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Use of soluble sperm extract to improve cloning efficiency in zebrafish

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ABSTRACT During somatic cell nuclear transfer (SCNT), egg activation is required to initiate embryonic development. In zebrafish cloning, the reconstructed egg is activated by exposing it to hypotonic water. Egg activation using water-only is not capable of activating the same intracellular calcium release as fertilization which is required for proper embryonic development. Here we test whether the use of soluble sperm extract (SSE) can properly modulate the activation of reconstructed eggs during SCNT. We microinjected SSE from genomic-inactivated zebrafish sperm into unfertilized eggs and reconstructed eggs right after somatic cell nuclear transfer. We also evaluated the most effective approach for SSE microinjection. Microinjection of SSE (with 0.68 mg/ml of protein concentration) into non-activated eggs through the micropyle induced parthenogenetic development beyond the blastula stage, whereas all water-only activated eggs failed to enter the cleavage period. Microinjection of SSE at 1 mg/ml of protein concentration into non-activated reconstructed egg improved the developmental rate of cloned embryos in comparison to non-injected control clones. The cumulative survival time of cloned embryos injected with SSE was significantly longer than reconstructed eggs activated following sham injection (P<0.01). No significant difference was found among controls (P=0.32). SSE benefits both parthenogenesis and the survival cloned embryos which have never been reported in zebrafish. Further work is necessary to define the functional component(s) of SSE as well as the physiological pathway, to understand its principle of action and advance the utilization of SSE in cloning.

KEY WORDS: *zebrafish, soluble sperm extract, somatic cell nuclear transfer, cloning*

Introduction

Somatic cell nuclear transfer (SCNT), is an artificial reproductive technique used to produce cloned animals. It consists of transferring a somatic nucleus into an enucleated oocyte that will subsequently develop like a naturally conceived zygote. The enucleated oocyte is assumed to contain reprogramming factors necessary to remodel the somatic cell's nucleus into an embryonic one. SCNT has been used to create transgenic animals (Cibelli *et al.*, 1998). In 2002, Lee and colleagues were the first to describe SCNT in zebrafish, using a protocol that was later improved by Siripattarapravat and colleagues (2009). Successful cloning, however, occurs only rarely in most species. Zebrafish is not an exception since only two to three percent of reconstructed zebrafish embryos survived to become adult fish. Among the most critical factors known to impact the efficiency of

Abbreviations used in this paper: SCNT, somatic cell nuclear transfer; SSE, soluble sperm extract; SSE-high, SSE with high protein concentration; SSE-low, SSE with low protein concentration; SSE-micropyle, microinjection of SSE through a micropyle; SSE-diffusion, microinjection of SSE by an electronic microinjector; UV-sperm, ultraviolet-irradiated sperm.

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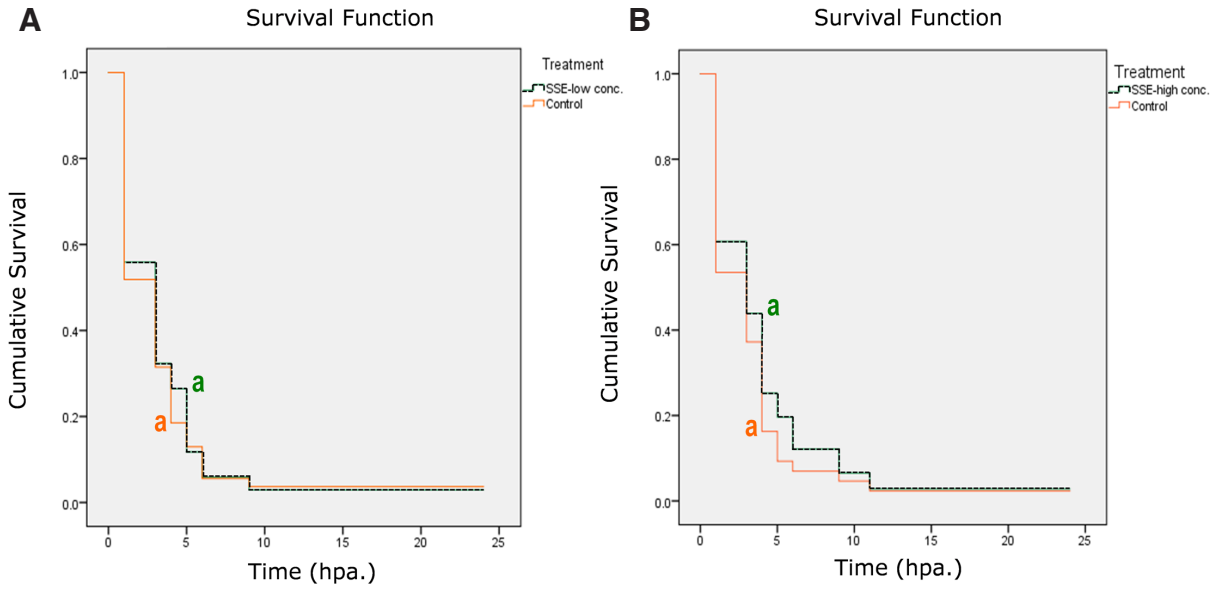

