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Morphokinetics of cloned mouse embryos treated with epigenetic drugs and blastocyst prediction

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Abstract

Time-lapse monitoring of somatic cell nuclear transfer (SCNT) embryos may help to predict developmental success and increase birth and embryonic stem cells (ESC) derivation rates. Here, the development of ICSI fertilized embryos and of SCNT embryos, non-treated or treated with either psammaplin A (PsA) or vitamin C (VitC), was monitored, and the ESC derivation rates from the resulting blastocysts were determined. Blastocyst rates were similar among PsA-treated and VitC-treated SCNT embryos and ICSI embryos, but lower for non-treated SCNT embryos. ESC derivation rates were higher in treated SCNT embryos than in non-treated or ICSI embryos. Time-lapse microscopy analysis showed that non-treated SCNT embryos had a delayed development from the second division until compaction, lower number of blastomeres at compaction and longer compaction and cavitation durations compared with ICSI ones. Treatment of SCNT embryos with PsA further increased this delay whereas treatment with VitC slightly reduced it, suggesting that both treatments act through different mechanisms, not necessarily related to their epigenetic effects. Despite these differences, the time of completion of the third division, alone or combined with the duration of compaction and/or the presence of fragmentation, had a strong predictive value for blastocyst formation in all groups. In contrast, we failed to predict ESC derivation success from embryo morphokinetics. Time-lapse technology allows the selection of SCNT embryos with higher developmental potential and could help to increase cloning outcomes. Nonetheless, further studies are needed to find reliable markers for full-term development and ESC derivation success.

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Introduction

The development of optimized systems for time-lapse monitoring of embryos during *in vitro* culture has allowed embryo morphokinetic analysis to become easier and more available without compromising embryo viability. As both embryo morphology and kinetics of development have been closely correlated with embryo quality and developmental competence (Wong *et al*. 2010, Kirkegaard *et al*. 2012), time-lapse monitoring is a promising tool to increase implantation and full-term development rates. Actually, it has already improved clinical results in human assisted reproduction technologies (Rienzi *et al*. 2011, Aparicio *et al*. 2013, Findikli & Oral 2014).

The precise timing and duration of embryo preimplantational development events have been studied in several non-human mammalian species, mainly bovine, hamster and mice, and candidate markers of viability have been proposed (Kirkegaard *et al*. 2012). The majority of these studies have been performed with fertilized embryos, but data on the morphokinetics of somatic cell nuclear transfer (SCNT) embryos is still scarce. SCNT is a technology with many potential applications, as it allows the generation of both cloned animals (reproductive cloning) and nuclear transfer embryonic stem cell (ntESC) lines (therapeutic cloning) from somatic cell nuclei (Niemann & Lucas-Hahn 2012, Tachibana *et al*. 2013). However, its present use is hindered by its low efficiency. SCNT embryos suffer from low implantation and full-term development rates, especially in the mouse, which are mainly due to a defective reprogramming of the somatic nucleus (Yang *et al*. 2007). In order to improve nuclear reprogramming, the use of epigenetic modifiers has been incorporated into SCNT protocols and this has significantly increased both reproductive and therapeutic cloning rates (Wakayama & Wakayama 2010, Ogura *et al*. 2013). For example, both the histone deacetylase inhibitor (HDACi) psammaplin A (PsA) and vitamin C (VitC), a potent antioxidant and a cofactor of jumonji-domain-containing histone demethylases (JHDMs) and ten–eleven translocation (TET) methylcytosine dioxygenases, have recently proved to improve mouse cloning efficiency (Mallol *et al*. 2014, 2015). In spite of this,

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SCNT efficiency is still low and more improvements are needed to increase the applicability of this cloning technology. In this sense, the development of a non-invasive method, based on morphokinetic parameters, for the selection of the most competent SCNT embryos prior to embryo transfer or embryo seeding could improve the birth rates per transferred embryo and the establishment of ntESC lines per seeded blastocyst respectively.

Owing to the obvious differences between fertilized and SCNT embryos, it is expected that both their kinetics of development and the potential predictive markers of developmental success significantly differ. It is well known that cloned embryos suffer from developmental delay and lower cell counts but, to the best of our knowledge, only two studies have analyzed the morphokinetics of SCNT embryos in detail. Balbach *et al.* (2012) reported that the second (two-cell and three-cell stages) and the third (four-cell stage) cell cycles were extended in SCNT B6C3F1 mouse embryos when compared with ICSI ones, but that only parameters later than the four-cell stage could predict development to the blastocyst stage with a reasonable accuracy (>66.7%). In spite of this, when embryos were classified as fast- or slow-cleaving according to the time spent until they reached the three-cell stage, they observed that fast-dividing embryos were more successful at forming blastocysts but less successful at producing ntESC lines than slow-dividing embryos. Fetal formation, however, was not different between fast- and slow-dividing embryos. Similarly, Mizutani *et al.* (2012) reported that SCNT B6D2F1 mouse embryos were delayed from the two-cell stage, but they observed that the speed of development was not a key factor for their full-term development, except for the very slow-developing ones. Yet, the developmental potential of the cloned embryos could be predicted based on the number of cells at 70 h post-activation. It is important to note that in the two aforementioned studies (Balbach *et al.* 2012, Mizutani *et al.* 2012), the monitored embryos were exposed to both white and fluorescent light and the developmental potential of the SCNT mouse embryos was negatively affected. In addition, none of these studies provided information on the timing of two key events in preimplantation development, such as compaction and cavitation. On the other hand, given the reported differences among mouse strains in cloning efficiencies and morphokinetic parameters (Wakayama & Wakayama 2010, Ogura *et al.* 2013, Wolff *et al.* 2013), investigations should be extended to other mouse strains to detect differences/similarities among them in the kinetics of development of cloned embryos and the determination of markers of developmental potential.

Furthermore, even though the treatment of cloned embryos with epigenetic modifiers is at present a routine addition to SCNT protocols, it is not clear whether these treatments alter embryo kinetics and in which way. Until now, only general observations have been reported, arising from the periodical observation of the cultured embryos at given time points. For example, Li *et al.* (2008) observed a delay in SCNT pig morula compaction when embryos were treated with the HDACi trichostatin A (TSA). In contrast, Dai *et al.* (2010) described that the onset of cavitation occurred earlier in SCNT mouse embryos treated with the HDACi m-carboxycinnamic acid bishydroxamide, and Huang *et al.* (2011*a*) reported that SCNT pig embryos treated with the HDACi valproic acid (VPA) reached the blastocyst stage earlier than non-treated ones.

