

# The absence of a $\text{Ca}^{2+}$ signal during mouse egg activation can affect parthenogenetic preimplantation development, gene expression patterns, and blastocyst quality

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## Abstract

A series of  $\text{Ca}^{2+}$  oscillations during mammalian fertilization is necessary and sufficient to stimulate meiotic resumption and pronuclear formation. It is not known how effectively development continues in the absence of the initial  $\text{Ca}^{2+}$  signal. We have triggered parthenogenetic egg activation with cycloheximide that causes no  $\text{Ca}^{2+}$  increase, with ethanol that causes a single large  $\text{Ca}^{2+}$  increase, or with  $\text{Sr}^{2+}$  that causes  $\text{Ca}^{2+}$  oscillations. Eggs were co-treated with cytochalasin D to make them diploid and they formed pronuclei and two-cell embryos at high rates with each activation treatment. However, far fewer of the embryos that were activated by cycloheximide reached the blastocyst stage compared to those activated by  $\text{Sr}^{2+}$  or ethanol. Any cycloheximide-activated embryos that reached the blastocyst stage had a smaller inner cell mass number and a greater rate of apoptosis than  $\text{Sr}^{2+}$ -activated embryos. The poor development of cycloheximide-activated embryos was due to the lack of  $\text{Ca}^{2+}$  increase because they developed to blastocyst stages at high rates when co-treated with  $\text{Sr}^{2+}$  or ethanol. Embryos activated by either  $\text{Sr}^{2+}$  or cycloheximide showed similar signs of initial embryonic genome activation (EGA) when measured using a reporter gene. However, microarray analysis of gene expression at the eight-cell stage showed that activation by  $\text{Sr}^{2+}$  leads to a distinct pattern of gene expression from that seen with embryos activated by cycloheximide. These data suggest that activation of mouse eggs in the absence of a  $\text{Ca}^{2+}$  signal does not affect initial parthenogenetic events, but can influence later gene expression and development.

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## Introduction

During fertilisation in mammals, the sperm triggers a series of repetitive  $\text{Ca}^{2+}$  oscillations that last for several hours and eventually stop around the time of pronuclear formation (Cuthbertson *et al.* 1981, Kline & Kline 1992, Jones *et al.* 1995, Marangos *et al.* 2003). These sperm-induced  $\text{Ca}^{2+}$  oscillations release the egg from meiotic arrest by targeting cyclin B for destruction by the proteasome (Hyslop *et al.* 2004). Similar to meiotic resumption, the  $\text{Ca}^{2+}$  increase at fertilisation is responsible for triggering cortical granule exocytosis and pronuclear formation. These events are part of the initial egg activation. A  $\text{Ca}^{2+}$  increase during fertilization is known to be essential for stimulating egg activation because inhibition of  $\text{Ca}^{2+}$  oscillations with the  $\text{Ca}^{2+}$  chelator BAPTA-AM completely prevents these early events of development (Kline & Kline 1992). The role

of  $\text{Ca}^{2+}$  in egg activation is also underlined by the fact that most parthenogenetic stimuli cause a  $\text{Ca}^{2+}$  increase in the egg. Chemicals treatments such as 7% ethanol, ionomycin, or injection of  $\text{Ca}^{2+}$  cause a single prolonged rise in  $\text{Ca}^{2+}$  (Cuthbertson *et al.* 1981, Colonna *et al.* 1989, Swann & Ozil 1994). Incubation of eggs in  $\text{Sr}^{2+}$ -containing media causes a series of  $\text{Ca}^{2+}$  oscillations (Kline & Kline 1992, Swann & Ozil 1994, Liu *et al.* 2002). Whilst the single rise in  $\text{Ca}^{2+}$  induced by agents such as ethanol is an effective stimulus for aged mammalian eggs, triggering  $\text{Ca}^{2+}$  oscillations appears to be more efficient in stimulating egg activation (Ozil 1998, Ducibella *et al.* 2002). Consequently, the series of  $\text{Ca}^{2+}$  oscillations at fertilization seems to have a role in guaranteeing that both the early (e.g. cortical granule exocytosis) and the later events of activation (pronuclear formation) are stimulated in a timely and effective sequence (Ducibella *et al.* 2002).

There is evidence that the  $\text{Ca}^{2+}$  oscillations in mammalian eggs may affect development beyond the first cell cycle. In rabbit eggs, the amplitude and duration of  $\text{Ca}^{2+}$  transients can be manipulated by means of electrical field pulses (Ozil 1990). Such methods have been used to demonstrate that the pattern of  $\text{Ca}^{2+}$  transients during parthenogenetic activation, influences the numbers of embryos reaching compacted morula and blastocyst stages (Ozil 1990, Ozil & Huneau 2001). Applying extra  $\text{Ca}^{2+}$  transients to rabbit eggs after fertilization has also been shown to influence the number of embryos that successfully implant (Ozil 1998). In the mouse egg, it has been shown that imposing different patterns of  $\text{Ca}^{2+}$  transients during activation and the first cell cycle can affect the number of cells in the inner cell mass of the resulting blastocysts (Bos-Mikich *et al.* 1997), however, it is not clear how the  $\text{Ca}^{2+}$  transients during the first cell cycle of development could affect the later events. Since  $\text{Ca}^{2+}$  oscillations of different frequencies can exert specific effects on gene expression in somatic cells (Dolmetsch *et al.* 1998), it is possible that some of the long-term effects of egg activation are mediated through the EGA. However, as yet this idea is untested. Furthermore, there have been no data to show whether the pattern of  $\text{Ca}^{2+}$  increase during egg activation can affect the pattern of gene expression in mammalian embryos.

One of the difficulties in trying to study an effect of  $\text{Ca}^{2+}$  transients upon embryo development is the fact that a  $\text{Ca}^{2+}$  increase is normally associated with, and necessary for, both fertilization and parthenogenetic activation. Consequently, it is difficult to demonstrate whether an effect of  $\text{Ca}^{2+}$  changes on the later embryo development are independent of the actions of  $\text{Ca}^{2+}$  on starting development. One potential method to separate the effect of  $\text{Ca}^{2+}$  on activation from an effect on later development is to use a method of starting development that does not rely upon  $\text{Ca}^{2+}$  increase. Protein synthesis inhibitors such as cycloheximide and puromycin can activate mouse eggs after prolonged periods of incubation (Siracusa *et al.* 1978, Moses & Kline 1995). Eggs activated with cycloheximide, form pronuclei, have been reported to undergo cleavage divisions. Cycloheximide is also reported to be an effective activator without inducing any increase in  $\text{Ca}^{2+}$  (Moses & Kline 1995). Protein synthesis inhibitors probably activate mammalian eggs by blocking the continuous synthesis of cyclin B that is required to stimulate the cell cycle protein kinase CDK1. Inhibitors of protein kinases such as roscovitine also activate mammalian eggs (Phillips *et al.* 2002). Although both cycloheximide and roscovitine-activated eggs undergo cleavage division, the later development of these embryos has not been documented systematically.

In this study, we have used cycloheximide to activate mouse eggs in the absence of a  $\text{Ca}^{2+}$  increase. The development of these eggs was compared to eggs

that were exposed to  $\text{Sr}^{2+}$  or ethanol in addition to cycloheximide. Our data suggest that a  $\text{Ca}^{2+}$  increase during egg activation plays a role in the completion of cell divisions that lead to blastocyst formation. The effects we see do not appear to be due to an alteration in the timing or the amount of global gene expression during EGA. However, we use microarray analysis to show that the lack of a  $\text{Ca}^{2+}$  increase during activation appears to lead to differences in the pattern of genes expressed in embryos at the mid preimplantation stages.