Fig. 1. Survival analyses of cloned embryos derived from two different protein concentrations of soluble sperm extract (SSE) *(experimental step B). Cumulative survival times of clones derived from SCNT injected with SSE batches containing 0.2 mg/ml (SSE-low)* **(A),** *and 1 mg/ml (SSE-high)* **(B)** *concentration of protein carried out by the SSE-micropyle procedure. Log-rank tests showed no significant differences between SSE-low and control (P = 0.66) groups, or between SSE-high and control (P=0.27) groups. Time is indicated in hours postactivation (hpa).*

SCNT, is getting the recipient oocyte/egg to engage in biochemical events leading to resumption of meiosis and egg activation in the absence of sperm. Unlike fertilized zygote, clone embryo lacks not only paternal genome contribution but also the cytoplasmic portion of the sperm head, and that plays an important part for egg activation.

In zebrafish, egg activation spontaneously occurs when the egg contacts hypotonic water (Wu and Kinsey, 2000) and we have adopted this activation in the cloning protocol. This activation event triggers the resumption of meiosis and cytoplasmic rearrangement (Siripattarapravat *et al.*, 2009). A spontaneously activated egg precedes morphological changes and forms a blastodisc, which, although is morphologically indistinguishable from a one-cell zygote, undergoes no further division. However, only in some cases fragmentation is observed, resembling asymmetrical cell cleavage at the blastodisc. While both spontaneous activation and fertilization release intracellular calcium, they exhibit differences as at what site its release starts (Sharma and Kinsey, 2008). In less than 30 seconds after sperm fusion, release of intracellular calcium is first detected at the cortex of an egg's cytoplasm; it then propagates as a wave to the center of the cytoplasm. In spontaneously activated eggs, there is a lack of a calcium release at their cortex, coinciding with an absence of Fyn kinase activity, an upstream element of the calcium cascade that only appears in fertilized eggs (Wu and Kinsey, 2000). In the absence of sperm, calcium releasing dynamics are incomplete, leading to a developmental failure of the sort that occurs during spontaneous activation.

Role of sperm in triggering intracellular calcium-release is evolutionary conserved, ranging from mammals (Fissore *et al.*, 1993) to ascidian (Runft and Jaffe, 2000). As demonstrated in the mouse model, following injection into an unfertilized oocyte, the cytosolic fraction of sperm is responsible for calcium oscillations (Gordo *et al.*, 2000). These experiments were later validated in horse, human, and cow where parthenogenetic development was induced in unfertilized oocytes (Bedford *et al.*, 2003; Rogers *et al.*, 2004; Ross *et al.*, 2008). Moreover, sperm extract can promote the *in vitro* activation of cloned horse embryos, and doubling the rates of embryonic development when compared to artificial egg activation using chemicals alone (Hinrichs *et al.*, 2007).

We hypothesize that any procedure capable of emulating the sperm's role in egg activation, is likely beneficial to the development of cloned zebrafish. In this study, we evaluated whether the delivery of zebrafish soluble sperm extract (SSE) could activate the egg. We compared the developmental potential of the SSE activated eggs to those activated gynogenetically, i.e., using genome-inactivated sperm to fertilize the egg. We determined the amount of SSE needed to deliver as to mimic more closely an amount of a fertilized sperm. Finally, we used SSE during SCNT, and analyzed the survival rate of cloned zebrafish at specific stages of development. Here our data show that injection of the soluble fraction of sperm into unfertilized eggs promotes spontaneous activation resulting in the derivation of parthenogenetic embryos that survive until blastula stage. We also report the lengthening of the cumulative survival time of cloned zebrafish using SSE.

