. Quantification of H2AX139ph, RAD51 and 53BP1 proteins in donor cells exposed to UV light. Representative immunoblots and protein quantification of H2AX139ph (A), RAD51 (B), and 53BP1 (C) in donor cells that were unexposed (Control) and exposed to UV light for 10 seconds (UV). The abundance of each protein was calculated in relation to the loading control, beta actin (β-actin). Asterisk indicates statistical difference from control group ($P<0.05$).

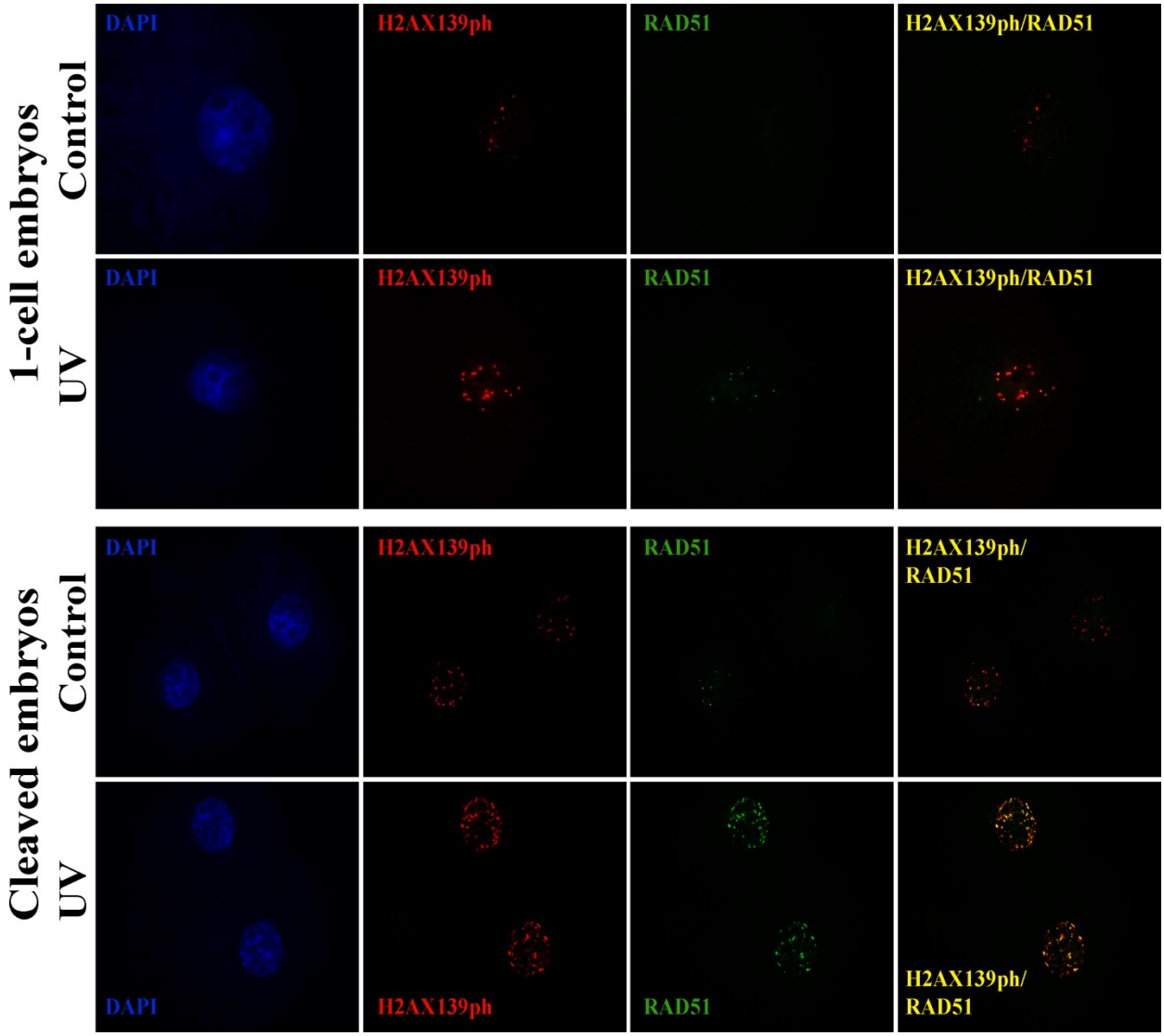

Figure 5.9.3. Immunodetection of H2AX139ph and RAD51 proteins in the nuclei of 1-cell and cleaved SCNT embryos. Representative fluorescent image of SCNT embryos reconstructed with donor cells that were unexposed (Control) or exposed to UV light for 10 seconds (UV). Nuclei are stained in blue, H2AX139ph foci are stained in red, RAD51 foci are stained in green and the sites of H2AX139ph/RAD51 co-localization are stained in yellow.

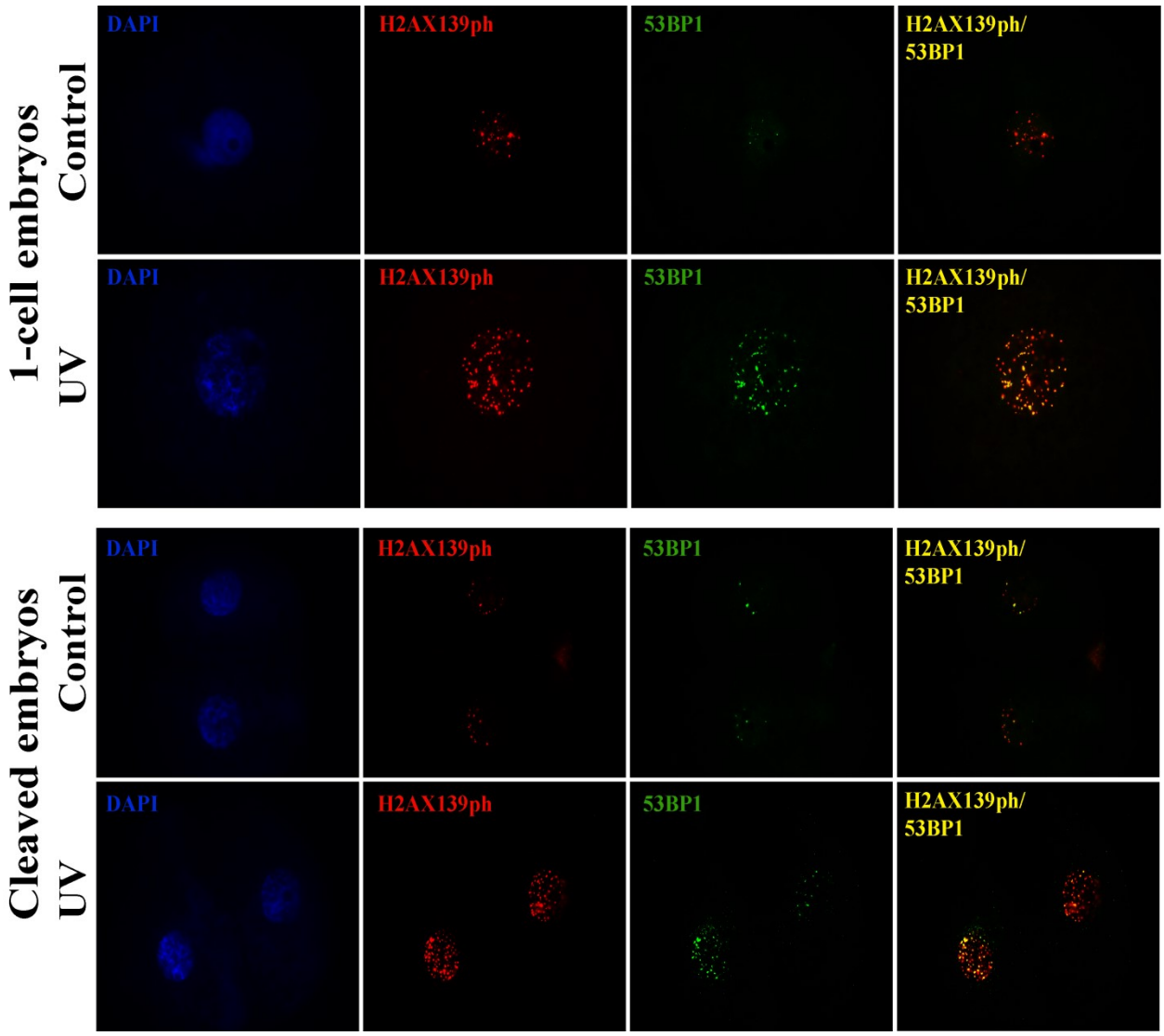

Figure 5.9.4. Immunodetection of H2AX139ph and 53BP1 proteins in the nuclei of 1-cell and cleaved SCNT embryos. Representative fluorescent image of SCNT embryos reconstructed with donor cells that were unexposed (Control) or exposed to UV light for 10 seconds (UV). Nuclei are stained in blue, H2AX139ph foci are stained in red, 53BP1 foci are stained in green and the sites of H2AX139ph/53BP1 co-localization are stained in yellow.

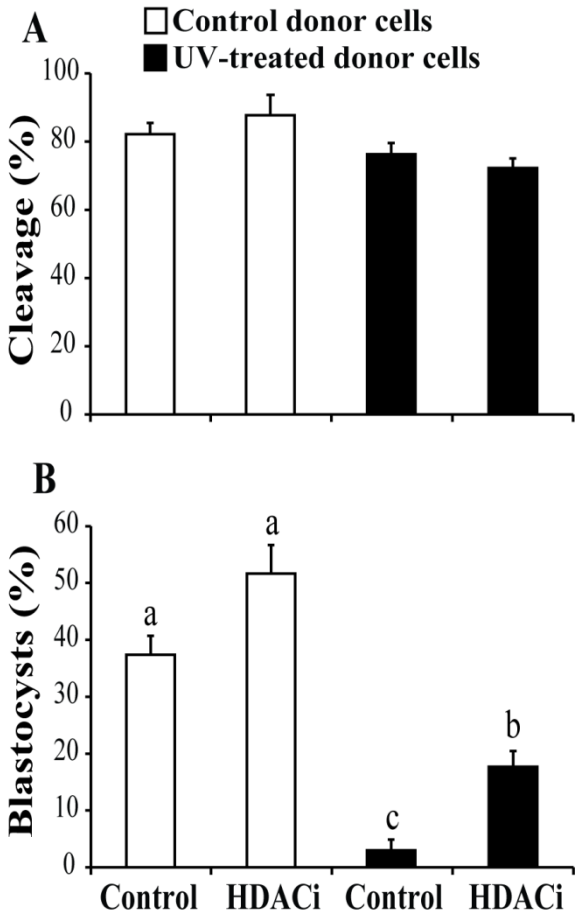

Figure 5.9.5. Effect of HDACi treatment on development of SCNT embryos reconstructed with control and UV-treated donor cells. Cleavage (A) and blastocyst (B) rates of SCNT embryos reconstructed with control (white bars) or UV-treated (black bars) donor cells, and treated with HDACi or vehicle (Control). Data are from four replicates with a minimum of 20 embryos in each group. Different letters indicate significant differences between groups ($p<0.05$).

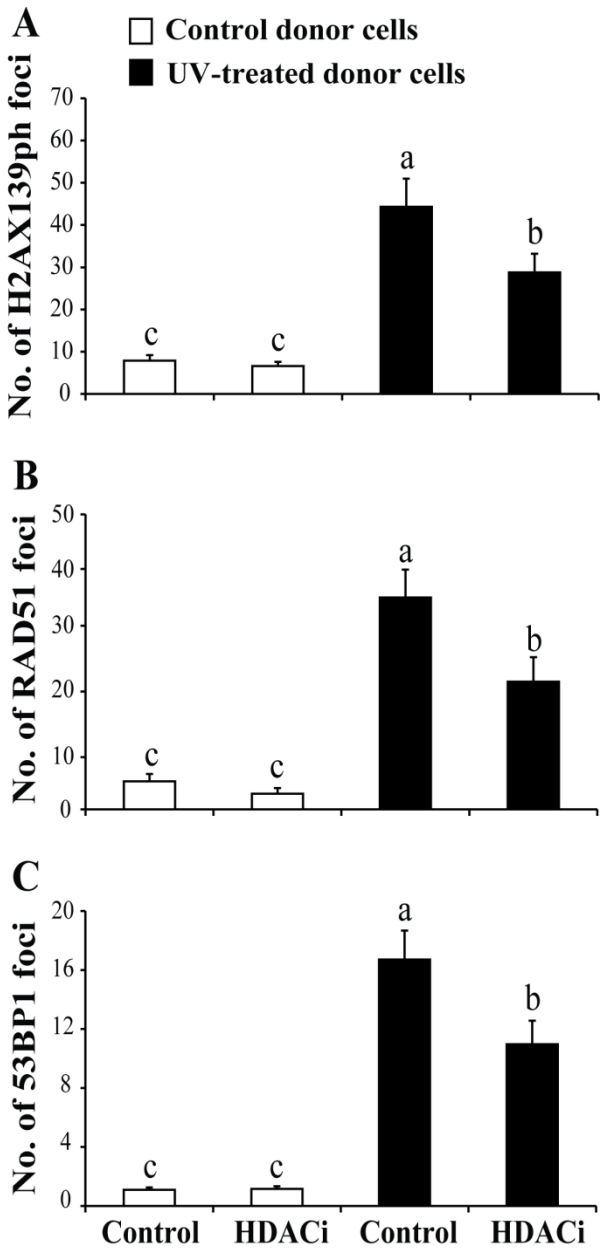

Figure 5.9.6. Quantification of H2AX139ph, RAD51 and 53BP1 proteins in the nuclei of cleaved SCNT embryos. Number of foci for H2AX139ph (A), RAD51 (B) and 53BP1 (C) proteins in the nuclei of cleaved SCNT embryos. Embryos were reconstructed with control (white bars) or UV-treated donor cells (black bars), and were treated with HDACi or vehicle (Control). The experiment was repeated three times and the minimal number of embryos analysed in each group was 24. Different letters indicate significant differences between groups ($p<0.05$).

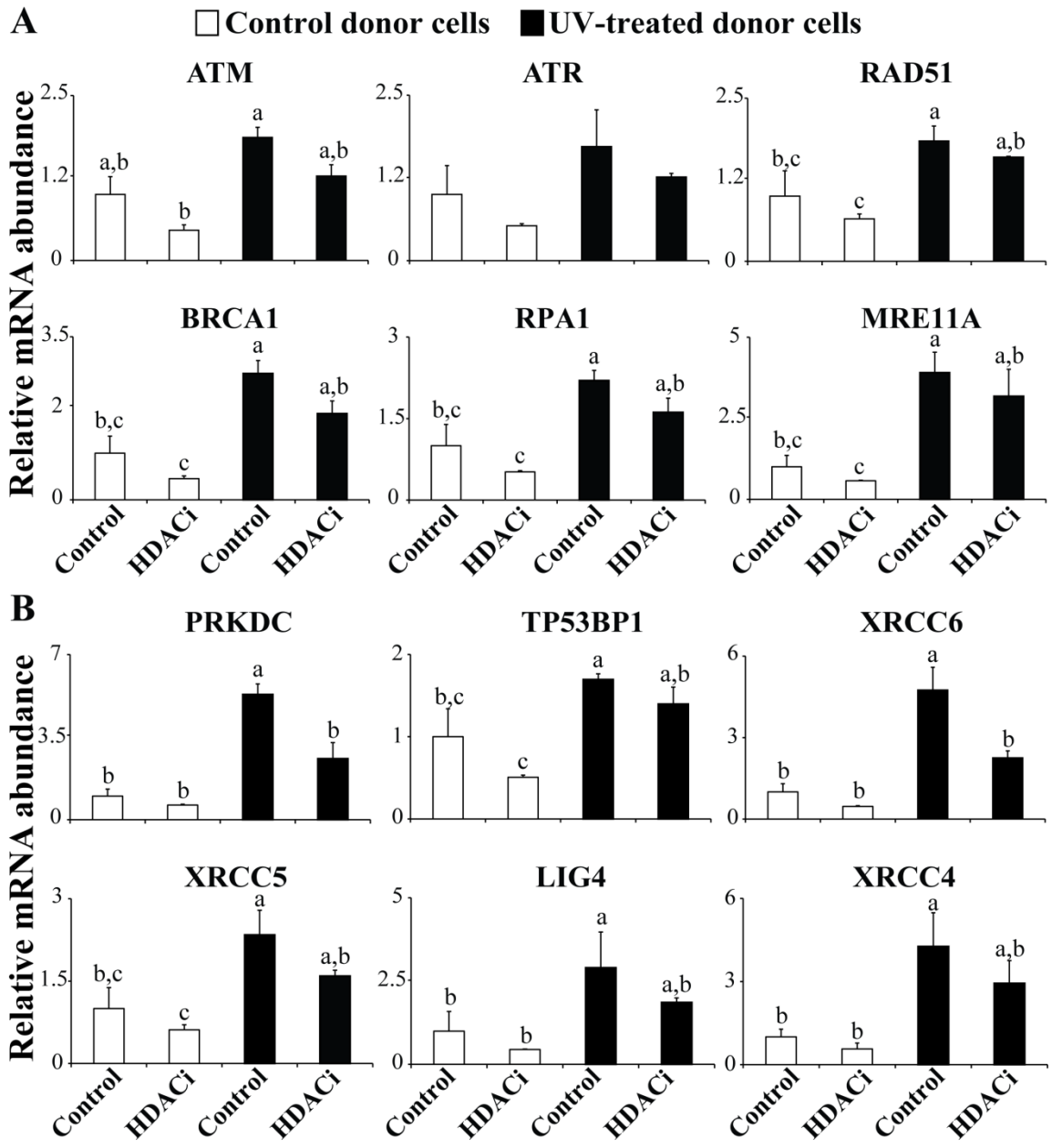

Figure 5.9.7. Relative mRNA abundance for genes involved in Homologous Recombination (A) and Nonhomologous End-Joining (B) pathways in day 5 SCNT embryos. Embryos were reconstructed with control (white bars) or UV-treated donor cells (black bars), and were treated with HDACi or vehicle (Control). The mRNA abundance was calculated relative to the reference

gene ACTB. Data are from three replicates with 20 embryos in each group. Different letters indicate significant differences between groups ($p<0.05$)

