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CDK inhibitor SU9516 induces tetraploid blastocyst formation from parthenogenetically activated porcine embryos

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Abstract
Objective To examine the effect of SU9516, a cyclin-dependent kinase inhibitor, on the induction of tetraploid blastocyst formation in porcine embryos by parthenogenetic activation.
Results Karyotype analysis of blastocysts showed that in the SU9516-treatment group 56% were tetraploid, whereas in the cytochalasin B (CB) group 67% were diploid. The level of maturation-promoting factor (MPF) in stimulated embryos treated with 10 μM SU9516 for 4 h was lower than in embryos treated with CB group (103 vs. 131 pg/ml). The mRNA expression levels of Nanog significantly increased in SU9516-treated embryos than CB group.
Conclusion SU9516 can induce tetraploid blastocyst formation at high efficiency. SU9516 can significantly influence the in vitro developmental competence of porcine parthenogenetically activated embryos by influencing the level of MPF and the gene related apoptosis and pluripotency.

Keywords Embryos · Maturation-promoting factor · Pigs · Polar body · Parthenogenetic activation · SU9516 · Tetraploid embryos

Qing Guo and Long Jin contributed equally to this study.

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Introduction

Tetraploid embryos have been widely used in animal reproduction. Chimeric mouse embryos can be induced by reconstructing diploid and tetraploid blastomeres, diploid cells contributed to the inner cell mass, whereas most tetraploid cells contributed to the trophectoderm (Kang et al. 2009). Tetraploid animals must be generated experimentally to determine the effect of polyploidy on growth and development because spontaneous embryonic tetraploidy is rare (Wen et al. 2014). SU9516, an indolinone derivative, binds to and selectively inhibits the activity of cyclin-dependent kinases (CDKs), including CDK4, and especially CDK1 and CDK2 (Lane et al. 2001). SU9516 might, therefore, enable production of tetraploid embryos by inhibiting CDK2 to influence centrosome duplication. In embryos, G_2-M transition is mainly regulated by the cytoplasmic maturation-promoting factor (MPF), a complex of cytochalasin B (CB) and CDK1 (Liu et al. 2013). During metaphase II (MII) arrest, MPF activity remains high level.

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Oscillation of the intracellular calcium concentration influences the activity of the MPF and the cytostatic factor (Somfai and Hirao 2011). This results in the progress of development of oocytes from the MII stage to the interphase. Therefore,SU9516 might improve the development of pig embryos by inhibiting the activity of CDK1 to decrease the level of MPF.

The anti-neoplastic, CDK-inhibitory agent SU9516 was investigated for its ability to induce blastocyst formation in parthenogenetic activation (PA) porcine embryos. SU9516 decreased the level of MPF and induced formation of a high proportion of tetraploid blastocysts compared with CB. These results represent the first use of anti-neoplastic agents to produce tetraploid embryos that can then be used for blastocyst complementation by stem cells or to produce chimeric embryos in animal reproduction.

Materials and methods

Ethics

This research was carried out in according with the Ethics Committee of Yanbian University.

Chemicals

All chemicals and reagents were purchased from Sigma, unless otherwise noted. SU9516 was purchased from Selleck Chemicals (Houston, TX, USA).

Maturation of oocytes

Oocyte maturation was performed as described by Funahashi et al. (1997). The mature eggs with first polar bodies were collected and used following experiment.

Parthenogenetic activation of oocytes

All the selected matured oocytes with extruded first polar body were subjected to a single direct current pulse of 1.5 kV/cm for 60 μs in 0.28 M mannitol containing 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.1% polyvinyl alcohol (PVA). After the electrical stimulation, oocytes were washed three times with NCSU-37 supplemented with 4 mg bovine serum albumin/ml. Subsequent incubations and treatments varied for different experiments.

