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Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term

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Abstract

The Ca^{2+} oscillations initiated by the fertilizing sperm (but terminating concomitant with pronucleus formation) apparently ensure that the events constituting egg activation occur in the correct temporal order; early events (e.g., cortical granule exocytosis) require fewer oscillations than later events (e.g., recruitment of maternal mRNA). Whether the Ca^{2+} signaling events impact long-term development, in particular development to term, is unknown. Using fertilized eggs that have undergone the first few Ca^{2+} oscillations, we developed procedures that result either in inhibiting or stimulating the natural pattern of Ca^{2+} signaling of inseminated eggs. Although the incidence of development to the blastocyst stage is unaltered by these procedures, fewer offspring are born following embryo transfer, indicating that developmental competence of the blastocysts is reduced. Interestingly, embryo transfer experiments reveal that when the natural regime of Ca^{2+} oscillations is precociously interrupted, the incidence of implantation is compromised whereas hyper-stimulation of Ca^{2+} signaling events compromises post-implantation development. Moreover, although there was no major difference in the overall growth rates of the offspring, those obtained following hyper-stimulation exhibited a far greater variability in their weight. Analysis of global patterns of gene expression by microarray analysis revealed that ~20% of the transcripts are mis-regulated when too few oscillations are experienced by the embryo and EASE analysis indicates that genes preferentially involved in RNA processing and polymerase II transcription are differentially affected. In addition, a set of genes involved in cell adhesion is also mis-expressed and could thus be mechanistically linked to the observed reduced implantation. Only about 3% of the transcripts were mis-regulated following hyper-stimulation, and EASE analysis indicates that genes preferentially involved in metabolism are differentially affected. *In toto*, these results indicate that a range Ca^{2+} signaling events following fertilization (an excess or reduction) has long-term effects on both gene expression and development to term.

Introduction

In mammals, following sperm–egg plasma membrane fusion, a diffusible sperm-specific phospholipase C, PLC-ζ, triggers in the egg a series of Ca^{2+} oscillations (Knott et al., 2005; Saunders et al., 2002; Swann et al., 2004) whose frequency, amplitude, and duration are species-specific, thereby generating a Ca^{2+} signature (Jones, 1998; Stricker, 1999). The Ca^{2+} is released from an IP_3-sensitive pool (Miyazaki et al., 1992) and the oscillations terminate with association of the PLC-ζ with the pronucleus (PN) (Larman et al., 2004; Yoda et al., 2004); PLC-ζ has a nuclear localization signal and eggs expressing a PLC-ζ with a mutated nuclear localization signal continue to exhibit Ca^{2+} oscillations following PN formation (Larman et al., 2004).

Release of Ca^{2+} from the IP_3-sensitive Ca^{2+} pool is essential for egg activation, because inhibiting its release with a monoclonal antibody that blocks the IP_3 receptor (Miyazaki et al., 1992) inhibits both early and late events of egg activation, i.e., cortical granule exocytosis, cell cycle resumption, and recruitment of maternal mRNAs (Xu et al., 1994). Calmodulin-modulated protein kinase II (CaMKII) is likely a major target of the released Ca^{2+} because expressing a constitutively active form of CaMKII, but not PKC, results in egg activation in the

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absence of any increase in the concentration of intracellular-free Ca^{2+} (Madgwick et al., 2005). CaMKII, whose activity changes in parallel with the changes in intracellular Ca^{2+} (Markoulaki et al., 2003, 2004), may do this by summing the amount of Ca^{2+} released during the course of egg activation (Ducibella et al., 2002; Ozil et al., 2005; Tóth et al., 2006).

The role of Ca^{2+} oscillations in egg activation and development has been hampered by the inability to manipulate experimentally the regime of oscillations without detrimental effects on development. Our ability to manipulate experimentally the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in unfertilized eggs by electric field pulses circumvents this historical problem. Results of experiments using this technique suggest that the oscillations encode information that drives both short- and long-term events of egg activation (Ducibella et al., 2002; Ozil et al., 2005). For example, in mice, cortical granule exocytosis, which is an early event of egg activation, require 4–8 Ca^{2+} oscillations, whereas completion of meiosis II and maternal mRNA recruitment, which are later events, require 16–24 oscillations (Ducibella et al., 2002).

Ca^{2+} oscillations may also have long-term consequences on development. In rabbit parthenogenotes, a similar fraction of the embryos cleaved whether they received 4 or 8 Ca^{2+} pulses of similar amplitude and frequency, but implantation was compromised in those that received 8 pulses (Ozil and Huneau, 2001). The latter study, however, used parthenogenotes, which cannot develop to term (McGrath and Solter, 1984), and hence this study was not able to assess the long-term consequences of Ca^{2+} oscillations on development to term. Thus, the role, if any, of Ca^{2+} signaling events that occur during the first few hours following insemination on development to term remains unresolved.

We report here a method that directly intervenes at the level of intracellular Ca^{2+} concentration in which following the first few Ca^{2+} oscillations induced by the fertilizing sperm, the endogenous Ca^{2+} oscillations are either terminated or overridden and an experimentally determined pattern imposed. We report that, in either case, development to the blastocyst stage is unaffected, but development to term is compromised. The basis for this differs; implantation is compromised when the natural pattern of signaling events is prematurely interrupted, and post-implantation development is compromised when the stimulation of the $[Ca^{2+}]_{cyt}$ oscillations is too high. The basis for these developmental differences likely reflects differences in the global patterns of gene expression in the blastocysts, as assessed with Affymetrix microarrays.

Materials and methods

Egg collection, embryo culture, and media used

Fertilized eggs were obtained from superovulated F1 (C57BL/6×CBA) female mice (6–8 weeks old) mated to F1 males. Females were superovulated by i.p. injection of 8 IU of PMSG (Folligon® Intervet, Angers, France) followed 48 h later by injection 7.5 IU of hCG (Chorulon® Intervet, Angers, France) to induce ovulation. Eggs were recovered in HEPES-buffered M2 medium (Sigma M7167) and cumulus cells were removed with 0.1% hyaluronidase (Sigma H-3506). Two types of eggs were recovered. In Group 1, freshly fertilized eggs were collected from oviducts at precisely 15 h post-hCG injection. Only eggs that displayed a fertilization cone and the early phase of second PB extrusion were selected (see Fig. 1) and subjected to the two treatments described below. In Group 2, fertilized eggs were collected at precisely 22 h post-hCG injection. Only eggs that displayed two PN were subjected to the experimental treatments. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Eggs were cultured in M16 medium supplemented with 4 mg/ml of bovine serum albumin (Sigma A-3311). The medium was equilibrated with 5% CO_2. When Ca^{2+}-free medium was required for inhibiting Ca^{2+} release from intracellular stores, Ca^{2+} was replaced by Mg^{2+}.