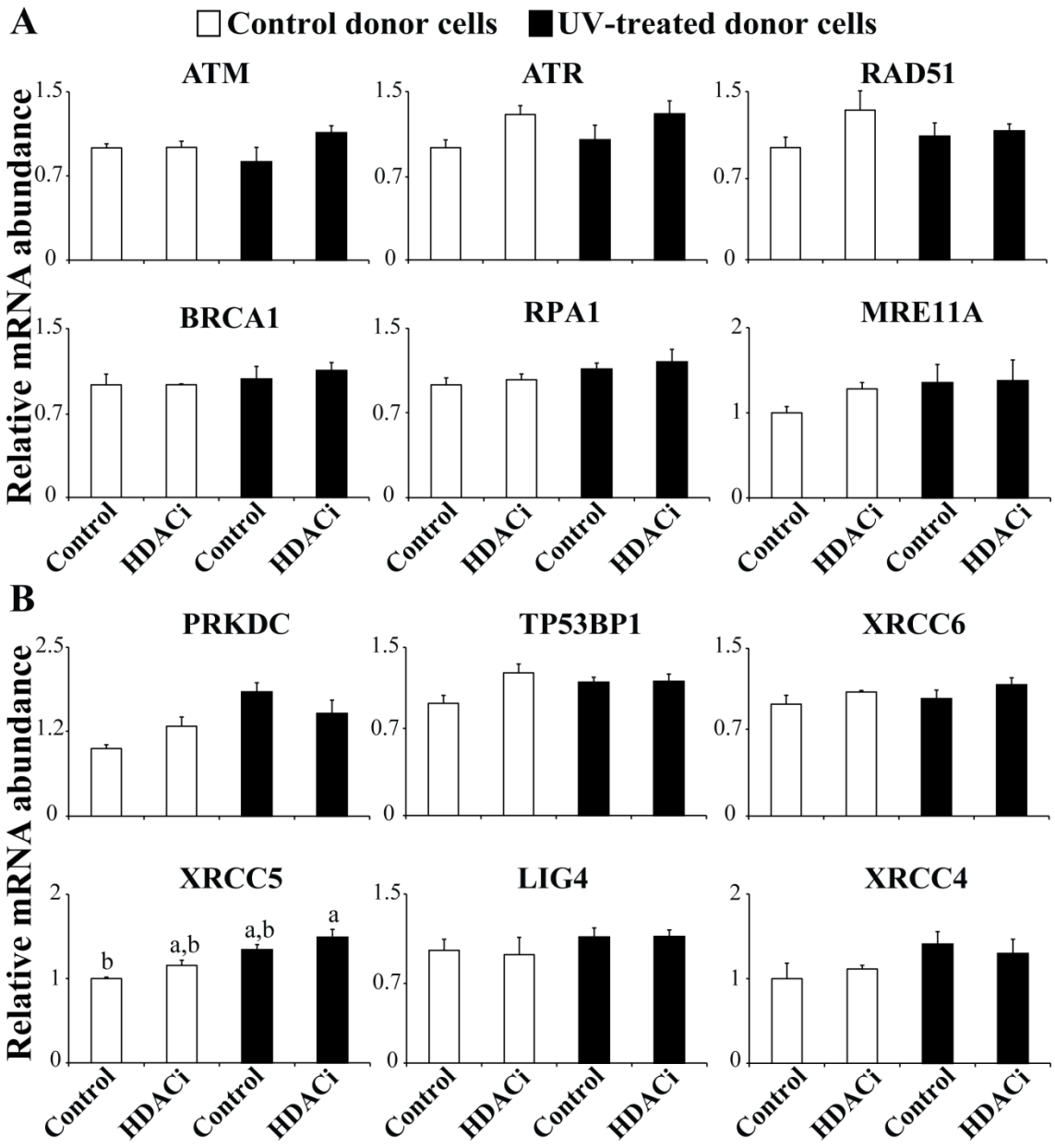

Figure 5.9.8. Relative mRNA abundance for genes involved in Homologous Recombination (A) and Nonhomologous End-Joining (B) pathways in day 7 SCNT embryos. Embryos were reconstructed with control (white bars) or UV-treated donor cells (black bars), and were treated with HDACi or vehicle (Control). The mRNA abundance was calculated relative to the reference gene ACTB. Data are from three replicates with 10 embryos in each group. Different letters indicate significant differences between groups ($p<0.05$).

5.10. Table

Table 5.10.1. Primers used for quantitative real-time PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Accession No.
RAD51	CTTCGGTGGAAGAGGAGAGC	CGGTGTGGAATCCAGCTTCT	NM_001123181.1
BRCA1	TGCTAAATCCGGAACAAAACACA	CTGGTGGAACGATCCAGAGAT	XM_003358030.1
ATM	CCGGTGTTTTGGGAGAGTGT	CTTCCGACCAAACTCAGCGT	NM_001123080.1
MRE11A	GGAGGATGTTGTCCTGGCTG	AGACGTTCCCGTTCTGCATT	XM_003129789.2
ATR	TGAGCTCCAGTGTTGGCATC	GCCAGTTCTCAGTGTGGTCA	XM_003132459.3
RPA1	TCAGATCCGTACCTGGAGCA	TGAACGCAGTGGCTCTGATT	XM_003131822.1
PRKDC	ATTCTTTGTCGGGAGCAGCA	CCTAGCTGTGTGGCACATGA	XM_001925309.4
XRCC5	CTGGCATCTCGCTGCAATTC	GAAAGGAGGGTCCATGGTGG	XM_003133649.2
LIG4	AGCTAGACGGCGAACGTATG	CCTTCCTGTGGGGAAACTCC	XM_003131089.2
TP53BP1	GGGAAAGGGGGAGTTCGTG	CTCACGCTCGTGCTAGAGAT	XM_001925938.4
XRCC4	ATGGCTTCACAGGAGCTTCA	ATGTTTTCAGCTGGGCTGTG	XM_003123760.2
XRCC6	ACGGAAGGTGCCCTTTACTG	TGCAGCACTGGGTTCTCAAA	NM_001190185.1
ACTB	GCAGATGTGGATCAGCAAGC	GAATGCAACTAACAGTCCGCC	XM_003124280.2

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CONNECTING STATEMENT 3

Previous studies have shown that the induction of DNA DSBs in either male or female pronuclei reduce the cleavage and blastocyst rates (Wang et al., 2013b). Moreover, mouse zygotes derived from DNA-damaged sperm have the first cleavage delayed (Gawecka et al., 2013). It was was shown that the checkpoint proteins Chek1 and 2 are activated in response to DNA DSBs and promote cell cycle arrest to allow enough time for DNA repair before DNA replication (Bartek and Lukas, 2003, Stracker et al., 2009, Reinhardt and Yaffe, 2013). Interestingly, these checkpoints are also activated in response to DNA DSBs during early embryo development (Wang et al., 2013a, Wang et al., 2013b). Therefore, early embryos also have mechanisms to stop or delay the cell cycle in response to DNA damage. Indeed, in the first manuscript of this thesis (Chapter 4) we observed that fast-developing embryos have fewer DNA DSBs than slow-developing embryos. Those findings suggest that the presence of DNA DSBs affects the first cleavage kinetics and further embryo development. Therefore, in the third manuscript of this thesis (Chapter 6) we investigate the occurrence of DNA DSBs in early- and late-cleaving embryos and its effects on later development to reach the blastocyst stage.

CHAPTER 6

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The incidence of DNA double-strand breaks is higher in late-cleaving and less developmental competent porcine embryos

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6.1. Abstract

Studies in different species, including human, mice, bovine, and swine, demonstrated that early-cleaving embryos have higher capacity to develop to the blastocyst stage and produce better quality embryos with superior capacity to establish pregnancy than late-cleaving embryos. It has also been shown that experimentally-induced DNA damage delays embryo cleavage kinetics and reduces blastocyst formation. To gain additional insights into the effects of genome damage on embryo cleavage kinetics and development the present study was conducted to compare the occurrence of DNA double-strand breaks (DSBs) and the expression profile of genes involved in DNA repair and cell cycle control between early- and late-cleaving embryos. Porcine oocytes matured in vitro were activated and then early- (before 24h) and late-cleaving (between 24 and 48h) embryos were identified and cultured separately. Developing embryos on days 3, 5 and 7 were used to evaluate the total cell number, presence of DSBs by counting the number of immunofluorescent foci for phosphorylated histone H2A.x (H2AX139ph) and RAD51 proteins, and to quantify transcripts of genes involved in DNA repair and cell cycle control by qRT-PCR. Early-cleaving embryos had fewer DSBs, lower transcript levels for genes encoding DNA repair and cell cycle checkpoint proteins, and more cells than late-cleaving embryos. Interestingly, at the blastocyst stage, embryos that developed from early- and late-cleaving groups have similar number of DSBs as well as transcript levels of genes induced by DNA damage. This indicates that only embryos with less DNA damage and/or superior capacity for DNA repair are able to progress to the blastocyst stage. Collectively, findings in this study revealed a negative correlation between the occurrence of DSBs and embryo cleavage kinetics, and embryo developmental capacity to the blastocyst stage.

6.2. Introduction

Over the last few decades, assisted reproductive technologies (ARTs) have been widely used for the treatment of infertility in humans and also in farm animal breeding programs. In vitro embryo production (IVP), which involves the manipulation and culture in vitro of gametes and early developing embryos, is among the widely used ARTs. Despite decades of research investigating embryo biology and testing culture conditions for increasing IVP efficiency, only a minor proportion (<50%) of in vitro produced embryos develop to the blastocyst stage. Although the in vitro culture environment still requires optimization (Lonergan et al., 2006, Lazzari et al., 2010), there is evidence showing that even transferring in vitro matured (IVM) and fertilized (IVF) embryos into the reproductive tract of host females results in lower development to the blastocyst stage compared to embryos that are naturally matured, fertilized and developed in vivo (Rizos et al., 2002, Gad et al., 2012). This suggests that the current culture conditions may not be the main factor responsible for reduced developmental capacity of IVP embryos. There is evidence that developmental capacity to reach blastocyst stage is mainly determined by the oocyte quality (Blondin et al., 2002, Rizos et al., 2002, Nivet et al., 2012).

Early-cleaving embryos provide higher rates of blastocyst formation compared to late-cleaving embryos. This has been confirmed by several studies using diverse embryo production methods in different species including human embryos produced by intra-cytoplasmic sperm injection (Sakkas et al., 1998), bovine (Yadav et al., 1993, Lonergan et al., 1999) and porcine (Booth et al., 2007) embryos produced by IVF, and porcine embryos produced by parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT) (Coutinho et al., 2011, Isom et al., 2012). Moreover, early-cleaving embryos were shown to have higher capacity to establish pregnancies (Edwards et al., 1984, Meseguer et al., 2011, Sugimura et al., 2012). These studies confirmed that

the kinetics of embryo cleavage is a good indicator of both pre- and post-implantation development.

Even though it is now well accepted that early-cleaving embryos are better equipped to sustain development, the cellular and molecular features involved in this process have not been fully characterized. Differences between early- and late-cleaving embryos have been reported in terms of gene expression (Fair et al., 2004, Mourot et al., 2006), mRNA polyadenylation (Brevini et al., 2002), chromatin remodeling (Bastos et al., 2008), presence of cleaved caspase 3 (Coutinho et al., 2011), and metabolic profiles (Lee et al., 2015). We have observed that porcine embryos, derived by IVF or SCNT, that achieved 4-cell stage by 48h of culture had lower number of DNA double-strand breaks (DSBs) than those that remain at 2-cell stage (Bohrer et al., 2013). This correlation suggests that late-cleaving embryos have either higher incidence of DNA damage or lower capacity for DNA repair compared to early-cleaving embryos.

There is evidence from previous studies indicating that normal embryo development depends upon the maintenance of genome integrity. For example, laser microbeam-induced DNA damage in mouse zygotes reduced embryo cleavage and development to the blastocyst stage (Wang et al., 2013b). In addition, mouse zygotes derived from DNA-damaged sperms undergo cell cycle arrest at the G2/M border and their first cleavage was delayed (Gawecka et al., 2013). It has also been shown that the cell cycle checkpoint kinase 1 (Chk1) and 2 (Chk2) were activated in zygotes having increased DNA damage (Wang et al., 2013a, Wang et al., 2013b). Studies with somatic cells have shown that these checkpoint proteins are activated in response to DSBs and induce cell cycle arrest, which is necessary to allow enough time for DNA repair to take place before segregation (Bartek and Lukas, 2003, Stracker et al., 2009, Reinhardt and Yaffe, 2013).

In somatic cells, DSBs are recognized as the most important form of DNA damage and their defective repair can result in mutations, genome instability or cellular death (Rich et al., 2000, Khanna and Jackson, 2001, Furgason and Bahassi el, 2013, Dicks et al., 2015). Homologous recombination (HR) and nonhomologous end-joining (NHEJ) are the two main pathways responsible for DSBs repair (Karran, 2000). After DSBs recognition, the protein kinases ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) or DNA-dependent protein kinase (DNA-PK) phosphorylate the histone H2A.x (H2AX139ph or γH2AX) at the sites of DNA damage (Burma et al., 2001, Stiff et al., 2004, Stiff et al., 2006). This facilitates the access of proteins involved in DNA-damage signaling and repair (Lee et al., 2010). Moreover, H2AX139ph serves as a platform for the accumulation of DNA repair proteins involved in either the HR (e.g., RAD51, MRE11, BRCA1) or NHEJ (e.g., 53BP1, KU80, DNA ligase IV, XRCC4) repair pathways around the damaged sites (Paull et al., 2000, McManus and Hendzel, 2005). To date, the incidence and consequences of DSBs have not been thoroughly investigated during early embryo development. Therefore, our objectives in this study were to: i) assess the occurrence of DSBs in early- and late-cleaving embryos; ii) investigate if transcripts encoding DNA repair and cell cycle checkpoint proteins are differently regulated in embryos with different developmental capacities; and iii) determine if embryos with higher developmental capacity produce blastocysts with lower number of DSBs.

6.3. Materials and Methods

6.3.1. Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma–Aldrich, Oakville, ON, Canada).

6.3.2. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were obtained from a local abattoir (Olymel S.E.C./L.P., Saint-Esprit, QC, Canada) and transported to the laboratory in 0.9% NaCl at 32 to 35°C. Follicles ranging from 3 to 6 mm in diameter were aspirated using an 18-gauge needle. Only cumulus-oocyte complexes (COCs) surrounded by a minimum of three cumulus cell layers and having an evenly granulated cytoplasm were selected for in vitro maturation (IVM). Groups of 30 COCs were cultured in 0.1 mL of maturation medium under mineral oil in a humidified atmosphere of 5% CO2 and 95% air at 38.5°C. Maturation medium consisted 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). After 22 to 24 h of maturation, oocytes were transferred to the same IVM medium, but without LH, FSH and dbcAMP, for an additional 20 to 22 h under the same conditions. Cumulus cells were removed by pipetting in TCM 199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% of hyaluronidase and then oocytes were parthenogenetically activated (PA) and cultured.

6.3.3. Oocyte activation and embryo culture

Selected oocytes were exposed to ionomycin (15 μM) for 5 minutes, then transferred to 10 mM of strontium chloride in Ca^{2+}-free porcine zygote medium (PZM-3) supplemented with cytochalasin B (7.5 μg/ml) and cycloheximide (10 μg/ml) for 4 h. Oocytes were then washed in PZM-3 medium supplemented with 3 mg/mL BSA (fatty acid-free) and cultured in a humidified atmosphere of 5% CO2 and 95% air at 38.5 ºC. Early- and late-cleaving embryos were selected at 24h and 48h after activation, respectively, and cultured separately. Culture medium was supplemented with 10% FBS on day 5 and blastocyst rate was evaluated on day 7.

6.3.4. Immunofluorescence Staining

Developing embryos at day 3, 5 and 7 were fixed for 15-20 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS), and then permeabilized in 1% Triton X-100 in PBS during 30 minutes at 37°C. Samples were incubated for 1 h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then incubated overnight with primary antibodies (H2AX139ph, Millipore, Billerica, MA, USA, 1:1000; RAD51, Santa Cruz Biotechnology, Dallas, Texas, USA, 1:500) diluted in blocking solution. Embryos were incubated for 1 h at room temperature in the presence of secondary antibodies anti-mouse IgG Cy3-conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:1000 or anti-rabbit IgG Alexa Fluor 488 (Life technologies) diluted 1:1000. DNA was stained by exposing the samples to 10 ∝g/ml of 4',6-diamidino-2-phenylindole (DAPI; Life technologies) in blocking solution for 20 minutes. Samples were then mounted on microscope slides using a drop of Mowiol. Slides were kept in a dark box and examined by epifluorescence using a Nikon eclipse 80i

microscope (Nikon, Tokyo, Japan). Images were captured at 400x or 600x magnification using a Retiga 2000R monochromo digital camera (Qimaging, Surrey, BC, Canada). The number and size of H2AX139ph foci were evaluated in each nucleus using the SimplePCI Imaging Software (Compix Inc., Sewickley, PA, USA). Only foci larger than 0.3 μm^3 were counted as a site of DSBs (McManus and Hendzel, 2005, Bohrer et al., 2013). The number of RAD51 foci co-localized with the H2AX139ph foci, which indicate sites of DSBs engaged in the HR repair pathway, were also counted (Paull et al., 2000, McManus and Hendzel, 2005, Bohrer et al., 2014).