Experimental Protocols

Zebrafish

The zebrafish used in this study were maintained with the approval of the Institutional Animal Care and Use Committee (IACUC), Michigan State University. We obtained the AB and Tuebingen strains from the Zebrafish International Resource Center (ZIRC), receiving 50 pairs per line; the fish had an average age of six to ten months old. Fifty pairs of GloFish, Tg(*mylz2*:EGFP), were purchased from a local pet store. We generated the TAB

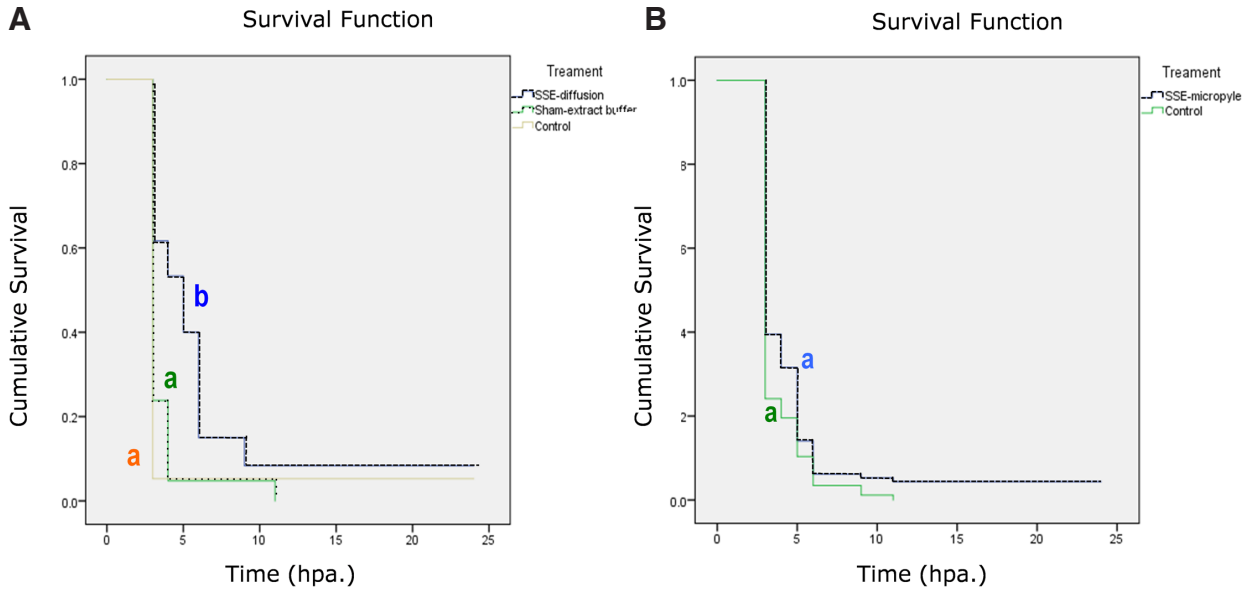

Fig. 2. Survival analyses of cloned embryos derived from two different microinjection approaches *(experimental step C). Cumulative survival times of clones derived from SCNT injected with SSE batch containing 1 mg/ml concentration of protein, using the SSE-diffusion method* **(A)** *and the SSE-micropyle method* **(B)**. *The log-rank test showed a significant difference between the SSE-diffusion and the control groups (P<0.01), while no significant difference was observed between the sham and control groups (P=0.32). The log-rank test showed no significant difference (P = 0.17) between the SSE-micropyle and control groups. Time is indicated in hours postactivation (hpa).*

crossbreed by crossing AB and Tuebingen strains. Adult TAB fish were then used as egg donors for all experiments in this study.