Karyotype analysis of activated embryos

After electrical stimulation, embryos were treated with 10 μM SU9516 or 5 μg CB/ml for 4 h and then cultured in in vitro culture (IVC) medium for 7 days to the blastocyst stage. Karyotype analysis was carried out on 150 blastocysts for each treatment. Porcine PA embryos were treated with 0.2 μg demecolcine/ml for 6 h in 5% CO_2 at 38 °C to arrest cell division at metaphase. Blastocysts were then added to a hypotonic solution (0.075 M KCl) for 5 min at 37 °C. Swollen blastocysts were fixed on a clean glass slide immersed in stationary liquid (methanol/acetic acid = 3:1 v/v). Chromosome spreads were kept at 25 °C for a few minutes, and then stained with 10% (v/v) Giemsa for 4–5 min before imaging. Haploid, diploid, tetraploid, and hybrid polyploid karyotypes were recorded.

In vitro MPF activity assay

The effects of SU9516 treatment on the activity of maturation-promoting factor (MPF) were examined. The optimal dose in terms of the effect on MPF activity was 10 μM for 4 h. Control oocytes were cultured IVC medium containing 5 μg CB/ml without SU9516. The activity of MPF was determined in 50 oocytes per treatment group for three times. Oocytes were washed several times in phosphate-buffered saline (PBS) supplemented with 0.1% PVA, and then placed into 100 μl PBS droplets. Oocytes were repeatedly aspirated using a fine needle with a smaller diameter than the oocytes until the cells ruptured. Samples were centrifuged at $\sim 1000 \times g$ for 15 min at 4 °C. Supernatants were collected and stored at 20 °C. The activity of MPF was detected using the porcine MPF ELISA Kit (Kexing, Shanghai, China). 40 μl dilution buffer was added to each well that was precoated with purified anti-porcine MPF antibody and 10 μl oocyte extract was added. Plates were incubated for 30 min at 37 °C in the dark and washed thoroughly with buffer five times. Horseradish peroxidase conjugate reagent (50 μl) was added to each well, except the blank well. Plates were again incubated for 30 min at 37 °C in the dark and washed five times. TMB substrate solution was added to each

well and plates were incubated for 15 min at 37 °C in the dark. Sulfuric acid (50 µl) was added to each well to terminate the reaction. The color change was measured at 450 nm within 15 min of the reaction being terminated. The concentration of MPF in the sample was determined using a standard curve.

Gene expression analysis by quantitative real-time PCR

Total RNA was extracted from groups of 30 porcine PA blastocysts induced by SU9516 and 33 porcine PA blastocysts induced by CB using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. First-strand complementary DNA(cDNA) was synthesized by reverse transcription of mRNA using the oligo(dT) 12-18 primer and SuperScript III reverse transcriptase (Invitrogen). Real-time PCR amplification was conducted with a QuantiTect SYBR Green PCR kit (Finnzymes, Espoo, Finland) on a RotorGene 2000 real-time PCR System (Applied Biosystems, Foster City, CA,USA). Each real-time PCR reaction mixture contained 1 µl cDNA,10 µl SYBR, 0.5 µl Rox, 7.5 µl of nuclease-free water and 0.5 µl of the appropriate forward and reverse primers (Supplementary Table 1) in 20 µl. All tests were conducted in triplicate. Relative gene expression data were analyzed using quantitative real-time PCR and the $2^{-\Delta\Delta C_T}$ method.

Statistical analysis

All data were obtained from more than three replicates. Data expressed as percentages were analyzed using the Chi square test, and nuclei numbers were analyzed by the *t* test (independent samples) using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). P values ≤ 0.05 were considered statistically significant.

Results

Effect of SU9516 on second polar body (PB2) extrusion of porcine PA embryos

Maximum development of porcine PA blastocysts in vitro was with 10 µM SU9516 for 4 h (data not shown). The effect of SU9516 on PB2 extrusion in activated embryos was determined in comparison with CB and with no treatment. In both the treatment group with SU9516 and the control group with 5 µg CB/ml, PA embryo types were mostly 'nuclei with PB1' that did not extrusion PB2, and only a few were 'nuclei with PB1 and PB2' that extruded PB2 (Table 1). By contrast, in the untreated group, embryos were mostly 'nuclei with PB1 and PB2' that extruded PB2 (Table 1).