6.3.5. RNA purification and qRT-PCR

Total RNA was purified from groups of 30 (day 3), 20 (day 5) and 10 (day 7) embryos using the PicoPure RNA Isolation Kit (Life Technologies) according to manufacturer's instructions. RNA was treated with DNase I (Qiagen, Mississauga, ON, Canada) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR (qPCR) reaction was carried out using a CFX384 Real-Time Detection System (Bio-Rad, Mississauga, ON, Canada), iQ SYBR Green Supermix (Bio-Rad), 400 nM of primers and 2 µl of cDNA. Primers (Table 1) were designed using Primer-Blast and specificity was confirmed using BLAST (NCBI, Bethesda MD, USA). Common thermal cycling parameters (5 minutes at 95ºC and 40 cycles of 15 seconds at 95ºC and 30 seconds at 60ºC) were used to amplify each transcript, and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene and expression was normalized to the mean abundance of internal control genes *ACTB* and *H2A*. All reactions used for quantification had efficiency between 90 -110%, R^2 ≥0.98 and slope values from -3.6 to -3.1.

6.3.6. Statistical analyses

Statistical analyses were performed using the JMP Software (SAS Institute, Inc., Cary, NC, United States). Continuous data were analyzed using ANOVA and the means were compared using Student's t-test or Tukey–Kramer Honestly Significant Difference test for single or multiple comparisons, respectively. All experiments were repeated at least three times and data are expressed as mean ± s.e.m. Differences were considered statistically significant if $P<0.05$.

6.4. Results

6.4.1. Embryo development and total cell numbers

We first confirmed the superior developmental potential of early-cleaving embryos in our embryo production system. The proportions of early- (before 24h) and late- (between 24 and 48h) cleavage were 36.6% and 24.4%, for a total embryo cleavage of 61% (Fig. 1A). Early-cleaving embryos developed to the blastocyst stage at a higher frequency than late-cleaving embryos (70.8% vs. 45%, respectively; Fig. 1B). In addition, early-cleaving embryos fixed on days 3, 5 or 7 of culture had higher cell numbers than late-cleaving embryos (Fig. 1C-E). The superior quality of early-cleaving embryos was particularly evident on days 5 and 7 of development, when they had approximately four times more cells than late-cleaving embryos.

6.4.2. Presence of DSBs in early- and late-cleaving embryos

The objective in this experiment was to evaluate if early- and late-cleaving embryos on days 3, 5 and 7 of development have different incidence of DSBs. This was performed by counting the number of fluorescent foci larger than 0.3 μm^3 for the DNA damage response (DDR) protein, H2AX139ph. We detected lower number of H2AX139ph foci in the nuclei of early- than late-cleaving embryos on days 3 (Fig. 2A-B) and 5 (Fig. 2C-D) of development. Interestingly, there was no difference in the number of DSBs between early- and late-cleaving embryos that developed to blastocyst stage on day 7 (Fig. 2E-F). To further confirm the presence of DSBs we also counted the number of fluorescent foci for the DNA repair protein RAD51 that were co-localized with H2AX139ph. As expected, we observed higher number of RAD51 foci in late-cleaving embryos on both day 3 (Fig 3A-B) and day 5 (Fig 3C-D) of development.

6.4.3. Transcript levels for DNA repair and cell cycle checkpoint genes in early- and late-cleaving embryos

In this experiment, mRNA abundance for genes involved in the HR and NHEJ pathways of DNA repair were compared between early- and late-cleaving embryos on days 3, 5 and 7 of development. As shown in Fig. 4, mRNA levels of genes involved in the HR pathway were less abundant in early-cleaving embryos on day 3 (*RAD51, RAD52* and *MRE11A*) and day 5 (*ATM, ATR, RAD51, RAD52* and *MRE11A*) than in late-cleaving embryos. Interestingly, in embryos that developed to the blastocyst stage by day 7, none of the assessed genes had different transcript levels between early- and late-cleaving (Fig. 4). Similarly, transcripts abundance for genes involved in the NHEJ pathway (Fig. 5) were higher in late-cleaving embryos at day 3 (*XRCC6* and

LIG4) and day 5 (*PRKDC, XRCC4, XRCC5, XRCC6* and *LIG4*), but there was no difference in mRNA levels for any of the studied genes when comparing embryos that developed to the blastocyst at day 7. Analyses of transcripts for the cell cycle checkpoint genes *CHEK1* and *CHEK2*, which encode the checkpoint proteins Chk1 and Chk2, respectively, revealed that late-cleaving embryos had higher mRNA levels for *CHEK1* on days 3 and 5 and for *CHEK2* on day 5 compared to early-cleaving embryos (Fig. 6). The transcript abundance for both *CHEK1* and *CHEK2* were not different between late- and early-cleaving embryos on day 7 of development (Fig. 6).

6.5. Discussion

Findings from this study provide evidence that DSBs affect early embryo cleavage kinetics and development to the blastocyst stage. Using a well-established model for embryo developmental competence based on the time to first embryo cleavage, we showed that early-cleaving and more developmentally competent embryos have less DSBs than late-cleaving and less developmentally competent embryos. We also found that early-cleaving embryos have lower transcript levels for genes encoding proteins that participate in either the HR or the NHEJ pathways for DNA repair. In addition, transcript levels of the cell cycle checkpoint proteins Chk1 and Chk2 were more abundant in late- compared to early-cleaving embryos. More importantly, we found that early- and late-cleaving embryos that can develop to the blastocyst stage have similar number of DSBs per nuclei and transcript profiles of genes involved in DSBs repair, despite of the relative lower number of cells observed in the late-cleaving group.

A number of studies have shown that early-cleaving embryos of several species and produced by different protocols have superior developmental capacity and produce better quality blastocysts than late-cleaving embryos (Yadav et al., 1993, Sakkas et al., 1998, Booth et al., 2007, Coutinho et al., 2011, Isom et al., 2012). However, the cause and mechanisms behind the altered cell cleavage and development have not been sufficiently characterized. Our findings in this study revealed that the presence of DSBs may represent an important constraint for embryo cell cleavage kinetics and development. Indeed, by analyzing the number of H2AX139ph fluorescent foci larger than 0.3 μm^3 and the number of RAD51 foci (Paull et al., 2000, McManus and Hendzel, 2005, Bohrer et al., 2014), we demonstrated that early-cleaving embryos have less DSBs than late-cleaving embryos on days 3 and 5 of development. As expected, all the RAD51 foci were co-localized with the H2AX139ph foci, which confirm that this protein involved in the HR pathway for DSBs was recruited to the damaged sites marked by the H2AX139ph foci. The remaining H2AX139ph foci not co-localized with RAD51 foci were also higher in the late- compared to early-cleaving embryos on days 3 and 5 of development. This suggests that those foci would be engaged in the NHEJ repair pathway and therefore co-localized with other DNA repair proteins. Indeed, we have recently shown that H2AX139ph foci are co-localized with DNA repair proteins of both pathways in developing porcine embryos (Bohrer et al., 2014), which suggests that both pathways are involved in DNA repair during early embryo development. In line with these observations are findings of previous studies indicating that the induction of DSBs in mouse zygotes delayed embryo cleavage and impaired development to the blastocyst stage (Gawecka et al., 2013, Wang et al., 2013b). In addition, activation of checkpoint proteins and cell cycle arrest at the G2/M transition has been reported after fertilization with DNA-damaged sperms in mice (Wang et al., 2013a). Along with these previous observations, our findings suggest that slow cell-

cleavage during early embryo development is likely a cell response to allow enough time for DSBs repair to take place, thus preventing the segregation of DNA mutations to the somatic or germ cell lineages in the developing embryo. This could compromise fetal development and cell metabolism as suggested by previous studies (Lane et al., 2014).

There are two main possible reasons that could explain the difference in the number of DSBs between early- and late-cleaving embryos, the higher incidence of damages in the late-cleaving or the superior capacity for repair in the early-cleaving. To further investigate the differences related to DSBs between early- and late-cleaving embryos we have quantified the transcript levels of genes encoding important proteins required for DNA repair at different stages of embryo development. Previous studies have reported the presence of transcripts for DNA repair genes in oocytes and early developing embryos (Menezo et al., 2007, Jaroudi et al., 2009), which suggests that the products of these genes are required during all stages of embryo development. In support to this hypothesis are the findings of our previous studies confirming that DNA repair genes are regulated in early developing embryos in response to increased DSBs induced by ultraviolet exposure (Henrique Barreta et al., 2012, Bohrer et al., 2014). The present study revealed that transcript levels for a number of DNA repair genes were more abundant in late- than early-cleaving embryos at either day 3 or day 5 of development. Moreover, we observed that late-cleaving embryos can up-regulate DNA repair genes of both HR and NHEJ pathways, which further supports a role of both pathways during early embryo development. Since the average number of cells of late-cleaved embryos on day 3 of culture was less than 4, we believe that the up-regulation of genes involved in DNA repair occurred before the major activation of the embryonic genome, which occurs between 4 to 8 cells in the porcine embryo (Tomanek et al., 1989, Hyttel et al., 2000, Cao et al., 2014). This is in line with our previous observations that late-

cleaved bovine embryos undergo premature chromatin remodeling compatible with increased transcriptional activity [19]. Based on these findings we propose that the higher number of DSBs in the late-cleaving embryos is more likely due to an increased incidence of damages rather than a decreased capacity for DNA repair.

To gain additional insights into the effects of DSBs on embryo cell cleavage and development, we compared the mRNA abundance of genes encoding the cell cycle checkpoint proteins Chk1 and Chk2 between early- and late-cleaving embryos. Chk1 and Chk2 are activated by the ATM and ATR kinases in response to DNA damage (Liu et al., 2000, Melchionna et al., 2000). Once activate, Chk1 and Chk2 promote the degradation of the phosphatases Cdc25A and Cdc25C by inducing the phosphorylation of the serine residues 123 and 216, respectively (Peng et al., 1997, Sørensen et al., 2003). Lack of Cdc25A and Cdc25C activity prevents the dephosphorylation and activation of cyclin-dependent kinases leading to cell cycle arrest. It is known that the cell cycle arrest is an induced response to ensure the repair of damaged DNA can take place before its replication and segregation in cleaving cells (Donzelli and Draetta, 2003). In this study, we found higher abundance of transcripts encoding the Chk1 in late-cleaving embryos at day 3 and 5 and the Chk2 at day 5. This indicates that the slow-cleavage and reduced cell numbers observed in the late-cleaving embryos were likely due to the activation of cell cycle checkpoints in response to damaged DNA. Supporting this are previous reports confirming the activation of Chk1 and Chk2 in mouse zygotes in response to induced DNA damage (Wang et al., 2013a, Wang et al., 2013b).

An interesting finding of the present study is that blastocysts derived from early- and late-cleaving embryos had similar number of DSBs and as well as transcript levels of genes encoding DNA repair and cell cycle checkpoint proteins. This is in line with our previous observations in

embryos produced by somatic cells nuclear transfer, whereas the induction of DNA damage by ultraviolet treatment significantly reduced embryo development but those that achieved the blastocyst stage had similar number of DSBs and levels of transcripts encoding DNA repair genes (Bohrer et al., 2014). These findings support the hypothesis that only the embryos with less DNA damage and/or superior capacity for DNA repair are able to progress to the blastocyst stage. In line with this are previous findings by Wang et al. (2013b) indicating that mouse blastomeres with increased number of DSBs undergo apoptosis before the blastocyst stage.

In conclusion, data from this study indicate that the presence of DSBs can alter the kinetics of embryo cell cleavage and development to the blastocyst stage. We have also shown that transcripts for DNA repair and cell cycle checkpoint genes are up-regulated in late-cleaving embryos, which suggest that the high number of DSBs is more likely a consequence of increased DNA damage than defective DNA repair. Finally, our findings provide evidence that only embryos with less DNA damage and/or superior capacity for DNA repair are able to achieve the blastocyst stage. Because DSBs can result in DNA mutations and genome instability, which might be transmitted to the body tissues and also passed on to next generation, we propose that the selection of fast cleaving embryos may reduce the risks for segregation of altered genomes with threatening consequences for later life and health.

6.6. Acknowledgements

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6.7. Figures

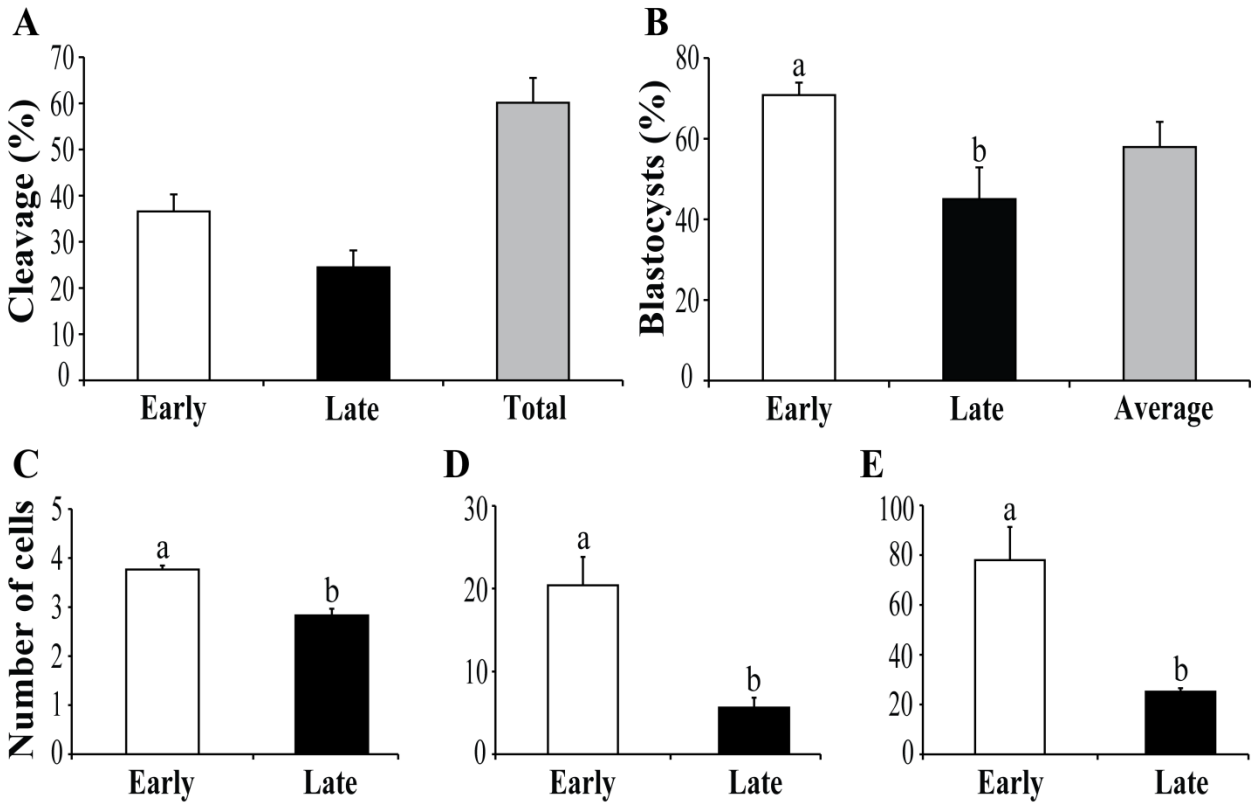

Figure 6.7.1. Development and number of cells in embryos derived from early- and late-cleaving groups. A) Proportion of early-, late- and total embryo-cleavage from activated oocytes. B) Proportion of development to the blastocyst stage from early, late and total cleaved embryos. Data are from four replicates with a minimum of 30 embryos in each group. C-E) Cell number in early- and late-cleaving embryos fixed on day 3 (C), 5 (D) and 7 (E) of culture. Data are from three replicates and the minimum number of embryos analyzed in each group was 40, 20 and 10 for day 3, 5 and 7, respectively. Different letters or asterisk indicate significant difference between groups ($p<0.05$).

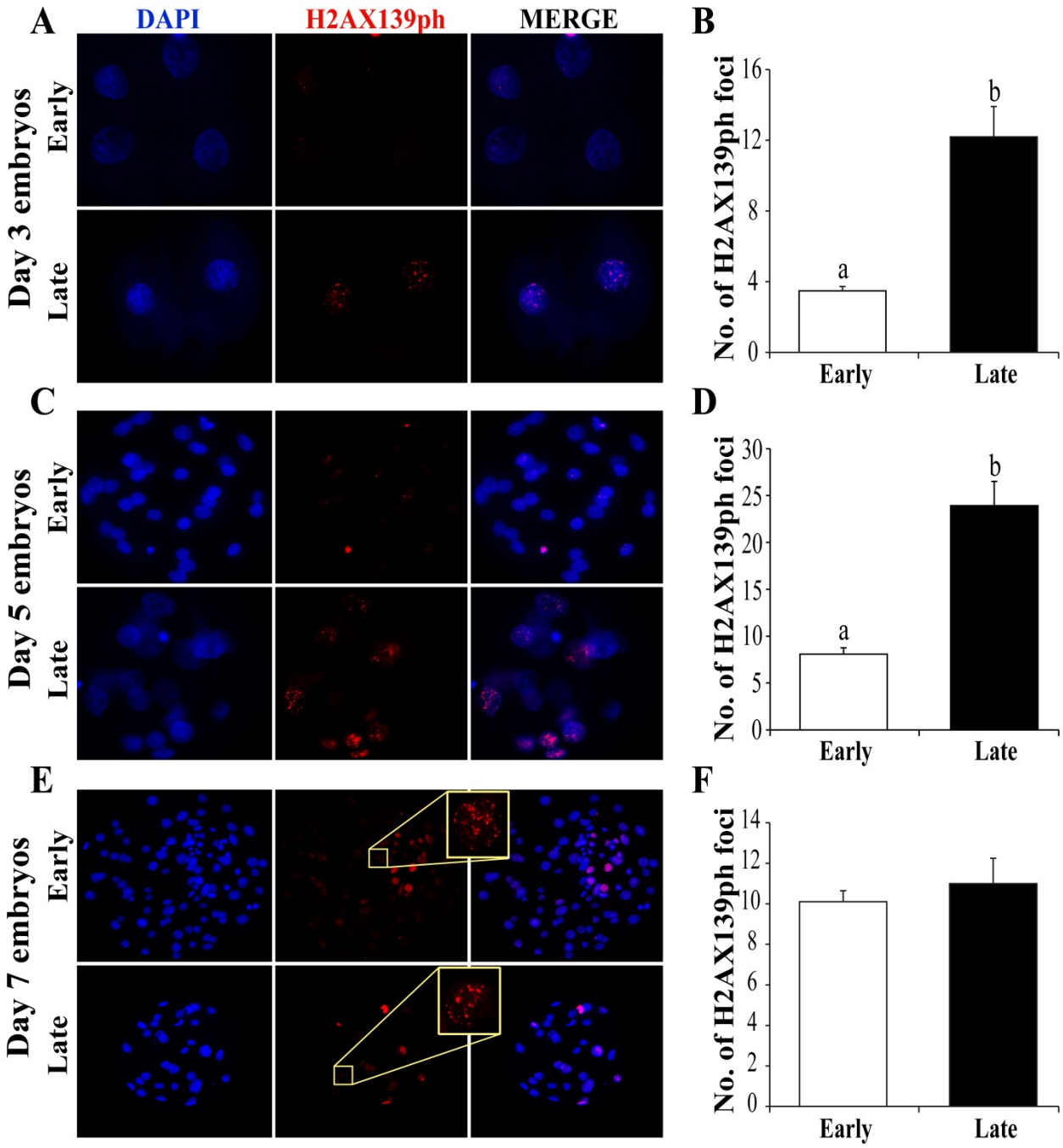

Figure 6.7.2. Number of fluorescent foci for H2AX139ph in the nuclei of early- and late-cleaving embryos. Representative fluorescent images of early- and late-cleaving embryos fixed on day 3 (A), 5 (C) and 7 (E) of culture. Nuclei are stained in blue and H2AX139ph foci are stained in red.