Preparation of soluble sperm extract

Following the protocol described by Westerfield (2007), we collected the milt of twenty to thirty transgenic males, Tg(*mylz2*:EGFP). Pool milt was evaluated for its sperm concentration using hemocytometer, and sperm quality was assessed by established criteria (Hagedorn and Carter, 2011) (supplementary protocol 1). We genomically inactivated sperm with UV radiation (900 microjoules/cm^2; Walker *et al.*, 2009) suspended in extract buffer-1 prior to sonication. Later, extract buffer-2 was added to the lysate. We then centrifuged sperm lysate (16,100 G at 4˚C for 30 minutes), collected the liquid phase (i.e., the SSE), and measured the SSE's protein concentration using Nanodrop spectrophotometer before storing it at -80˚C (supplementary protocol 1).

Gynogenesis and ploidy manipulation

UV-sperm were prepared from transgenic males [Tg(*mylz2*:EGFP)]. To produce diploid gynogenetic embryos, we performed *in vitro* fertilization (IVF) of wild-type (WT) eggs (TAB crossbreed) with UV-irradiated sperm (Walker *et al.*, 2009) and subjected the embryos to a ploidy manipulation technique known as heat shock (Westerfield, 2007). After applying the heat at a specific period of time, fertilized eggs were cultured in 28.5˚C maintenance chamber. We recorded the number of developing embryos and observed the morphological appearances as well as the head-to-tail length of the embryos (supplementary protocol 2).

Soluble sperm extract microinjections

We conducted two approaches of microinjection in the present study. We microinjected SSE into non-activated eggs through the micropyle (SSE-micropyle), a sperm entry site. This approach as well as the manipulation instruments are similar to the protocol of cell transplantation used in the SCNT process (Siripattarapravat *et al.*, 2016). This approach yielded a range for the average injected volume of 1.56 to 1.76 nl. We designed a second approach to microinjecting SSE (SSE diffusion) in order to emulate the normal chronology of natural fertilization. We used an electronic microinjector (FemtoJet, Eppendorf) together with a specific custom-made injection needle, and a gel-coated manipulation dish. An automatic direction controller (InjectMan, Eppendorf) was used to perform the microinjection at the spherical center of the egg, and then the extract was allowed to diffuse from the injection site to the cytoplasmic rim. To obtain a precise timing for microinjection, we validated the diffusion time using Time-lapse fluorescent imaging. This approach yielded an average volume of injection of 4.21 nl (supplementary protocol 3).

Somatic cell nuclear transfer

Crossbred TAB Female were carefully selected as an egg donor. Nucleus donor cells were obtained by manually dissecting the tail tips of naturally fertilized Tuebingen embryos at the twenty-somite stage. Following the SCNT procedure by Siripattarapravat (2016), we collected the non-activated eggs and maintained them in Chinook salmon ovarian fluid (CSOF). We stained its DNA and subjected the egg to laser genomic inactivation followed by microinjection of donor cell via the micropyle. After incubating the reconstructed eggs in CSOF for 15 minutes, we placed them in embryo medium (10 percent HBSS (v/v) in sterile water) to spontaneously activate the eggs. We recorded the number of live embryos observed at each designated stage of development. Finally, we performed life table analyses with log rank tests in SPSS Statistics (version 24, IBM).

TABLE 1

EXPERIMENTAL PLAN

Step	Experiments	Protein concentration of SSE	Delivery approaches	Control group(s)
A	SSE induces parthenogenetic development	0.68 mg/ml	Microinjection via micropyle	- Parthenogenesis by spontaneous activation - Haploid gynogenesis by UV-sperm fertilization - Diploid gynogenesis by Heat-shock
B	Evaluation of SCNT efficiency following supplemented with two different protein concentrations of SSE	0.2 mg/ml	Microinjection via micropyle	- Normal SCNT
		1 mg/ml	Microinjection via micropyle	- Normal SCNT
C	Evaluation of SCNT efficiency following supplemented with SSE by two different delivery approaches	1 mg/ml	Diffusion	- Delivery SSE buffer with diffusion approach - Normal SCNT
		1 mg/ml	Microinjection via micropyle	- Normal SCNT

Sperm soluble extract was primarily tested for its ability to induce parthenogenetic development of non-activated unfertilized eggs (A), and then the applications of SSE in cloning process were evaluated varying by the differences of protein concentration of SSE (B) and the methods of delivery (C).