Effect of SU9516 on the development of porcine PA embryos and blastocyst quality

Porcine PA embryos were treated with 10 µM SU9516 or 5 µg CB/ml as a control. Twelve hours after electrical stimulation 91% (294/322) of SU9516 treated and 91% (298/330) embryos had formed a pronucleus. However, there are two pronuclei in the SU9516 group significantly higher than untreated group (76 vs. 15%, $P < 0.05$; Table 2). Embryos that developed to the blastocyst stage were collected and evaluated after 7 days. The proportion of embryos that developed to the blastocyst stage was significantly higher in the group treated with 10 µM SU9516 than in the control (48 vs. 26%, $P < 0.05$; Table 3; Fig. 1a, d). The number of nuclei in each blastocyst was counted under fluorescence microscopy. Mean cell numbers of blastocysts were significantly lower in the treatment group than in the control group (Table 3; Figs 1b, e, 2).

Karyotype analysis of porcine PA blastocysts induced by SU9516 and CB

Embryos were treated with 10 µM SU9516 or CB for 4 h after electrical stimulation and then cultured IVC medium for blastocyst development for 7 days. Karyotype analysis was carried out on 100 blastocysts in SU9516 group, yielding 43 slides with scattered chromosomes and 56 without, and on 88 blastocysts in the CB group, yielding 32 slides with scattered chromosomes and 56 without. The numbers of haploid, diploid, tetraploid, and hybrid polyploid karyotypes were recorded from the scattered chromosomes. Tetraploidy was the most common karyotype observed in the treatment group (Table 4; Fig. 1c), whereas diploidy was the most common in the CB group (Table 4; Fig. 1f).

Table 1 The appearance of PB2 in different treatment groups embryos after 2 h of elctrical plus treated with SU9516, cytochalasin B (CB) or not

Group	No. of embryos examined (No.exp.)	Nuclei + PB1 (%)*	PB1 + PB2 (%)**	Died (%)
SU9516 (10 μM)	145 (3)	115 (79.3)[a]	21 (14.5)[a]	9 (6.2)[a]
CB (5 μg/ml)	151 (3)	116 (76.8)[a]	22 (14.6)[a]	13 (8.6)[a]
Non-treatment	154 (3)	20 (13)[b]	126 (81.8)[b]	8 (5.2)[a]

The values with different superscripts (a–b) in the same column are significantly ($p < 0.05$) different

No.exp. Number of experiments

* PB1 refers to embryos with nuclei and the first polar body

** PB1 + PB2 refers to embryos with nuclei and first and second polar body

Table 2 Pronucleus (PN) formation in porcine embryos treated with SU9516 or not

SU9516	Embryos cultued, (No.exp.)	Activated embryos, N (%)		Nonactivated embryos, N (%)
		1 PN	2 PN	
(+)	322 (3)	49 (15.2)[a]	245 (76.1)[b]	28 (8.7)[a]
(−)	328 (3)	250 (76.2)[b]	48 (14.6)[a]	30 (9.1)[a]

The values with different superscript (a–b) in the same column are significantly ($p < 0.05$) different

1 PN one pronucleus, *2 PN* two pronucleus, *No.exp* Number of experiments

Table 3 Effect of SU9516 treatment on the in vitro development of PA embryos

Group	No. of embryos cultured	Two-cell and four-cell stage (%)	Blastocyst stage (%)	Mean ± SEM of cells per blastocyst
SU9516	610	550 (90.1)[a]	290 (47.5)[a]	34.4 ± 17.5[b]
Cytochalasin B	630	570 (90.5)[a]	165 (26.2)[b]	45.1 ± 10.6[a]

The values with different superscripts (a–b) in the same column are significantly ($p < 0.05$) different

Effect of SU9516 on the level of MPF in porcine PA embryos

MPF in mature oocytes is important for the development of activated embryos. Oocytes can be released from MII arrest by various physical and chemical stimuli that change the level of MPF. The level of MPF was detected in embryos treated with 10 μM SU9516 or with 5 μg CB/ml. The level of MPF in PA embryos treated with 10 μM SU9516 for 4 h was significantly lower than in the CB-treated embryos (103 vs. 131 pg/ml; Fig. 3).