Average number of H2AX139ph foci per nuclei in early- and late-cleaving embryos at day 3 (B), 5 (D), and 7 (F) of culture. Data are from three replicates and the minimum number of embryos analyzed in each group was 40, 20 and 10 for day 3, 5 and 7, respectively. Different letters indicate significant differences between groups ($p<0.05$).

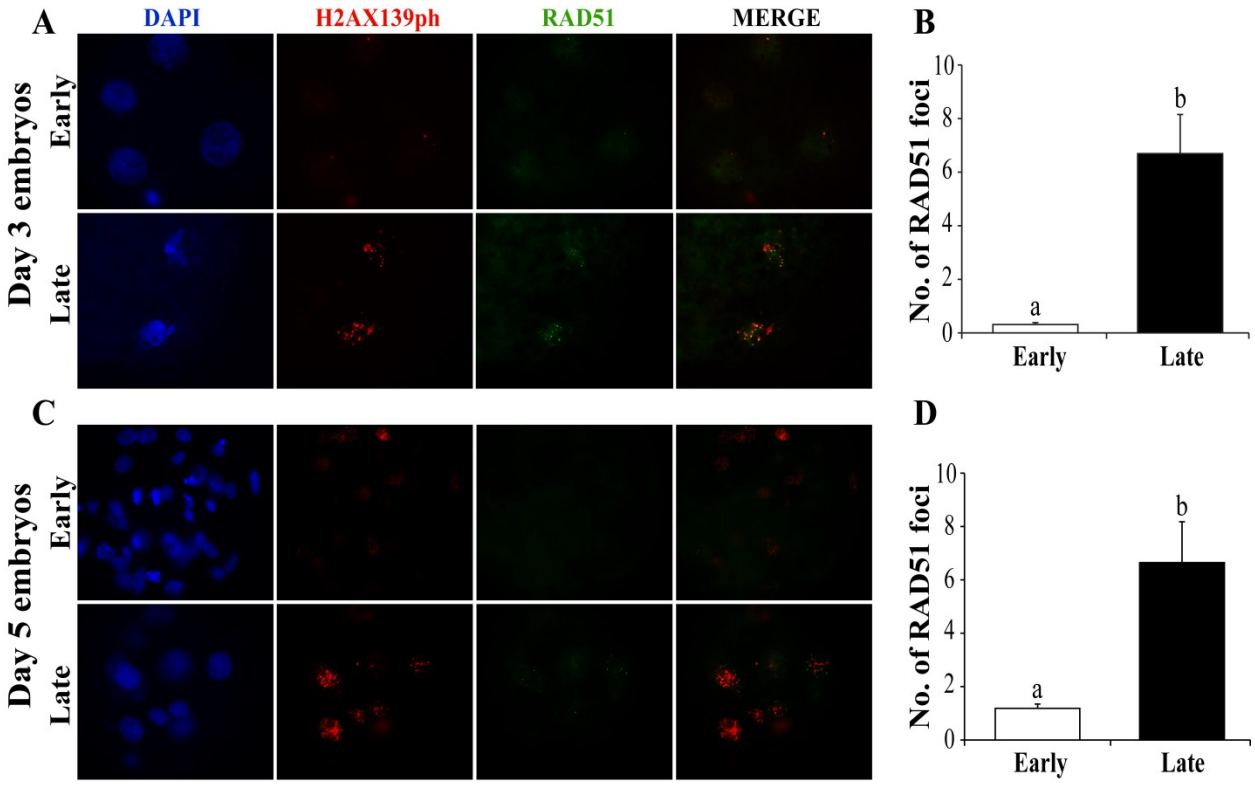

Figure 6.7.3. Co-localization of fluorescent foci for RAD51 and H2AX139ph in the nuclei of early- and late-cleaving embryos. Representative fluorescent images of early- and late-cleaving embryos fixed on day 3 (A) and 5 (C). Nuclei are stained in blue, H2AX139ph foci are stained in red and RAD51 foci are stained in green. Average number of RAD51 foci per nuclei in early- and late-cleaving embryos fixed on day 3 (B) and 5 (D) of culture. Data are from three replicates and

the minimum number of embryos analyzed in each group was 40 and 20 for days 3 and 5, respectively. Different letters indicate significant differences between groups ($p<0.05$).

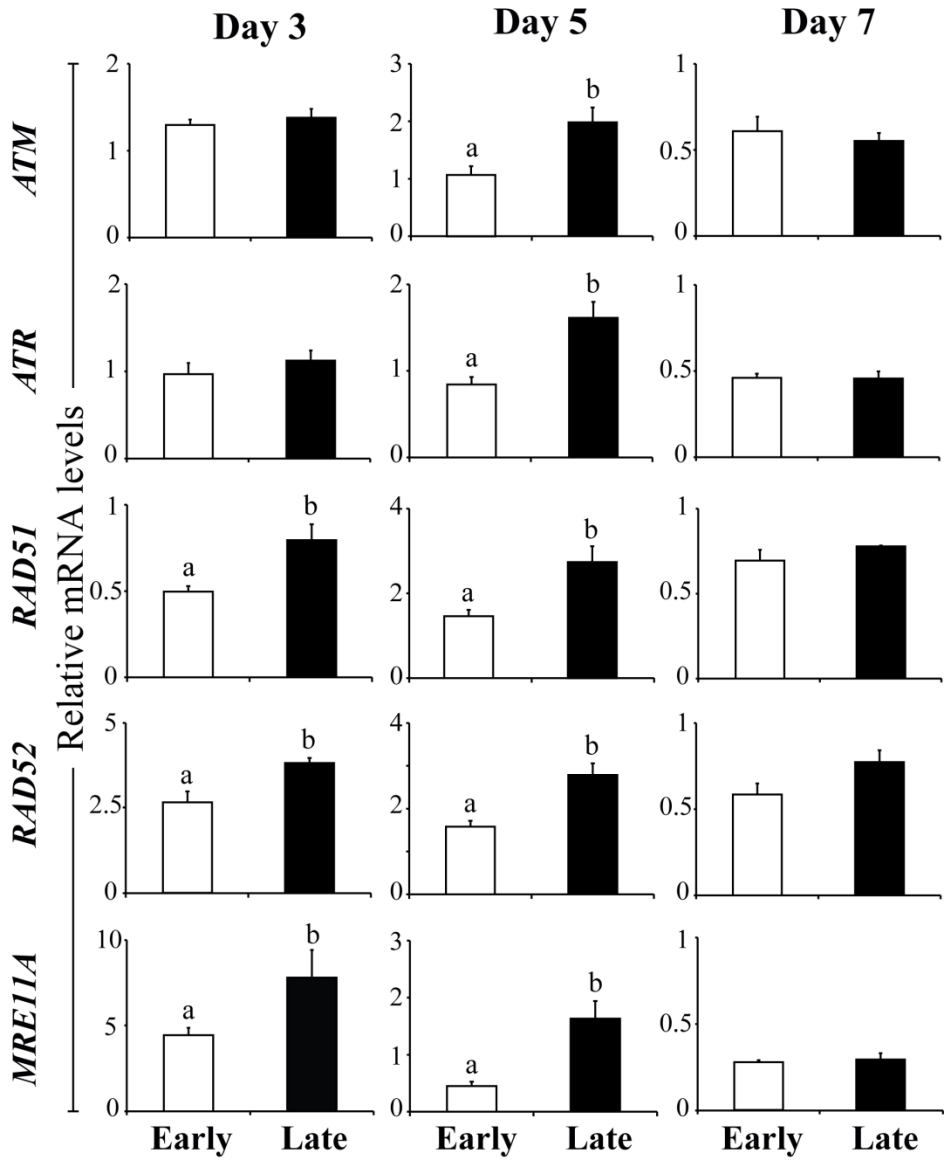

Figure 6.7.4. Relative mRNA abundance of genes involved in the homologous recombination pathway for DNA repair in early- and late-cleaving embryos collected on day 3, 5 and 7 of culture. The mRNA abundance was calculated relative to the average of the reference genes *ACTB* and

H2A. Data are from three replicates with 30, 20 and 10 embryos collected on day 3, 5 and 7, respectively. Different letters indicate significant differences between groups ($p<0.05$).

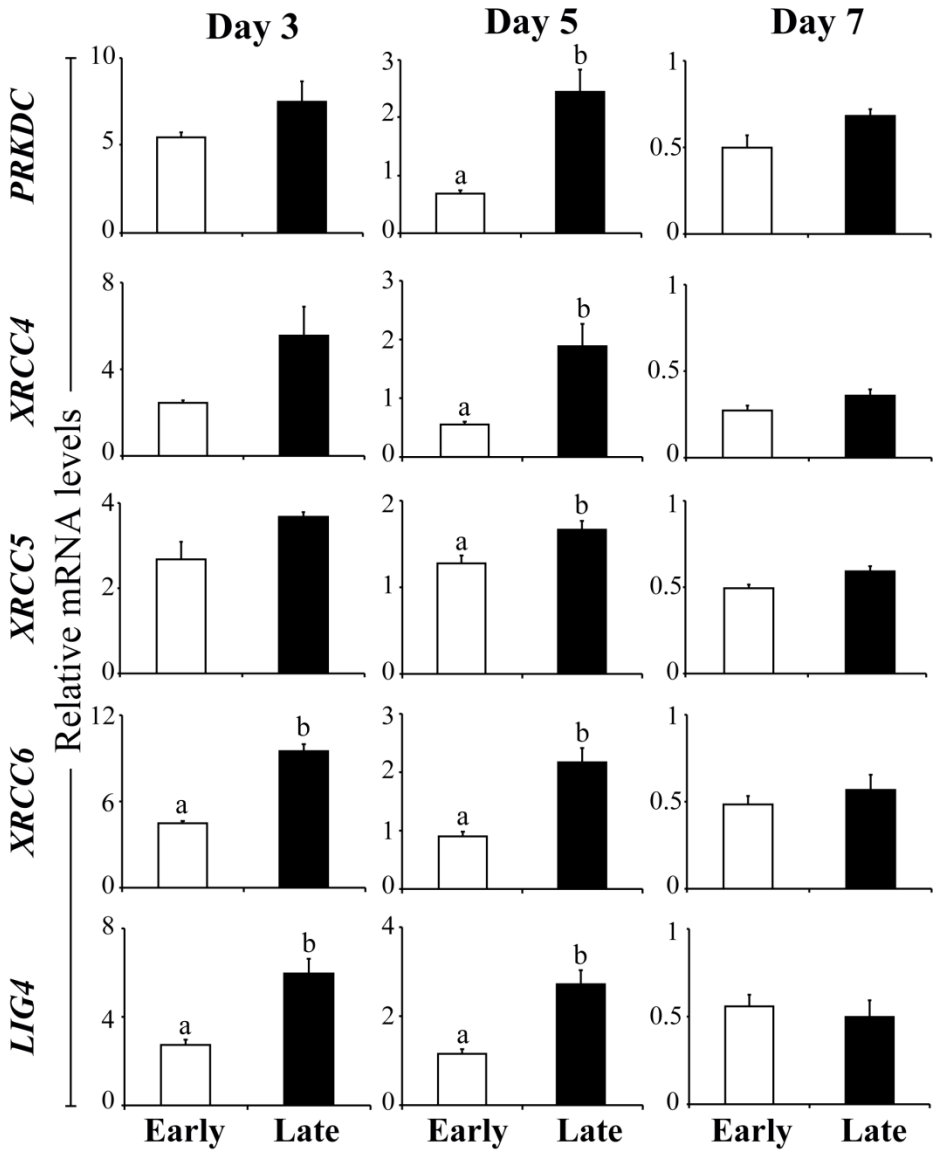

Figure 6.7.5. Relative mRNA abundance of genes involved in nonhomologous end-joining pathway for DNA repair in early- and late-cleaving embryos collected on day 3, 5 and 7 of culture. The mRNA abundance was calculated relative to the average of the reference genes *ACTB* and

H2A. Data are from three replicates with 30, 20 and 10 embryos collected on day 3, 5 and 7, respectively. Different letters indicate significant differences between groups ($p<0.05$).

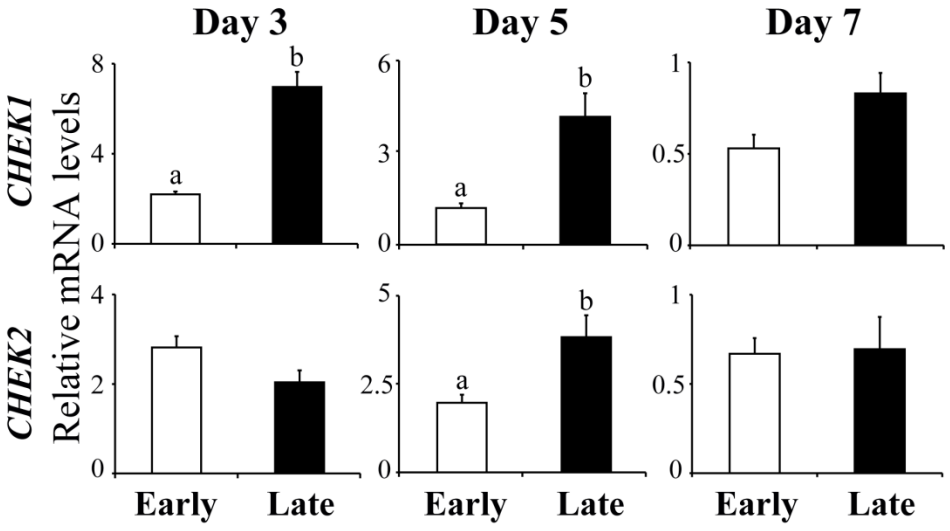

Figure 6.7.6. Relative mRNA abundance of *CHEK1* and *CHEK2* in early- and late-cleaving embryos collected on day 3, 5 and 7. The mRNA abundance was calculated relative to the average of the reference genes *ACTB* and *H2A*. Data are from three replicates with 30, 20 and 10 embryos on day 3, 5 and 7, respectively. Different letters indicate significant differences between groups ($p<0.05$).

6.8. Table

Table 6.8.1. Primers used for quantitative real-time PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Accession No.
ATM	CCGGTGTTTTGGGAGAGTGT	CTTCCGACCAAACTCAGCGT	NM_001123080.1
ATR	TGAGCTCCAGTGTTGGCATC	GCCAGTTCTCAGTGTGGTCA	XM_003132459.3
RAD51	CTTCGGTGGAAGAGGAGAGC	CGGTGTGGAATCCAGCTTCT	NM_001123181.1
RAD52	ATTCAGCAAGGGATGCCCAC	TAGGGCAAGGGCGTTTTCTT	XM_003358103.2
MRE11A	GGAGGATGTTGTCCTGGCTG	AGACGTTCCCGTTCTGCATT	XM_003129789.2
PRKDC	ATTCTTTGTCGGGAGCAGCA	CCTAGCTGTGTGGCACATGA	XM_001925309.4
XRCC4	ATGGCTTCACAGGAGCTTCA	ATGTTTTCAGCTGGGCTGTG	XM_003123760.2
XRCC5	CTGGCATCTCGCTGCAATTC	GAAAGGAGGGTCCATGGTGG	XM_003133649.2
XRCC6	ACGGAAGGTGCCCTTTACTG	TGCAGCACTGGGTTCTCAAA	NM_001190185.1
LIG4	AGCTAGACGGCGAACGTATG	CCTTCCTGTGGGGAAACTCC	XM_003131089.2
CHEK1	TGCCCTTTGTGGAAGACTGG	ACTGCAACTGCTTCCTCAGT	XM_003130047.2
CHEK2	GCCTGTGGTGAGGTGAAACT	TGCTGGATCTGCCTCTCTCT	NM_001137638.1
ACTB	GCAGATGTGGATCAGCAAGC	GAATGCAACTAACAGTCCGCC	XM_003124280.2
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	XM_001927727.2

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CONNECTING STATEMENT 4

The findings published in the previous manuscripts of this thesis (Chapters 4, 5 and 6) showed that the occurrence of DNA DSBs is an important factor affecting early embryo development and cell reprogramming in SCNT embryos. Therefore, it is important to understand the mechanisms involved in DNA DSBs repair during early embryo development. Studies in somatic cells have shown that the activation of both the HR and the NHEJ pathways is important for genome integrity maintenance and cellular health (Takata et al., 1998, Couëdel et al., 2004). Other studies have shown that the HR pathway is more important than the NHEJ pathway for DNA DSBs repair in embryonic stem cells (Essers et al., 2000). Interestingly, it has been shown that both the HR and the NHEJ pathways are active in mouse embryos (Derijck et al., 2008). In addition, our previous studies (chapter 5 and 6) have shown that early embryos respond to DNA DSBs by increasing the mRNA abundance for genes involved in DNA DSBs repair by both the HR and the NHEJ pathways. However, it has not been determined if both pathways are crucial for DNA DSBs repair during early embryo development. Therefore, in the fourth manuscript of this thesis (Chapter 7) we investigate whether the HR and the NHEJ pathways are both required for DNA DSBs repair and their regulation during early embryo development**.**

CHAPTER 7

This manuscript is being prepared for submission to the "Development" journal for publication.

