Phosphorylated histone H2A.x in porcine embryos produced by IVF and somatic cell nuclear transfer

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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 characterized the presence of H2AX139ph during *in vitro* development of porcine embryos produced by IVF and somatic cell nuclear transfer (SCNT). Pronuclear stage embryos produced by IVF had, on average, 9.2 H2AX139ph foci per pronucleus. The number of H2AX139ph foci was higher in the 2-cell-stage embryos than in the 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 (ION) alone than in those activated with ION and strontium chloride (ION + Sr²⁺). A negative correlation was found between the percentage of H2AX139ph-positive cells and the total number of cells per embryo in day 7 blastocysts produced by IVF or SCNT. Based on the detection of H2AX139ph foci, the findings of this study indicate that DSBs occur in a high proportion of porcine embryos produced by either IVF or SCNT; fast-cleaving embryos have fewer DSBs than slow-cleaving embryos; the oocyte activation protocol can affect DNA integrity in SCNT embryos; and better-quality blastocysts have fewer DSBs. We propose that the presence of H2AX139ph foci can be a useful marker of embryo quality.

Reproduction (2013) **146** 325–333

Introduction

Embryo technologies such as IVF and somatic cell nuclear transfer (SCNT) have been applied to produce embryos in a number of species. Because pigs 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 & Niemann 2004, Kuwaki *et al.* 2005, Aigner *et al.* 2010). However, the development of IVF and SCNT porcine 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 & 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 cytoplasm, which can compromise embryo development (Campbell 1999, Fulka & Fulka 2007). Intra-oocyte calcium oscillations induced by the sperm trigger oocyte activation during normal fertilization (Fissore *et al.* 1992). Strontium chloride (Sr²⁺) has been shown to

induce calcium oscillations in oocytes, and it has, consequently, been used for oocyte activation in different species such as mouse, rat, bovine, and ovine species (Kline & Kline 1992, Kishikawa *et al.* 1999, Méo *et al.* 2004, Tomashov-Matar *et al.* 2005, Zhang *et al.* 2005, Choi *et al.* 2013). We have previously shown that porcine oocyte activation using a combination of ionomycin (ION) and Sr²⁺ results in superior development of SCNT embryos compared with oocyte activation using ION alone (Che *et al.* 2007).

Currently, there is limited information 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 on the occurrence of DNA damage. In somatic cells, the histone H2A.x has been shown to be indispensable for the preservation of genome integrity because it participates in the process of DNA double-strand break (DSB) repair. H2A.x is a member of the H2A histone family including H2A1, H2A2, and H2AZ, which help in the package and organization of DNA into chromatin (West & Bonner 1980). H2A.x is immediately activated by the 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 & Ohnishi 2005, Stiff *et al.* 2006). In ~30 min of DSB formation, phosphorylated histone H2A.x (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 larger than $0.30\ \mu\text{m}^3$, indicate the sites of DSBs because they are tightly co-localized with DNA-damage signaling and repair proteins (Paull *et al.* 2000, McManus & 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 a few studies that have reported 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 has been shown to impair cellular reprogramming and reduce the efficiency of production of 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 i) investigate the occurrence of large H2AX139ph foci as indicators of DSBs during *in vitro* development of porcine embryos produced by IVF and SCNT; ii) evaluate the effect of the protocol used for oocyte activation on the occurrence of H2AX139ph foci in SCNT embryos; and iii) assess whether the presence of H2AX139ph foci is correlated with development and number of cells per embryo at cleavage and blastocyst stages.

Materials and methods

Chemicals

Unless otherwise indicated, all chemical reagents were purchased from Sigma Chemical Company (Sigma-Aldrich).

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 sterile 0.9% NaCl at 30–35 °C. Follicles having a diameter of 3–6 mm 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 cumulus 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. The maturation medium consisted of TCM-199 (Life Technologies), supplemented with 20% porcine follicular fluid, 0.1 mg/ml cysteine, 10 ng/ml epidermal growth factor (Life

Technologies), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.5 µg/ml luteinizing hormone (LH) (SIOUX Biochemical, Inc., Sioux Center, IA, USA), 0.5 µg/ml follicle stimulating hormone (FSH) (SIOUX Biochemical, Inc.), and 20 µg/ml gentamicin (Life Technologies). After 22–24 h of maturation, oocytes were transferred to the same IVM medium, but without LH and FSH, for an additional 20–22 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 the oocytes were used for IVF or SCNT.