Effect of SU9516 on the expression of genes involved apoptosis and pluripotency

To investigate gene expression patterns of tetraploid blastocyst induced by SU9516 and diploid blastocyst induced by CB group during porcine PA early embryonic development, the expression levels of three genes related to pluripotency (Oct4, Sox2 and Nanog) and of two genes related to apoptosis (Bcl2, and Bax) were determined on 7-day-old embryos. The mRNA expression levels of Bax and Bcl2 were significantly decreased in SU9516-treated group than CB group ($P < 0.05$; Fig. 4). At the same time, the mRNA expression levels of Oct4, and Sox2 were slightly increased in SU9516-treated group embryos than CB group, however, the mRNA expression levels of Nanog significantly increased in SU9516-treated embryos than control group ($P < 0.05$; Fig. 4).

Discussion

Spontaneous duplication of the mammalian genome occurs in approx. 1% of fertilizations (Eakin and

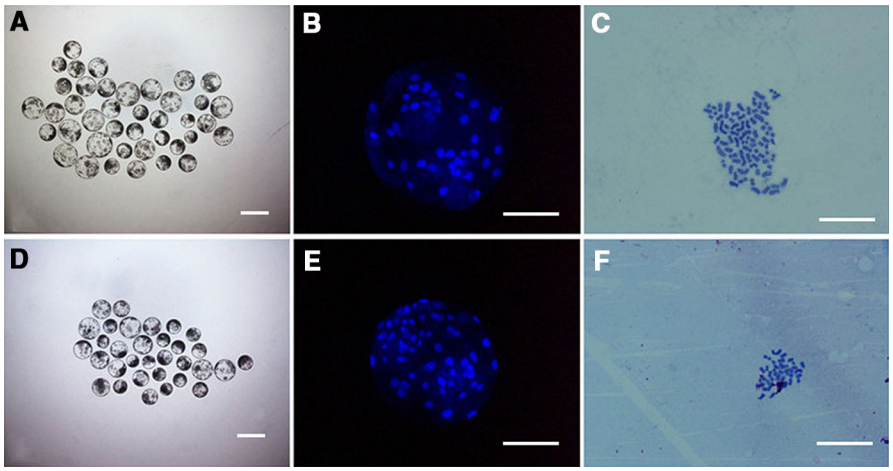

Fig. 1 **a** Porcine blastocysts derived from the SU9516-treated group. *Scale bar* 100 μM. **b** An image of a 7 day SU9516-treated parthenogenetically activated porcine embryo stained with Hoechst 33342. *Scale bar* 100 μM. **c** Tetraploid karyotype from SU9516 treated blastocysts. *Scale bar* 50 μM. **d** Porcine blastocysts derived from the cytochalasin B (CB)-treated group. *Scale bar* 100 μM. **e** An image of a 7 day CB-treated parthenogenetically activated porcine embryo stained with Hoechst 33342. *Scale bar* 100 μM. **f** diploid karyotype from CB treated blastocysts. *Scale bar* 50 μM