The main purpose of the present work was to obtain a detailed description of the morphokinetics of ICSI and SCNT B6CBAF1 mouse embryos, from the one-cell to the blastocyst stages, using a commercially available time-lapse monitoring system which has been shown not to compromise the development of fertilized mouse embryos (Pribenszky *et al.* 2010). Furthermore, we aimed to analyze the effect of PsA and VitC treatments on the morphokinetics of SCNT embryos. Finally, we sought to identify morphokinetic parameters of ICSI and non-treated or treated SCNT embryos which allow predicting their successful development to the blastocyst stage and embryonic stem cell (ESC) lines establishment.

Materials and methods

Unless otherwise indicated, all reagents were purchased from Sigma.

Animals

Mouse care and procedures were conducted according to the protocols approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament de Medi Ambient i Habitatge of the Generalitat de Catalunya.

Hybrid B6CBAF1 (C57Bl/6×CBA/J) mice aged 6–12 weeks were used as sperm, oocytes, and cumulus cells donors. All animals were purchased from Charles River (L'Arbresle, France).

Generation of ICSI and SCNT embryos

The generation of ICSI and SCNT embryos, using cumulus cells as nuclear donors, was performed as previously described (Mallol *et al.* 2015). Latrunculin A (5 μM; Santa Cruz Biotechnology) was used as an inhibitor of actin polymerization during micromanipulation and parthenogenetic activation of SCNT oocytes.

Treatment of SCNT embryos

PsA (Santa Cruz Biotechnology) was dissolved in DMSO to prepare a 3 mM stock solution and stored frozen. VitC was dissolved in tri-distilled water to prepare a 5 mg/ml stock solution, filtered, and stored at 4 °C for 2 weeks. The final

concentrations were prepared by dilution of the stock solutions in either the activation or culture media. Embryos were exposed to 10 µM PsA or 100 µM VitC during 6 h of activation and 10 h of the posterior culture (16 h treatment). At the end of the treatment, embryos were extensively washed in drops of KSOM with amino acids (MR-106-D, Millipore) and transferred to a new culture dish.

Embryo culture and time-lapse monitoring

The time-lapse monitoring system used (PrimoVision, Vitrolife, Göteborg, Sweden) is a compact digital inverted microscope placed inside a regular incubator and connected to a controlling unit outside the incubator. It uses low intensity green light (550 nm) and has very short exposure times (<1 s) for image acquisition. The system was set to take a digital picture of the embryos every 5 min.

Embryos were cultured in the microwells of a specially designed well-of-the-well PrimoVision embryo culture dish (Vitrolife). The wells were filled with 30 µl of KSOM with amino acids culture medium and covered with oil. Each well contained a single embryo and each dish contained 16 embryos from the same experimental group. Several replicates of each group were produced on separate weeks. The dishes were placed inside the incubator on the time-lapse monitoring microscope at 37 °C and 5% CO_2, and kept undisturbed until 120 h after fertilization/parthenogenetic activation. The only exception were SCNT embryos treated with PsA or VitC, which were moved out of the incubator for media change at 16 h post-activation and then transferred to the same microwell position of another PrimoVision dish containing fresh KSOM with amino acids culture medium before being returned to the incubator.

Evaluation of morphokinetic parametres

Image sequences acquired for each embryo were analyzed using the PrimoVision Analyzer Software (Vitrolife) to determine the precise timing of several developmental events, specifically of the disappearance of pronuclei (PNd), each cell division until compaction (div1–7), appearance of nuclei at the two-cell stage (Na), compaction (comp), and cavitation (cav) (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). From these data, the duration of many developmental events was calculated, as intervals between the time points analyzed (Fig. 1 and Supplementary Table 2). As ICSI embryos were placed on the PrimoVision right after injection, whereas SCNT embryos were placed after the 6 h activation period, the starting point for time-lapse monitoring was not equivalent between the two groups of embryos. For this reason, the timing of the first event in common between the two groups (PNd) was set as time zero for all groups of embryos. Thus, the time of all events, except for PNd, was expressed as minutes post-PNd.

Aside from time points and durations, the frequencies of several qualitative parameters were also annotated, such as the occurrence of a direct division from one- to three- or four-cell (directdiv) or of an abnormally early compaction at the two-cell stage (comp2C), and the presence of fragmentation (frag) or blastomeres with asymmetric sizes (uneven) at any developmental stage. In addition, the number of cells at the beginning of compaction (cellscomp), excluding comp2C embryos, and the frequency of embryos undergoing compaction between three- and seven-cell stage were also recorded (Fig. 1).

Establishment of ESC lines

ESC lines were derived from monitored embryos that reached the blastocyst stage. After removal of the zona pellucida with acid Tyrode solution, blastocysts were seeded onto a feeder cell monolayer of mouse embryonic fibroblasts (ECACC, Porton, Salisbury, UK) inactivated with 10 µg/ml mitomycin C (Invitrogen) during 3 h. The derivation medium used consisted of DMEM (Invitrogen) supplemented with 100× non-essential amino acids (Invitrogen), 100 µM 2β-mercaptoethanol (Invitrogen), 20% (v/v) knockout serum replacement (Invitrogen), 10 µl/ml penicillin (Gibco), 10 µl/ml streptomycin (Gibco), and 5×10^3 units/ml leukemia inhibitory factor (Millipore). The culture medium was changed every 48 h. Blastocysts were kept in culture (37 °C, 5% CO_2) until outgrowths were observed ~7 days after seeding. Outgrowths of embryonic cells were then mechanically disaggregated in the presence of a 1:10 dilution of Trypsin (Gibco) in Hanks Balanced Salt Solution (Invitrogen),