IVF

Matured oocytes were washed thrice 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 & Day 1997). Groups of 20 oocytes were then placed in 90 µl drops of IVF medium covered with mineral oil. Spermatozoa were prepared from fresh semen samples of a proven fertile boar. The spermatozoa were washed twice with IVF medium by centrifugation (800 g) for 3 min, and the sperm pellet was resuspended in IVF medium. Oocytes in each drop were fertilized by adding 10 µl of the sperm preparation with the concentration adjusted to 10 000 spermatozoa per oocyte. The 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).

Somatic cell nuclear transfer

Cumulus-free oocytes selected with a polar body were cultured in TCM-199 medium supplemented with 0.4 µg/ml demecolcine and 0.05 M sucrose for 60 min. This treatment resulted in a protrusion in the ooplasmic membrane that contained the metaphase chromosomes (Yin *et al.* 2002). The oocytes were then transferred into Tyrode's lactate–pyruvate–HEPES medium (5 mM glucose, 113 mM NaCl, 3.2 mM KCl, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 2 mM NaHCO₃, 20 mM lactate, 10 mM HEPES, and 0.3% polyvinyl alcohol (PVA), pH 7.4) supplemented with 7.5 mg/ml cytochalasin B (CB) for 5–10 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 in a 0.28 M mannitol solution supplemented with 50 µM CaCl₂, 100 µM MgSO₄, and 0.1% PVA. The reconstructed oocytes were cultured in TCM-199 medium supplemented with 3 mg/ml BSA for 1 h before activation.

Oocyte activation

The reconstructed oocytes were activated using the following protocols (Che *et al.* 2007):

- i) ION: the oocytes were exposed for 5 min to 15 µM ION in TCM-199 and then transferred to PZM-3 medium supplemented with CB (7.5 g/ml) and cycloheximide (CHX; 10 g/ml) for 4 h.

- ii) $\text{ION} + \text{Sr}^{2+}$: the oocytes were exposed to 15 M ION for 5 min and then transferred to 10 mM Sr^{2+} in PZM-3 (Ca^{2+} free) + 7.5 g/ml of CB and 10 g/ml of CHX for 4 h. After activation, the oocytes were washed and cultured in PZM-3 medium.

Embryo culture

The IVF or SCNT oocytes were washed thrice in PZM-3 medium supplemented with 3 mg/ml BSA (fatty acid free) and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 °C. The cleavage rate was determined 48 h after IVF or activation. The cleaved embryos were transferred to a fresh drop of PZM-3 medium, and on day 5 of culture, 10% fetal bovine serum was added, and the embryos were maintained in culture until day 7.

Western blotting

In vitro produced porcine embryos (65 blastocysts) and porcine fetal fibroblasts were lysed in Laemmli buffer (Bio-Rad) containing Phosphatase and Protease Inhibitor Cocktail (G-Biosciences, St Louis, MO, USA). Total lysate was resolved and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% BSA in Tris-buffered saline with 0.1% Tween, the membranes were incubated overnight with anti-phospho-H2A.x (Ser139) mouse MAB (dilution 1:1000; Millipore, Billerica, MA, USA) or with anti- β -actin rabbit polyclonal primary antibodies (1:5000; Abcam, Inc., Toronto, ON, Canada). Subsequently, the membranes were incubated for 1 h with peroxidase-conjugated anti-mouse (1:3000) or anti-rabbit (1:10 000) secondary antibodies (Cell Signaling, Boston, MA, USA). The membranes were incubated for 2 min with reagents provided with the Immun-Star WesternC Chemiluminescent Kit, and images were captured using the ChemiDoc MP System (Bio-Rad).