Intracellular Ca^{2+} concentration

To measure the change in intracellular Ca^{2+} caused by the treatment, eggs were incubated for 15 min at 37 °C under 5% CO_2 in air with 20 μM fura 2-acetoxymethyl dye (Fura 2-AM, Molecular Probes no. F1221). The dye was first dissolved in DMSO containing Pluronic F-127 (Molecular Probes no. P3000) (final concentration, 0.08% v/v and 0.016%, respectively) and then diluted in M16 medium; the final concentration of dimethylsulfoxide was 0.016% w/v. The eggs were placed on the stage of a Nikon TE2000 inverted microscope, which was fitted with a Fluor 40× oil objective. The individual eggs were held in the chamber by a holding micropipette as previously described (Ozil and Swann, 1995). The optical field was illuminated with a 75-W Xenon arc lamp and wavelengths were selected at 340±5 nm and 380±5 nm by using a Cairn Optoscan monochromator. A long pass emission filter (upper 520 nm, Nikon Kawasaki, Japan) was used to transmit emitted light from the fura 2 fluorochrome to a Photometric CoolSNAPES Monochrome digital camera (Roper Scientific, USA). Emission wavelengths recorded at 340 and 380 nm and intracellular Ca^{2+} levels were recorded in terms of a ratio of fluorescence (F340/F380), which increases with rising intracellular Ca^{2+}. The whole process was controlled by MetaFluor software v6.2.

Stimulation and inhibition of Ca^{2+} signaling events

The non-Fura-2-loaded eggs were placed in a microfluidic perfusion chamber that allows for both rapid washing in nonionic media and electric field pulse (Ozil et al., 2005). Briefly, a 10-s wash in isotonic and low ionic strength medium (60 g/l glucose (Aristar grade, BDH no. 452134B)) containing or not containing 100 μM Ca^{2+} allows rapid diffusion of the ionic content from the perivitelline space prior to electrical pulsation. Following the electrical pulse, the cells are rapidly washed with M16 medium to ensure membrane healing. The rapidity of the two washing steps, which takes about few hundred milliseconds, made it possible to control simultaneous Ca^{2+} influx in a batch of eggs when 100 μM Ca^{2+} ions were present in the glucose solution. The electrical pulse was

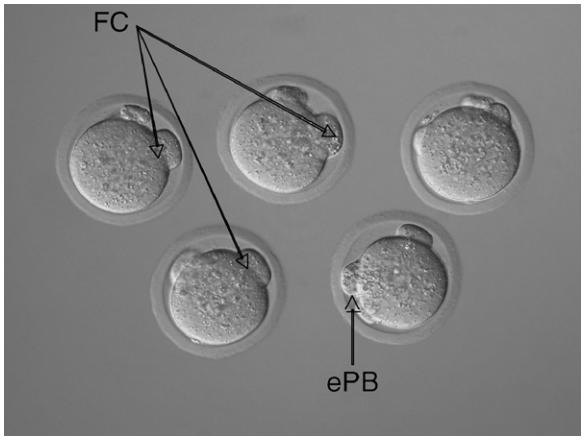

Fig. 1. Photomicrographs of recently inseminated eggs. Freshly inseminated egg showing the fertilization cone (FC) and emitting second polar body (ePB) as is seen using a dissecting microscope of egg selection.

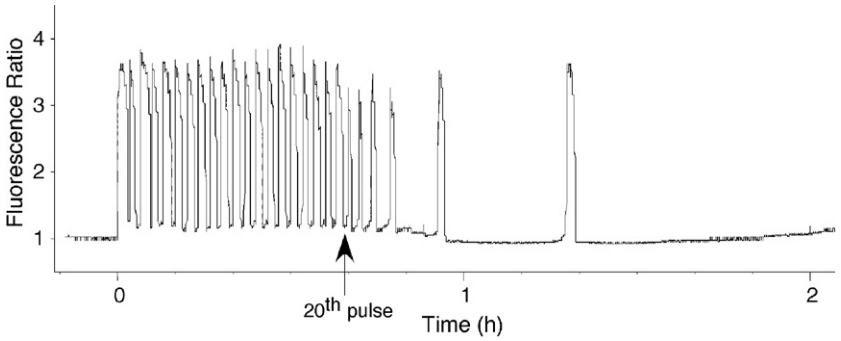

Fig. 2. Calcium oscillatory pattern following 20-p treatment of newly inseminated eggs. Each electric field pulse causes a highly reproducible Ca^{2+} signal and efficient Ca^{2+} reuptake that follows Ca^{2+} release. Note that reuptake is not fully completed during the pulse because the baseline remains slightly higher, but after cessation of the treatment, the spontaneous oscillations terminate with a return to the baseline. In this record 6, spontaneous oscillations occurred after the 20th pulse.

made up of a series of alternative pulses of 1.6 kV cm^{-1} amplitude of 45 μs duration at 5 μs intervals for 300 μs. The eggs from Groups 1 and 2 were subjected to a series of 20 pulses delivered every 2 min for 40 min. This treatment is hereafter referred to as the 20-p treatment. Note that when 100 μM Ca^{2+} was added to the glucose solution that an increase in $[Ca^{2+}]_{cyt}$ was observed after each electric field pulse (Fig. 2). In contrast, in the absence of Ca^{2+} no increase in $[Ca^{2+}]_{cyt}$ was observed following the pulse (Fig. 3), but rather an endogenous series of oscillations was observed that were uncoupled to the electric field pulse.

To inhibit Ca^{2+} signaling, eggs from Groups 1 and 2 were placed in a microfluidic perfusion chamber and perfused at 37 °C for 5 h with a M16 Ca^{2+}-free medium at a flow rate of 2.2 μl/s (see Fig. 4). This treatment is hereafter referred to as the CFW (calcium-free wash) treatment. The medium in the chamber was continuously replenished at 37°C from a glass syringe that was loaded with M16 medium equilibrated with 5% CO_2 in air. The flow rate of 2.2 μl/s assures a constant pH in the chamber.

After either treatment in the microfluidic chamber, the eggs were replaced inside an incubator and cultured in M16 medium at 37°C under 5% CO_2 in air. For every experimental replicate, some untreated eggs were cultured in the incubator (and not in the microfluidic chamber) and then transferred to recipient to assess their developmental potential.

Embryo transfer

Experimental and control embryos were transferred to recipients the same day of the experiment to minimize the time spent *in vitro*. By allowing the embryos to develop for the majority of time *in vivo* following embryo transfer, we minimized the known detrimental effects of embryo culture on gene expression and development (Rinaudo and Schultz, 2004). Female F1 mice (C57BL/6×CBA), 8–13 weeks old, were used as recipients. The recipient mice were mated with vasectomized males the night before transfer. All treated eggs at the two-PN stage were transferred to the left oviduct of each recipient female on the day on which a vaginal plug was found (respectively, day 0 of the pregnancy) and 3 to 11 PN-embryos were transferred (average = 8.2 ± 0.3 eggs/recipient). The number of offspring was recorded for every experimental group.