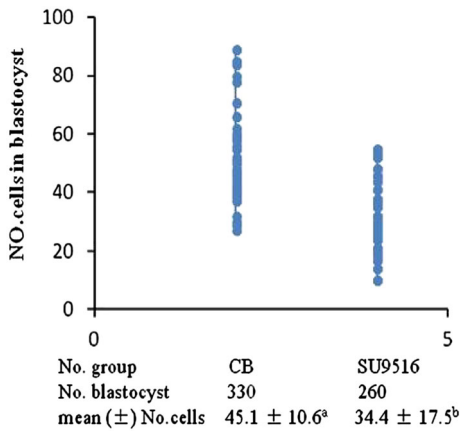

Fig. 2 Total number of cells per blastocyst. Total number of cells per blastocyst derived from porcine PA embryos treated with CB or SU9516 on Day 7. The values with *different superscripts* (*a–b*) in the same column are significantly ($p < 0.05$) different

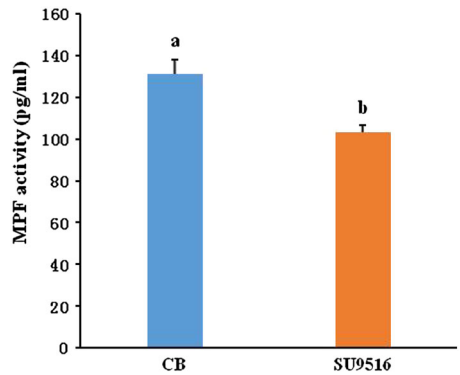

Fig. 3 The level of maturation-promoting factor (MPF) in porcine embryos treated with SU9516 for 4 h.It is show that SU9516 can significantly reduce the activity of MPF in porcine PA embryo compared with control. *Bars* with *different superscripts letters* (*a*, *b*) in each column indicate significantly differences (SU9516 or CB; $p < 0.05$). Error bars indicate standard deviation. The experiment was replicated three times

Table 4 Karyotype analysis of porcine PA blastocysts

Group	Blastocyst number (No.exp.)	Karyotype analysis of blastocyst			
		Haploid (%)	Diploid (%)	Tetraploid (%)	Hybrid polyploid (%)
SU9516	43 (3)	6 (14.0)[a]	8 (18.6)[a]	24 (55.8)[b]	5 (11.6)[a]
Cytochalasin B	32 (3)	3 (9.4)[a]	22 (68.8)[b]	5 (15.6)[a]	2 (6.3)[a]

The values with different superscripts (a–b) in the same column are significantly ($p < 0.05$) different

No.exp. Number of experiments

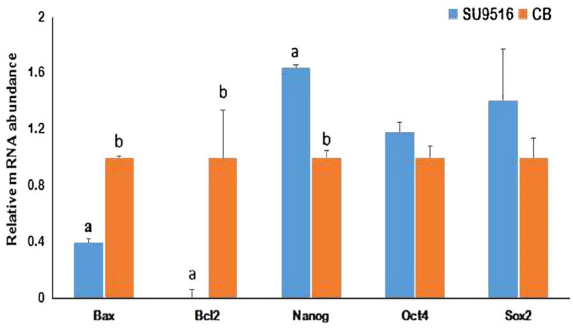

Fig. 4 Relative mRNA expression of genes related to pluripotency and apoptosis in porcine PA blastocyst induced by SU9516 or CB. Relative mRNA expression of genes related to pluripotency and apoptosis in porcine PA blastocyst induced by SU9516 or CB were examined. The mRNA abundance was calculated relative to the reference gene GAPDH. Bars with different superscripts letters (a, b) in each column indicate significantly differences (Oct4, Sox2, and Nanog). Error bars indicate standard deviation. The experiment was replicated 3 times

Behringer 2003). The most direct route to produce tetraploidy is the physical injection of a donor diploid nucleus into a single-celled fertilized oocyte by using micromanipulators (Bromhall 1975). These tetraploid embryos exhibit morphologically normal blastocyst formation but with fewer cells than in corresponding diploid embryos because of a delay in one round of cell division (Pfeiffer et al. 2012). The polyploid embryo has been used to provide insight into the regulation of cell size, cell number, and rates of cell cleavage in early conceptuses. Most notably, tetraploid embryos are commonly used to rescue embryonic lethality as a result of defective extraembryonic phenotypes in laboratory mouse strains, as well as to enable the production of mice directly from ESCs (Gertsenstein 2015). The tendency of ESCs to contribute to the embryonic lineages combined with tetraploid complementation of extraembryonic phenotypes may also be exploited to enable the production of embryos nearly 100% derived from ESCs (Gao et al. 2008).