Immunofluorescence staining

Samples of IVF and SCNT embryos at 1- and 2–4-cell stages and day 7 blastocysts were fixed for 15–20 min in 4% paraformaldehyde and permeabilized in 1% Triton X-100 in PBS for 30 min at 37 °C. The samples were incubated for 1 h at room temperature in a 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 antibodies (Millipore) diluted (1:1000) in the blocking solution. The embryos were then washed thrice for 20 min each with the 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 antibodies (Jackson ImmunoResearch Laboratories, Baltimore, PA, USA). After this period, DNA was stained by exposing the samples to 10 $\mu\text{g/ml}$ of 4',6-diamidino-2-phenylindole (Life technologies) in the blocking solution for 20 min. The samples were then washed thrice (20 min each) with the blocking solution and mounted on microscope slides using a drop of Mowiol. The slides were kept in a dark box and examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon). Images were captured at

200 $\times$, 400 $\times$, or 600 $\times$ magnification using a Retiga 2000R monochrome digital camera (Qimaging, BC, Canada) and the SimplePCI Imaging Software (Compix, Inc., Sewickley, PA, USA). Nuclei were individually evaluated, and H2AX139ph foci larger than 0.30 μm^3 , which indicates the sites of DNA DSBs (McManus & Hendzel 2005, Grenier *et al.* 2012), were counted. The number of H2AX139ph foci was determined in IVF embryos at the pronuclear stage (at 10, 15, and 20 h post-fertilization (hpf)), SCNT embryos at 20 h post-nuclear transfer (hpnt), and both IVF and SCNT cleaved embryos (2- and 4-cell stages) and day 7 blastocysts at 48 h. The results are presented as average number of H2AX139ph foci per nucleus or the proportion of nuclei with <10 foci, $\geq$ 10 foci, and total positives.

Statistical analyses

All analyses were performed using the JMP Software (SAS Institute, Inc., Cary, NC, USA). 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. Data were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. The correlations were done using simple linear regression analyses, and the nominal data were analyzed using the χ^2 test. Differences were considered to be statistically significant at the 95% level ($P < 0.05$).

Results

Presence of H2AX139ph in IVF embryos

The specificity of H2AX139ph antibody was confirmed by western blotting using porcine fetal fibroblasts and porcine embryos on 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 9.2 foci per pronucleus (Fig. 1B). The average number of foci was numerically higher but not statistically significant in zygotes fixed at 15 and 20 h compared with those fixed at 10 hpf ($P = 0.096$; Fig. 1C). H2AX139ph foci were found in most of the zygotes regardless of the time they were fixed after fertilization (Fig. 1D). Based on the average number of foci counted per pronucleus (approximately ten), we decided to classify H2AX139ph-positive nuclei as those with less than and more than ten foci. The proportion of pronuclei with <10 foci compared with that of pronuclei with $\geq$ 10 foci was not significantly different in zygotes fixed at 10, 15, and 20 hpf (Fig. 1D).

Cleaved embryos fixed at 48 hpf were grouped into 2-cell-stage embryos (slow cleaving) and 4-cell-stage embryos (fast cleaving). The mean number of H2AX139ph foci per nucleus (Fig. 2A) was higher

Figure 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 20 h after IVF. Arrows indicate H2AX139ph foci larger than $0.30 \mu\text{m}^3$. (C) Mean number of H2AX139ph foci in the pronuclei of porcine zygotes fixed 10, 15, and 20 h after IVF. (D) Percentage of zygotes with H2AX139ph foci (<10 foci, ≥ 10 foci, and total positives) 10, 15, and 20 h after IVF. Results shown in (C) and (D) were not significantly different ($P > 0.05$).

($P = 0.011$) in embryos with two cells (16.7 foci) than in those with four cells (6.0 foci; Fig. 2B). H2AX139ph foci were detected in most of the nuclei of both the 2- and 4-cell-stage embryos (Fig. 2C). The proportion of nuclei presenting more than ten foci was also higher ($P = 0.043$) in embryos with two cells (46.4%) than in those with four cells (21.8%; Fig. 2C). Embryos with four cells had a higher proportion of nuclei with <10 foci than those with ≥ 10 foci (Fig. 2C).