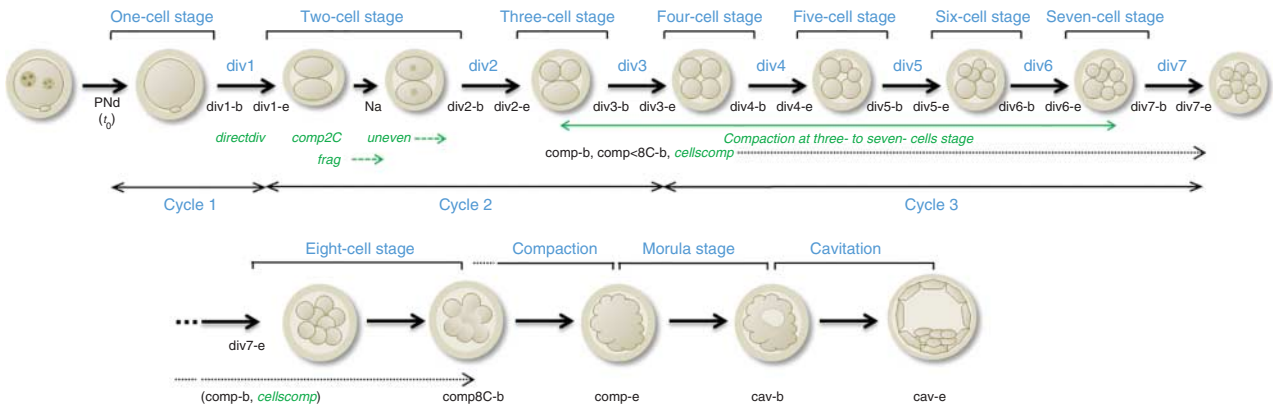

Figure 1 Schematic view of the *in vitro* development parameters analyzed, from pronuclei disappearance (PNd) to blastocyst formation. Time-points appear in black, durations in blue, and qualitative parameters in green. For a detailed description of each parameter see Supplementary Tables 1 and 2.

using a mouth-controlled Pasteur pipette to move the aggregates in and out of the tip of the pipette until reducing them to smaller cell clumps of a few single cells. Passaging was performed approximately six times until ESC lines were considered to be established.

Characterization of ESC lines

ESC lines were first characterized by a morphological criterion. In this sense, lines with colonies containing round cells with a small amount of cytoplasm and without the presence of differentiated cells were classified as ESC-like lines. Then, pluripotency of the selected ESC-like lines was assessed by immunofluorescence analysis of two specific markers of undifferentiated cells, OCT4 and SOX2. Briefly, ESC-like colonies were fixed in 4% paraformaldehyde during 15 min, washed three times in a PBS solution and permeabilized in a PBS solution containing 0.5% (v/v) Triton X-100, 3% (v/v) goat serum, and 0.2% (v/v) sodium azide during 30 min. Then, colonies were incubated overnight at 4 °C with primary antibodies, washed three times in PBS solution and incubated with the corresponding secondary antibody during 2 h at room temperature. Primary antibodies used were rabbit polyclonal anti-Sox2 (1:200, AB5603; Millipore) and rabbit monoclonal anti-Oct4 (1:50, A13998; Life Technologies). Secondary antibodies were Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 chicken anti-mouse IgG (A-11037 and A-21200; Life Technologies), both used at 6 μg/ml. Finally, the samples were stained with Hoechst 33258 (Life Technologies) at 10 μg/ml as a nuclear counterstain, mounted, and examined with an epifluorescence microscope (Olympus BX60). Digital images were acquired on Isis Software version 5.3.3.9 (MetaSystems, Boston, MA, USA).

In vitro *differentiation of ESC lines*

To confirm their pluripotency, ESC lines were subjected to differentiating culture conditions to obtain cells of the three germ layers: ectoderm, endoderm, and mesoderm. ESC lines were subcultured onto gelatin pre-coated dishes in the absence of feeder cells and LIF, and knockout serum replacement was substituted with FCS (Fisher Scientific). Culture was done at 37 °C in a 5% CO_2 atmosphere and the medium was changed every 48 h. After 7 days, the cells were fixed for immunofluorescence analysis of specific differentiation markers: the neuroepithelial stem cell protein (nestin), characteristic of the ectodermal layer; alphafetoprotein (AFP), characteristic of endoderm; and alpha-smooth muscle actin (α-SMA), characteristic of mesoderm. Fixation and immunofluorescence procedures were the same used for the characterization of the undifferentiated ESC lines. Primary antibodies used were rabbit polyclonal anti-nestin (1:250, ab5968; Abcam), rabbit polyclonal anti-AFP (1:400, A000829-2, Dako, Barcelona, Spain), and mouse monoclonal anti-α-SMA (1:400, A5228; Sigma). Secondary antibodies, used at 6 μg/ml, were Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 chicken anti-mouse IgG (Life Technologies). Finally, the cells were stained with Hoechst 33258 at 10 μg/ml as a nuclear counterstain. Mounted samples were examined with an epifluorescence microscope (Olympus BX60) and digital images were acquired on Isis Software version 5.3.3.9 (MetaSystems).

Statistical analysis

Each experiment was repeated at least five times and the results obtained were pooled and analyzed with SAS v9.3 Software (SAS Institute, Inc., Cary, NC, USA).

First, a bivariate analysis was performed using the group variable as the independent one. For qualitative variables χ^2 or Fisher exact tests were used, and for quantitative variables the non-parametric Kruskal–Wallis test was used. Pairwise comparisons with Bonferroni's correction were performed using a Mann–Whitney *U* test. In all cases, $P<0.05$ was considered statistically significant.

Secondly, for the prediction of blastocyst formation and of ESC derivation, bivariate logistic regression models were obtained for each variable, and those with $P<0.05$ were included in a multivariate logistic regression model. The final models were obtained using the backward method for variable selection, taking into account possible multicollinearity effects among variables. Receiver operating characteristic (ROC) curves were used to test the predictive value of each model and their area under the curve (AUC) was calculated. Values of the variables providing the maximum sensitivity and specificity were also determined. Finally, *F*-score was calculated for determining accuracy of each predictor model:

$$\left(F = 2 \times \frac{\text{precision} \times \text{sensitivity}}{\text{precision} + \text{sensitivity}}\right).$$

Results

Blastocyst formation and ESC derivation rates

Non-treated SCNT embryos showed significantly lower rates of blastocyst formation than control ICSI embryos (Table 1). Treatment of SCNT embryos with either PsA or VitC improved developmental potential, resulting in blastocyst rates equivalent to those of ICSI embryos.

ESC lines were derived from all four groups of embryos (Table 2). Both PsA and VitC treatments increased the rates of blastocyst attachment to the feeder monolayer, outgrowth formation at day 7, and ESC lines establishment compared with non-treated SCNT embryos, despite the differences were only significant for the VitC group. All the ESC lines obtained were positive for the pluripotency markers analyzed and, after differentiation, were positive for the differentiation markers of the three germ layers (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Morphokinetic differences between ICSI and non-treated SCNT embryos

A graphic representation of the time points and durations of *in vitro* development events, from PNd to cavitation, of

Table 1 *In vitro* development of ICSI embryos and SCNT embryos non-treated (NT) or treated with psammaplin A (PsA) or vitamin C (VitC).