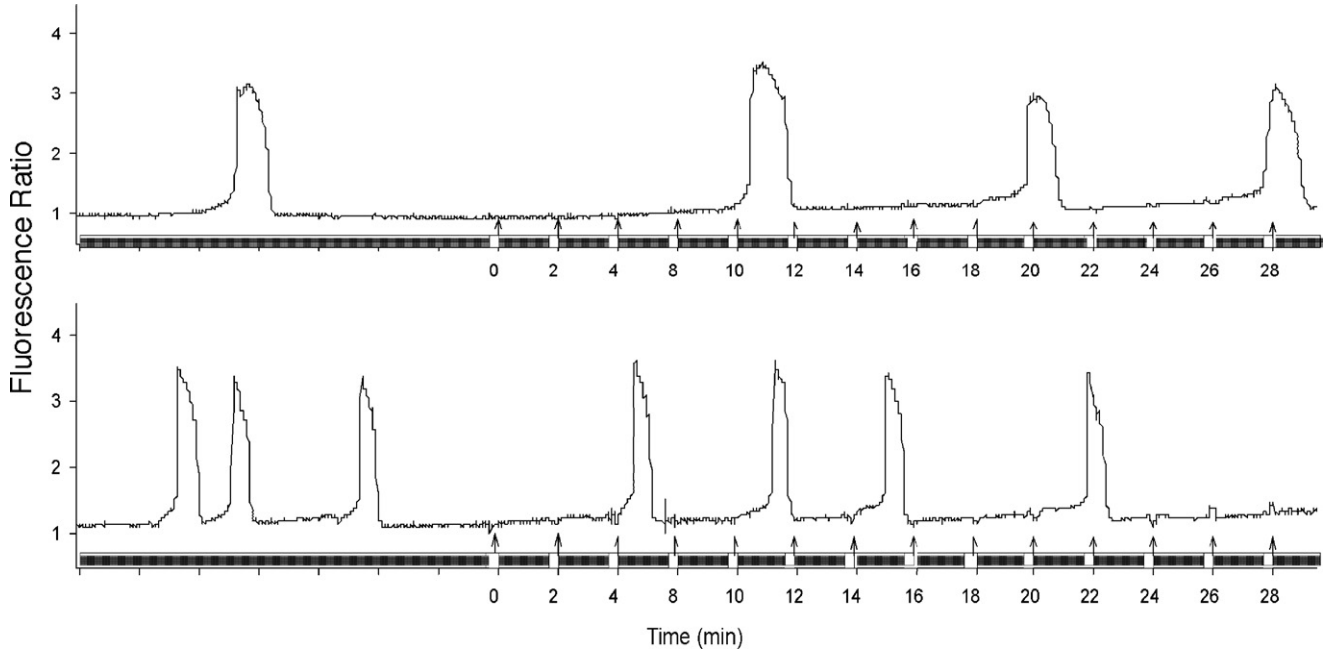

Fig. 3. Calcium oscillatory pattern in newly inseminated eggs subjected to a series of electrical pulses in glucose-containing medium lacking Ca^{2+}. Shown are two traces in which a newly fertilized egg that displayed spontaneous oscillations was treated every 2 min with electrical pulses (arrows) of 1.6 kV cm^{-1} for 300 μs in glucose-containing medium lacking Ca^{2+}. A 10 s washing (white rectangle insert in the bottom line before the arrow) was performed before each electrical pulse; the remainder of the time the eggs were perfused in M16 medium containing 1.7 mM Ca^{2+}. Note that the Ca^{2+} signal (Ca^{2+} released) is not coupled with the electrical pulse. The records demonstrate that the innocuousness of the methodology.

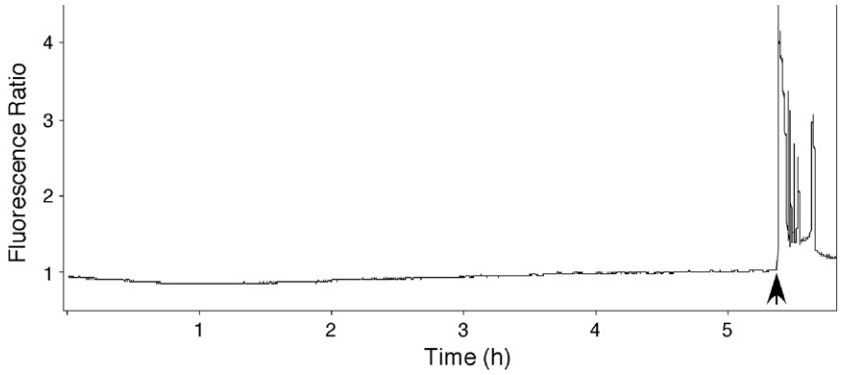

Fig. 4. Intracellular Ca^{2+} profile following CFW treatment of a newly inseminated egg. At the end of the treatment, the Ca^{2+}-free medium was replaced with M16 media (1.7 mM Ca^{2+}) and the egg was subjected to one electric pulse (arrow) to cause a single Ca^{2+} influx. The record shows that the egg generates a series of dampened oscillations that are a landmark of fertilization and activity the sperm-derived PLC-ζ.

Assessment of development at D3.5 and 10 of pregnancy

Preimplantation and post-implantation development was examined at D3.5 and D10, respectively. At D3.5, the uterine horns of the recipients were flushed, the embryos were recovered and cultured 10 h in KSOM medium at 37°C in 5% CO_2 (KSOM, Specialty Media no. MR-106-D). The time spent in culture was sufficient so that all of the embryos completed the morula-blastocyst transition. For microarray analyses, 20 early-mid-cavitating blastocysts (experimental and control) were transferred to 20 μl of lysis buffer (Pan et al., 2005). The samples were then immediately frozen and stored at −80°C. For embryo transfer experiments, the number of implantation sites was recorded by the presence of deciduae and the presence of fetuses was recorded at D10 (Fig. 1S).

Growth rate of the newborns

Starting 1 week after delivery, newborns were weighed every week until they were 8 weeks old. At 4 weeks, the pups were weaned, and males and female in each litter were separated.

Statistical analysis

The data were analyzed using SigmaStat 3.0 software and plotted using SigmaPlot 8.0 software. Statistical significance was assessed using Chi-square, Fisher or *t*-test; $p<0.05$ was considered to be statistically significant.

RNA extraction, labeling, and hybridization

Total RNA was extracted from 20 blastocyts, amplified, labeled, and fragmented as previously described (Pan et al., 2005). The yield of biotinylated cRNA for each replicate was 45–70 μg and the RNA samples were submitted to the Penn Microarray Facility and hybridized to MOE430 v2 GeneChips, which contain about 39,000 transcripts that cover most of the mouse genome. Quality control parameters (e.g., % present, 3′/5′ ratio) are found in Table S1 and indicate that the microarray data generated were of high quality.