In day 7 blastocysts produced by IVF (Fig. 3A), H2AX139ph was detected in 49.8% of the nuclei, whereas 36.6% of the positive cells had <10 foci and 13.1% had ≥ 10 foci ($P < 0.001$; Fig. 3B). Interestingly, there was a negative correlation ($r^2 = 0.6834$; $P < 0.001$) between the percentage of cells with H2AX139ph foci and the total number of cells per blastocyst (Fig. 3C).

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 the 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 the 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 (two vs four cells) and the activation protocol (Fig. 5). The proportion of nuclei with less than ten foci was higher in the 4-cell-stage embryos (38.8%) than in the 2-cell-stage embryos (10.5%) that were activated with ION. There was no significant difference between the 2- and 4-cell-stage embryos in the group activated with ION+ Sr^{2+} (Fig. 5A). The proportion of nuclei with ≥ 10 foci was higher in the 2-cell-stage embryos (42.1 and 35.2%) than in the 4-cell-stage embryos (11.1 and 8%) 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

Figure 2 Presence of H2AX139ph foci in cleaved embryos fixed 48 h after IVF. (A) Representative fluorescent images showing the presence of H2AX139ph foci in the 2- and 4-cell-stage embryos. (B) Mean number of H2AX139ph foci in the 2- and 4-cell-stage embryos (a vs b, $P = 0.011$). (C) Percentage of nuclei with H2AX139ph foci (<10 foci, ≥ 10 foci, and total positives) from the total number of evaluated nuclei. * $P = 0.043$ and ** $P = 0.004$.

Figure 3 Presence of H2AX139ph in blastocysts on day 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 the 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, and total positives) from the total number of evaluated nuclei. ($P < 0.05$) between the groups. (C) Correlation between the percentage of H2AX139ph-positive nuclei and the total number of nuclei in day 7 blastocysts ($P < 0.001$).

the proportion of nuclei presenting H2AX139ph foci. The proportion of nuclei with more than ten foci (38.8 vs 16%; Fig. 5A) and the total number of H2AX139ph-positive nuclei (50 vs 24%; Fig. 5C) were significantly higher in the 4-cell-stage embryos activated with ION than in those activated with ION + Sr^{2+} .

In blastocysts produced by SCNT, the percentage of nuclei with < 10 H2AX139ph foci (27.2 vs 12.0%) and the total number of H2AX139ph-positive nuclei (43.7 and 20.7%) were significantly higher in SCNT blastocysts produced with the ION activation protocol than in

those produced with the ION + Sr^{2+} activation protocol (Fig. 6A). There was a negative correlation ($r^2 = 0.6284$; $P < 0.001$) between the percentage of cells with H2AX139ph foci and the total number of cells in SCNT blastocysts (Fig. 6B).

Discussion

The phosphorylation of histone H2A.x is essential to promote DNA DSB repair by acting as a platform for the accumulation and retention of important proteins involved in signaling and repair mechanisms (Kinner *et al.* 2008). Despite its importance in DSB 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 as follows: i) DSBs are detected during all stages of *in vitro* embryo development; ii) fast-developing embryos at 48 h (four cells) have fewer DSBs than slow-developing embryos (two cells); iii) the oocyte activation protocol affects the occurrence of DSBs in embryos produced by SCNT; and iv) better-quality blastocysts with a higher cell number have fewer DSBs than low-quality blastocysts.

It has been shown that H2AX139ph foci larger than $0.30 \mu\text{m}^3$ indicate the sites of DNA DSBs and co-localize

Figure 4 Presence of H2AX139ph in 1-cell-stage SCNT embryos fixed 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. White and black bars represent embryos activated with ION and ION + Sr^{2+} respectively. Differences between the ION and ION + Sr^{2+} groups were not statistically significant ($P > 0.05$).