Group	*n*	No. of embryos developed to (%)			
		Two-cell	Four-cell	Morula	Blastocyst
ICSI	68	68 (100)*	67 (98.5)*	64 (94.1)*	48 (70.6)*
SCNT–NT	83	78 (94.0)*	71 (85.5)†,‡	72 (86.7)*	38 (45.8)†
SCNT–PsA	75	72 (96.0)*	57 (76.0)†	55 (76.4)†	42 (58.3)*,†
SCNT–VitC	93	92 (99.0)*	85 (91.4)‡	78 (84.8)*,†	56 (60.9)*,†

Values with different symbols (*, †, and ‡) differ significantly within the same column ($P<0.05$).

all embryos analyzed in the ICSI and SCNT groups is shown in Fig. 2, and the values and statistical comparisons are shown in Supplementary Table 3, see section on supplementary data given at the end of this article.

In non-treated SCNT embryos the beginning of the second division was significantly delayed and the duration of this division was twice as much as in the ICSI group. As a result, the durations of the two-cell and three-cell stages and of the cleavage cycle 2 were significantly longer in SCNT embryos than in ICSI ones. The beginning of divisions four to seven was also delayed in SCNT embryos when compared with ICSI ones and the duration of divisions five and six was longer, resulting in a significantly longer cleavage cycle 3. Therefore, SCNT embryos showed a developmental delay in comparison with ICSI embryos, starting from the second division.

Abnormally early comp2C was observed in both groups of embryos at equivalent levels (Supplementary Table 3), and embryos showing comp2C either arrested or decompacted and continued to develop. In contrast, embryos starting compaction after the two-cell stage never decompacted afterwards. Thus, the beginning of compaction (comp-b) was annotated only for embryos compacting from the three-cell stage onwards, and we distinguished compaction initiation before the eight-cell stage (comp<8C-b) from compaction initiation at the eight-cell stage (comp8C-b). Indeed, most non-treated SCNT embryos underwent compaction at three- to seven-cell stages, in contrast to ICSI embryos, which mostly compacted at eight-cell. Therefore, the median number of blastomeres at the beginning of compaction was significantly lower in SCNT embryos compared with ICSI ones (Fig. 3 and Supplementary Table 4, see section on supplementary data given at the end of this article). In addition, most SCNT embryos compacting before the eight-cell stage did so at four cells, whereas in the ICSI group it occurred mostly at six to seven cells. In agreement with this, comp<8C-b occurred chronologically earlier in non-treated SCNT embryos than in ICSI ones (Fig. 3A and Supplementary Table 3). However, in embryos compacting at the eight-cell stage the timing of compaction beginning (comp8C-b) was delayed in non-treated SCNT embryos when compared with ICSI embryos, according to their delayed development. Notably, in ICSI embryos comp<8C-b occurred chronologically later than comp8C-b, whereas the opposite situation was found in SCNT embryos. The durations of both compaction and cavitation were longer in SCNT embryos than in ICSI ones (Fig. 2A, Supplementary Table 3, and Supplementary Video).

Fragmentation was commonly observed in both ICSI and non-treated SCNT embryos, and not significantly different (Supplementary Table 3).

Effect of PsA and VitC treatments on SCNT embryo morphokinetics

The PsA treatment resulted in a significant delay of PNd in comparison with the other two groups of SCNT embryos and in significantly longer durations of the one-cell stage and the first cycle when compared with ICSI embryos, due to a delay in the beginning and ending of the first division (Fig. 2 and Supplementary Table 3). Similar to non-treated SCNT embryos, PsA- and VitC-treated ones were also significantly delayed in comparison with ICSI embryos from the beginning of the second division until the beginning of compaction, but significant differences were observed among groups depending on the treatment. In general, PsA-treated embryos were slower than non-treated ones at initiating and completing all cell divisions, whereas VitC-treated embryos showed an accelerated development in comparison with non-treated ones and more similar to that of ICSI embryos. Indeed, differences between the two groups of treated SCNT embryos were detected at all timings of cell divisions (div2-b–div7-e) and in the durations of the two-cell stage and cycle 2, which were longer in PsA-treated than in VitC-treated embryos.

Compaction at three- to seven-cell stages was also detected in treated SCNT embryos at a higher frequency than in ICSI ones, but its occurrence was significantly reduced in VitC-treated embryos when compared with PsA-treated and non-treated ones. In spite of this, the median number of blastomeres at the beginning of compaction was similar among all groups of SCNT embryos (four cells), and lower than in ICSI ones (eight cells) (Fig. 3 and Supplementary Table 4).

As for the timing of compaction initiation, both comp<8C-b and comp8C-b occurred earlier in VitC-treated embryos than in PsA-treated ones, according to their faster pace of development, but no differences were

Table 2 ESC derivation from ICSI blastocysts and SCNT blastocysts non-treated (NT) or treated with psammaplin A (PsA) or vitamin C (VitC).

Group	*n*	Attached 24–48 h (%)	Outgrowths day 7 (%)	ESC lines (%)
ICSI	28	16 (57.1)*	7 (25.0)*	5 (17.9)*,†
SCNT–NT	33	16 (48.5)*	6 (18.2)*	5 (15.2)*
SCNT–PsA	42	27 (64.3)*	19 (45.2)*,†	13 (31.0)*,†
SCNT–VitC	40	38 (95.0)†	24 (60.0)†	16 (40.0)†

Values with different symbols (* and †) differ significantly within the same column ($P<0.05$).