## Materials and Methods

### Egg and embryo handling

Female MF1 strain mice of 21–24 days were super-ovulated by i.p. injection of pregnant mare serum gonadotrophin and human chorionic gonadotrophin (hCG) (both from Intervet, Milton Keynes, UK) as described previously (Lawrence *et al.* 1997). Eggs were collected from oviducts 14–16 h posthCG injection and maintained in HEPES-buffered potassium simplex optimised medium (HKSOM) buffer (Summers *et al.* 2000). Parthenogenetic activation in a  $\text{Ca}^{2+}$ -dependent manner was carried out by exposing eggs to 7% ethanol for 7 min, or by incubation for 2 h in media with 10 mM  $\text{Sr}^{2+}$ . Parthenogenetic activation in a  $\text{Ca}^{2+}$ -independent manner took place by incubating eggs in 20  $\mu\text{g}/\text{ml}$  cycloheximide for 4 h. When eggs were activated using a combined treatment, they were first incubated in cycloheximide for 2 h, embryos were then placed in HKSOM medium containing cycloheximide and  $\text{Sr}^{2+}$  for the next 2 h. Alternatively, eggs were incubated in cycloheximide for 2 h, removed to a medium containing cycloheximide and 7% ethanol for 7 min at room temperature, and then returned to cycloheximide media for 1 h 53 minutes. All eggs were cultured in media containing 2  $\mu\text{g}/\text{ml}$  cytochalasin D during 2 hours of their activation protocol in order to produce diploid parthenotes. Cytochalasin D itself does not cause any alteration in the intracellular  $\text{Ca}^{2+}$  in eggs (McAvey *et al.* 2002, K Swann, unpublished observations). Activated eggs were cultured at 37 °C in KSOM/AA in 5%  $\text{CO}_2$  (Summers *et al.* 2000).

### $\text{Ca}^{2+}$ measurements

The eggs were loaded with 2  $\mu\text{M}$  Fura-2 AM (Sigma) for 15 min. Zona pellucidae were removed with acidified Tyrode's solution and eggs transferred to a heated chamber on to the stage of a Nikon Diaphot microscope. Fluorescence ratios from eggs were recorded as described previously (Lawrence *et al.* 1997, Halet *et al.* 2004).

### **Monitoring genome activation with a luciferase reporter gene**

EGA was assayed by monitoring luminescence from embryos injected with the luciferase reporter gene pGL3 (Promega) as described previously (Miranda *et al.* 1993, Ram & Schultz 1993). Pronuclei of *in vivo* fertilised or *in vitro*-activated parthenotes were injected with 50 ng/μl of pGL3-control vector DNA in the buffer containing 120 mM KCl and 20 mM Hepes. After injection, the pronucleate embryos were incubated in HKSOM media containing luciferin (100 μM). The luminescence from groups of embryos was monitored continuously for 25 h using a 10×0.5 NA (numerical aperture) objective and an imaging photon detector system supplied by Science Wares (www.sciencewares.com). This system uses an inverted microscope with an Imaging Photon Detector (Photek Ltd, St Leonards on Sea, UK).

### **Differential staining of inner cell mass and trophectoderm**

Zona pellucidae were removed from blastocysts (103 h after activation) with acidified Tyrode's solution and blastocysts were then exposed to 100 μg/ml fluorescein isothiocyanate (FITC)-labelled wheat germ lectin (WGA, Sigma) for 20 min at 37 °C followed by fixation in 4% paraformaldehyde in PBS at room temperature for 30 min. Blastocysts were then exposed to 0.05% Triton-X in PBS for 1 h followed by incubation in 5 μg/ml propidium iodide for 1 h. After permeabilisation and staining, blastocysts were washed in PBS before being viewed on a confocal microscope (model LSM510, Carl Zeiss, UK, Welwyn Garden City, UK). Fluorescein was excited with a 488-nm line of an Argon laser with emission at 450–490 nm, and propidium iodide was excited with at 543 nm with a He-Ne laser with emission at 585–615 nm.

### **Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) labelling to assay apoptosis**

Mouse blastocysts were washed through drops of PBS containing 1 mg/ml poly vinyl propylene (PVP) and then fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Following fixation blastocysts were permeabilised in PBS containing 0.5% Triton-X (Sigma) for 1 h. Positive controls were incubated in 5 U/ml RQ1 DNase for 20 min at 37 °C. Blastocysts were then washed in PBS/PVP and incubated in fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TUNEL reagents, Roche) for 1 h in the dark at 37 °C. In order to count the number of cells in the blastocyst, the nuclei were stained by incubation in 5 μg/ml propidium iodide and 50 μg/ml RNase A for 1 h in the dark at room temperature. Blastocysts were then washed in PBS/PVP and mounted on a glass slide in 10% glycerol. Slides were viewed on a confocal microscope

as explained previously for fluorescein and propidium iodide. FITC-labelled cells were counted as apoptotic cells and propidium iodide-stained nuclei were counted as total number of live cells. The apoptotic index was calculated by the total number of TUNEL-stained cells divided by the total number of cells in each blastocyst.

### **Embryo collection for microarray work**

Eggs were activated parthenogenetically using Sr<sup>2+</sup> or cycloheximide as described previously. After 60 h of culture, only eight-cell embryos exhibiting good morphology were selected. Batches of 34 eight-cell embryos were washed four times through drops of HKSOM media and rapidly frozen in 4 μl of the same media. Eight-cell embryos were stored at –80 °C until they were used for RNA extraction.

### **RNA extraction labelling and hybridisation on the NIA 22K 60-Mer Oligo microarray**

Two batches of 34 embryos were collected for each group of cycloheximide and Sr<sup>2+</sup>-activated eight-cell embryos. Total mRNA was extracted from each group of embryos using a Quickprep micro poly-A RNA Extraction Kit (Amersham) and linear acrylamide as a carrier (Ambion, Austin, TX, USA). Total RNA samples from each extraction were labelled with Cy3-dye by two-round linear amplification labelling reaction for cRNA targets using a Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA). Quantification of target was determined using a microscale spectrophotometer (NanoDrop, Wilmington, DE, USA). Universal mouse reference RNA (Stratagene, La Jolla, CA, USA) was labelled with Cy5-dye by a one-round amplification-labelling reaction and used as a control for all hybridization reactions, allowing cross-comparisons between all data sets. The cRNA targets from both the sets of parthenogenetically activated embryos and universal mouse reference RNA were hybridised on the NIA 22k 60-mer oligo microarray (Carter *et al.* 2003). The data presented are from two biological and two technical replicates.

### **Analysis of microarray data**

The intensity of 21 045 gene features per array was extracted from scanned microarray images using Feature Extraction 5.1.1 software (Agilent Technologies, Palo Alto, CA, USA). Statistically significant genes were determined quantitatively using the ANOVA-false discovery rate (ANOVA-FDR)=10% (Sharov *et al.* 2005). Assignment of gene function was carried out using gene ontology (GO) terms (Ashburner *et al.* 2000), which characterized genes into basic groups such as cell cycle and cell adhesion. GO

terms were used in conjunction with GenMapp/MAPP-Finder (Doniger *et al.* 2003) to identify the genes and functional groups associated with embryos parthenogenetically activated with  $\text{Sr}^{2+}$  or cycloheximide. The microarray data are available from the public databases (GEO and ArrayExpress) and the website: <http://lgsun.grc.nia.nih.gov/microarray/data.html>.

## Results

### **Development of embryos stimulated with- or without- a $\text{Ca}^{2+}$ increase**

To stimulate parthenogenetic activation of mouse eggs, we used cycloheximide and  $\text{Sr}^{2+}$  or ethanol. Consistent with previous studies (Siracusa *et al.* 1978, Moses & Kline 1995, Moos *et al.* 1996), we found that eggs treated with cycloheximide formed pronuclei, but they failed to show any  $\text{Ca}^{2+}$  increase during a 4-h incubation (Fig. 1a). Other agents such as the protein kinase inhibitor roscovitine have also been reported to activate mouse eggs in the absence of a  $\text{Ca}^{2+}$  increase (Phillips *et al.* 2002). However, we found that they were not as effective, on their own, in activating eggs as cycloheximide, so they were not used in further studies (data not shown). In contrast to cycloheximide treatment,  $\text{Sr}^{2+}$  treatment caused a series of  $\text{Ca}^{2+}$  oscillations and 7% ethanol caused a singular  $\text{Ca}^{2+}$  increase (Fig. 1b and c). Both of these treatments also lead to pronuclear formation. These data confirm previous findings and show that, under our conditions, we can stimulate activation with agents that cause no  $\text{Ca}^{2+}$  increase, a single large  $\text{Ca}^{2+}$  increase, or a series of  $\text{Ca}^{2+}$  oscillations.