Results

Soluble sperm extract pushed parthenogenetically activated eggs through a cleavage stage

To test whether SSE could promote parthenogenesis, we microinjected SSE through the micropyle of unfertilized metaphase II eggs. The amount of injected SSE was closely equaled to the amount of extract from one sperm cell (Table 1 – step A and Table S1). Treated eggs were activated immediately after microinjection by placing them in embryo medium, a process designed to emulate how UV-sperm acts when used to produce haploid and diploid gynogenetic zebrafish. We used a group of spontaneously activated eggs as a control. The developmental ratios (Table 2) were calculated from four replications each of SSE microinjection (n=186) and spontaneous activation (n=120), and three replications each of haploid production (n=90) and heat-shocked gynogenesis (n=151).

Embryos from all treatments displayed signs of normal egg activation, as same as the IVF control group; exception being the group of eggs that were spontaneously activated only. Within the first 40 minutes, spontaneously activated eggs remained at the one-cell stage, though 29.2 percent (35/120) of eggs formed an asymmetrical two-cell-like structure with an undefined furrow (Figure S1B); none of these eggs managed to develop further. By the period of cleavage (2-cell stage), the haploid and diploid gynogenotes (developmental percentage at 85.6 and 92.7 respectively) had developed much further than the SSE-micropyle microinjection and spontaneous activation groups which developmental percentages were noted at 26.3 and 0, respectively (Table 2)

Although the SSE-microinjection group had a low developmental ratio, it was markedly different from the spontaneously activated egg counterpart which never underwent symmetrical two-cell division and all died off. SSE-microinjected eggs continued cell division: 12.9 percent (24/186) reached the four-cell stage (Fig. S1C), and 0.5 percent (1/186) reached the 128-cell stage (Fig. S1D). At three hours postfertilization, the percentage of developing embryos derived from both UV-sperm and heat-shocked embryos was comparable to the IVF control (data not shown). Haploid characteristics were initially noticeable at 24 hpf in all embryos fertilized by UV-sperm, including deformed heads, pericardial enlargement, and short body lengths (Fig. S2D). In the gynogenesis group, a few embryos showed signs of haploidy at 24 hpf, indicating a possible failure of artificial diploidization. While 13 of 151 (8.6%) heat-shocked embryos survived until they were two days old, only 1 embryo (0.7%) grew into an adult. Embryos derived from SSE-microinjection and gynogenesis were GFP negative, indicating no sperm genomic DNA contribution. Control IVF embryos were GFP positive (Fig. S2A).

Efficacy of soluble sperm extract

To evaluate the effect of SSE on cloned embryos, SSE batches containing 0.2 mg/ml and 1 mg/ml concentrations of protein, representing low-concentration SSE (SSE-low) and high-concentration SSE (SSE-high) were injected into cloned embryos immediately after donor cell transplantation (Table 1 – step B and Table S1) and compared to our previously reported method of activation for cloned embryos (Siripattarapravat *et al.*, 2016). All reconstructed eggs spent 15 minutes incubating in CSOF as a part of the standard protocol and then got spontaneously activated in embryo medium. Embryos in the SSE-low group (n=68) developed at slightly higher percentages during the cleavage-to-midblastula period (26.5 to 55.9 %) than the control (n=54) group, which had development percentages ranging from 18.5 to 51.9 %; however, we observed no marked differences later (Table 3 – step B/ SSE-low).

Embryos in the SSE-high group (n=107) developed better than those of the control group (n=43), with a higher percentage of clones surviving at almost every stage observed (Table 3 – step B/SSE-high). Although the percentage of development was higher in SSE-high group than control, there was no statistical difference (P=0.27) in terms of the cumulative survival time of clones (Fig.