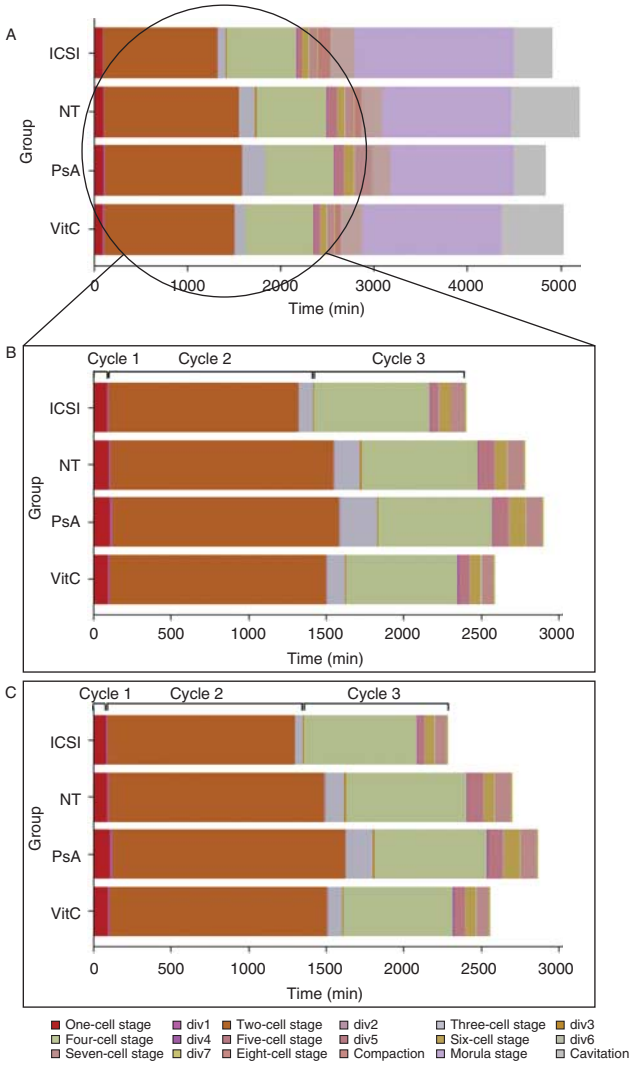

Figure 2 Graphical representation of the median duration of *in vitro* development events in ICSI and SCNT embryos non-treated (NT) or treated with psammaplin A (PsA) and vitamin C (VitC). (A) Data of all embryos analyzed, from pronuclei disappearance (PNd) to cavitation. For compaction, only embryos compacting at the eight-cell stage were considered. (B) Enlarged image of early events, from PNd to division 7 (div7), for all embryos analyzed. (C) Enlarged image of early events, from PNd to div7, only for embryos that reached the blastocyst stage.

found between treated and non-treated SCNT embryos (Fig. 3A and Supplementary Table 3). Comp < 8C-b also occurred earlier in VitC-treated embryos when compared with ICSI ones. Furthermore, in both groups of treated SCNT embryos the timing of comp < 8C-b was chronologically earlier than comp8C-b, similar to non-treated SCNT embryos but contrary to ICSI embryos.

With regards to the length of the morphogenetic events, a reduction in the duration of compaction and of cavitation by the VitC and the PsA treatments respectively, was observed, making these values more similar to those of ICSI embryos (Fig. 2A and Supplementary Table 3).

VitC-treated SCNT embryos showed more fragmentation than ICSI embryos and PsA-treated SCNT embryos (Supplementary Table 3).

Morphokinetic parameters of ICSI and SCNT embryos that reached the blastocyst stage

When only the embryos that developed to the blastocyst stage were considered, significant differences among groups in morphokinetic parameters were globally

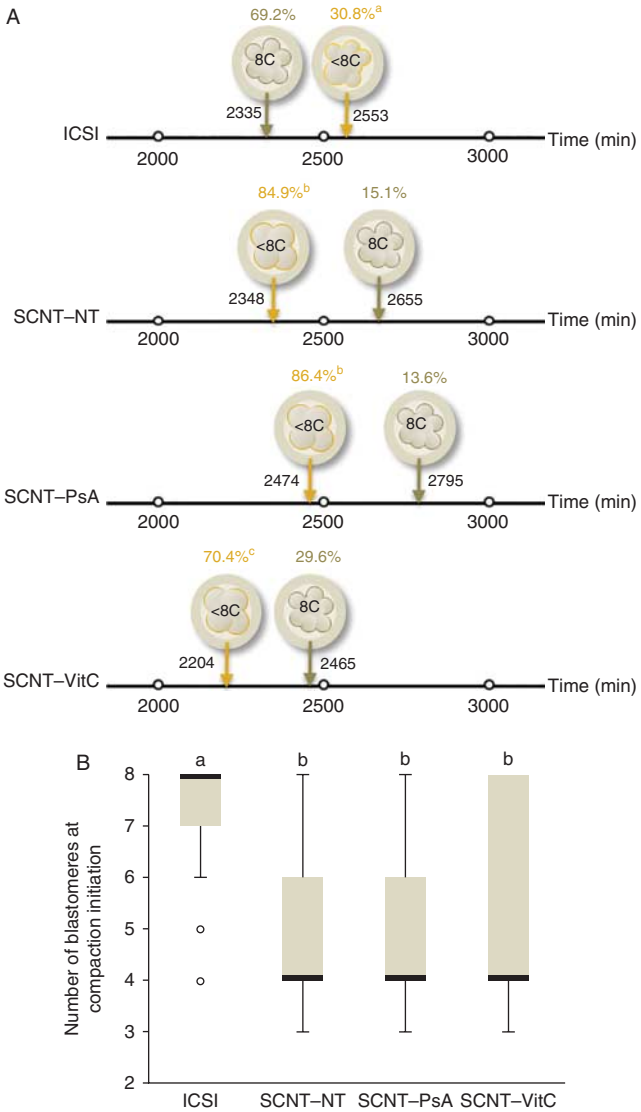

Figure 3 Compaction initiation in ICSI embryos and SCNT embryos non-treated (NT) or treated with psammaplin A (PsA) and vitamin C (VitC). (A) Percentage of embryos in each group initiating compaction before (<8C) and at the eight-cell stage (8C), and timing of compaction initiation (min). Different superscripts (a, b and c) denote significant differences ($P<0.05$) among groups in the percentage of embryos initiating compaction before the eight-cell stage. (B) Box-and-whisker plots depicting the median number of blastomeres at the time of compaction. Different letters (a and b) on top of each box denote significant differences ($P<0.05$) among groups.

maintained (Fig. 2C and Supplementary Table 5, see section on supplementary data given at the end of this article). Compared with the previous analyses including all the embryos, significant differences among groups were lost for some of the parameters analyzed while others emerged (compare Fig. 2B and C, and Supplementary Tables 3 and 5). Notably, the delayed development of PsA-treated SCNT embryos in comparison with non-treated ones was evidenced earlier, at div2-b instead of div4-b, and lasted until div6-e, instead of div5-e. As a result, the two-cell stage and cycle 2 were significantly longer in PsA-treated embryos that developed to blastocysts than in non-treated ones. In contrast, differences in the timing of division beginning and ending (from div4-b to div7-e) between non-treated and VitC-treated SCNT embryos disappeared when only embryos developing to the blastocyst stage were considered. The frequency of embryos undergoing compaction between the three- and seven-cell stages was still much higher in all groups of SCNT embryos (63.6–81.0%) than in ICSI embryos (18.8%) (Supplementary Table 4).