Microarray data analysis

Microarray Analysis Suite 5.0 (MAS, Affymetrix) was used to quantify microarray signals with default analysis parameters and global scaling to target mean=200. GeneChip tabular data are available at the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo). The MAS metrics output was loaded into GeneSpring v7.2 (Silicon Genetics) with per-chip normalization to the 50th percentile and per-gene normalization to the median. Condition tree clustering was used to identify relationships among the global gene expression levels of samples. To minimize false positives, only the genes called as "Present" in at least 3 out of 4 replicates for each treatment group were used for Statistical Analysis of Microarrays (SAM). Because four biological replicates provided sufficient statistical power and confidence levels to detect a 1.4-fold difference in RNA abundance (Zeng et al., 2004), genes with significant differences of 1.4-fold or greater between conditions were identified at a 5% false discovery rate (FDR) using SAM. The resulting gene lists were then imported into Expression Analysis Systematic Explorer (EASE, version 2.0) to analyze the gene ontology for overrepresentation (Hosack et al., 2003). The EASE score was calculated for likelihood of overrepresentation of annotation classes and only selected biological processes with an EASE score less than 5% are shown. Note that, on the Affymetrix GeneChip used, each probe set represents one gene but many Unigenes are represented more than once by different probe sets.

Results

Experimental design and rationale

We have validated a method that permits experimental but non-detrimental intervention on the cytosolic Ca^{2+} in eggs (Ducibella et al., 2002; Ozil et al., 2005). The experimentally induced oscillations largely mimic those that occur following insemination and afford a powerful tool to assess the role of Ca^{2+} signaling events that comprise egg activation. Nevertheless, the approach does not faithfully mimic the first Ca^{2+} oscillation, which is of longer duration than subsequent oscillations and is characterized by a series of mini-spikes at its peak (Saunders et al., 2002). In addition, because the treatment results in parthenogenotes, the phenomenon of genomic imprinting precludes the ability to assess the long-term effects of the experimentally manipulated Ca^{2+} oscillations, i.e., development to term.

To circumvent these limitations, we developed the following procedure. Use of eggs fertilized *in vivo* bypasses the aforementioned problems, i.e., the first transient occurs and displays its hallmark traits and the presence of a paternal genome permits development to term. In addition, using eggs fertilized *in vivo* would circumvent any unforeseen problems that could arise from either fertilization *in vitro* or intracytoplasmic sperm injection (ICSI) (Kimura and Yanagimachi, 1995). The problem that arises, however, is that when eggs inseminated *in vivo* are isolated, the investigator does not know precisely how many Ca^{2+} transients have occurred. Therefore,

we estimated the approximate number of Ca^{2+} transients at the time of egg collection as follows:

Fertilization triggers the first Ca^{2+} oscillation that lasts some 3–7 min (average is 5 min), that are followed by a series of Ca^{2+} transients that occur about every 20 min and whose duration is ~1 min for 5 h, which is the time when PN formation occurs (Deguchi et al., 2000). The total duration of the Ca^{2+} transients is comprised of the initial 5 min spike and 15 spikes of ~1 min duration, i.e., the total time that cytosolic Ca^{2+} is elevated is ~20 min.

Previous work suggests that early telophase occurs 60 min following fertilization – these eggs have a clearly defined fertilization cone – and the second polar body is emitted ~30 min later (Deguchi et al., 2000). As described under Materials and methods, the inseminated eggs that possess a fertilization cone are culled and used for the experiments. By the time of completion of formation of the second polar body, the eggs have experienced the initial Ca^{2+} transient (5 min) and 2–4 subsequent transients and therefore have "seen" a cumulative Ca^{2+} signal of 7–9 min duration, which we estimate is 30–50% of the total duration of elevated cytosolic calcium.

Continuous perfusion of these eggs with Ca^{2+}-free medium prior to PN formation results in cessation of Ca^{2+} oscillations, which require extracellular Ca^{2+} that is necessary to replenish the intracellular Ca^{2+} stores (Igusa and Miyazaki, 1983; Kline and Kline, 1992; McGuinness et al., 1996; Mohri et al., 2001). Alternatively, transfer of these eggs to the experimental chamber permits over-riding the endogenous Ca^{2+} oscillations initiated by the fertilizing sperm. A high frequency (2 min between pulses) of stimulation is used to override the spontaneous Ca^{2+} oscillations that are triggered by any Ca^{2+} influx – freshly inseminated eggs display a very active Ca^{2+}-induced Ca^{2+} release (CICR) – and the sperm-derived PLC-ζ.

Effect of inhibiting or stimulating Ca^{2+} signaling events on development to blastocyst stage

Results from our previous studies examined the effect of the number of Ca^{2+} oscillations or the total amount of Ca^{2+} released (including amounts in substantial excess to that normally released) on events of egg activation and early cleavage (Ducibella et al., 2002; Ozil et al., 2005). Although results from another study using the rabbit model indicated that cleavage, at least to the 8-cell stage, is independent of the number of Ca^{2+} oscillations once the concentration of released Ca^{2+} reaches a threshold to trigger egg activation (Ozil and Huneau, 2001), parthenogenotes were used. Thus, the effect of the number of Ca^{2+} oscillations or total amount of released Ca^{2+} sufficient to activate an egg and induce cleavage to the blastocyst stage in normal embryos remains unresolved. Accordingly, we first addressed this issue using our newly described protocols, which compared to controls, results in fewer oscillations (CFW) or releases too much Ca^{2+} (20-p).

Recently fertilized eggs were harvested and subjected to either the CFW or 20-p protocol prior to PN formation. The embryos were then transferred to pseudopregnant mothers and flushed from the uteri when the embryos were late morulae. In this and experiments described below, the range in the number of embryos transferred was similar for each treatment and for each experiment a control was performed. The embryos were then cultured for 10 h and the number that developed to the blastocyst was determined. Results of these experiments demonstrated that relative to controls, there was no apparent difference in the incidence of development to the blastocyst stage (Table 1). In addition, the blastocysts in all groups appeared morphologically similar (data not shown).

Effect of inhibiting or stimulating Ca^{2+} signaling events on development to term

Although there was no apparent effect of a premature termination of Ca^{2+} signaling or hyper-stimulation on development to the blastocyst stage, the developmental competence of these blastocysts was not established. It is now widely recognized that development to the blastocyst stage is not an accurate indicator of developmental competence, e.g., the developmental competence of cloned blastocysts is markedly reduced (Campbell et al., 2005; Latham, 2005). Our experimental protocol permits assessment of the developmental competence of the resulting blastocysts by using embryo transfer because the blastocysts possess both maternal and paternal genomes.

Results of these experiments demonstrated that both treatments applied before PN formation resulted in reduced numbers of offspring when compared to controls (Table 2, Group 1). When accounting for only those embryo transfers that resulted in a pregnancy, 72% of the transferred control embryos developed to term. In contrast, about 50% of the transferred embryos generated by either protocol developed to term, which reflects about a 30% overall decrease relative to controls (of which ~70% developed to term). Of note is that a transient but statistically significant decrease in weight in the 20-p group was noted at 3 weeks following birth (Fig. 5A); the overall growth rate of offspring derived from either protocol was similar to that of controls. Strikingly, the variation in body weight was much greater in offspring derived from the 20-p protocol when compared to either the control or CFW-treatment group (Figs. 5B–D).