Double-strand DNA breaks are mainly repaired by the homologous recombination pathway in early developing embryos

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7.1. Abstract

The occurrence of DNA DSBs is known to affect early embryo development. The HR and the NHEJ are the main pathways involved in DNA DSBs repair in somatic cells. However, whether the HR and the NHEJ pathways are required and their relative importance for DNA DSBs repair during early embryo development has not been well investigated. Therefore, the objective of this study was to access the importance of the HR and the NHEJ pathways for DNA DSBs repair during early embryo development. We observed that chemical inhibition of HR or both pathways, but not NHEJ alone, increased the number of DNA DSBs, reduced blastocyst formation and increased the proportion of apoptotic cells in UV-exposed embryos. We also observed that knockdown of factors involved in the activation of the HR (ATM and ATR) or both (ATM, ATR and DNA-PK)

pathways, profoundly reduced blastocyst formation and quality. Interestingly, ATM knockdown increased the number of DNA DSBs, reduced blastocyst formation and quality and prevented ICM formation. Moreover, ATM knockdown increased the mRNA levels for genes involved in DNA DSBs repair from both the HR and the NHEJ pathways. These findings indicate that the HR is the main pathway involved in DNA DSBs repair, and it is mainly activated by ATM during early embryo development.

Key words: pre-implantation embryos, in vitro, DNA damage, DNA repair, H2AX139ph.

7.2. Introduction

Normal embryo development is a primary condition of fertility in humans and domestic animals. It also a limiting factor for application of assisted reproductive technologies based on in vitro embryo culture since most of embryos stop developing before reaching the blastocyst stage. While the causes of embryo arrest are many, there is recent evidence from studies in different species indicating that DNA double-strand breaks (DSBs) contribute to embryo arrest and death (Simon et al., 2014, Bohrer et al., 2015, Juan et al., 2015). Indeed, induction of DSBs decreased cleavage and blastocyst rates in fertilized and somatic cell nuclear transfer embryos (Henrique Barreta et al., 2012, Wang et al., 2013b, Bohrer et al., 2014). DNA DSBs can be caused by both endogenous (e.g., by-products of cellular metabolism, collapsed DNA replication forks), and exogenous (e.g., ultraviolet (UV) light, ionizing irradiation, chemicals) factors (Lindahl and Wood, 1999, De Bont and van Larebeke, 2004, Grigaravičius et al., 2009, Mu et al., 2011).

Studies with somatic cells revealed that DSBs are the most deleterious form of DNA damage because their defective repair can induce genome instability, harmful mutations and cellular death (Rich et al., 2000, Khanna and Jackson, 2001, Peterson and Côté, 2004, Furgason and Bahassi el, 2013). Homologous recombination (HR) and nonhomologous end-joining (NHEJ) are the two main pathways involved in DNA DSB repair (Karran, 2000, Stiff et al., 2004, Stiff et al., 2006). The HR pathway is activated by the proteins ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3 related (ATR), and the NHEJ by the DNA-dependent protein kinase (DNA-PK) (Stiff et al., 2004, Stiff et al., 2006). During DNA repair by the HR pathway, ATM is recruited to DSBs sites by the Mre11–Rad50–Nbs1 (MRN) complex (Lee and Paull, 2005). The recruitment of ATR depends on the ATR-interacting protein (ATRIP) and the replication protein A (RPA)-bound single-stranded DNA (Falck et al., 2005). During DNA repair by the NHEJ pathway, the heterodimer protein ku70/80 senses and binds to the double-stranded DNA ends and rapidly recruits DNA-PK to the damaged sites (Hammarsten and Chu, 1998, Mari et al., 2006). Within minutes after their activation, the kinases ATM, ATR or DNA-PK phosphorylate the C-terminal serine 139 of the histone H2AX (H2AX139ph or γH2AX) around the DSBs sites (Rogakou et al., 1998, Stiff et al., 2004, Stiff et al., 2006). H2AX139ph expands over large chromatin extensions (megabases) and form docking sites where proteins involved in DSB repair by the HR (e.g., RAD51, BRCA1, RAD52) or NHEJ (e.g., 53BP1, LIG4, XRCC4) pathways accumulate (Rogakou et al., 1999, Paull et al., 2000, McManus and Hendzel, 2005).

During normal development of the mammal embryo, the first cell lineage specification forms the trophectoderm (TE) and inner cell mass (ICM) at the blastocyst stage, which will give rise to placenta and body tissues, respectively (Papaioannou, 1982). Effective mechanisms to maintain genome integrity, especially in the ICM, are critical for normal development and grow

of a healthy offspring. Early-developing embryos can quickly respond to DSBs by inducing the phosphorylation of histone H2A.x (H2AX139ph) and activation of the checkpoint proteins Chek1 and Chek2, which may block the cell cycle to prevent the segregation of DSBs in the dividing cells (Mu et al., 2011, Wang et al., 2013a, Wang et al., 2013b). It has established from studies in somatic cells that the HR pathway promotes more accurate DSBs repair because it uses an intact sister chromatid as a template (Li and Heyer, 2008). It was observed that the HR is the predominant pathway for DNA DSBs repair in pluripotent embryonic stem cells (Yang et al., 2004). Therefore, we hypothesized that early developing embryos would preferentially activate the HR pathway to repair DNA DSBs and thus decrease the risks of segregating altered genomes. Although embryos respond to DNA damage by upregulating the expression of genes involved in both the HR or the NHEJ pathways (Henrique Barreta et al., 2012, Bohrer et al., 2015), it has not been determined if both pathways are required for DNA DSBs repair in early embryos before the first cell lineage specification. Therefore, our objectives in this study were to: i) investigate the relative contribution of each pathway for DNA DSBs repair in early developing embryos; and ii) to determine if ATM, ATR and DNA-PK kinases are required for the activation of DNA DSBs repair pathways during early embryo development.

7.3. Materials and Methods

7.3.1. Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma–Aldrich, Oakville, ON, Canada).

7.3.2. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were obtained from a local abattoir (Olymel S.E.C./L.P., Saint-Esprit, QC, Canada) and transported to the laboratory in 0.9% NaCl at 32 to 35°C. Follicles ranging from 3 to 6 mm in diameter were aspirated using an 18-gauge needle. Only cumulus-oocyte complexes (COCs) surrounded by a minimum of three cumulus cell layers and having an evenly granulated cytoplasm were selected for in vitro maturation (IVM). Groups of 30 COCs were cultured in 0.1 mL of maturation medium under mineral oil in a humidified atmosphere of 5% CO2 and 95% air at 38.5°C. Maturation medium consisted 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). After 22 to 24 h of maturation, oocytes were transferred to the same IVM medium, but without LH, FSH and dbcAMP, for an additional 20 to 22 h under the same conditions. Cumulus cells were removed by pipetting in TCM 199 HEPES-buffered medium (Life Technologies) supplemented with 0.1% of hyaluronidase.

7.3.3. Parthenogenetic activation (PA)

Matured oocytes were exposed to ionomycin (15 µM) for 5 minutes, then transferred to 10 mM of strontium chloride in Ca2+-free porcine zygote medium (PZM-3) supplemented with cytochalasin B (7.5 µg/ml) and cycloheximide (10 µg/ml) for 4 h. Oocytes were then washed and in vitro cultured.

7.3.4. In vitro fertilization (IVF)

Matured oocytes were washed three times with IVF medium (113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2.2H2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 0.1% BSA) (Abeydeera and Day, 1997). Groups of 20 oocytes were then placed in 90 µl drops of IVF medium covered with mineral oil. Sperm was prepared from fresh semen samples of a proven fertile boar. Sperms were washed twice in IVF medium by centrifugation (800 rpm) for 3 minutes and the sperm pellet was resuspended with IVF medium. Oocytes in each drop were fertilized by adding 10 µl of the sperm preparation with concentration adjusted to 1,000 spermatozoa per oocyte. Oocytes were co-incubated with the sperm for 4 h and then collected, washed twice to remove unattached sperm cells and then in vitro cultured.

7.3.5. Somatic cell nuclear transfer (SCNT)

Matured oocytes were cultured in TCM199 supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 60 minutes. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes (Yin et al., 2002). Oocytes were then transferred to TCM 199 HEPES-buffered medium supplemented with 2 mg/ml of BSA (fatty acid free), 20 µg/ml gentamicin and 7.5 mg/mL cytochalasin B for 5 to 10 minutes and enucleated by removing the protruded oocyte chromatin together with the first polar body. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte and electrically fused using a single DC pulse of 1.6 kV/cm for 70 µs. Electrofusion was performed into a 0.28 M mannitol solution supplemented with 50 µM CaCl2, 100 µM MgSO4 and 0.1% PVA. Reconstructed oocytes

were cultured in TCM199 medium supplemented with 3 mg/mL BSA for 1 h before parthenogenetic activation.

7.3.6. Embryo Culture

Embryos were cultured in PZM-3 medium (Yoshioka et al., 2002) in a humidified atmosphere of 5% CO2 and 95% air at 38.5 ºC. Embryo cleavage rates were determined after 24 and 48 h of culture and blastocyst rates on day 7. Culture medium was supplemented with 10% FBS on day 5 of culture.

7.3.7. Inhibition of DNA repair pathways

To inhibit the HR pathway embryos were treated with inhibitors of ATM (10 μM KU-55933; Selleck Chemicals, TX, US) and ATR (10 μM VE-821; Selleck Chemicals). To inhibit the NHEJ pathway embryos were treated with an inhibitor of DNA-PK (1 μM NU7441; Selleck Chemicals). The inhibitors KU-55933, VE-821 and NU7441 are referred as KU, VE and NU, respectively, for short. In the first experiment, oocytes were PA and then cultured in PZM-3 medium with vehicle (Dimethyl sulfoxide – DMSO) or in the presence of inhibitors of both HR and NHEJ (KU + VE + NU) pathways for 24, 48, 96 or 168 h. In the groups cultured with the inhibitors for less than 168 h (day 7), embryos were washed twice and cultured in PZM-3 medium with vehicle until day 7. In the second experiment, embryos produced by PA, IVF or SCNT were cultured with vehicle, inhibitors of HR (KU + VE), inhibitor of NHEJ (NU) or inhibitors of both HR and NHEJ (KU + VE + NU) during the 7 days of culture.

7.3.8. UV light exposure

To induce DNA damage, PA the embryos were placed in 35 mm culture dishes (Corning, Tewksbury, MA, US) containing 1 ml of PZM-3 medium and exposed to UV light for 0 (control group), 10, 30 or 60 s inside a biological safety cabinet (Thermo Scientific™ 1300 Series Class II). After UV treatment embryos were washed and cultured for 7 days in PZM-3 medium in the presence of vehicle or inhibitors of DNA repair pathways. For the quantification of H2AX139ph, 2-4 cell stage embryos produced by PA were exposed to UV light during 10 s and then cultured with vehicle or inhibitors of both pathways (KU + VE + NU). Embryos were collected at 1, 2, 4 or 8 h after UV exposure, fixed and immunostained to detect H2AX139ph.

7.3.9. ATM, ATR and DNA-PK knockdown

For the knockdown experiments, Dicer-substrate short interfering RNAs (DsiRNAs) were designed (Custom DsiRNA Design Tool) and synthetized by IDT (Integrated DNA Technologies, Inc., Iowa, US). Specificity was confirmed using BLAST (NCBI, Bethesda MD). Matured oocytes were microinjected with approximately 10 pl of 25 μM diluted sense and antisence DsiRNAs targeting two different sequences of ATM (ATM1-2), ATR (ATR1-2), DNA-PK (DNA-PK1-2) or negative control (Table 1). Microinjection was performed in TCM 199 HEPES-buffered medium supplemented with 2 mg/ml BSA (fatty acid free) and 20 μg/ml gentamicin using an inverted Nikon microscope (Nikon, Tokyo, Japan) equipped with a micromanipulator system (Narishige International, Long Island, NY, US). After microinjection, the oocytes were PA and in vitro cultured. Knockdown efficiency was evaluated by assessing the relative mRNA abundance

of ATM, ATR and DNA-PK by qPCR at days 3 and 5 after DsiRNAs microinjection. ATM protein abundance was evaluated by immunofluorescence in embryos at day 5 after microinjection.

7.3.10. Immunofluorescence Staining

Developing embryos were fixed for 15-20 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS), and then permeabilized in 1% Triton X-100 in PBS during 30 minutes at 37°C. Samples were incubated for 1 h at room temperature in blocking solution (3% BSA and 0.2% Tween-20 in PBS), and then incubated overnight with primary antibodies for H2AX139ph (05-636; Millipore, Billerica, MA, US), cleaved caspase 3, 53BP1 (9661, 4937, respectively, Cell Signaling, Danvers, MA, US), ATM, RAD51 and SOX-2 (sc-7230, sc-8349 and sc-17320, respectively, Santa Cruz Biotechnology, Dallas, Texas, USA) using recommended dilutions in blocking solution. Embryos were incubated for 1 h at room temperature in the presence of secondary antibodies, anti-mouse IgG Cy3-conjugated (115-165-146, Jackson ImmunoResearch Laboratories, West Grove, PA, US), anti-rabbit or anti-goat IgG Alexa Fluor 488 (ab150077 and ab150133, respectively, Abcam Inc. Toronto, ON, Canada) diluted 1:1000 in blocking solution. DNA was stained by exposing the samples to 10 μg/ml of 4',6-diamidino-2-phenylindole (DAPI; Life technologies) in blocking solution for 20 minutes. Samples were then mounted on microscope slides using a drop of Mowiol. Slides were kept in a dark box and examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan). Images were captured at 200x, 400x or 600x magnification using a Retiga 2000R monochromo digital camera (Qimaging, Surrey, BC, Canada). Fluorescence intensity (FI) for H2AX139ph was quantified in each nucleus and for ATM in the whole embryo using the SimplePCI imaging software (Compix Inc., Sewickley, PA, US). The same software was used to determine the number of DNA DSBs by counting the number of

H2AX139ph foci lager than 0.3 µm3 in each nucleus (Bohrer et al., 2013). The number of fluorescent foci for 53BP1 and RAD51 co-localized with the H2AX139ph foci were also counted (Bohrer et al., 2014).

7.3.11. Western Blot

Porcine fetal fibroblasts were lysed in Laemmli buffer (Bio-Rad, Mississauga, ON, Canada) containing proteases inhibitor cocktail (G-Biosciences, St. Louis, MO, US). Total lysate was size fractionated by SDS-PAGE 7.5% gel and electroblotted onto nitrocellulose membranes (Bio-Rad). After blocking with 5% BSA or skim milk powder in Tris-buffered saline (TBS) + 0.1% of Tween, pH 7.6, membranes were incubated overnight with primary antibodies for ATM (sc-7230; Santa Cruz Biotechnology, Dallas, TX, US) 1:500 and Beta-actin (ab8227; Abcam Inc. Toronto, ON, Canada) 1:5000 diluted in blocking solution. Membranes were incubated for 1 h with peroxidase-conjugated secondary antibody anti-mouse (7076; Cell Signaling) diluted 1:3000 or anti-rabbit (ab6721; Abcam) diluted 1:10.000 in blocking solution, and then for 5 minutes with Immun-Star WesternC Chemiluminescent Kit. Images were captured using the ChemiDoc MP System (Bio-Rad) and bands were quantified using the Image Lab 3.0 software (Bio-Rad).

7.3.12. RNA purification and qRT-PCR

Total RNA was purified from groups of 30 (day 3) and 20 (day 5) embryos using the PicoPure RNA Isolation Kit (Life Technologies) according to manufacturer's instructions. RNA was treated with DNase I (Qiagen, Mississauga, ON, Canada) and reverse transcribed using SuperScript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR (qPCR)

reaction was carried out using a CFX384 Real-Time Detection System (Bio-Rad, Mississauga, ON, Canada), iQ SYBR Green Supermix (Bio-Rad), 400 nM of primers and 2 µl of cDNA. Primers (Table 2) were designed using Primer-Blast and specificity was confirmed using BLAST (NCBI, Bethesda MD, US). Common thermal cycling parameters (5 minutes at 95ºC and 40 cycles of 15 seconds at 95ºC and 30 seconds at 60ºC) were used to amplify each transcript, and melting-curve analysis was used to verify the specificity of reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for each gene and expression was normalized to the mean abundance of internal control genes ACTB and H2A. All reactions used for quantification had efficiency between 90 -110%, R2 ≥0.98 and slope values from -3.6 to -3.1.

7.3.13. Statistical analyses

Statistical analyses were performed using the JMP Software (SAS Institute, Inc., Cary, NC, United States). Continuous data were analyzed using ANOVA and the means were compared using Student's t-test or Tukey–Kramer Honestly Significant Difference test for single or multiple comparisons, respectively. All experiments were repeated at least three times and data are expressed as mean ± s.e.m. The proportion of cells positive to cleaved caspase 3 in day 5 and day 7 embryos was analyzed using the chi-square test. Differences were considered statistically significant if $P<0.05$.