When intra-group differences were analyzed, significant differences in many morphokinetic parameters were detected between embryos that developed to the blastocyst stage and those that did not, especially in the ICSI group (Fig. 2 and Supplementary Table 6, see section on supplementary data given at the end of this article). In general, divisions began and ended earlier and cell stages and cycles were shorter in embryos that gave rise to blastocysts than in those that did not (Fig. 2B and C), though the differences were not significant in all cases. Indeed, only three parameters showed consistent significant differences in all groups between embryos that developed to the blastocyst stage and those that did not: cav-b, fragmentation, and cellscomp. In particular, embryos that reached the blastocyst stage had an earlier start of cavitation than those that did not, and despite fragmentation was not incompatible with blastocyst formation, the percentage of fragmented embryos was higher in embryos that did not reach this stage (Supplementary Table 6). Moreover, although undergoing compaction between the three- and seven-cell stages did not prevent blastocyst formation, the occurrence of this phenomenon was significantly higher and the median number of blastomeres at the time of compaction was significantly lower in embryos that arrested at previous stages of development (Supplementary Table 4). Although less consistent, parameters related to the third division, such as div3-b, div3-e, duration of div3, and duration of the three-cell stage, also seem important for blastocyst development because all groups of embryos showed significant differences in one or more of these parameters between embryos that formed blastocysts and embryos that did not.

Prediction of developmental success from morphokinetic parameters

In search of early parameters that could predict *in vitro* developmental success in SCNT embryos, irrespective of whether they were treated or not, we found that the time point div3-e was a good predictor of blastocyst development. To increase the sensitivity and specificity, several prediction models were formulated, in which the timing of div3-e was combined with other good predictor variables, specifically the duration of compaction and/or the presence of fragmentation (Table 3 and Fig. 4). Interestingly, these models could also predict development to the blastocyst stage in ICSI embryos with high sensitivity and specificity. All selected models showed similar AUC values for each of the SCNT

Table 3 Prediction models of *in vitro* developmental success based on the timing of the end of the third division (div3-e) in ICSI embryos and SCNT embryos non-treated (NT) or treated with psammaplin A (PsA) and vitamin C (VitC).

				Simplified prediction model (with threshold)				
Group	**Prediction model**	***n***	**AUC**	div3-e threshold (min)	AUC	Se	Sp	*F*-score
ICSI	div3-e	64	0.71*	1400	0.64	70.8	56.2	0.76
	div3-e + compaction	64	0.82*,†	1500	0.82	79.2	87.5	0.86
	div3-e + frag	64	0.92†,‡	1600	0.92	83.3	100.0	0.91
	div3-e + compaction + frag	64	0.95‡	1600	0.91	93.8	87.5	0.95
SCNT–NT	div3-e	70	0.76	1675	0.69	68.4	68.8	0.70
	div3-e + compaction	70	0.77	1530	0.73	68.4	78.1	0.73
	div3-e + frag	70	0.84	1530	0.81	73.7	90.6	0.81
	div3-e + compaction + frag	70	0.84	1530	0.80	81.6	78.1	0.82
SCNT–PsA	div3-e	52	0.72	2070	0.81	92.9	70.0	0.93
	div3-e + compaction	52	0.75	2000	0.82	83.3	80.0	0.89
	div3-e + frag	52	0.81	2100	0.81	73.8	90.0	0.84
	div3-e + compaction + frag	52	0.81	2100	0.81	73.8	90.0	0.84
SCNT–VitC	div3-e	76	0.59	1640	0.60	65.4	54.2	0.70
	div3-e + compaction	76	0.61	1640	0.63	67.3	58.3	0.72
	div3-e + frag	76	0.68	1500	0.61	63.5	58.3	0.69
	div3-e + compaction + frag	76	0.70	1500	0.65	63.5	66.7	0.71

Frag, fragmentation; AUC, area under the curve; Se, sensitivity, Sp, specificity.
Values with different symbols (*, †, and ‡) differ significantly within the group ($P<0.05$).

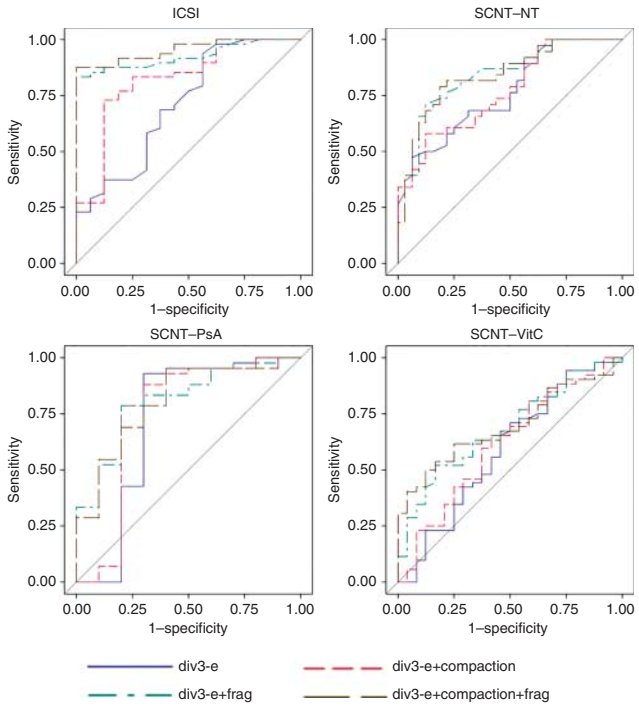

Figure 4 ROC curves for the prediction models based on the timing of the end of the third division (div3-e) alone, or in combination with the presence of fragmentation (frag) and/or the duration of compaction. In these curves, the true positive rate (sensitivity) is plotted against the false positive rate (1 − specificity) at various threshold settings. The closer the curve is to the upper left corner (sensitivity = 1 and specificity = 1), the higher the accuracy of the model (larger area under the curve). NT, non-treated; PsA, psammaplin A; VitC, vitamin C.

groups, but in the ICSI group those including fragmentation showed the highest AUC values (Table 3 and Fig. 4).

In order to being able to classify embryos according to their developmental potential, we selected the threshold value that maximized the sensitivity and specificity of each model and developed a hierarchical classification for each group (Table 3). The threshold values varied among groups, in accordance with existing differences in cleavage timing, as previously described. Thus, PsA-treated SCNT embryos were the most delayed (Fig. 2) and their threshold values were accordingly the highest (Table 3). As an example, in order to validate the prediction model including div3-e and the presence of fragmentation in PsA-treated SCNT embryos, the hierarchical classification represented in Fig. 5 would be used.