Cellular and developmental impact of the methodology

The reduced development to term could have been due to the treatment procedures *per se* and not a consequence of manipulating Ca^{2+} signaling events. The experiments described below strongly suggest that such was not the case.

Table 1
Effect of treatments on development to blastocyst stage

Treatment	# replicates	# eggs transferred	# recipients	# D3.5 blastocysts (%)
Control	13	684	38	475 (69)
CFW	9	321	17	200 (62)
20-p	4	219	12	163 (74)

Table 2
Effect of treatments on development to term

Treatment	Group 1: fertilized eggs treated before PN formation						Group 2: fertilized eggs treated after PN formation					
	# replicates	# eggs transferred	# recipients	# pregnant recipients (%)	# newborns (%)	Rate of newborn from pregnant recipient (%)	# replicates	# eggs transferred	# recipients	# pregnant recipients (%)	# newborns (%)	Rate of newborn from pregnant recipient (%)
Control	12	153	18	17 (94)	106 (69)	106/147 (72)	6	146	15	12 (80)	78 (53)	78/116 (67)
CFW	7	145	19	15 (79)	60 (41)[a]	60/117 (51)[b]	3	148	15	10 (67)	69 (47)	69/98 (70)
20-p	5	106	13	8 (62)	28 (26)[a, c]	28/65 (43)[b]	4	235	25	16 (64)	66 (28)[d]	66/150 (44)[e]

[a] The rates of newborn of the experimental embryos are significantly lower than the control group, Chi-square test ($p<0.001$).
[b] The rate of newborn among pregnant recipient of two treatments groups are significantly lower than the control group, Chi-square test ($p<0.001$).
[c] The rate of newborn obtained from embryos that experienced the 20-p treatment is significantly lower than the one obtained from those that experienced the CFW Chi-square test ($p=0.02$).
[d] The rate of newborn obtained from embryos that experienced the 20-p is significantly lower than the two other groups, Chi-square test ($p<0.001$).
[e] The rate of newborn among pregnant recipient that received the 20-p embryos is significantly lower than the two other groups, Chi-square test ($p<0.001$). Chi-square test ($\alpha=0.05$).

The CFW treatment likely depleted Ca^{2+} stores, and in fact such was the case. Treating fertilized eggs that were subjected to the CFW treatment with ionomycin before PN formation resulted in release of less Ca^{2+} than control fertilized eggs (Fig. 6A). Thus, the observed reduced development to term could have been due to a low intracellular Ca^{2+} concentration, e.g., by reducing the rate of protein synthesis during this period of time (Lawrence et al., 1998; Paschen and Doutheil, 1999). This was unlikely to be the case because perfusion of fertilized eggs with Ca^{2+}-free medium for 5 h following PN formation, which substantially depleted Ca^{2+} (Fig. 6B), had no apparent effect on development to term (Table 2, Group 2). Moreover, the resting levels of $[Ca^{2+}]_{cyt}$ were essentially the same before PN formation for the CFW and control groups (0.80 and 0.82, respectively), as well as after PN formation for the CFW and control groups (0.75 and 0.80, respectively). Thus, although the CFW treatment reduced the size of the intracellular Ca^{2+} store, cytoplasmic Ca^{2+} appeared to be buffered and relatively unaffected. These results strongly suggest that the effects of this treatment on gene expression and developmental competence (see below) are linked to suppressing the last Ca^{2+} transients in the cytosol (Fig. 4) rather than a consequence of markedly reducing $[Ca^{2+}]_{cyt}$ in freshly inseminated eggs.

Eggs subjected to 20-p following PN formation displayed a decrease incidence of development to term, the decrease being quite similar to that observed when the 20-p was given prior to PN formation (Table 2, Group 2). A series of control experiments minimized the possibility that the treatment by

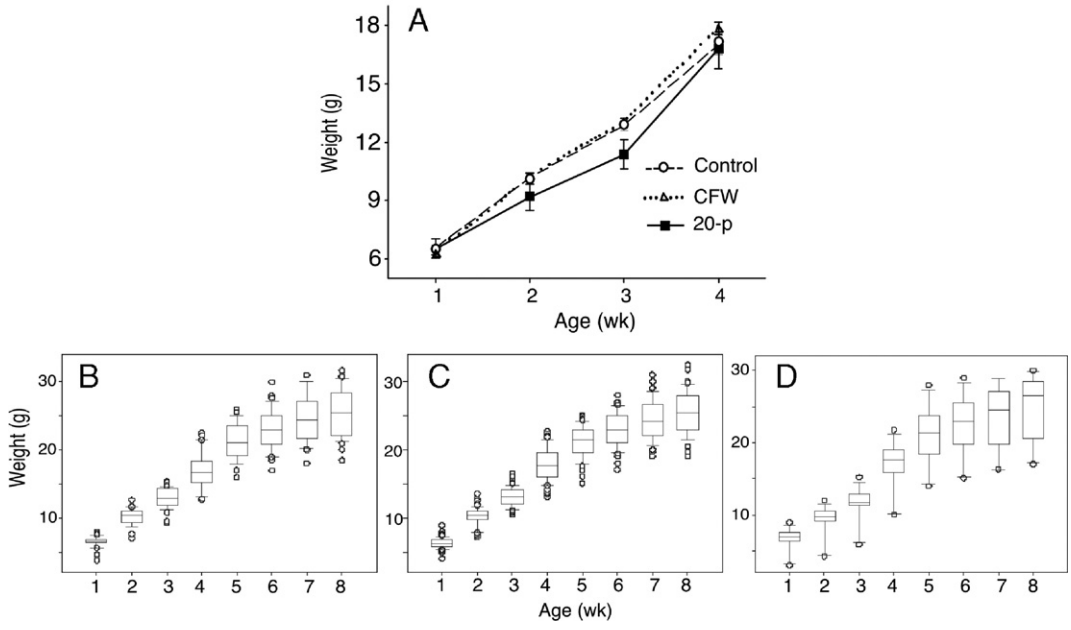

Fig. 5. Post-natal growth rate of offspring following either CFW or 20-p treatment. (A) Growth rate during first 4 weeks after birth. The difference between the 20-p group ($n=12$) and the CFW ($n=58$) and control ($n=34$) at 3 weeks is significant. Data are mean±SEM. The size variation of the three treatments during growth is shown in panels B–D. The box plot graphs shows the range of weight values recorded every week for 2 months for the control (B), CFW (C), and 20-p (D) treatments. The height of each box shows the range within which 50% of the values fell, and the median value is represented by a transverse bar inside the box. The error bars above and below the box indicate the 90th and 10th percentile. In addition, the outlying values are represented by a circle.