7.4. Results

7.4.1. Inhibition of DNA DSBs repair pathways affects embryo development to the blastocyst stage

In the first experiment, we evaluated development (cleavage and blastocyst rates) of PA embryos treated with inhibitors of HR and NHEJ pathways (Fig. 1A). Inhibition of both pathways did not affect the embryo cleavage rates at 24 and 48h but development to the blastocyst stage decreased in embryos cultured in the presence of the inhibitors for 96 and 168 h. To evaluate the effect of each pathway, embryos produced by PA, IVF and SCNT were cultured for 7 days in the presence of the inhibitors (Fig. 1B). The inhibition of both pathways significantly reduced blastocyst development. Despite of not been statistically different, blastocyst formation tended to be lower in the HR compared to the NHEJ inhibited groups. Since the response to the inhibitors was similar between the three embryo production models, all the subsequent studies were performed with PA embryos.

7.4.2. Inhibition of HR or both pathways reduce development of embryos exposed to UV light

Exposure of PA embryos to UV light for 10 s or more delayed cleavage and reduced blastocyst formation (Fig. 2A). Inhibition of HR or both repair pathways significantly decreased development to blastocyst stage of embryos that were exposed to UV light for 10 s (Fig. 2B). Embryos exposed to UV light had increased fluorescence intensity for H2AX139ph than control embryos (Fig. 2C). As expected, culture of UV-treated embryos in the presence of DNA repair

inhibitors delayed H2AX phosphorylation (1 h after UV), but increased its accumulation (8 h after UV), which indicates more DNA damage (Fig. 2D).

7.4.3. Inhibition of DNA repair pathways increase DNA DSBs accumulation and cell death in developing embryos

The number of H2AX139ph foci at days 3 and 5 of culture were counted in control and UV-treated embryos to determine the effect repair inhibitors on the occurrence of DNA DSBs (Fig. 3A). The number of DSBs was higher in day 3 and 5 embryos cultured with inhibitors of each or both repair pathway, with the exception of day 3 control embryos treated with inhibitors of the HR pathway and day 5 UV-exposed embryos treated with the inhibitor of the NHEJ pathway (Fig. 3B). Presence of cleaved caspase 3 (Fig. 3C) revealed that inhibitors of the HR or both pathways increased the rate of cell death in UV-treated at day 5 embryos, and in both control and UV-treated embryos at day 7. Inhibition of the NHEJ pathway did not increase embryo cell death in either control or UV-treated embryos (Fig. 3D).

7.4.4. DsiRNA-mediated Knockdown of DNA DSBs repair pathways decreases embryo development and quality

To further investigate the importance of each pathway for DNA DSBs repair during early embryo development and to confirm that the results obtained with the inhibitors were not caused by non-specific effects, DsiRNAs targeting ATM, ATR and DNA-PK mRNAs were microinjected into PA oocytes. Knockdown efficiency was >80% at day 3 and >60% at day 5 after microinjection (Fig. 4A). Immunofluorescence staining confirmed the reduction of ATM protein in embryos

injected with DsiRNAs targeting ATM mRNA (Fig. 4B-C). The specificity of the anti-ATM antibody was confirmed by western blotting using a protein sample extracted from pig fibroblast cells (Fig. 4D). Similarly to findings using inhibitors, DsiRNA-mediated knockdown of the HR pathway (ATM and ATR) or both pathways (ATM, ATR and DNA-PK) decreased not only embryo development but also the total number of cells in embryos that developed to the blastocyst stage (Fig. 5A). The knockdown of ATM and ATR separately revealed that the effect on development and cell number was mainly mediated by ATM than ATR (Fig. 5B and C). Moreover, by immunofluorescence staining using an anti-SOX-2 antibody, we observed absence of inner cell mass (ICM) in blastocysts derived from oocytes injected with DsiRNAs targeting ATM mRNA (Fig. 5B and D).

7.4.5. ATM Knockdown increases the number of DNA DSBs and mRNA abundance of genes involved in DNA repair and cell cycle control

The effect of DsiRNA-mediated knockdown of ATM, ATR and DNA-PK on the occurrence of DNA DSBs and gene expression was assessed in day 5 embryos. We evaluate the number fluorescent foci larger than 0.3 μm^3 for the DNA damage response protein H2AX139ph and the number of fluorescent foci for the DNA damage repair proteins 53BP1 and RAD51 that were colocalized with H2AX139ph foci (Fig 6A and B). ATM knockdown increased approximately 2 times the number of DNA DSBs, indicated by the number of H2AX139ph fluorescent foci larger than 0.3 μm^3 (Fig 6A and C) in day 5 embryos. The number of 53BP1 foci was also increased in ATM knockdown embryos, which suggests that the NHEJ pathway was upregulated (Fig 6A and C). On the other hand, DNA-PK knockdown increased the number of RAD51 foci, which suggests an upregulation of the HR pathway (Fig. 6B and C). We also found

that ATM knockdown increased mRNA abundance of genes involved in DNA DSBs repair by HR (*ATM*, *ATR*, *RAD51*, *MRE11A*, *BRCA1* and *RAD52*) and NHEJ (*PRKDC*, *XRCC4*, *XRCC5*, *XRCC6* and *LIG4*) pathways, as well as cell cycle checkpoint genes (*CHEK1* and *CHEK2*) in day 5 embryos (Fig. 7A, B and C). On the other hand, none of those genes were significantly up regulated in ATR and DNA-PK knockdown embryos.

7.5. Discussion

Findings from this study indicate that DNA DSBs are mainly repaired by the HR pathway during early embryo development. Using two experimental approaches, exposure to chemical inhibitors and microinjection of DsiRNA, we first demonstrated that attenuation of the HR pathway reduced embryo development and quality, and increased DNA DSBs and cell apoptosis. In addition, we found that the activation of the HR pathway in early developing embryos relies more on ATM than ATR activity.

It is well established that both the HR and the NHEJ pathways are important for DNA DSBs repair and preservation of genome integrity and cell viability in somatic cells (Takata et al., 1998, Couëdel et al., 2004). On the other hand, it has been proposed that embryonic stem cells mainly use the HR pathway for DNA DSBs repair (Essers et al., 2000). In early developing embryos, the two repair pathways are active (Derijck et al., 2008, Bohrer et al., 2015) and genes involved in both pathways are upregulated in response to UV-induced DNA damage (Henrique Barreta et al., 2012, Bohrer et al., 2014). Nonetheless, whether both pathways are crucial for DNA DSBs repair during early embryo development, and if there is a compensatory response between the pathways still need to be determined. Findings from this study added new information to

explain how the two pathways contribute to DNA DSBs repair in pre-implantation embryos. First, using chemical inhibitors that were previously tested in somatic cells (Hickson et al., 2004, Leahy et al., 2004, Reaper et al., 2011) we shown that downregulation of both repair pathways markedly reduced development to the blastocyst stage of pig embryos produced by PA, IVF or SCNT. Our findings also suggested that both pathways contribute to the repair process since the inhibition of each pathway separately did not significantly decrease embryo development compared to non-treated embryos. On the other hand, when the incidence of DNA damage was increased by UV exposure, suppression of the HR pathway alone significantly hampered embryo development and quality and increased accumulation of DNA DSBs and cell death. None of those features was different from the control group in UV-exposed embryos treated with the inhibitor of the NHEJ pathway, which indicate a less preeminent role of the NHEJ pathway compared to the HR pathway.

To further characterize the role of each pathway, DsiRNAs were used to knockdown the expression of DNA DSB-induced kinases responsible for the activation of the HR and NHEJ repair pathways. Knockdown of the HR or both repair pathways severely reduced blastocyst formation and quality. This confirmed the finding using chemical inhibitors that DNA DSBs are mainly repaired by the activation of the HR pathway in early developing embryos. It is known that the HR pathway is the predominant form of DNA DSBs repair in S/G2 phase cells and that the NHEJ pathway is more active in non-replicating G0/G1 phase cells (Takata et al., 1998, Delacôte and Lopez, 2008). The fact that dividing blastomeres in early stage embryos are essentially deprived of G1 phase and enter the S phase immediately after the end of the M phase (Mukherjee, 1976, Smith and Johnson, 1986, Schoenbeck et al., 1992) would explain the primary role of the HR pathway. This is further supported by studies showing that components of the HR pathway, including ATM and Rad51 proteins, have important effects on genome stability during embryo

development in different species (Perez et al., 2006, Zha et al., 2008, Kujjo et al., 2010, Kujjo et al., 2012).

It is known that both ATM or ATR kinases can activate the HR pathway (Harper and Elledge, 2007), but their role in DNA DSBs repair during early embryo development has not been characterized. Using DsiRNAs targeting ATM or ATR mRNA, we observed that ATM knockdown had a more prominent effect on blastocyst development and quality than ATR knockdown. Indeed, ATM knockdown significantly increased DNA DSBs accumulation and prevented accumulation of the DNA repair protein RAD51, which has a major role in the HR pathway. This indicate that ATM activity is required for the activation of the HR pathway in early developing embryos. Interestingly, we observed that ATM knockdown prevented ICM formation in embryos that developed to the blastocyst stage, which suggest that DNA DSBs accumulation affects cell differentiation and pluripotency. In line with this, previous studies shown that DNA DSBs reduced the expression of pluripotency genes (e.g., NANOG), induced embryonic stem cell differentiation (Lin et al., 2005), and impaired cell reprogramming (Marion et al., 2009).

To further characterize the role of the HR and NHEJ repair pathways and their activation kinases on DNA DSBs repair during early embryo development, the expression profile of genes involved in the two repair pathways was evaluated in embryos injected with DisRNAs. We observed that ATM knockdown but not ATR or DNA-PK knockdown promoted an increase in mRNA abundance of genes involved in either HR or NHEJ repair pathways, as well as cell cycle checkpoint genes. This is in line with our previous studies showing that genes of both pathways are upregulated in embryos having increased incidence of DNA DSBs (Bohrer et al., 2014, Bohrer et al., 2015).

In conclusion, this study revealed that during early embryo development: i) the HR is the main pathway involved in DNA DSBs repair; and ii) the HR pathway is primarily activated by the ATM kinase.

7.6. Acknowledgements

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7.7. Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

7.8. Funding

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7.9. Figures

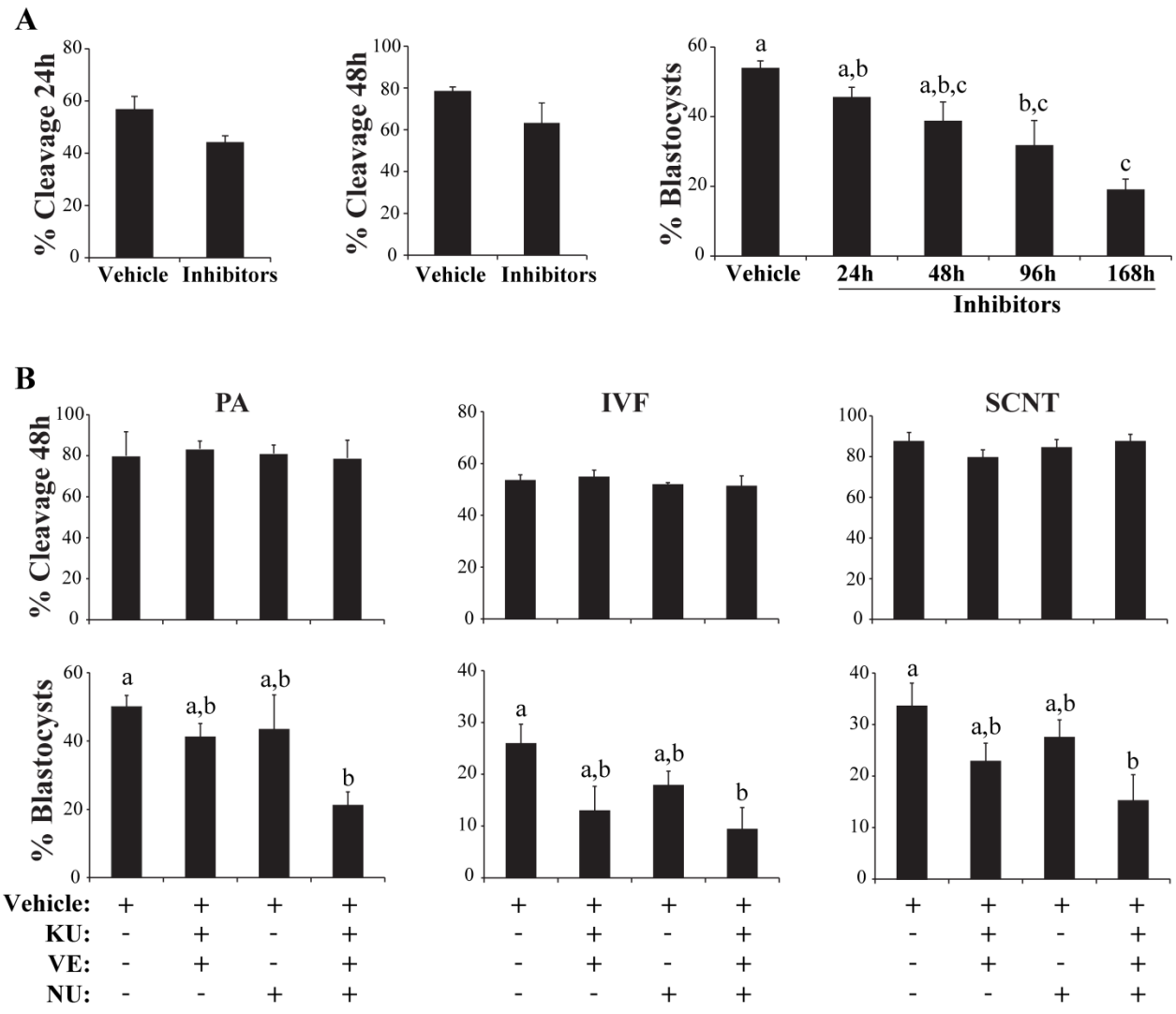

Fig. 7.9.1- Effect of inhibiting DNA DSBs repair pathways on embryo development. (A) Cleavage and blastocyst rates of PA embryos treated with inhibitors of both HR and NHEJ pathways (KU + VE + NU) for 24, 48, 96 or 168h. (B) Cleavage (48h) and blastocyst (day 7) rates of PA, IVF and SCNT embryos treated with inhibitors of HR (KU + VE), NHEJ (NU) or both pathways (KU + VE + NU) for 168h. Data are from 4 replicates with at least 25 embryos per treatment in each replicate. Different letters indicate significant differences between treatments ($P < 0.05$).

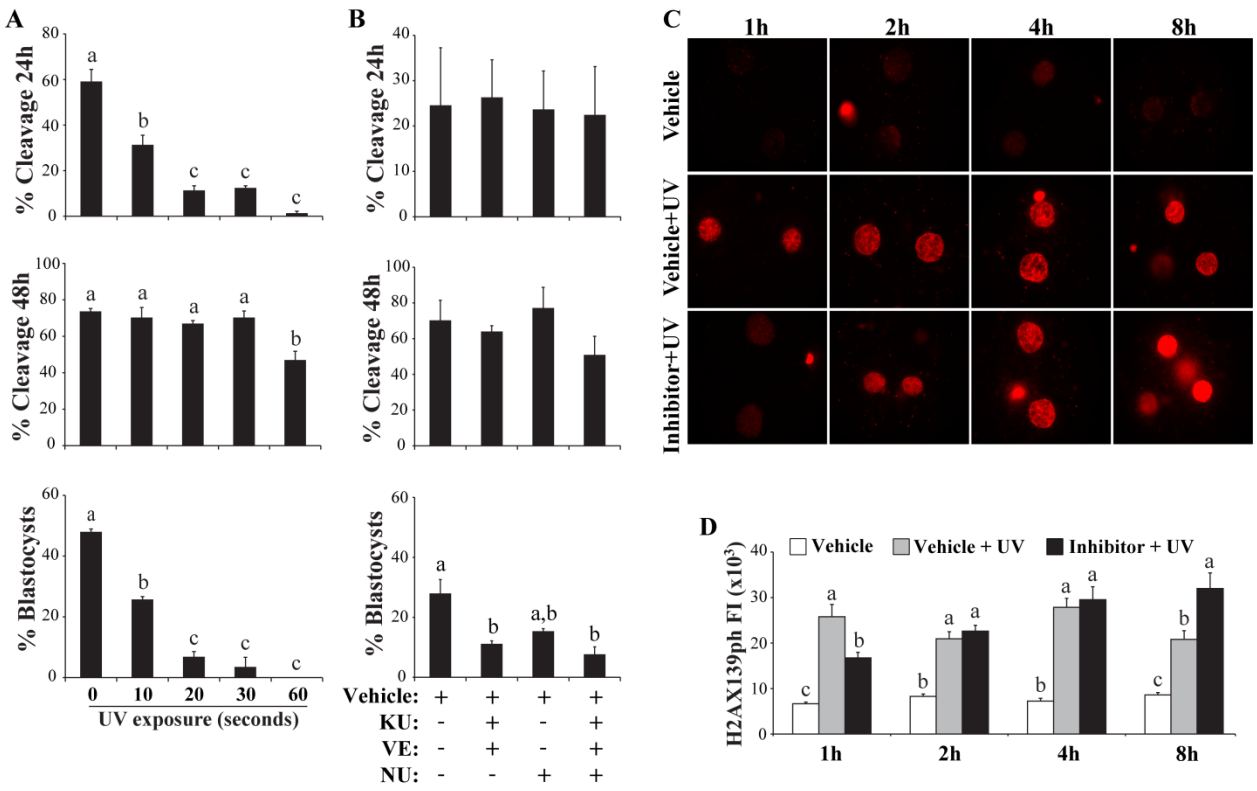

Fig. 7.9.2- Effect of inhibiting DNA DSBs repair pathways in embryos with increased DNA damage induced by UV light exposure. (A) Cleavage and blastocyst rates of embryos exposed to UV light for 0 (control), 10, 20, 30 or 60 sec immediately after PA activation. (B) Cleavage and blastocyst rates of embryos exposed to UV light for 10 sec and treated with inhibitors of HR (KU + VE), NHEJ (NU) or both (KU + VE + NU) pathways. (C) Representative images (original magnification x600) of the immunofluorescent signal for H2AX139ph (red) in 2-cell stage embryos fixed at 1, 2, 4 or 8h after exposure to UV light for 10 sec and treatment with inhibitors of both HR and NHEJ (KU + VE + NU) pathways. (D) Quantification of the fluorescence intensity (FI) for H2AX139ph in the nuclei of 2-cell embryos at 1, 2, 4 and 8h after UV exposure. FI was compared between treatments within time. Embryo development data are from 4 replicates with at least 25 embryos per treatment in each replicate. H2AX139ph quantification was performed with

at least 30 embryos per treatment. Different letters indicate significant differences between treatments ($P < 0.05$).