Including the thresholds in the prediction models, an AUC or an *F*-score higher than 0.80 could be obtained in all groups, except for SCNT–VitC, with at least one of the proposed models. Blastocyst development of VitC-treated embryos was indeed the most difficult to predict, as the maximum AUC value achieved was 0.65 (Table 3). However, in this group, an alternative prediction model taking into account div3 duration, comp-e and the presence of fragmentation could be more accurate ($n = 77$, AUC = 0.84). Using this alternative model, 76.6% of embryos would be correctly classified (sensitivity = 75.5 and specificity = 79.2).

Morphokinetic parameters of ICSI and SCNT embryos that gave rise to ESC lines and prediction of ESC derivation success

Contrary to the numerous differences in morphokinetic parameters observed among ICSI and SCNT embryos that were capable of developing to the blastocyst stage, only one parameter was found to be different among the four groups when only the embryos that gave rise to ESC lines were considered: the timing of div2-e (Supplementary Table 7, see section on supplementary data given at the end of this article). In particular, the timing of div2-e was significantly delayed in SCNT embryos with regards to the ICSI group, although the difference was only significant for VitC-treated embryos. However, it must be pointed out that sample sizes for these analyses were small and this would have precluded achieving significance for many other parameters in which the timings, durations, and frequencies clearly differed among groups. A similar situation occurred when intra-group differences were analyzed (Supplementary Table 8).

Unfortunately, none of the variables studied resulted in a significant *P* value for ESC establishment prediction. However, in the PsA- and VitC-treated groups, where the sample sizes were higher, we observed that embryos with longer five-cell stage and div7 durations respectively, gave rise to ntESC lines, in contrast to the fastest ones (Supplementary Table 8).

Discussion

In the present study, we have performed a comprehensive time-lapse monitoring analysis of the *in vitro* development of SCNT B6CBAF1 mouse embryos, both untreated and treated with epigenetic modifiers, up to the blastocyst stage. Blastocyst rates of monitored

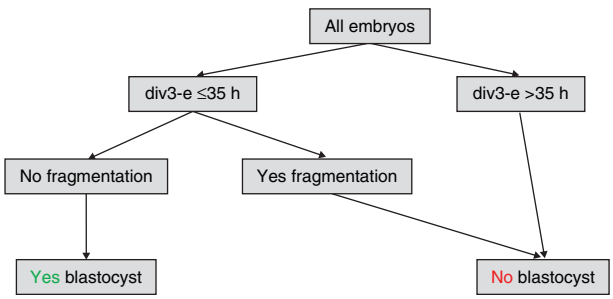

Figure 5 Hierarchical classification of SCNT embryos treated with psammaplin A according to their *in vitro* developmental success, based on the time point of the end of the third division (div3-e) and the presence of fragmentation (AUC 0.81).

embryos were similar, or even higher, than those previously obtained in our laboratory for non-imaged ICSI and SCNT embryos cultured in the same incubator (Mallol *et al*. 2015), confirming that embryo development was not compromised by the use of the PrimoVision imaging system, as already reported by Pribenszky *et al*. (2010) for IVF mouse embryos, which are not as sensitive as SCNT embryos to culture conditions (Gao *et al*. 2003).

In comparison with ICSI embryos, non-treated SCNT embryos were delayed in their division timing from the second division, and this delay was maintained until compaction. In spite of this, only the duration of the two- and the three-cell stages, but not of the other cleavage stages, was significantly longer in SCNT embryos. Studies in other mouse strains have also reported a slower development of SCNT embryos, with extended durations of the two-, three-, and four-cell stages in comparison with ICSI embryos (Balbach *et al*. 2012, Mizutani *et al*. 2012). As somatic differentiated cells show longer cell cycles than early embryos (Hörmanseder *et al*. 2013), the reason for this delay in the early stages of development could be attributed to a somatic memory, due to the defective reprogramming of the transferred somatic nucleus. In addition, as the delay appears at the two-cell stage, when embryonic genome activation (EGA) takes place in the mouse (Zuccotti *et al*. 2000), it is possible that the incomplete EGA caused by defective reprogramming in SCNT embryos (Inoue *et al*. 2006, Vassena *et al*. 2007) could affect the expression of key cell cycle genes, resulting in reduced levels of the corresponding proteins (Sebastiano *et al*. 2005), and thus limiting and slowing the progression of cell cycle (Dadi *et al*. 2006).

Another key event in the development of SCNT embryos detected in our study was compaction. Compaction before the eight-cell stage was detected in the majority of SCNT embryos, but it was much less frequent in ICSI embryos. These results are in agreement with those of Li *et al*. (2011), which reported that 87% of SCNT B6D2F1 mouse embryos compacted between three- and seven-cell stages. Remarkably, in our study compaction before the eight-cell stage in ICSI embryos occurred chronologically later than compaction at the eight-cell stage, but the reverse situation was observed in all groups of SCNT embryos. In addition, most ICSI embryos compacting before the eight-cell stage did so at six to seven cells, whereas in the SCNT groups it occurred mostly at four-cell stage. Therefore, the reasons behind this abnormal compaction might be different between the two populations of embryos. It has been proposed that a clock mechanism controls the timing of preimplantation developmental events, and that it could be coupled to the rounds of DNA replication or to chromatin and DNA epigenetic modifications (Kojima *et al*. 2014). In this context, a deregulation of this clock caused by defective reprogramming of the somatic nucleus could explain the observed alterations in the timing of compaction in SCNT embryos. If this was the case, then cloned embryos compacting at the eight-cell stage would have undergone better reprogramming than cloned embryos compacting before the eight-cell stage, and this in fact would agree with their higher potential to form blastocysts.

We also analyzed the effect of PsA and VitC treatments on the morphokinetics of SCNT mouse embryos. While both treatments increased blastocyst rates, ntESC lines derivation (the present study), and development to term (Mallol *et al*. 2014, 2015), they showed opposite effects on embryo morphokinetics. On the one hand, PsA treatment caused a more pronounced delay in the development of SCNT embryos, which was more evident when comparing embryos that developed to the blastocyst stage. Our results are in agreement with previous general observations with other HDACis, such as VPA (VerMilyea *et al*. 2009) and TSA (Li *et al*. 2008), suggesting that the slowing effect observed here could be common for all HDACis. Interestingly, other factors affecting nuclear reprogramming, such as caffeine or vanadate, also have an effect on cloned embryo developmental kinetics (Kwon *et al*. 2008). As most HDACi treatments, including PsA, improve nuclear reprogramming in SCNT embryos (Mallol *et al*. 2014, 2015), HDACi-induced retardation of the speed of development could be related to the time needed for this more extensive nuclear reprogramming to occur. In fact, differences in the speed of development between non-treated and PsA-treated SCNT embryos were mainly detected in the timing of the first divisions, when the bulk of nuclear reprogramming is supposed to take place (Simonsson & Gurdon 2004, Vassena *et al*. 2007).