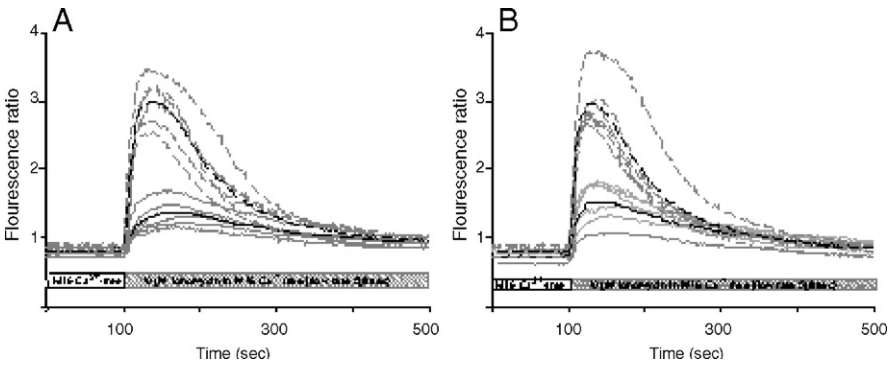

Fig. 6. Comparison of ionomycin-induced Ca^{2+} transients in freshly fertilized eggs (A) or eggs at PN stage (B) subjected to CFW treatment. (A) The solid grey lines represent the Ca^{2+} profiles of 6 newly fertilized eggs that were subjected to the CFW treatment and then subjected to a wash in Ca^{2+}-free medium containing 10 μM ionomycin. The dark line represents the average values of the 6 individual records. The dashed grey lines represent the Ca^{2+} profiles of 6 untreated but fertilized eggs monitored at the same time as the treated group. The dark dashed line represents the average values of these 6 records. (B) The same recording but with eggs that have been subjected to CFW after PN formation. The solid lines represent the response to ionomycin of 6 eggs subjected to the CFW treatment and the dark line represents the average record. The dashed lines show the response of the untreated but fertilized egg subjected to the ionomycin treatment at the same time. The bars under the graphs represent the washing protocol. The peak ratios of the CFW-treated eggs significantly differed from controls ($p<0.001$, Student's *t*-test) but not different between them (Student's *t*-test). The amplitude of the ionomycin-induced Ca^{2+} signals was recorded simultaneously on an experimental and a fertilized egg held by two pipettes and perfused with Ca^{2+}-free M16 medium containing 10 μM ionomycin (flow rate of 5 $\mu l\text{-}s^{-1}$).

itself compromised development as opposed to being simply due to perturbing Ca^{2+} signaling events. The two records shown in Fig. 3 addressed the effect of the washing process and electrical pulses and demonstrated that (1) subjecting a fertilized egg every 2 min to an electrical pulse of 1.6 kV cm^{-1} in a non-conductive solution did not induce any perturbation in the natural Ca^{2+} regime, (2) the washing protocol efficiently cleared all ions from the culture medium before the next electrical pulse and prevented any membrane damage brought about by residual ions, and (3) the high rate of live young from fertilized eggs subjected to this treatment (35 out of 63, i.e., 56% that was not significantly different from the control group) ensured that these interventions did not cause any side effects. Hence under these experimental conditions, the physiological effect relies solely on the Ca^{2+} concentration present in the glucose solution. In this case, the transmembrane Ca^{2+} influx can itself be deleterious. To ascertain if this was the case, we subjected freshly fertilized eggs to a treatment that approximated the physiological number of Ca^{2+} spikes, i.e., 24 pulses of 1.4 kV cm^{-1} every 8 min in Ca^{2+}-free medium (see Fig. 7). The high incidence of live young obtained after such treatment (61 out of 99, 62%, Chi-square not significantly different from the control) strongly suggested that the combination of washing protocols and the electric field pulses was not deleterious *per se*, but rather the level of $[Ca^{2+}]_{cyt}$ experienced by the eggs during the process of fertilization was responsible for the observed developmental effects.

Effect of inhibiting or stimulating Ca^{2+} signaling events on implantation and post-implantation development

The reduced developmental potential of the experimental eggs could be due to reduced implantation, reduced fetal development, or a combination. The observation that the 20-p protocol (Group 1) resulted in offspring that displayed greater size variation than their CFW counterparts (Group 1) suggested that the reduced developmental competence observed with the two protocols could result from differential effects on implantation and fetal development. To determine the basis for the observed reduced developmental potential, embryos

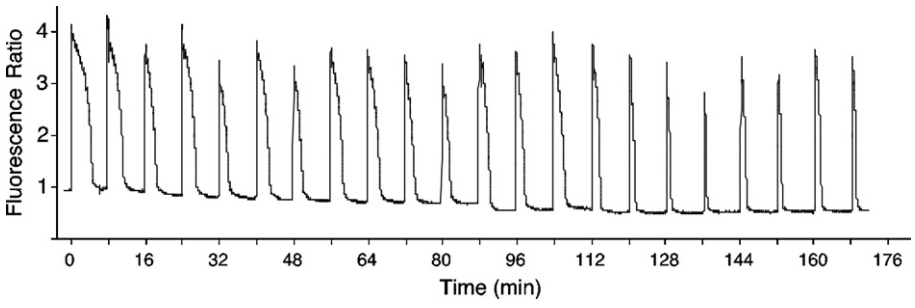

Fig. 7. Calcium pattern of freshly fertilized egg subjected to the CFW treatment and a regime of intracellular Ca^{2+} stimulation by a series of 24 electrical pulses. This record shows that the CFW treatment suppresses the spontaneous oscillations during the interval between Ca^{2+} stimulation, thereby making it possible to impose a pattern that approximates the physiological number of Ca^{2+} transients. Nevertheless, the width of the Ca^{2+} signal transient is progressively shortened with time indicating that less Ca^{2+} is released; this agrees with the progressive decrease of the intracellular Ca^{2+} due to depletion of the intracellular Ca^{2+} shown in Fig. 5. Shown in the recording are 22 of the 24 pulses that were administered.

were treated prior to PN formation using one of the two protocols and their development was assessed on D10 by counting the number of fetuses per number of implantation sites and the number of fetuses per number of eggs transferred for those mice that became pregnant (Table 3).

The CFW treatment resulted in a significant decrease in implantation relative to controls (53% vs. 83%). Of those embryos that implanted 74% developed into fetuses, which was similar to the 84% of control embryos that implanted and developed into fetuses. Thus, although implantation was compromised, the developmental competence those embryos that did implant appeared unaffected.

A different situation was observed with embryos generated by the 20-p protocol. In this case, there was no significant difference in the incidence of implantation relative to the control (74% vs. 83%). The developmental competence of these embryos, however, was reduced because only 54% of them developed into a fetus, compared to 84% of the controls. Thus, the decrease in development to term observed following the two treatments was achieved by different mechanisms. As expected, the number of fetuses per implantation site in those mice that were pregnant (70% (38/54), 40% 923/58), and 40% (20/50) for the control, CFW, and 20-p groups, respectively) was remarkably similar to the incidences of development to term in the same treatment group (Table 2).