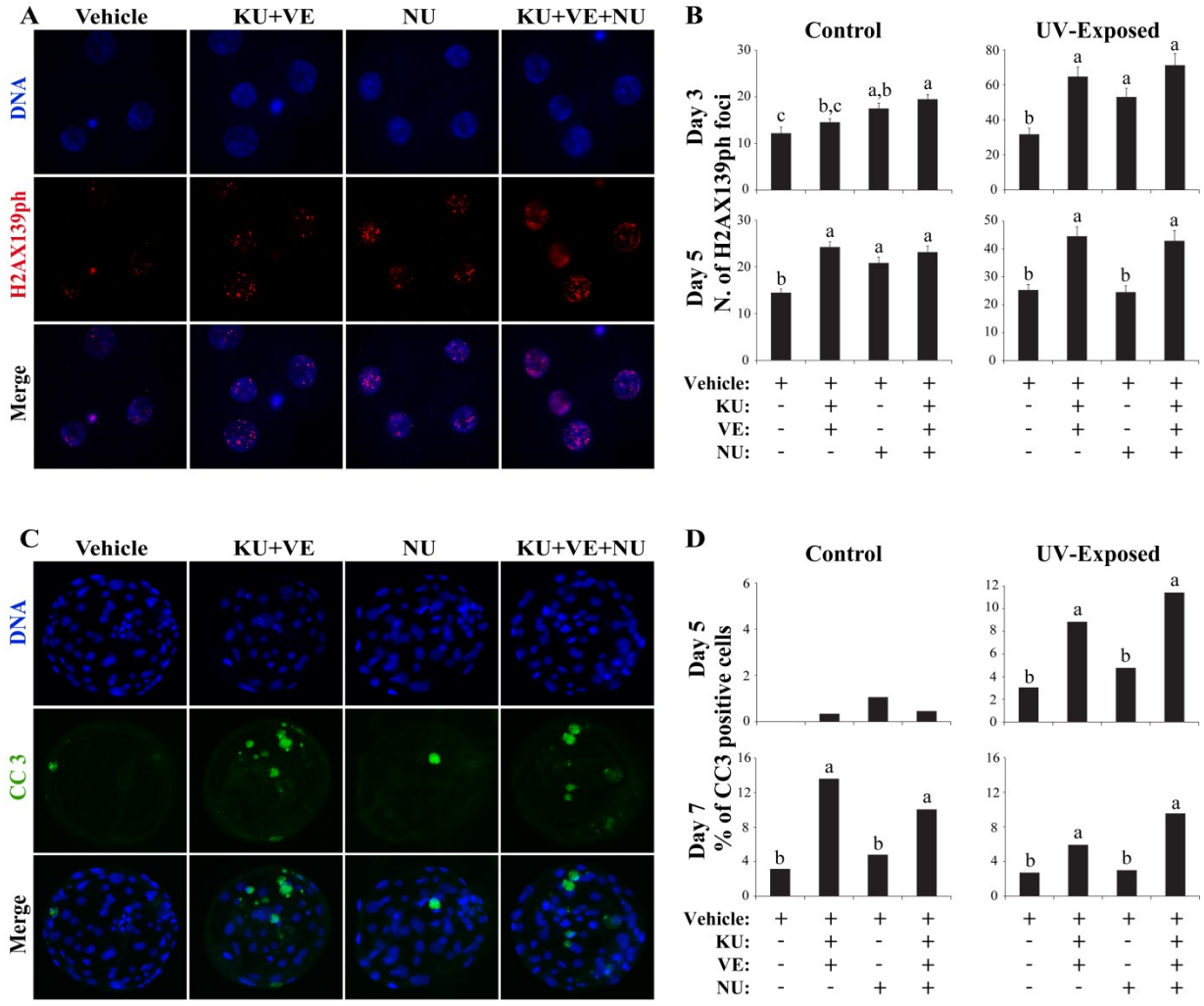

Fig. 7.9.3- Number of H2AX139ph foci and cleaved-caspase 3 positive cells in embryos treated with inhibitors of DNA DSBs repair pathways. (A) Representative fluorescent images (original magnification x600) of day 3 embryos treated with inhibitors of HR (KU + VE), NHEJ (NU) or both (KU + VE + NU) pathways. (B) Number of H2AX139ph foci in control and UV-exposed embryos at day 3 and 5 of culture in the presence of inhibitors of HR (KU + VE), NHEJ (NU) or

both (KU + VE + NU) pathways. (C) Representative fluorescent images (original magnification x 200) of day 7 blastocysts cultured with inhibitors of HR (KU + VE), NHEJ (NU) or both (KU + VE + NU) pathways. (D) Proportion of cleaved-caspase 3 positive cells in control and UV-exposed embryos at day 5 and 7 of culture in the presence of inhibitors of HR (KU + VE), NHEJ (NU) or both (KU + VE + NU) pathways. At least 20 embryos were used in each treatment to quantify H2AX139ph and cleaved-caspase 3. Different letters indicate significant differences between groups ($P < 0.05$).

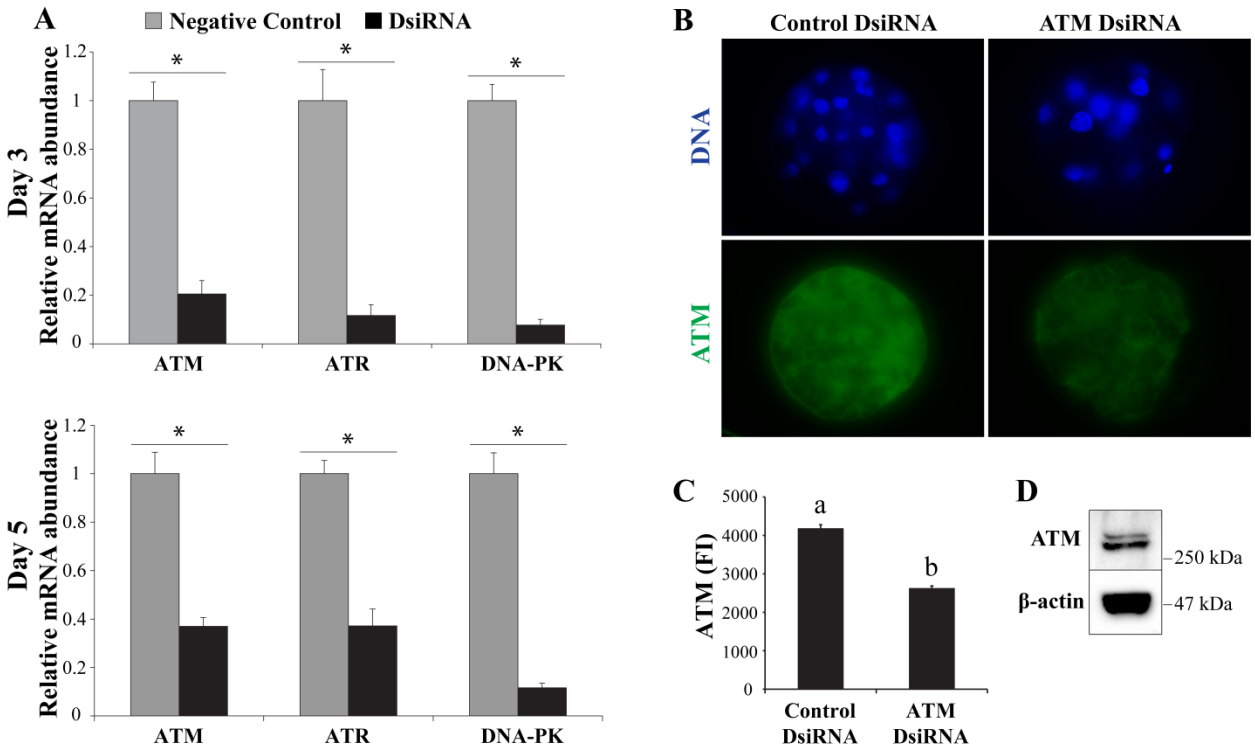

Fig. 7.9.4- Knockdown of ATM, ATR and DNA-PK by intraoocyte injection of DsiRNAs. (A) Relative mRNA abundance of ATM, ATR and DNA-PK in day 3 and day 5 embryos after microinjection of a negative control or two specific DsiRNAs targeting ATM, ATR or DNA-PK mRNA sequences. (B) Representative fluorescent images of day 5 embryos that were

microinjected with negative control or ATM-targeting DsiRNAs. Original magnification X400. (C) Fluorescence intensity (FI) for ATM protein in day 5 embryos that were microinjected with negative control or ATM-targeting DsiRNAs. (D) Immunoblots showing protein bands for ATM and β-actin in porcine fetal fibroblasts confirming antibody specificity. mRNA abundance was determined from three replicates with 20 embryos in each treatment and replicate. mRNA abundance was calculated relative to the average of the reference genes ACTB and H2A. Quantification of ATM protein is from a minimum of 25 embryos in each treatment. Asterisks or different letters indicate significant differences between groups ($P < 0.05$).

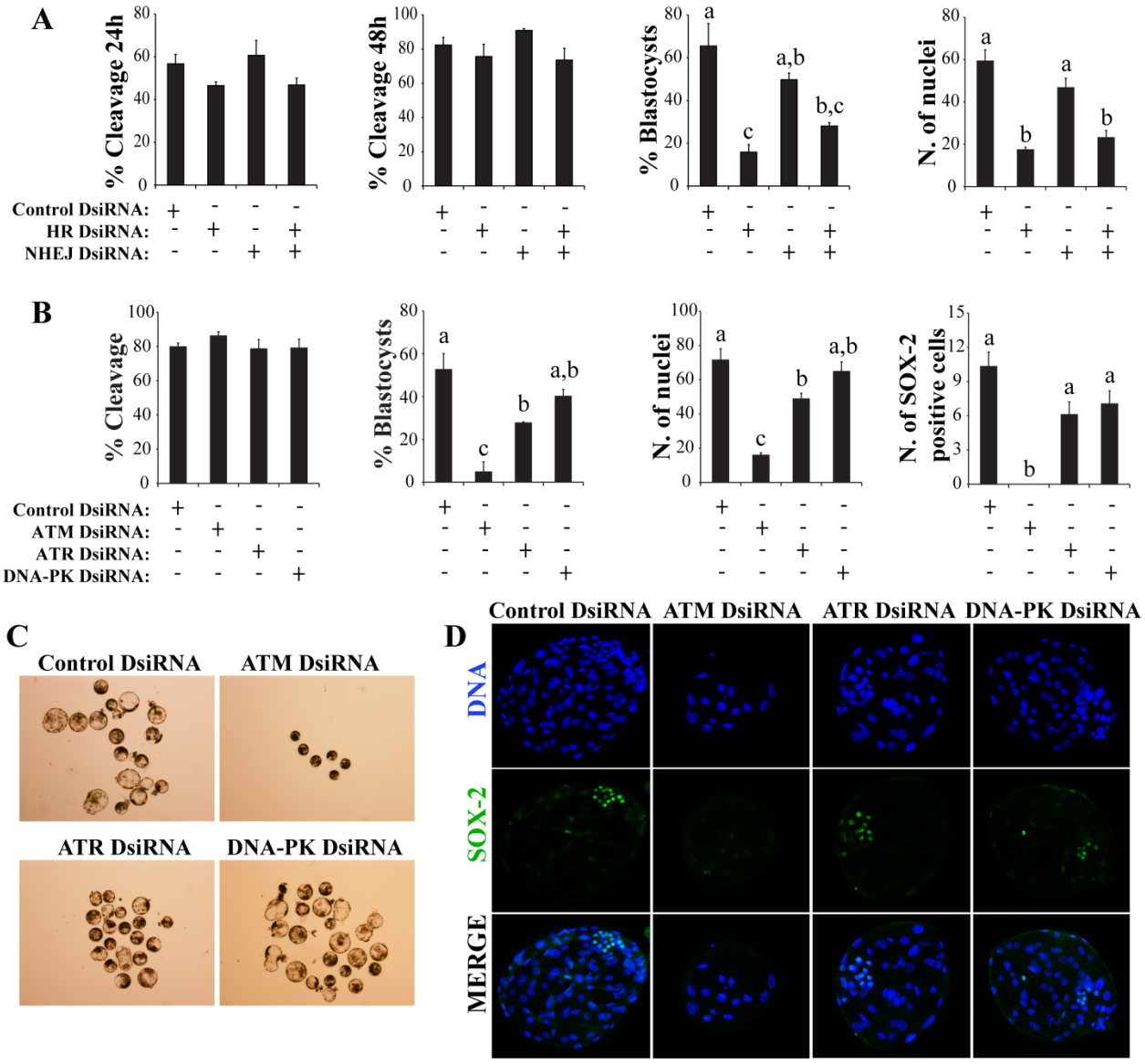

Fig. 7.9.5- Effect of ATM, ATR and/or DNA-PK knockdown on embryo development and quality. (A) Cleavage and blastocyst rates and number of cells in embryos derived from oocytes injected with DsiRNAs targeting HR, NHEJ or both (HR + NHEJ) DNA repair pathways. (B) Effect of DsiRNAs targeting ATM, ATR or DNA-PK on cleavage and blastocyst rates, total number of cells and number of SOX-2 positive cells in day 7 blastocysts. (C) Representative images of day 7 embryos derived from oocytes injected with control, ATM, ATR or DNA-PK targeting DsiRNAs. (D) Representative fluorescent images of embryos derived from oocytes injected with control,

ATM, ATR or DNA-PK targeting DsiRNAs. Embryo development data are from three replicates with a minimum of 30 embryos per treatment in each replicate. Total cell number and number of SOX-2 positive cells were determined with a minimum of 15 blastocysts from each treatment. Different letters indicate significant differences between groups ($P < 0.05$).

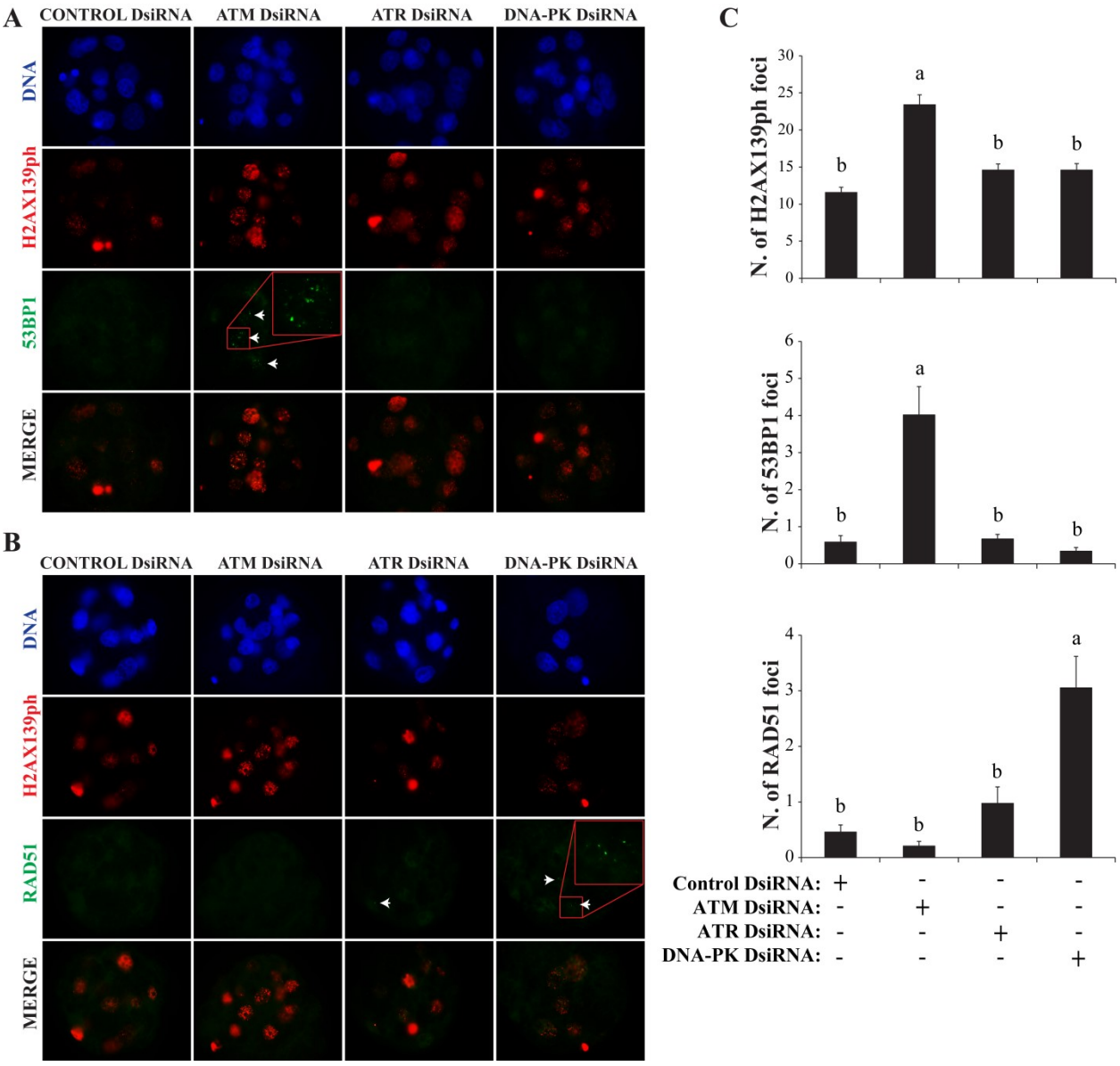

Fig. 7.9.6- Effect of ATM, ATR or DNA-PK knockdown on the number of foci for H2AX139ph, 53BP1 and RAD51 proteins. (A) Representative fluorescent images (original magnification x400) showing colocalization of H2AX139ph and 53BP1 proteins in day 5 embryos that were injected with control, ATM, ATR or DNA-PK targeting DsiRNAs. Nuclei are stained in blue, H2AX139ph in red and 53BP1 in green. Arrows indicate 53BP1 foci. (B) Representative fluorescent images (original magnification x400) showing colocalization of H2AX139ph and RAD51 proteins in day 5 embryos that were injected with of control, ATM, ATR or DNA-PK targeting DsiRNAs. Nuclei

are stained in blue, H2AX139ph in red and RAD51 in green. Arrows indicate RAD51 foci. (C) Average number of H2AX139ph, 53BP1 and RAD51 foci per nuclei in day 5 embryos. Data are from a minimum of 20 embryos in each group. Different letters indicate significant differences between treatments ($P < 0.05$).