Figure 5 Effect of the activation protocol on the occurrence of H2AX139ph in SCNT embryos fixed 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 the 2- and 4-cell-stage embryos. White and black bars represent embryos activated with ION and ION+Sr²⁺ respectively. Asterisks indicate significant differences ($P<0.05$) between the groups.

with DNA-repair proteins including RAD51, BRCA1, 53BP1, NBS1, MRE11A, KU80, XRCC4, and DNA ligase IV (Paull *et al.* 2000, McManus & Hendzel 2005). In this study, we observed the presence of large H2AX139ph foci in a high proportion of nuclei in porcine embryos evaluated on day 1 (pronuclear stage), day 2 (2- and 4-cell stages), and day 7 (blastocyst stage) of *in vitro* culture. This suggests that porcine embryos might rely on H2AX139ph to promote DNA-damage repair during all stages of development from the zygote

to the blastocyst stage. Although H2AX139ph foci have also been reported to be present in zygotes and cleaved embryos in rats (Barton *et al.* 2007, Grenier *et al.* 2012), there are probably differences among species since H2AX139ph foci have been detected in cleaved embryos but not in the zygotes of mice (Adiga *et al.* 2007, Yukawa *et al.* 2007).

This study revealed that cultured porcine embryos have a high incidence of DSBs, which can probably 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 the 2-cell-stage embryos than in the 4-cell-stage embryos. Moreover, we observed a higher proportion of nuclei with ≥10 foci in the 2-cell-stage embryos than in the 4-cell-stage embryos. It has been shown that fast-cleaving porcine embryos produce more and better-quality blastocysts than slow-cleaving embryos (Dang-Nguyen *et al.* 2010, Coutinho *et al.* 2011). Similarly, fast-developing porcine

Figure 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 on day 7. White and black bars represent embryos activated with ION and ION+Sr²⁺ respectively. Asterisks indicate significant differences ($P<0.05$) between the ION and ION+Sr²⁺ groups. (B) Correlation between the percentage of H2AX139ph-positive nuclei and the total number of nuclei in day 7 SCNT blastocysts ($P<0.001$).

embryos produced by SCNT have a higher potential to support embryo development than slow-developing embryos (Isom *et al.* 2012). Therefore, based on current observations, it appears that a higher incidence of DSBs delays early-embryo cleavages and compromises later-embryo development and quality.

Our findings based on blastocysts, produced both 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 the 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). The 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 (Finkelstein *et al.* 2001), but in some cases it can lead to the activation of apoptotic pathways (Zhivotovsky & Kroemer 2004, Cook *et al.* 2009). Our observation that embryos with a higher proportion of H2AX139ph-positive cells have a lower cell number suggests that the checkpoint pathways are also induced in porcine embryos to repair/preserve genome integrity. The overall findings of this study were very similar for embryos produced by IVF and SCNT, which further supports an important effect of DSBs during *in vitro* embryo development.

This study provides evidence that the occurrence of DSBs may represent an important component impairing the development and efficiency of embryo production by SCNT. In somatic cells, DNA damage has been shown to reduce efficiency in the production of iPS cells (Marion *et al.* 2009). Moreover, H2AX139ph has been shown to be important for maintaining self-renewal of mouse embryonic and iPS cells (Turinetti *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 activation of oocytes with ION + Sr²⁺ result in superior development of SCNT porcine embryos compared with activation with ION alone (Che *et al.* 2007). One possibility is that Sr²⁺ treatment promotes a higher physiological response in the oocytes and consequently reduces the detrimental effects of cell cycle incompatibilities 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 this study, we investigated whether Sr²⁺ treatment affected the occurrence of DSBs in SCNT embryos. Interestingly, Sr²⁺ reduced the percentage of nuclei with less than ten H2AX139ph foci and the total

number of positive nuclei in the 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 + Sr²⁺. These observations demonstrate that better rates of embryo production from oocytes activated by Sr²⁺ 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, to our knowledge, characterized the presence of H2AX139ph during *in vitro* development of porcine embryos produced by IVF and SCNT. Based on the presence of H2AX139ph foci, we confirmed that DSBs occur during all stages of culture from zygote to the blastocyst stage. We also observed that fast-developing embryos have fewer DSBs than slow-developing embryos, as well as the existence of a negative correlation between the number of cells with DSBs and the total number of cells in the blastocyst-stage embryos. Moreover, we provide evidence that the activation protocol influences the occurrence of DSBs in the cleavage-stage embryos 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.

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.

Funding

R C Bohrer was supported by a scholarship from the Science Without Borders Program of the Brazilian Council of Scientific and Technological Development (CNPq).

Acknowledgements

The authors thank 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.

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Received 21 June 2013

Accepted 11 July 2013