Effect of inhibiting or stimulating Ca^{2+} signaling events on gene expression in blastocysts

The previous results demonstrated a differential effect of the CFW and 20-p treatments on the ability of blastocysts to implant or develop post-implantation. We hypothesized that the two treatments ultimately led to differences in gene expression, which underlie the observed differences. To explore this possibility, global patterns of gene expression in control and treated blastocysts (Group 1) were analyzed by microarrays.

To minimize false positives, only transcripts called as "Present" in at least 3 out of 4 replicates in each group were taken as detected. A total of 15340, 16304, and 17075 probe sets were detected in the control, 20-p, and CFW groups, respectively, and this translates to 9953, 10259, and 10608 Unigenes. An unsupervised hierarchical cluster analysis using all genes detected revealed that each group clustered independently, indicating that the global gene transcription was significantly affected by the treatments in developing embryos, and a clustering dendogram revealed that the 20-p group was more similar to the control than the CFW treatment group (Fig. 8). The number of differentially expressed probe sets was much higher for the CFW treatment group than the 20-p treatment group when compared to the control (Table 4). Noteworthy is that the majority of differentially expressed transcripts were down-regulated in the CFW group, whereas the majority were up-regulated in the 20-p group. Gene lists for the transcripts that exhibit a 2-fold difference in expression are found in Table S2. It should also be noted that similar results were obtained when all transcripts detected on the chip were subjected to clustering analysis (data not shown).

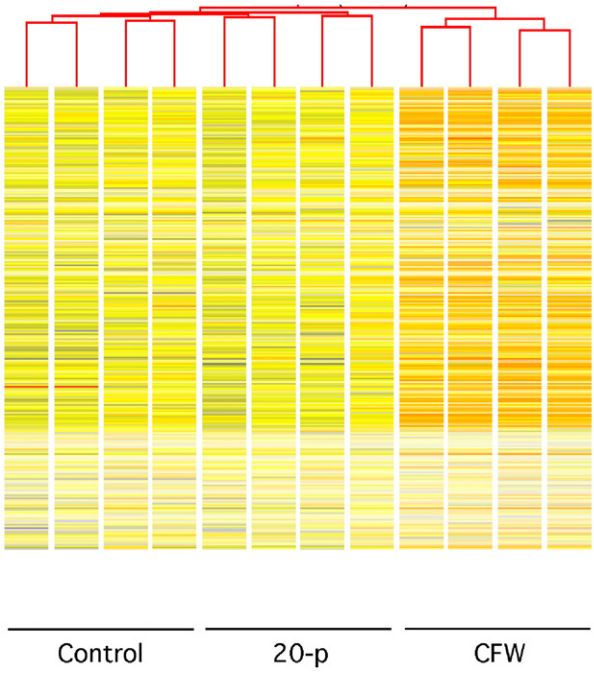

Fig. 8. Hierarchical cluster analysis. Hierarchical clustering of all samples obtained from control, 20-p, CFW treatment groups is shown.

The differentially expressed transcripts at the 1.4-fold level were subjected to EASE analysis. EASE facilitates interpreting gene lists derived from microarray experiments by providing statistical methods (reported as an EASE score) for discovering

Table 3
Effect of treatments on implantation and post-implantation development

Treatment	# replicates	# egg transferred	# recipients	# pregnant recipients (%)	# implantations (%)	Rate of implantation of pregnant recipients (%)	# normal fetuses (%)	# resorptions (%)
Control	4	54	6	6 (100)	45 (83)	45/54 (83)	38 (84)	8 (18)
CFW	3	72	11	9 (82)	31 (43)[a]	31/58 (53)[b]	23 (74)	8 (26)
20-p	3	70	7	5 (71)	37 (53)[a]	37/50 (74)	20 (54)[c]	18 (49)[d]

Note that for both the control and 20-p groups that the percentages of the number of normal fetuses and resorptions do not add up to 100% because in both cases two fetuses were found at a single implantation site.

[a] Implantation rates of the two treated groups are significantly lower than the control group, Chi-square test ($p<0.001$).

[b] Implantation rates of pregnant recipient that receive the CFW embryos are significantly lower than the two other groups, Chi-square test ($p\leq0.045$).

[c] The rate of normal fetuses per implantation of the embryos that experienced the 20-p treatment is significantly lower than the control group, Chi-square test ($p=0.006$).

[d] The rate of fetuses resumptions among the 20-p embryos is significantly lower than the control group, Chi-square test ($p=0.006$). Chi-square test ($\alpha=0.05$).

Table 4
Pair-wise comparison of number of genes differentially expressed

Pair-wise comparison	Probesets detected	Unigenes detected	Probesets expressed differentially (~5%FDR)								
			1.4×			2×			5×		
			Total	Up	Down	Total	Up	Down	Total	Up	Down
Control vs. 20-p	16,737	10,540	464	406	58	79	73	6	2	2	0
Control vs. CFW	17,597	10,926	3269	748	2521	240	82	158	6	6	0

biological themes within gene lists. EASE unmasks biological themes by identifying functional gene categories that are overrepresented (Hosack et al., 2003). Note that overrepresentation does not refer to the abundance of gene expression but rather describes a class of genes that have similar functions that appear more often in a list of interest than would be predicted by their distribution among all genes assayed (for more discussion about EASE, see Zeng et al., 2004).

EASE analysis revealed that genes involved in RNA polymerase II-mediated transcription, mRNA processing, and cell cycle/proliferation were preferentially mis-expressed in blastocysts derived following the CFW treatment (Table 5). In contrast, genes involved in metabolism were preferentially mis-expressed following the 20-p treatment. Thus, differences in the Ca^{2+} oscillatory pattern differentially alter the global pattern of gene expression in the resulting blastocysts such that different biological processes appear to be affected. Gene lists for each treatment (or group) for the EASE analysis are found in Table S3.

Cell adhesion molecules play an essential role in implantation. Accordingly, we analyzed expression of 347 genes known to be involved in adhesion. Of note was that expression of 35 genes involved in cell adhesion was significantly reduced by at least 40% in blastocysts derived from the CFW treatment, but not from the 20-p treatment. Among these genes are integrins, cadherins, and collagen (see Table S4 for a complete gene list). Whether these changes are linked to the observed decreased incidence of implantation remains to be established. Last, the developmental potential of only a subpopulation of blastocysts is compromised. Therefore, the observed differences in gene expression would likely be more pronounced if one could identify and analyze this subpopulation.

Table 5

Gene category	List hits	EASE score
EASE of selected biological processes for CFW treatment group [a]		
RNA splicing	20	2.87E-03
Cell cycle	78	5.56E-03
Transcription from pol II promoter	34	1.48E-02
RNA processing	32	2.30E-02
EASE of selected biological processes for 20-p treatment group [a]		
Metabolism	63	2.48E-04
Macromolecule metabolism	34	4.80E-03
Lipid metabolism	10	5.83E-03
Carbohydrate metabolism	7	2.78E-02

[a] EASE was performed with genes that displayed a 1.4-fold difference in expression.