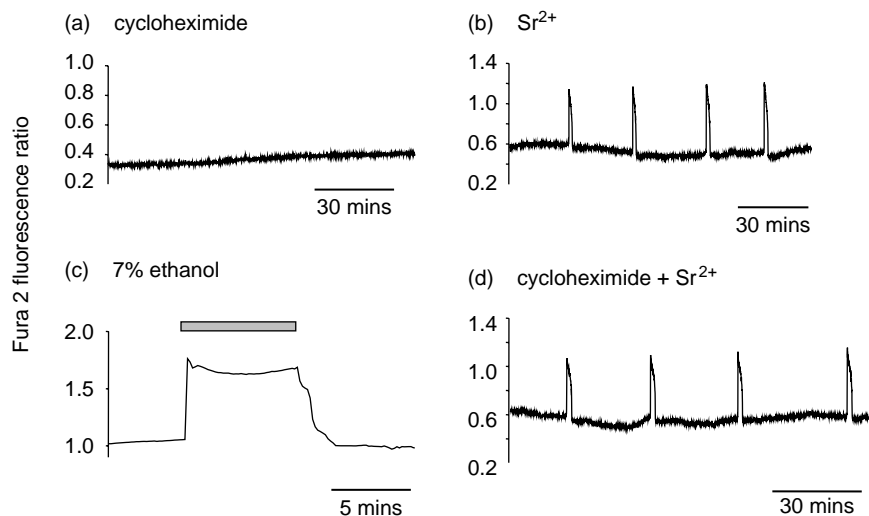
The overall degree of egg activation, as judged by pronuclear formation, was found to be similar with these three different treatments (Fig. 2). At 4 h after starting the activation treatments, a higher number of  $\text{Sr}^{2+}$ -activated embryos had two fully formed pronuclei (58%) when compared to cycloheximide or ethanol-activated embryos

(26%). However, by 6 h postactivation, a similar proportion (>75%) of embryos from all three treatment groups had two fully formed pronuclei (Fig. 2). At 6 h after activation, the majority of one-cell embryos (86%) produced by cycloheximide-induced activation underwent a temporary phenotypic stage in which they formed irregular shapes and furrows. This phenotype persisted for 1–2 h before the embryos returned to a well-defined spherical shape. Eggs activated with  $\text{Sr}^{2+}$  or ethanol alone did not produce embryos with this phenotype.

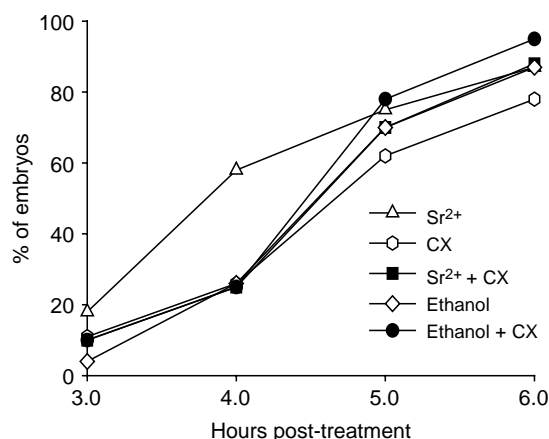
We found that egg activation by means of  $\text{Sr}^{2+}$ , cycloheximide, or ethanol was sufficient to produce high numbers of two-cell embryos. With cycloheximide and  $\text{Sr}^{2+}$  90%, and with ethanol 89% of the pronucleate, embryos reached the two-cell stage. Most embryos in each group also cleaved to the four-cell stage. These data are shown in detail in Table 1 and represents the continuation of development of the eggs presented in Fig. 2. However, despite rates of the initial development, a major difference was seen with the different parthenogenetic agents when embryos were cultured beyond the four-cell stage. The number of embryos reaching the eight-cell stage dropped dramatically when the embryos activated by means of cycloheximide were compared with those activated by  $\text{Sr}^{2+}$  or ethanol. The effect on activation was clearly related to events after the first cell cycle because we found that approximately 70% of the two-cell embryos reached the blastocyst stage when they had been activated by  $\text{Sr}^{2+}$  and ethanol (Table 1). In contrast, only 24% of two-cell embryos reached the blastocyst stage when they have been activated by cycloheximide (Table 1).

### **The poor development of embryos activated by cycloheximide is rescued by a $\text{Ca}^{2+}$ increase**

The relatively poor development of embryos after activation with cycloheximide could be due to their



**Figure 1**  $\text{Ca}^{2+}$  measurements in mouse eggs during parthenogenetic activation.  $\text{Ca}^{2+}$  was monitored in eggs by changes in the Fura-2 fluorescence ratio (see Materials and Methods). (a) An example of an egg treated with cycloheximide from the start of the recording (typical of 14/14 eggs); (b) an example of egg undergoing oscillations after addition of media containing 10 mM  $\text{Sr}^{2+}$  from the start of the trace (typical of 13/14 eggs); (c) an egg was exposed to 7% ethanol for 7 min starting at times indicated by the bar (similar to 5/5 eggs); (d) An egg had been exposed to cycloheximide for 2 h before being placed in media containing both  $\text{Sr}^{2+}$  and cycloheximide at the start of the recording (typical of 16/16 eggs).



**Figure 2** Egg activation and development with cycloheximide, Sr<sup>2+</sup> and ethanol. The timing of pronuclear formation during parthenogenetic activation is shown for mouse eggs treated with Sr<sup>2+</sup>, cycloheximide (CX) or ethanol, or combinations of thereof. The data are taken from 10 replicates with a total of 505 eggs for Sr<sup>2+</sup> treatment, 575 eggs for cycloheximide treatment, 321 eggs for treatment with Sr<sup>2+</sup> plus cycloheximide, 290 eggs for ethanol treatment and 270 eggs for treatment with ethanol plus cycloheximide.

lack of Ca<sup>2+</sup> signal (as shown in Fig. 1a). To test this hypothesis, we treated eggs with combinations of cycloheximide and agents that can cause a Ca<sup>2+</sup> increase in eggs, either Sr<sup>2+</sup> or ethanol. Sr<sup>2+</sup> media is reported to be less effective in causing Ca<sup>2+</sup> oscillations as embryos progress through the first cell cycle and we found that when eggs were incubated for 4 h in cycloheximide, many of them failed to respond to generate Ca<sup>2+</sup> oscillations in the Sr<sup>2+</sup> media (data not shown). However, Fig. 1d shows that treating eggs with Sr<sup>2+</sup> media was still effective in causing Ca<sup>2+</sup> changes after they had been incubated for 2 h in cycloheximide and hence this protocol was adopted for subsequent activation studies. We found that the combined treatments of cycloheximide with either Sr<sup>2+</sup> or ethanol were associated with similar times for pronuclear formation as that seen with each activating agent on its own (Fig. 2). When eggs were treated with cycloheximide first and then with Sr<sup>2+</sup> plus cycloheximide for 2 h, the numbers of embryos developing to the eight-cell stage (77%) were

also comparable to embryos activated by Sr<sup>2+</sup> alone (Table 1). We also found similar results when eggs were treated with cycloheximide and ethanol compared to activation with ethanol alone (Table 1). Hence, these data show that Ca<sup>2+</sup>-elevating agents reversed the detrimental effect of cycloheximide activation upon embryo development. This shows that the poor development of embryos activated by cycloheximide is unlikely due to its effect upon protein synthesis *per se*, but can be explained by the lack of a Ca<sup>2+</sup> increase during the activation phase.