On the other hand, VitC treatment slightly accelerated the kinetics of embryo development. Besides improving the developmental potential of SCNT embryos (Jeong *et al*. 2006, Huang *et al*. 2011*b*, Chawalit *et al*. 2012, Kere *et al*. 2013, Mallol *et al*. 2015), VitC also enhances the generation of induced pluripotent stem cells (iPSCs) from both mouse and human somatic cells, and this enhancement is also accompanied by an accelerated timing of iPSC generation (Esteban *et al*. 2010). The effect of VitC on iPSC generation seems to be independent of its antioxidant activity and has been attributed to epigenetic changes induced by the increase of JHDMs and TET activities (Wang *et al*. 2011, Blaschke *et al*. 2013, Chen *et al*. 2013*a*, Yin *et al*. 2013). In this context, it is possible that VitC promotes some epigenetic changes in SCNT embryos that may ease EGA and the expression of cell cycle genes, accelerating cell division. However, the epigenetic effect of VitC on cloned embryos remains elusive (Chawalit *et al*. 2012, Mallol *et al*. 2015) and, if it occurs, it may not be as extensive as that of PsA (Mallol *et al*. 2015). This could explain that the kinetics of PsA-treated SCNT embryos was much slower than that of the VitC-treated ones.

Alternatively, because both PsA and VitC have pleiotropic effects, their influence on the speed of development of SCNT treated embryos could be attributed to other functions, not related with their epigenetic effects. For instance, VitC is a well-known antioxidant and it has been reported that embryos exposed to VitC show reduced levels of reactive oxygen species (Wang *et al*. 2002, Hu *et al*. 2012, Li *et al*. 2014) and increased reduced glutathione content (Wongsrikeao *et al*. 2007, Zhang *et al*. 2010, Mallol *et al*. 2015), which lead to lower levels of apoptosis (Jeong *et al*. 2006, Hu *et al*. 2012, Li *et al*. 2014), parameters which have been related to reduced speed of embryo development (Favetta *et al*. 2004, 2007, Wale & Gardner 2010). Thus, the antioxidant effect of VitC could reduce DNA damage and checkpoint arrest, accelerating cell cycle progression in SCNT embryos.

When only those embryos that reached the blastocyst stage were considered, differences between all groups of SCNT and ICSI embryos were still evident, reinforcing the idea that the precise kinetic parameters which allow blastocyst formation differ among groups, according to their developmental pace. However, in general, embryos developing to the blastocyst stage showed faster kinetics of development, in agreement with other published results in both SCNT (Balbach *et al*. 2010, 2012, Mizutani *et al*. 2012) and fertilized mouse embryos (Arav *et al*. 2008, Pribenszky *et al*. 2010) and in fertilized bovine and human embryos (Lechniak *et al*. 2008, Ivec *et al*. 2011). In our study, the timing of div3-e (i.e. formation of the four-cell embryo) was identified as an early good predictor parameter for blastocyst development for all groups of embryos and, alone or combined with the duration of compaction and/or the presence of fragmentation, allowed the prediction of blastocyst development with high accuracy. Although a good prediction was possible using only embryo kinetics information, the addition of morphological data, such as the presence of fragmentation, allowed us to increase the accuracy of the prediction models in all groups of embryos, achieving *F*-score values higher than 0.70. In a time-lapse study of fertilized mouse embryos, Pribenszky *et al*. (2010) also found that the probability of reaching the blastocyst stage was significantly reduced if fragmentation occurred during development. In contrast to our results, Balbach *et al*. (2012) could not identify predictor parameters for SCNT embryos before the four-cell stage, and even when combining two or more earlier parameters the accuracy values (*F*-scores) for the prediction models were lower than 0.50. The different mouse strain, embryo culture conditions or imaging conditions could be the cause of the differing results when compared with our study. Nevertheless, when they used the timing to divide to three-cell stage to discriminate between slow- and fast-developing embryos, they found higher blastocyst rates in the faster ones.

Embryos that reached the blastocyst stage after time-lapse monitoring were used for ESC derivation. We were able to successfully derive ntESCs from PsA- and VitC-treated SCNT embryos, demonstrating that these treatments improve ntESC derivation, two- and 2.6-fold respectively, in addition to the previously reported improvement in full term development (Mallol *et al*. 2015). Other studies have already shown the positive effect of epigenetic modifiers on mouse PSCs generation (Dai *et al*. 2010, Esteban *et al*. 2010, Chen *et al*. 2013*b*). However, to the best of our knowledge, this is the first time that the effect of PsA and VitC treatments on ntESC derivation is analyzed, and the derivation rates achieved (31 and 40% respectively) are much higher than those previously reported with the HDACi VPA in the same mouse strain (11.6%) (Costa-Borges *et al*. 2011). Contrary to our success with prediction of blastocyst development, we failed to predict ESC derivation from embryo morphokinetics results, probably because of the more reduced sample size. In spite of this, we observed that SCNT embryos treated with PsA or VitC which gave rise to ntESC lines showed longer five-cell stage or div7 durations respectively, than those that did not. Balbach *et al*. (2012) also found that slow-developing SCNT embryos yielded higher ntESC derivation rates than fast-developing ones in the B6C3F1 mouse strain, but when considering the timing to divide to three-cell stage. According to these authors, pluripotency-related genes may be more efficiently reprogrammed in slow-dividing cloned embryos, favoring ntESC establishment.

In summary, our results demonstrate that *in vitro* development of SCNT mouse embryos is delayed in comparison with that of fertilized embryos, from the second division until compaction. Moreover, the majority of SCNT embryos initiate compaction at the four-cell stage and show longer compaction and cavitation durations than fertilized embryos. Treatment of SCNT embryos with either PsA or VitC increases their capacity to give rise to blastocysts and ntESC lines, but alters their developmental pace in opposing ways, suggesting that they act through different mechanisms. The development of both non-treated and treated SCNT embryos to the blastocyst stage could be predicted from early morphokinetic data with high accuracy, using the same parameters as for ICSI embryos. Thus, time-lapse monitoring is a promising tool to increase cloning outcomes. Nonetheless, further investigation is needed to test the reliability of the predictor models established here, and to determine whether they are able to improve embryo selection not only for blastocyst development but also for full-term development and ESC lines derivation.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0354.

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.

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