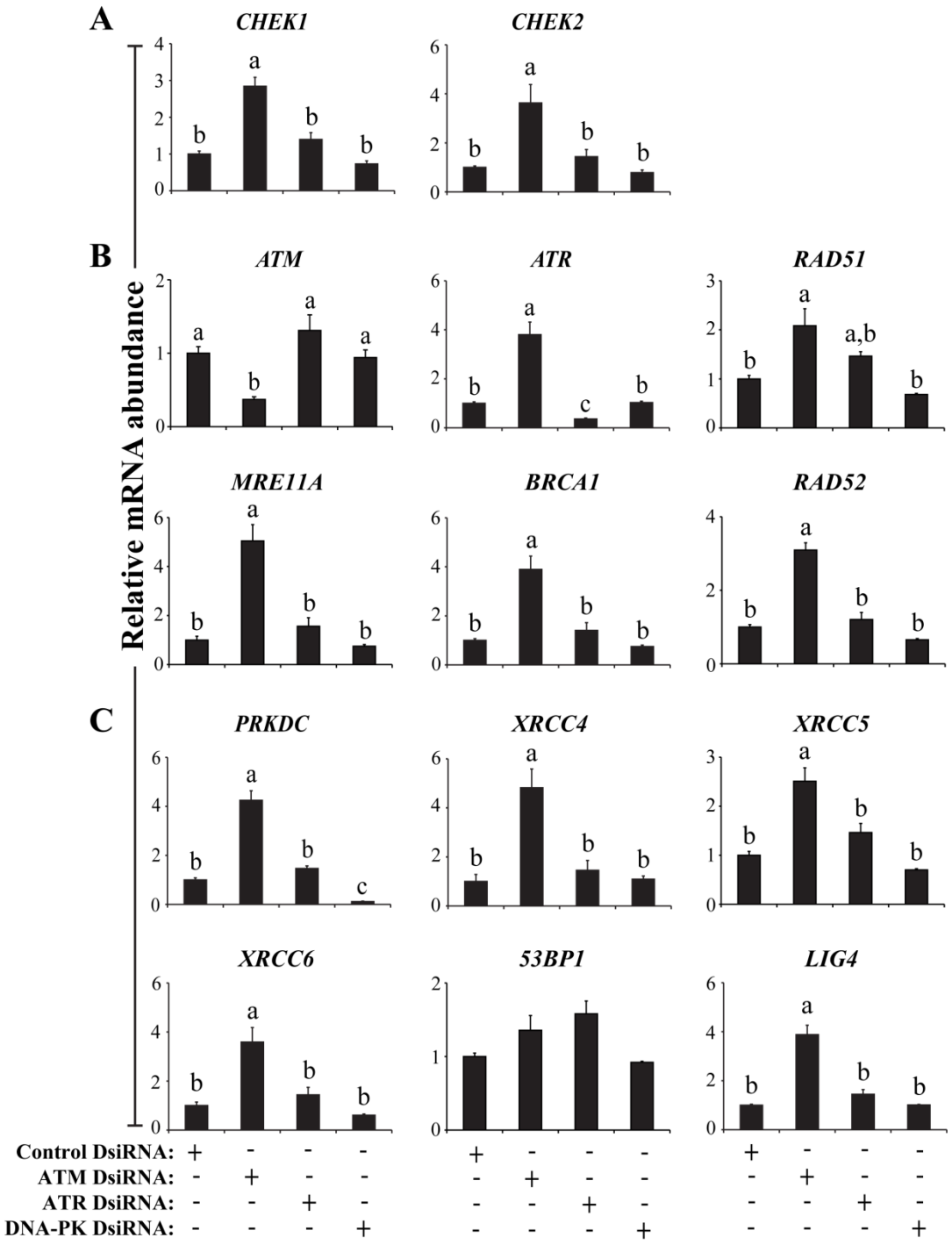

Fig. 7.9.7- Relative mRNA abundance of genes involved in cell cycle control in response to DNA damage (A), DNA DSBs repair by HR (B) and NHEJ (C) in day 5 embryos derived from oocytes injected with control, ATM, ATR or DNA-PK targeting DsiRNAs. mRNA abundance was

calculated relative to the average of the reference genes ACTB and H2A. Data are from 3 replicates with 15 embryos per treatment in each replicate. Different letters indicate significant differences between groups ($P < 0.05$).

7.10. Tables

Table 7.10.1. DsiRNAs used for knockdown experiments.

Target	Sense	Antisense
ATM 1	AAACAAAGUAUGAUGAGG	GGAUCACCCUCAUCAUAC
ATM 2	UCAUUAUUGUGGAAGAAU	CAAGGAGAUUCUUCGACA
ATR 1	GAAGAAACACACAUCGUG	GCACAAUCACGAUGUGUG
ATR 2	CCUGAAUGGCAUAAGCAG	GAUUCAGCUGCUUAUGCC
DNA-PK 1	UUAAUGAUACCGAUGGAU	CUAGGAAAUCCAUCGGUA
DNA-PK 2	GUCUGGAAGCAGAGCAA	GGAGAAGCUUGCUCUGC
Negative Control	CGUUAAUCGCGUAUAAUAC GCGUAT	AUACGCGUAUUAUACGCGAUU AACGAC

Table 7.10.2. Primers used for quantitative real-time PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Accession No.
ATM	CCGGTGTTTTGGGAGAGTGT	CTTCCGACCAAACTCAGCGT	NM_001123080.1
ATR	TGAGCTCCAGTGTTGGCATC	GCCAGTTCTCAGTGTGGTCA	XM_003132459.3
RAD51	CTTCGGTGGAAGAGGAGAGC	CGGTGTGGAATCCAGCTTCT	NM_001123181.1
RAD52	ATTCAGCAAGGGATGCCCAC	TAGGGCAAGGGCGTTTTCTT	XM_003358103.2
BRCA1	TGCTAAATCCGGAACAAAACACA	CTGGTGGAACGATCCAGAGAT	XM_003358030.1
MRE11A	GGAGGATGTTGTCCTGGCTG	AGACGTTCCCGTTCTGCATT	XM_003129789.2
PRKDC	ATTCTTTGTCGGGAGCAGCA	CCTAGCTGTGTGGCACATGA	XM_001925309.4
XRCC4	ATGGCTTCACAGGAGCTTCA	ATGTTTTCAGCTGGGCTGTG	XM_003123760.2
XRCC5	CTGGCATCTCGCTGCAATTC	GAAAGGAGGGTCCATGGTGG	XM_003133649.2
XRCC6	ACGGAAGGTGCCCTTTACTG	TGCAGCACTGGGTTCTCAAA	NM_001190185.1
TP53BP1	GCAGATGGACCCTACTGGAA	GGCTTTCAGGCTGAGAATCTT	XM_001925938.4
LIG4	AGCTAGACGGCGAACGTATG	CCTTCCTGTGGGGAAACTCC	XM_003131089.2
CHEK1	TGCCCTTTGTGGAAGACTGG	ACTGCAACTGCTTCCTCAGT	XM_003130047.2
CHEK2	GCCTGTGGTGAGGTGAAACT	TGCTGGATCTGCCTCTCTCT	NM_001137638.1
ACTB	GCAGATGTGGATCAGCAAGC	GAATGCAACTAACAGTCCGCC	XM_003124280.2
H2A	GGTGCTGGAGTATCTGACCG	GTTGAGCTCTTCGTCGTTGC	XM_001927727.2

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CHAPTER 8

8.1. GENERAL DISCUSSION AND CONCLUSIONS

The general objective of the studies reported in this thesis was to characterize the occurrence, consequences and repair mechanisms of DNA DSBs during early embryo development. In the first manuscript of this thesis we observed that swine embryos produced by IVF and SCNT have high incidence of DNA DSBs, which compromises cell cycle progression, embryo development and quality. We observed that fast-developing embryos have fewer DSBs than slow-developing embryos. It can be explained by studies in somatic cells showing that DNA damage response involves cell cycle coordination and arrest at G1/S or G2/M checkpoints to allow DNA DSBs repair before DNA replication and cell division (Aquilina et al., 1999). It gives more time for the affected cells to repair DNA damage before mitosis (Finkielstein et al., 2001). Interestingly, in this study we also observed a negative correlation between the percentage of cells with DNA DSBs and the total number of cells per embryo at blastocyst stage. It suggests that the occurrence of DNA DSBs is an important determinant of blastocyst quality.

In the first manuscript we also observed that SCNT embryos activated with strontium chloride and ionomycin have lower percentage of nuclei with DNA DSBs than SCNT embryos activated with ionomycin alone. Previous studies have shown that oocytes activated with strontium chloride and ionomycin result in superior development of SCNT swine embryos compared to those activated just with ionomycin (Che et al., 2007). It can be explained by the possibility that strontium chloride treatment promotes a more physiological response in the oocyte, and consequently reduces detrimental effects of cell cycle compatibilities between the transferred nucleus and the host cytoplasm. Abnormal cell cycle compatibilities are thought to affect chromatin replication, integrity and segregation in SCNT embryos (Campbell, 1999). Therefore,

the findings of our first manuscript also suggest that the inclusion of strontium chloride in the activation protocol during SCNT reduces the occurrence of DNA DSBs during activation and therefore improves early embryo development and blastocyst quality.

In the second manuscript, we assess the impact of DNA integrity on cell reprogramming in embryos produced by SCNT. We observed that the induction of DSBs reduced embryo development after SCNT. Previous studies have shown that genome integrity preservation is crucial for somatic cell reprogramming and production of iPS cells in human and mouse (Marion et al., 2009, González et al., 2013, Molina-Estevez et al., 2013). Moreover, studies have also shown that DNA damage affects cell cycle progression and compromises the normal early embryo development (Gawecka et al., 2013, Wang et al., 2013b). In line with these observations, our results using SCNT embryos provided further evidence that both cell reprogramming and early embryo development are severely affected when the genome integrity is disrupted.

In the second manuscript we also have found that HDACi treatment greatly increased development to the blastocyst stage of SCNT embryos produced from somatic cells with higher number of DNA DSBs. Moreover, HDACi treatment consistently reduced the number of DNA DSBs in cleaved SCNT embryos produced from somatic cells having increased number of DNA DSBs. HDACi treatment has been used to improve donor cell reprogramming and to increase the developmental capacity of SCNT embryos and the efficiency of animal cloning from somatic cells in various species including swine (Maalouf et al., 2009, Zhao et al., 2010, Wang et al., 2011b, Chen et al., 2013). Although the mechanisms by which HDACi promote cell reprogramming and development have not been completely characterized, it has been shown that HDACi treatment increases the acetylation levels of multiple lysine residues on histones, including H3K14, H4K5 and H4K8 in SCNT embryos (Iager et al., 2008, Martinez-Diaz et al., 2010, Zhao et al., 2010). It

is known from studies in somatic cells that acetylation of lysine residues, including H4K5, H4K12 and H4K16, by different histone acetyltransferases (HATs) is critical for DSBs repair (Tamburini and Tyler, 2005, Sharma et al., 2010, Tang et al., 2013, Yang et al., 2013). It has also been shown that during DNA damage response the HAT Gcn5 interact with H2AX139ph in the sites of DSBs and acetylate various lysine residues on the histone H3, including H3K9, H3K14, H3K18 and H3K23 (Lee et al., 2010). Interestingly, our findings indicate that HDACi treatment facilitates DNA DSBs repair in SCNT embryos. Therefore, chromatin remodeling during cell reprogramming after SCNT is not only important for the resetting of the epigenetic program but also to promote DNA DSBs repair and preserve the genome integrity for normal embryo development.

In the third manuscript, using a well-established model for embryo developmental competence based on the time to first embryo cleavage, we showed that early-cleaving and more developmentally competent embryos have less DNA DSBs than late-cleaving and less developmentally competent embryos. A number of studies have shown that early-cleaving embryos of several species and produced by different protocols have superior developmental capacity and produce better quality blastocysts than late-cleaving embryos (Yadav et al., 1993, Sakkas et al., 1998, Booth et al., 2007, Coutinho et al., 2011, Isom et al., 2012). However, the cause and mechanisms behind the altered cell cleavage and development have not been sufficiently characterized. Our findings in this study revealed that the presence of DNA DSBs might represent an important constraint for embryo cell cleavage kinetics and development. Interestingly, studies have shown that the presence of DNA DSBs activates checkpoint proteins that promote cell cycle arrest at the G2/M transition in mouse embryos fertilized with DNA-damaged sperms (Wang et al., 2013a). Therefore, our findings suggest that slow cell-cleavage during early embryo development is likely a cell response to allow enough time for DNA DSBs repair to take place,

thus preventing the segregation of DNA mutations to the somatic or germ cell lineages in the developing embryo.

In the third manuscript we also observed that the transcript levels for a number of DNA repair genes involved in DNA DSBs repair by both the HR and the NHEJ pathways and for checkpoints involved in cell cycle control in response to DNA DSBs were more abundant in late- than early-cleaving embryos at either day 3 or day 5 of development. Previous studies have reported the presence of transcripts for DNA repair genes in oocytes and early developing embryos (Menezo et al., 2007, Jaroudi et al., 2009), which suggests that the products of these genes are required during all stages of embryo development. Indeed, our previous studies have shown that genes involved in DNA repair are regulated in response to DNA DSBs during early embryo development (Henrique Barreta et al., 2012, Bohrer et al., 2014). Interestingly, findings from this manuscript suggest that early cleaving embryos are able to respond to DNA DSBs and, therefore, we propose that the higher number of DNA DSBs in the late-cleaving embryos is more likely due to an increased incidence of damages rather than a decreased capacity for DNA repair.

Finally, in our forth manuscript we investigated the importance of the HR and the NHEJ pathways for DNA DSBs repair during early embryo development. We observed that attenuation of the HR pathway reduced embryo development and quality, and increased DNA DSBs and cell apoptosis. Our findings are in line with previous studies with embryonic stem cells showing that the HR is relatively more important than the NHEJ pathway for DNA DSBs repair (Essers et al., 2000). It is known that the HR pathway is the predominant form of DNA DSBs repair in S/G2 phase cells and that the NHEJ pathway is more active in non-replicating G0/G1 phase cells (Takata et al., 1998, Delacôte and Lopez, 2008). The fact that dividing blastomeres in early stage embryos are essentially deprived of G1 phase and enter the S phase immediately after the end of the M

phase (Mukherjee, 1976, Smith and Johnson, 1986, Schoenbeck et al., 1992) would explain the primary role of the HR pathway. This is further supported by studies showing that components of the HR pathway, including ATM and Rad51 proteins, have important effects on genome stability during embryo development in different species (Perez et al., 2006, Zha et al., 2008, Kujjo et al., 2010, Kujjo et al., 2012).

It is known that both ATM or ATR kinases can activate the HR pathway (Harper and Elledge, 2007). Therefore, in our fourth manuscript we also investigated the importance of ATM and ATR kinases for DNA DSBs repair during early embryo development. We observed that ATM knockdown had a more prominent effect on blastocyst development and quality than ATR knockdown. Indeed, ATM knockdown significantly increased DNA DSBs accumulation and prevented accumulation of the DNA repair protein RAD51, which has a major role in the HR pathway. This indicate that ATM activity is required for the activation of the HR pathway in early developing embryos. Interestingly, we observed that ATM knockdown prevented ICM formation in embryos that developed to the blastocyst stage, which suggest that DNA DSBs accumulation affects cell differentiation and pluripotency. In line with this, previous studies shown that DNA DSBs reduced the expression of pluripotency genes (e.g., NANOG), induced embryonic stem cell differentiation (Lin et al., 2005), and impaired cell reprogramming (Marion et al., 2009).

In conclusion, findings from the studies reported in this thesis revealed that: 1) DNA DSBs occur in a high proportion of porcine embryos produced in vitro; 2) the presence of DNA DSBs is an important component affecting the cleavage kinetics and early embryo development; 3) HDACi improves somatic cell reprogramming by facilitating DNA DSBs repair during early development of SCNT embryos; 4) DNA DSBs are mainly repaired by the HR pathway during early embryo

development; and 5) ATM is the main proteins involved in the activation of the HR pathway during early embryo development.

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