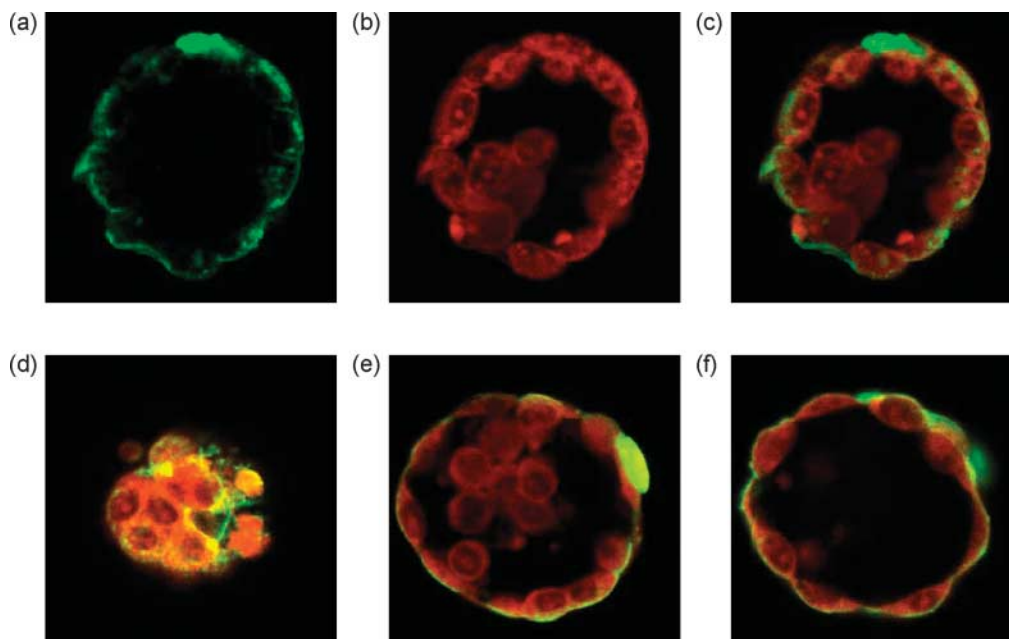
### Cell proliferation and apoptosis in parthenogenetic embryos

Although preimplantation development is compromised by activation with cycloheximide, about 20% of embryos reached the blastocyst stage. Previous studies of mouse embryos have assessed the quality of blastocysts by the number of cells and the degree of apoptosis (Hardy 1997). We first examined our blastocyst-stage embryos by differential staining to count the number of cells in the inner cell mass and trophectoderm. An example of a blastocyst stained with propidium iodide and FITC lectin is shown in Fig. 3. Figure 4 shows the results of analysis of embryos that had been activated by either Sr<sup>2+</sup> or cycloheximide. The data show that there was a significant reduction in the overall cell numbers in blastocysts that had been activated by cycloheximide compared with those activated by Sr<sup>2+</sup> (Fig. 4a). The difference in the number of trophectoderm cells was not significantly different between the two groups of embryos (Fig. 4b). However, a significant difference was seen in the number of cells in the inner cell mass where cycloheximide-activated embryos had, on an average, 8 compared with 14.3 cells in Sr<sup>2+</sup>-activated embryos (Fig. 4c). This led to a significant difference in the ratio of the inner cell mass number to the trophectoderm cell number in the two types of embryos (Fig. 4d). These data suggest that even those cycloheximide-activated embryos that develop to the blastocyst stages are compromised in the number of cells in the inner mass.

**Table 1** Preimplantation development after parthenogenetic activation.

Activation stimulus	Number of embryos at the two-cell	Four-cell	Eight-cell	Morula	Blastocyst
Sr <sup>2+</sup>	454	417 (92)	374 (82)	357 (79)	323 (71)
Cycloheximide	519	432 (83)	221 (43)*	183 (35)*	126 (24)*
Sr <sup>2+</sup> + cycloheximide	307	281 (92)	248 (81)	223 (73)	209 (68)
Ethanol	258	224 (87)	204 (79)	204 (79)	184 (71)
Ethanol + cycloheximide	253	217 (86)	203 (80)	193 (76)	160 (63)

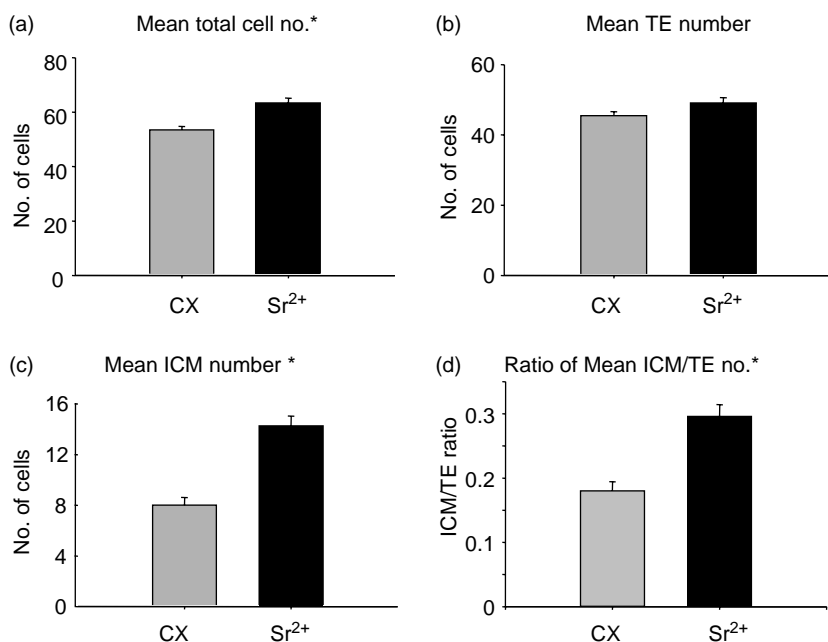
The data are from the same pool of eggs that was used to generate Fig. 2. The number of embryos that reached the two-cell stage was similar for each group and is used as the starting point for illustrating the data on later development. Numbers in parenthesis indicate the percentage of embryos developing from the two-cell stage. The asterisk next to the row of figures in the cycloheximide data indicates that the number of embryos is significantly different from those treated with Sr<sup>2+</sup> ( $P < 0.05$ , unpaired *t*-test).



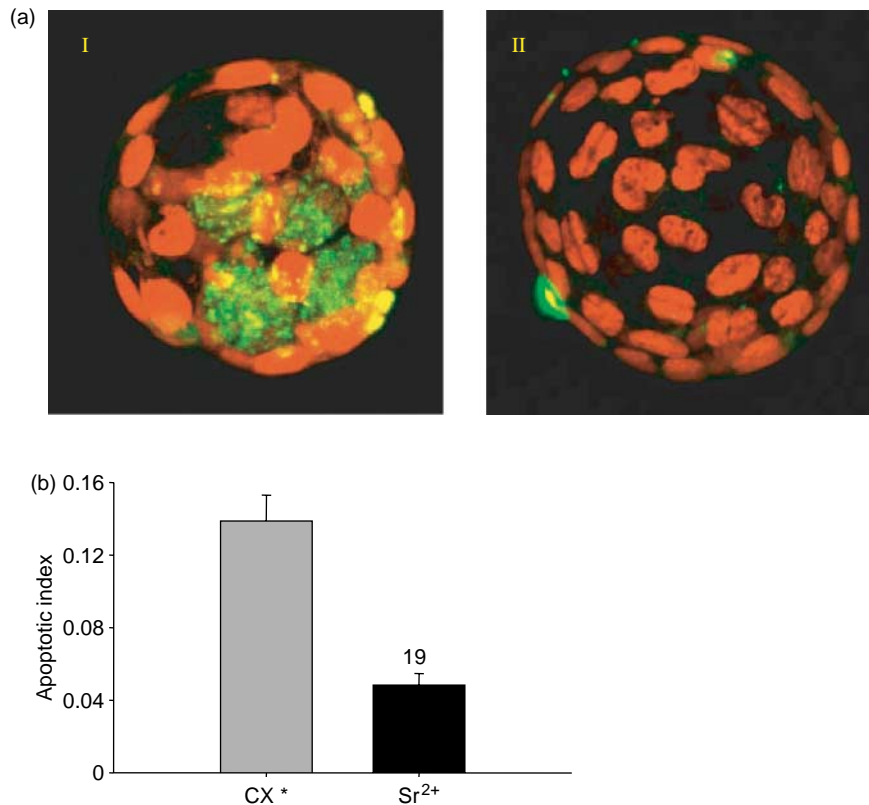
**Figure 3** Differential staining of cells in parthenogenetic blastocysts. Confocal images are shown of sample mouse blastocyst activated by cycloheximide. The image shows the trophoblast cells alone stained using WGA-FITC (a), the nuclei of all cells stained with propidium iodide (b), and the merged section is shown in (c). The images in (d)–(f) are sections through another blastocyst activated by  $Sr^{2+}$  illustrating the top of the blastocyst with all the trophoblast cells stained green and red (d), a layer further down showing the red inner cell mass nuclei in the middle (e), and a section directly afterwards showing the large cavity with trophoblast cells only (f).