The centrosome influences cell structure through the nucleation and organization of cytoplasmic microtubules (Webster 2002). The centrosome is duplicated once, and only once, during a normal cell cycle to give rise to two centrosomes that function as the spindle poles of the dividing cell (Kellogg 1989). The key stages of cell-cycle progression are governed by the subcellular location, and periodic activation and subsequent inactivation, of CDKs (Pines 1999). Both centrosome duplication and DNA replication are dependent on CDK2 activation and are blocked by the CDK2 inhibitors butyrolactone I and roscovitine (Keezer and Gilbert 2002).

SU9516, an inhibitor of CDK2, was used to improve the development of blastocysts and to induce formation of tetraploid blastocysts. SU9516 can improve the rate of development from embryo to blastocyst and prevent release of the PB2. Karyotype analysis of parthenogenetically activated blastocysts showed a distribution of 56% tetraploid, 19% diploid, 14% haploid, and 12% hybrid polyploid in the SU9516 treatment group. In the CB-treated control group, diploid blastocysts were most common, rather than tetraploid. The percentage of porcine PA embryos that developed to the blastocyst stage was about twofold higher with SU9516 treatment than with CB treatment. Treatment with 10 μM SU9516 for 4 h improved the development of porcine PA embryos most effectively, by inhibiting the level of MPF in the embryo. Blastocyst quality, judged by the total number of cells per blastocyst, was significantly different between the treatment group and the control group. The mean number of cells per blastocyst was significantly lower following SU9516 treatment than with CB treatment. This finding is in accordance with the results of a previous study showing that diploid blastocysts have significantly more cells than polyploid and haploid blastocysts (Ulloa et al. 2008).

SU9516 can inhibit many protein kinases, including CDK1, CDK4, and protein kinase C, but especially CDK2 (an important regulator of centrosome duplication). Our results showed that SU9516 can be used to increase the number of tetraploid blastocysts that can be generated in vitro and that can potentially be used in tetraploid compensation. SU9516 also inhibits CDK1, a component of MPF, thereby preventing cell division. Temporary inhibition of MPF with 10 μM SU9561 for 4 h increases the proportion of embryos that reach the blastocyst stage, possibly by enabling cytoplasmic maturation.

To investigate the effect of SU9516 on development of early embryoic development,we analyzed gene expression of Bax, Bcl2, and Oct4, Nanog, Sox2. Bax is a proapoptosis gene and Bcl2 gene is an antiapoptosis gene (Lowthert et al. 2012). Bax expression was reduced significantly, but Bcl2 gene expression was significantly lower compared with control. Important

genes, such as those related to pluripotency (Oct4, Nanog, Sox2), which affect the in vitro and in vivo developmental potential of PA embryos, were relatively highly expressed in the SU9516 group compared with the control group. These results showed that SU9516 treatment might not influence of apoptosis in pig embryoic development, but increase development competence by relatively highly expressed pluripotency gene in porcine embryos.

Conclusion

SU9516 can reduce the activity of maturation-promoting factor and increase the proportion of embryos that proceed to formation of porcine PA blastocysts. In addition, most of the blastocysts induced by SU9516 treatment are tetraploid karyotype and have significantly improved expression of pluripotency-related Nanog genes. These results represent the first use of an anti-neoplastic agent, SU9516, that can serve as an effective method for induction of tetraploid blastocyst formation in porcine PA blastocysts, which can be used for blastocyst complementation by stem cells or to produce chimeric embryos in animal reproduction.

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Supporting information Supplementary Table 1—Primer sequences used for gene expression analysis.

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