Discussion

The results described here constitute the first demonstration that the Ca^{2+} signaling events, which are restricted to the first few hours of development following fertilization and terminate with PN formation, can be manipulated with a sufficient level of accuracy and without detrimental effects to reveal that there are long-term effects on development to term. Although perturbing the Ca^{2+} oscillatory pattern does not impact development to the blastocyst stage, the developmental potential of the resulting blastocysts is reduced; premature termination of the signaling events results in blastocysts whose ability to implant is compromised, whereas hyperstimulation results in an increased incidence of post-implantation failure.

The most obvious and potentially important linkage between the Ca^{2+} signaling events and these differences in developmental potential is at the level of gene expression. In the preimplantation mouse embryo, there are three periods during which there are pronounced changes in gene expression; (1) the maternal-to-zygotic transition that initiates during the 1-cell stage and is essentially completed by the 2-cell stage, (2) compaction that occurs during the 8-cell stage and results in the formation of the first epithelium, and (3) blastocyst formation that entails the first overt events of cellular differentiation with the formation of the totipotent inner cell mass (ICM) and the differentiated trophectoderm.

The changes in gene expression that occur during these three developmentally critical transitions are likely interconnected. For example, activation of gene expression during the maternal-to-zygotic transition entails a dramatic reprogramming of gene expression (Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004; Zeng and Schultz, 2005) and presumably is required for those changes in gene expression that occur during compaction, which in turn are required for the changes in gene expression that accompany blastocyst formation. In other words, there is a linear and temporally coupled sequence of changes in gene expression. In fact, such waves of transcription have been observed in a microarray study (Hamatani et al., 2004). The correct execution of this cascade of changes in gene expression is believed to be required for successful development to the blastocyst stage and beyond. The results described here are consistent with the hypothesis that the Ca^{2+} signaling events that occur in the narrow window of time between the sperm-induced rise in intracellular Ca^{2+} and PN formation encode information that directs the initial reprogramming of gene expression. This reprogramming subsequently leads to the aforementioned

cascade of changes in gene expression, resulting in developmentally competent blastocysts.

Results from our previous studies indicated that the Ca^{2+} transients coordinate temporally events that constitute egg activation and thereby insure that these events occur in the proper temporal fashion. For example, cortical granule exocytosis, which leads to the *zona pellucida* block to polyspermy, requires fewer Ca^{2+} impulses than cell cycle resumption (Ducibella et al., 2002). Establishing a polyspermy block before cell cycle resumption minimizes the likelihood of supernumerary sperm participating in the impending cell cycle, and thereby generating a polyploid embryo. CaMKII appears to be the major target of Ca^{2+} action. CaMKII activity oscillates in parallel with the Ca^{2+} oscillations (Markoulaki et al., 2003, 2004) and expressing a constitutively active form of CaMKII results in all the events of egg activation in the absence of any change in intracellular Ca^{2+} (Madgwick et al., 2005). In fact, expressing a constitutively active form of CaMKII in eggs injected with a sperm whose PLC-ζ activity has been inactivated can result in offspring (Knott et al., 2006).

Genome activation, which initiates in the 1-cell embryo, requires recruitment of maternal mRNA (Aoki et al., 2003) and therefore is a late event of egg activation that likely requires the full complement of Ca^{2+} oscillations. Recruitment of maternal cyclin A2 mRNA, in collaboration with CDK2, appears to be a critical event because genome activation in 1-cell embryos is inhibited when maternal cyclin A2 mRNA is targeted by an siRNA, and microinjection of recombinant cyclin A2-CDK2 protein increases transcriptional activity in 1-cell embryos (Hara et al., 2005). The finding that the 20-p treatment group clusters with the control group is consistent with the proposal that reprogramming is a late event. Also consistent with this proposal is that there are significantly more mis-expressed genes following the CFW treatment. Thus, reprogramming of gene expression following CFW treatment may deviate significantly from what normally occurs, generating a ripple effect that is magnified in the blastocyst. Genes involved in transcription and RNA processing are preferentially mis-expressed in blastocysts following the CFW treatment. Of interest is that these types of genes are preferentially expressed during genome activation (Zeng et al., 2004; Zeng and Schultz, 2005). Future studies will examine reprogramming of gene expression in 2-cell embryos that are generated by the 20-p and CFW treatments.

The Fetal Origins of Disease (FOAD) hypothesis, a.k.a., the Barker hypothesis (Hales and Barker, 2001), proposes that fetal adaptations *in utero* to maternal under-nutrition or malnutrition can lead to specific diseases in the adult, including coronary heart disease, high blood pressure, and type II diabetes, i.e., perturbations occurring during early during development can have long-term effects in the offspring. FOAD can even be extrapolated back to preimplantation development. A low protein diet restricted to preimplantation stage in rats led to changes in birthweight, postnatal growth rate, hypertension, and organ/body-weight ratios in either male or female offspring (Kwong et al., 2000). Moreover, simply culturing preimplantation mouse embryos from the 2-cell to blastocyst stage prior to embryo transfer can result in behavioral alterations in the elevated zero maze (which reflects anxiety) and Morris water maze (which measure spatial memory) tasks in the offspring (Ecker et al., 2004), and embryo culture *in vitro* results in perturbations in the global pattern of gene expression (Rinaudo and Schultz, 2004). In fact, perturbations that occur during the 1-cell stage may become manifest only following implantation. For example, exposure of female mice to ethylene oxide under conditions in which the mutagen acts only during the early 1-cell stage can result in fetal abnormalities (Rutledge et al., 1992).

The finding that differences in the Ca^{2+} signaling pattern can have effects not only on implantation and post-implantation development but also long-term effects on the weight variation in the offspring suggests that FOAD can be extrapolated back to the first few hours following fertilization. If so, our findings have direct implications for use of Assisted Reproductive Technologies (ART) in treating human infertility; in developed countries it is estimated that 1–3% of children born are conceived by ART (Maher, 2005). Intracytoplasmic sperm injection (ICSI) is routinely used to treat male infertility. Of potential concern is that sperm used for ICSI and containing low amounts of PLC-ζ could trigger enough Ca^{2+} oscillations sufficient to initiate development but insufficient to support development to term. Also of concern is that ICSI entails breaching the plasma membrane, which results in a Ca^{2+} influx while plasma membrane integrity is being restored. The entering Ca^{2+} can trigger spontaneous Ca^{2+} transients due to CICR, and we have observed such high frequency Ca^{2+} oscillations after fertilization when membrane integrity is disrupted (Ozil, unpublished observations).

It is now recognized that there are risks associated with ART that range from an increase in loss-of-imprinting disorders (Maher, 2005) to birth defects (Hansen et al., 2005) to low birth weight even in singleton pregnancies (Bower and Hansen, 2005; De Geyter et al., in press). Future studies will explore the effect of manipulating Ca^{2+} oscillations on expression of imprinted genes and behavior of the offspring.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.041.

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