One possible explanation of a reduced inner cell mass in a blastocyst could be that more cells have undergone apoptosis in these embryos. We used TUNEL staining to assess apoptosis in our embryos (Brison & Schultz 1997). Figure 5a shows examples of parthenogenetic embryos stained with TUNEL reagents and propidium iodide.

Figure 5b shows the apoptotic index for embryos activated by  $Sr^{2+}$  or cycloheximide, where the index is a measure of the proportion of cells undergoing programmed cell death. The data indicate that there is an increase in apoptosis in embryo that were activated by cycloheximide compared with those activated



**Figure 4** Composition of blastocysts derived from cycloheximide or  $Sr^{2+}$ -induced egg activation. Bar charts with error bars showing differences in mean inner cell mass (A), mean trophoblast (B), mean total cell number (C) and ratio of ICM/TE (D) in blastocysts generated from either  $Sr^{2+}$  or cycloheximide-induced parthenogenetic activation. Each asterisk indicates that  $Sr^{2+}$  and cycloheximide groups are significantly different (Student's *t*-test,  $P < 0.01$ ). The data represent the mean numbers and s.e.m. from 4 replicate experiments. A total of 22 and 21 blastocysts derived from  $Sr^{2+}$  and CX-induced parthenogenetic egg activation were used for staining respectively.



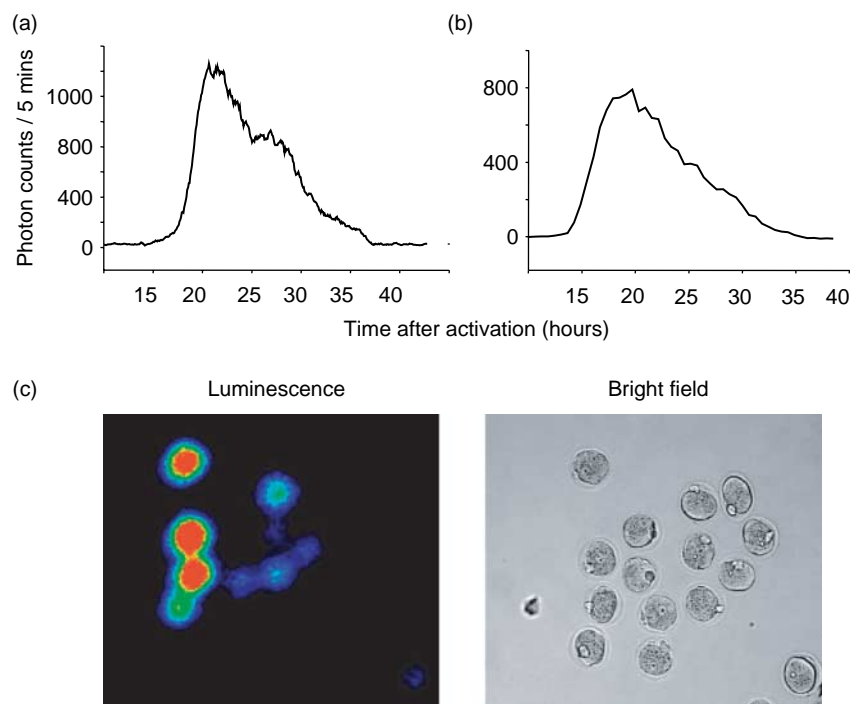
**Figure 5** Apoptosis in embryos activated by cycloheximide or Sr<sup>2+</sup>. The staining for apoptosis is illustrated in (a) which shows blastocysts derived from a cycloheximide- (i) or Sr<sup>2+</sup> (ii)-activated embryo. The red colour shows propidium iodide staining the nuclei of all cells and the green shows the fluorescein conjugated TUNEL reagent used as an indicator of apoptosis; (b) the bar chart shows the mean and s.e.m. for the apoptotic index of blastocyst parthenotes. A significantly larger apoptotic index occurs in blastocysts derived from cycloheximide activation ( $n=26$ ) compared to Sr<sup>2+</sup> activation ( $n=19$ ) ( $P<0.0001$ ). Blastocysts were from three biological replicates.

by Sr<sup>2+</sup>. These results are consistent with those obtained for the cell counts and suggests that any blastocysts that are generated without having undergone a Ca<sup>2+</sup> increase during egg activation are not of the same quality as those that have been exposed to Ca<sup>2+</sup> increases.

#### Parthenogenetic activation and the onset of EGA

EGA is essential for development and can be first detected at the one-cell stage. We investigated if EGA was relatively deficient in cycloheximide-activated embryos compared to those activated by Sr<sup>2+</sup>. We monitored total gene expression during EGA by imaging embryos injected with firefly luciferase-encoding DNA into pronuclei at the one-cell stage as described previously (Miranda *et al.* 1993, Ram & Schultz 1993). We found that gene expression during EGA in all embryos occurs in a bell-shaped pattern with an initial sharp rise to the peak followed by a slower and more staggered decrease in expression. The start of EGA occurred 15–20 h postactivation and this phase of gene expression continued for approximately 20 h. Figure 6a and b shows some examples of the pattern of luciferase

luminescence from Sr<sup>2+</sup> and cycloheximide-activated embryos respectively. Figure 6c shows a luminescence image of embryos expressing luciferase. The patterns of increased luciferase luminescence we observed in these embryos were similar to fertilized zygotes (data not shown). For the parthenogenetic embryos, we took the area under the curve of the luminescence as a quantitative way of measuring the total relative amount of gene expression. We measured a total sum of  $1.7 \pm 0.7 \times 10^5$  photons (s.e.m.,  $n=19$ ) from Sr<sup>2+</sup>-activated embryos and  $1.5 \pm 0.8 \times 10^5$  photons (s.e.m.,  $n=18$ ) from cycloheximide-activated embryos. We also took the time to reach half the maximum peak of luminescence as a measure of the rate of EGA onset. We found the half-time for the onset of EGA was  $9.6 \pm 0.8$  h (s.e.m.,  $n=19$ ) for Sr<sup>2+</sup>-activated embryos and  $10.9 \pm 0.9$  h (s.e.m.,  $n=18$ ) for cycloheximide-activated embryos. There was a considerable variation in the degree of EGA from one embryo to another, so we cannot exclude small differences in EGA in our embryos. However, these data do suggest that there is no major difference in the timing or overall degree of EGA in embryos activated with or without a Ca<sup>2+</sup> increase.



**Figure 6** EGA in parthenogenetic embryos using a luciferase reporter gene. The expression of luciferase is measured in one-cell embryos by the luminescence (photons/5 min). (a) A representative profile of luciferase expression from a  $\text{Sr}^{2+}$ -activated embryo and (b) a similar representative recording of expression from a cycloheximide-activated embryo. The time is measured after the start of the activation treatments; (c) luminescence and bright field image of some  $\text{Sr}^{2+}$ -activated embryos. The luminescence image is made from light integrated over 5 min at about 25 h after activation.

### Microarray analysis of gene expression in parthenogenetically activated embryos

It is possible that changes in gene expression underlie the poor development and the relatively poor quality of blastocysts of the cycloheximide-activated embryos. To gain insight into the effect of  $\text{Ca}^{2+}$  signals on later gene expression, we used eight-cell embryos activated with either  $\text{Sr}^{2+}$  or cycloheximide for expression profiling. A universal mouse reference RNA (Stratagene) was used and a pair-wise comparison of gene expression using microarray data was carried out to highlight differentially expressed genes in  $\text{Sr}^{2+}$  or cycloheximide-activated groups. Quantitative statistical analysis using a  $\text{FDR} \leq 10\%$  identified 807 genes that were differentially expressed between the two groups of embryos. There were 239 genes that were identified as being expressed at higher levels in the  $\text{Sr}^{2+}$ -activated group compared to 568 found to be expressed at higher levels in the cycloheximide-activated group. MAPPFinder (Doniger *et al.* 2003) was used to assign functional categories to differentially expressed genes by identifying the major GO-terms associated with them. As it is impossible to be conclusive about a function when very few genes are differentially expressed in a functional category, Tables 2 and 3 are compiled by only selecting those categories containing more than three differentially expressed genes in either group of embryos. The eight-cell embryos resulting from  $\text{Sr}^{2+}$ -activation had many genes

associated with the GO-terms 'cell proliferation, cell adhesion and ion transport' (Table 3), whereas eight-cell embryos resulting from cycloheximide activation were characterized by greater expression of genes associated with the GO-terms 'cell cycle, apoptosis and cell differentiation' (Table 2). It is important to note that a gene can be common to more than one biological process, for example RAD21 is associated with the 'cell cycle' and 'apoptosis' gene-expression pathways. The data in Tables 2 and 3 suggest that the presence or absence of a  $\text{Ca}^{2+}$  signal during egg activation does have a distinctive influence upon the pattern of gene expression during preimplantation development. Although there were differences in expression pattern for many GO groups, it was notable that cycloheximide-activated embryos show greater expression of genes associated with apoptosis. This is consistent with our other observations on cell numbers and TUNEL staining in intact blastocysts.

### Discussion

A number of previous studies in the mouse and rabbit have found that imposing different type of  $\text{Ca}^{2+}$  oscillations during egg activation can influence post-implantation embryo development (Ozil 1990, Ozil & Huneau 2001). This is the first study that compares the development of preimplantation embryos that have been activated using both  $\text{Ca}^{2+}$ -dependent and -independent means. This provides a simple and reproducible protocol



**Table 2** Genes that were more highly expressed in cycloheximide-activated embryos than in Sr<sup>2+</sup>-activated embryos.

Gene ontology classification	Protein (or gene) of interest	Fold change over Sr <sup>2+</sup> -activated embryos
Cell cycle	Spindlin ( <i>Spin</i> ); cyclin B3, ( <i>Ccnb3</i> ); cell division cycle 6 ( <i>Cdc6</i> ); cyclin-dependent kinase 7 ( <i>Cdk7</i> ); cyclin-dependent kinase 9 ( <i>Cdk9</i> ); cyclin-dependent kinase inhibitor 2D ( <i>Cdkn2d</i> ); MAP kinase 4 ( <i>Mapk4</i> ); polo-like kinase 4 ( <i>Plk4</i> ); double-strand-break repair protein rad21 homolog ( <i>Rad21</i> )	2.1;1.9;1.9;2.2;2.0;1.7;1.8;1.7;1.7
Microtubule-based processes	Cytoplasmic, light chain 1 ( <i>Dncl1</i> ); kinesin family member 11( <i>Kif11</i> ); kinesin family member 1b ( <i>Kif1b</i> ); kinesin family member 2c ( <i>Kif2c</i> ); kinesin family member 3C ( <i>Kif3c</i> ); tubulin, $\alpha$ 4 ( <i>Tuba 4</i> ); tubulin, $\beta$ 3 ( <i>Tubb3</i> ); bicaudal D homolog 2 ( <i>Bicd2</i> ); dynein, light intermediate chain 1 ( <i>Dncl1c</i> )	1.7;2.4;1.8;1.9;1.7;1.8;2.1;2.1;2.0
Cytoskeleton-dependent intracellular transport	Bicaudal D homolog 2 ( <i>Bicd2</i> ); Dynein, cytoplasmic, light chain I ( <i>Dncl1c</i> ); dynein, light intermediate chain 1 ( <i>Dncl1c</i> ); tubulin, $\alpha$ 4 ( <i>Tuba4</i> )	2.1;1.7;2.0;1.8
Microtubule motor activity	Kinesin family member 11 ( <i>Kif11</i> ); kinesin family member 1b ( <i>Kif1b</i> )	2.4;1.8
Apoptosis	Fas-associated factor 1 ( <i>Faf-1</i> ); death-associated protein kinase 2 ( <i>Dapk2</i> ); JAK 2 ( <i>Jak2</i> ); double-strand-break repair protein rad21 homolog ( <i>RAD21</i> ); zinc finger protein 346 ( <i>Zfp346</i> ); growth arrest and DNA-damage-inducible 45 $\gamma$ ( <i>Gadd45g</i> ); CCAAT/enhancer-binding protein (C/EBP) $\beta$ ( <i>Cebpb</i> ); caspase recruitment domain 4 ( <i>Card4</i> )	2.4;1.7;1.8;1.7;1.8;2.4;1.9;1.8
Cell growth	Suppressor of cytokine signalling 1 ( <i>Socs1</i> ); suppressor of cytokine signalling 2 ( <i>Socs2</i> ); suppressor of cytokine signalling 3 ( <i>Socs3</i> )	1.7;1.9;1.9
Cell proliferation	Cyclin-dependent kinase 9 ( <i>Cdk9</i> )	2.0
Organic acid transporter activity	Solute carrier family 1 (glutamate transporter) member 3 ( <i>Slc1a7</i> ); solute carrier family 38, member 4 ( <i>Slc38a4</i> ); solute carrier family 1 (glial high affinity glutamate transporter) member 3 ( <i>Slc1a3</i> )	1.8;2.0;1.9
Morphogenesis of epithelium	Xanthine dehydrogenase ( <i>Xdh</i> ); cadherin EGF LAG seven-pass G-type receptor 1 precursor ( <i>Celsr1</i> ); jagged 1 ( <i>Jag 1</i> )	1.8;1.9;2.0
Protein phosphorylation	Thymoma viral proto-oncogene 3 ( <i>Akt3</i> ); cyclin-dependent kinase 7 ( <i>Cdk7</i> ); cyclin-dependent kinase 9 ( <i>Cdk9</i> ); death-associated kinase 2 ( <i>Dapk2</i> ); discoidin domain receptor family, member 1 ( <i>Ddr1</i> ); eukaryotic translation initiation factor 2 $\alpha$ kinase 3 ( <i>Eif2ak3</i> ); JAK 2 ( <i>JAK 2</i> ); MAP kinase 14 ( <i>Mapk14</i> ); MAP kinase 4 ( <i>Mapk4</i> ); NIMA (never in mitosis gene a)-related expressed kinase 4 ( <i>Nek4</i> ); PKC, $\beta$ ( <i>Prkcb</i> ); protein kinase, cAMP-dependent regulatory, type II $\alpha$ ( <i>Prkar2a</i> ); polo-like kinase 4 ( <i>PLK4</i> )	1.8;2.2;2.0;1.7;2.0;2.0;1.8;1.9;1.8;2.4;1.9;1.8;1.7
ATP binding	ATP-binding cassette, sub-family B (MDR/TAP) member 9 ( <i>Abcb9</i> ); ATP-binding cassette, sub-family C ( <i>Abcc10</i> ); ATP citrate lyase ( <i>Acly</i> ); thymoma viral proto-oncogene 3 ( <i>Akt3</i> ); DNA segment, Chr 1, Pasteur Institute 1 ( <i>D1Pas1</i> ); death-associated kinase 2 ( <i>Dapk2</i> ); deoxycytidine kinase ( <i>Dck</i> ); discoidin domain receptor family, member1 ( <i>Ddr1</i> ); DEAD box polypeptide 27 ( <i>Ddx27</i> ); DEAD box polypeptide 28 ( <i>Ddx28</i> ); cell division cycle 6, ( <i>Cdc6</i> ); cyclin-dependent kinase 7 ( <i>Cdk7</i> ); cyclin-dependent kinase 9 ( <i>Cdk9</i> )	2.0;1.7;1.7;1.8;1.7;1.7;2.1;2.0;1.9;2.0;1.9;2.2;2.0
Cell differentiation	Ectodysplasin-A receptor ( <i>Edaradd</i> ); growth arrest and DNA-damage-inducible 45 $\gamma$ . ( <i>Gadd45g</i> ); JAK 2( <i>Jak2</i> ); xanthine dehydrogenase ( <i>Xdh</i> ); trans-acting transcription factor 3 ( <i>Sp3</i> ); interferon consensus sequence-binding protein 1 ( <i>Icsbp1</i> ); CCAAT/enhancer-binding protein, $\beta$ ( <i>Cebpb</i> ); suppressor of cytokine signalling 2 ( <i>Socs2</i> )	2.1;2.4;1.8;1.2;1.9;1.8;1.9;2.1
Integral to membrane	Melanocortin 2 receptor accessory protein ( <i>Mrap</i> ); RIKEN cDNA 1300007B12 gene ( <i>1300007B12Rik</i> ); RIKEN cDNA 1700018O18 gene ( <i>1700018O18Rik</i> ); RIKEN cDNA 1810055G02 gene ( <i>1810055G02Rik</i> ); RIKEN cDNA 4732495E13 gene ( <i>4732495E13Rik</i> ); RIKEN cDNA 5031400M07 gene ( <i>5031400M07Rik</i> )	1.7;2.2;2.4;2.4;2.0;2.1
Extracellular space	ADP-ribosyltransferase 5 ( <i>Art5</i> )	1.9
Morphogenesis of an epithelium	Cadherin EGF LAG seven-pass G-type ( <i>Celsr1</i> ); jagged1 ( <i>Jag1</i> )	1.9;2.0
Signal transduction	Ankyrin,1 erythroid ( <i>Ank1</i> ); EDAR (ectodysplasin-A receptor-associated death domain ( <i>Edaradd</i> ); fibroblast growth factor 18 ( <i>Fgf18</i> ); G protein, $\gamma$ 7 subunit ( <i>Gng7</i> ); human immunodeficiency virus type enhancer protein 2 ( <i>Hivep2</i> ); protein kinase, cAMP-dependent regulatory, type II $\alpha$ ( <i>Prkar2a</i> ); regulator of G-protein signalling 17 ( <i>Rgs17</i> ); Rhotekin ( <i>Rtkn</i> ); suppressor of cytokine signalling 1 ( <i>Socs1</i> ); suppressor of cytokine signalling 2 ( <i>Socs2</i> ); suppressor of cytokine signalling 3 ( <i>Socs3</i> )	1.8;2.1;2.2;1.9;2.3;1.8;1.9;2.1;1.7;1.9;1.9
Protein biosynthesis	RIKEN cDNA 2410005K20 ( <i>2410005k20Rik</i> ); eukaryotic translation initiation factor 2 $\alpha$ kinase 3 ( <i>Eif2ak3</i> ); growth arrest and DNA-damage-inducible 45 $\gamma$ ( <i>Gadd45g</i> ); double-strand-break repair protein rad21 homolog ( <i>Rad21</i> ); ribosomal protein S27-like ( <i>Rps27l</i> ); ribosomal protein L17 ( <i>Rpl17</i> )	1.8;2.1;1.9;1.7;2.0;1.7

for studying the specific influence of a Ca<sup>2+</sup> signal on development that can be separated from the proximal effect of Ca<sup>2+</sup> upon egg activation. Our developmental data showed that a larger number of embryos that

underwent Ca<sup>2+</sup> oscillations or a single Ca<sup>2+</sup> increase, developed to the eight-cell and blastocyst stages compared to those from the cycloheximide-activated group, which experienced no Ca<sup>2+</sup> increase. Blastocysts

**Table 3** Genes that were more highly expressed in Sr<sup>2+</sup>-activated embryos than in cycloheximide-activated embryos.

Gene ontology classification	Protein (or gene) of interest	Fold change over cycloheximide-activated embryos
Apoptosis	Mucin 2 ( <i>Muc 2</i> ); nuclear factor of $\kappa$ B light chain gene ( <i>Nfkb1</i> )	1.8;2.2
Cell proliferation	B-cell translocation gene 1, anti-proliferative ( <i>Btg1</i> ); platelet derived growth factor, $\alpha$ ( <i>Pdgfa</i> ); protein kinase, cAMP-dependent regulatory, type I, $\alpha$ ( <i>Prkar1a</i> ); ubiquitin-like, containing PHD and RING finger domains, ( <i>Uhrf1</i> )	2.0;1.7;2.0;1.8
Organic acid transporter activity	Solute carrier family 12, member 7 ( <i>Slc12a7</i> )	1.8
ATP binding	AXL receptor tyrosine kinase ( <i>Axl</i> )	1.6
Cell differentiation	MyoD family inhibitor ( <i>Mdfr</i> ); dedicator of cytokinesis 2 ( <i>DOCK2</i> ); transforming growth factor, $\beta$ receptor II ( <i>Tgfr2</i> )	1.9;2.1;2.7
Cell adhesion	CD36 antigen ( <i>CD36</i> ); contactin-associated protein 1 ( <i>Cntnap2</i> ); dermatopontin ( <i>Dsc1</i> ); glycoprotein (transmembrane) nmb ( <i>Gpnmb</i> ); protocadherin 7 ( <i>Pcdh7</i> ); protocadherin $\alpha$ 1 ( <i>Pcdha1</i> )	1.8;2.0;3.5;2.1;1.9;1.7
Ion transport	Transient receptor potential cation channel, subfamily C, member 7 ( <i>Trpc7</i> ); solute carrier family 12, member 7 ( <i>Slc12a7</i> ); sodium channel, voltage-gated, type VIII, $\alpha$ polypeptide ( <i>Scn8a</i> ); potassium inwardly rectifying channel J 11 ( <i>Kcnj11</i> )	3.7;1.8;2.3;2.0
Signal transduction	Regulator of G-protein signalling 9 ( <i>Rgs9</i> ); nuclear factor of $\kappa$ B light chain gene ( <i>Nfkb1</i> ); growth factor receptor-bound protein 2-associated protein 1 ( <i>Gab1</i> ); protein kinase, cAMP-dependent regulatory, type I, $\alpha$ ( <i>Prkar1a</i> )	1.9;2.2;2.4;2.0
Protein biosynthesis	Eukaryotic translation initiation factor 3, subunit 10 (theta) ( <i>Eif3s10</i> ); mitochondrial ribosomal protein L15 ( <i>Mrp115</i> ); ribosomal protein L10 ( <i>Rpl10</i> ); ribosomal protein L9 ( <i>Rpl9</i> ); ribosomal protein L29 ( <i>Rpl29</i> ); ribosomal protein L9 ( <i>Rpl9</i> ); small inducible cytokine subfamily E, member 1 ( <i>Scye1</i> ); ribosomal protein S3a ( <i>Rps3a</i> ); ribosomal protein S27a ( <i>Rps27a</i> )	3.3;1.9;2.3;2.6;2.0;2.6;2.0;1.9;2.4

that were generated after cycloheximide activation also had a reduced inner cell mass number compared to those seen after Sr<sup>2+</sup> activation. Furthermore, TUNEL staining of blastocysts demonstrated that a higher incidence of apoptosis occurred in blastocysts developed after cycloheximide activation. An increase in apoptosis has been demonstrated in blastocyst-stage embryos from mouse and a number of other mammalian species after either fertilization or parthenogenetic activation. Apoptosis is particularly prevalent in human blastocysts where it has been suggested that it is related to events occurring during the first cell cycle (Hardy *et al.* 2001). It is noteworthy that we found that either brief application of 7% ethanol or longer incubations in Sr<sup>2+</sup> media were able to rescue the poor development seen after cycloheximide activation. These are very different chemical agents applied for very different times, and yet they both share in common the ability to increase Ca<sup>2+</sup> in mouse eggs. Consequently, our data suggest that the presence of a Ca<sup>2+</sup> increase during egg activation is the relevant early event that has later effects upon cell cycle progression in embryos and development to the blastocysts stage.

The method we used to activate eggs in the absence of a Ca<sup>2+</sup> increase is incubation in cycloheximide. Although cycloheximide has been known to activate mammalian eggs for many years, there have been no systematic studies on the developmental capacity of cycloheximide-activated embryos. Some of the early studies on the cycloheximide activation would have

involved haploid embryos, which itself is known to lead to impaired preimplantation development (Liu *et al.* 2002). In our study, we used incubation in cytochalasin D for all parthenogenetic activation treatments and, since our activated embryos formed two pronuclei, they were diploid. Consequently, it is clear that there is a systematic impairment of development after cycloheximide activation. It has been reported that incubation of mouse eggs in cycloheximide can delay the onset of EGA (Wang & Latham 1997, Aoki *et al.* 2003). This might be seen as an explanation for why cycloheximide-treated embryos showed poor development up to the blastocyst stage. However, the studies where cycloheximide was shown to delay EGA-used treatments of 6–14 h, whereas in this study we incubated eggs only in cycloheximide for 4 h. More significantly, we showed that treating eggs with either Sr<sup>2+</sup> or ethanol could reverse the poor development seen with cycloheximide activation, and it seems unlikely that the ability to inhibit protein synthesis is reversed by ethanol or Sr<sup>2+</sup>. In addition, when we monitored EGA using a luciferase reporter we found that there was no significant difference in the onset, timing or degree of luciferase expression in parthenogenetic embryos that had been activated with Sr<sup>2+</sup> or cycloheximide. Consequently, our data suggest that the poor development of embryos activated by cycloheximide alone is due to a lack of a Ca<sup>2+</sup> increase. This implies that a Ca<sup>2+</sup> increase during activation has later consequences for preimplantation development.

There are several lines of evidence in somatic cells that cytosolic Ca<sup>2+</sup> increases can induce gene expression (Dolmetsch *et al.* 1998). The ability of Ca<sup>2+</sup> to exert an influence upon later embryo development could potentially be due to some effect upon EGA. The onset of EGA in mouse is set by a clock that is initiated during fertilization (Schultz 1993). Since activation during fertilization involves robust Ca<sup>2+</sup> oscillations, it is possible that the Ca<sup>2+</sup> signal is the trigger for the start of a 'zygotic clock'. However, our experiments to monitor EGA with a reporter gene found no significant difference in embryos activated either with or without a Ca<sup>2+</sup> increase. To monitor EGA, we used a luciferase-based reporter gene that contains an SV40 promoter that is activated by Sp1. The Sp1 transcription factor is thought to play a major role in stimulating EGA in mouse embryos (Worrad *et al.* 1994). Therefore, our data suggest that any Sp1-dependent EGA in mouse embryo has no strict requirement for a Ca<sup>2+</sup> increase during egg activation. It remains possible that any effect of Ca<sup>2+</sup> upon later development is mediated by other transcription factors. Alternatively, the initial phase of genome activation in mammals could be independent of Ca<sup>2+</sup> signalling and may instead reflect a genome-wide release from the inhibition of transcription (Schultz 1993, Zeng *et al.* 2004).

Although we could not detect any large-scale differences in gene expression, using an exogenous probe in one-cell embryos, we did find differences in the pattern of gene expression in later stages of development using microarray analysis. We chose to gain snapshot of the differences in our two main groups of embryos at the eight-cell stage to maximize the chances of detecting changes in genes. The eight-cell stage also represents the mid-preimplantation gene activation that is proximal to the point when poor development is evident in cycloheximide-activated embryos (Hamatani *et al.* 2004). This stage is also the prelude to the blastocyst stage where differences in gene expression will be relevant to the differences in blastocyst quality that we observed between the two groups of embryos.

Our microarray analysis of parthenogenetic embryos showed that a number of genes associated with the cell cycle were expressed at higher levels in eight-cell embryos from the cycloheximide-activated compared with Sr<sup>2+</sup>-activated embryos (Tables 2 and 3). Overexpression of some these cell cycle-related genes such as *Ccnb3*, *Cdk7* and *Cdkn2d* can have detrimental consequences for the cell cycle (Okuda *et al.* 1995, Nishiwaki *et al.* 2000, Nguyen *et al.* 2002). Consistent with our TUNEL staining, we found that cycloheximide-activated embryos also had higher expression of a number apoptosis-related genes. *Gadd45 g* in particular has been shown to cause cell cycle arrest and apoptosis *in vitro* (Mak & Kultz 2004), and overexpression of *RAD21* (Pati *et al.* 2002), *DAPK2* (Kawaii *et al.* 1999), *plk4* (Mundt *et al.* 1997) and *Cdk9* (Foskett *et al.* 2001)

render cells sensitive to apoptosis. The overexpression of these apoptotic related-genes could contribute to the increased incidence of apoptosis, we observed in blastocysts derived from cycloheximide activation.

The greater expression of genes such as *Sp3*, *Icsbp1*, *Cepbp*, *Socs2*, *Jak2* and *Xdh*, which are associated with 'cell differentiation', were also found to be associated with the cycloheximide-activated group of embryos (Table 3). Overexpression of *Sp3* can cause down-regulation of N-cadherin (Le Mee *et al.* 2005), which is important for the embryos during the process of compaction. However, expression of some other genes associated with cell adhesion, cell proliferation and ion transport pathways were also characteristic of Sr<sup>2+</sup>-induced activation. Embryos activated by Sr<sup>2+</sup> also showed a relative increase in levels of genes for 'ion transport' such as *Trpc7*, *Slc12a7*, *Scn8a* and *Kcnj11*. The high levels of *Trpc7* expression were notable because it codes for a Ca<sup>2+</sup> permeable cation channel, which is activated by diacylglycerol and Ca<sup>2+</sup> store depletion (Hoffman *et al.* 1999). Some genes related to the cell adhesion processes, such as *Pcdh7* and *Pcdha*, or cell proliferation, such as *Pdgfa*, were also identified as being expressed at higher levels in Sr<sup>2+</sup>-activated embryos. These genes could also play some role in promoting embryo development of Sr<sup>2+</sup>-activated embryos over cycloheximide activated ones.

It is not clear how the presence or absence of a Ca<sup>2+</sup> increase during activation could exert such an influence upon the pattern of gene expression in embryos at much later stages of preimplantation development. The Ca<sup>2+</sup> increase during egg activation is known to stimulate a number of protein kinases such as calmodulin-dependent protein kinase II and PKC (Halet *et al.* 2004, Markoulaki *et al.* 2004, Madgwick *et al.* 2005). These protein kinases are involved in causing the immediate events of egg activation such as meiotic resumption and cortical granule exocytosis. However, these or other Ca<sup>2+</sup>-dependent protein kinases may have other substrates that exert a long-term influence. This might involve protein phosphorylation leading to changes in protein synthesis that could have a selective effect upon particular genes during EGA. Since each wave of gene expression has an influence upon the next phase (Hamatani *et al.* 2004, Wang *et al.* 2004), any difference in the pattern of gene expression at these earlier stages could have a knock-on effect upon the pattern of expression during the mid-preimplantation stage of development. The exact form of the Ca<sup>2+</sup> increase during activation may not be critical because either ethanol or Sr<sup>2+</sup> rescue poor development seen with cycloheximide activation, and these agents cause a single large Ca<sup>2+</sup> increase or a series of Ca<sup>2+</sup> spikes respectively. This is consistent with other parthenogenetic studies that suggest that any stimulus that causes a sufficient amount of Ca<sup>2+</sup> release during activation is effective in stimulating early development (Toth *et al.* 2006).

One of the implications of our current findings is that the way the egg is activated can influence the pattern of gene expression and the way the embryo develops up to the blastocyst stage. This has some practical implications since mammalian embryos are prone to developmental arrest and apoptosis during preimplantation stages (Hardy 1997). So far, attention has been focussed upon the use of different types of culture media to optimise preimplantation development (Rinaudo & Schultz 2004). Our data raise the possibility that failure of embryos to cleave, or the poor quality blastocysts, after *in vitro* culture, may also be a consequence of a deficient  $\text{Ca}^{2+}$  signal during egg activation.

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