TABLE 2

PARTHENOGENETIC DEVELOPMENT OF ZEBRAFISH EGGS DERIVED FROM SSE SUPPLEMENTATION AND THE THREE CONTROLS FROM THE EXPERIMENTAL STEP A

Experimental groups (step A)	Developmental percentages (% ±SE) 2-cell (40 mpa)	4-cell (60 mpa)	128-cell (2 hpa)	50% eb (5 hpa)	D1	D2
SSE microinjected eggs	26.3±6.8	12.9±3.2	0.5±0.5	0.0	0.0	0.0
Spontaneously activated eggs	29.2±2.1	0	0	0	0	0
UV-sperm fertilized embryos	85.6±1.2	83.3±2.1	83.3±2.0	55.6±4.2	51.1±3.1	24.4±2.1
Heat-shocked embryos	92.7±19.5	87.4±21.7	78.8±26.4	51.0±22.7	35.1±15.6	8.6±3.1

Developmental percentage of SSE-microinjected eggs (n=186), spontaneously activated eggs (n=120), UV-sperm-fertilized eggs (n=90), and heat-shocked eggs (n=151). The percentage of embryos calculated at the designated developmental stages from the two-cell stage to two-day old, at indicated time either minutes postactivation (mpa) or hours postactivation (hpa). “eb” is an abbreviation for epiboly stage.

TABLE 3

DEVELOPMENTAL PERCENTAGES OF CLONED EMBRYOS RECEIVED THE SUPPLEMENTATION OF SSE ACCORDING TO THE EXPERIMENTAL PLAN

		Developmental percentages (% ±SE)							
Step	**Experiments**	**64-cell**	**256-cell**	**High**	**Sphere**	**50% eb**	**70% eb**	**Tail bud**	**D1**
B	- SSE-low	55.9±0.7	32.4±0.9	26.5±0.6	11.8±0.7	5.9±0.3	2.9±0.3	2.9±0.3	0
	- Control	51.9±0.9	31.5±1.2	18.5±0.3	13.0±0.7	5.6±0.6	3.7±0.7	3.7±0.7	1.9±0.3
	- SSE-high	60.7±2.0	43.9±0.3	25.2±1.0	19.6±1.0	12.1±0.3	6.5±0.3	2.8±0	1.9±0.3
	- Control	53.5±1.3	37.2±1.2	16.3±0.3	9.3±0.3	7.0	4.7±0.3	2.3±0.3	0
C	- SSE-diffusion	61.7±4.5	61.7±4.5	53.3±6	40±6	15±0.5	8.3±0.5	8.3±0.5	6.7
	- Sham injection	33.3±0.5	23.8±1.5	4.8±0.5	4.8±0.5	4.8±0.5	4.8±0.5	0	0
	- Control	26.3±1.5	10.5	10.5	10.5	10.5	5.3±0.5	5.3±0.5	0
	- SSE-micropyle	44.7±2.7	39.5±2.9	31.6±2.4	14±0.6	6.1±0.4	5.3±0.2	4.4±0.3	2.6±0.4
	- Control	39.1±1.8	24.1±1.7	19.5±1.5	10.3±0.7	3.4±0.6	1.1±0.2	0	0

In step B, reconstructed eggs were injected with SSE containing 0.2 mg/ml (SSE-low) and 1 mg/ml (SSE-high) concentration of protein by microinjection through the micropyle, and the data are shown as the average of three sessions of SCNTs and controls. In step C, reconstructed eggs were injected with SSE containing 1 mg/ml concentration of protein carried out by two different methods. For the SSE-diffusion approach, data are shown as the average of two trials of SCNT and controls. For the SSE-micropyle approach, data are shown as the average of five trials of SCNT and controls. Live clones were counted in a designated developmental stage starting from cleavage period until one day-old. "eb" is an abbreviation for epiboly stage.

1B). Similarly, survival analysis demonstrated that the cumulative survival time of clones from SSE-low (P=0.66) did not differ significantly from the control group (Fig. 1A). In other words, performing SSE injection through a micropyle following nuclear transfer had no significant effect on the survival time of clones. However, microinjection with SSE at high protein concentration did provide an overall benefit over microinjection with SSE at low protein concentration.

Time-controlled microinjection of soluble sperm extract and its efficacy

Two experimental groups differed in the delivery location of SSE, SSE-micropyle and SSE-diffusion, were investigated with the same batch of SSE 1 mg/ml concentration of protein (Table 1 – step C and Table S1). In SSE-micropyle group, the SSE was microinjected at the same time that the somatic cell nucleus was injected. To emulate fertilization, we modified the delivery procedure called SSE-diffusion that delivered the extract to the center and diffuse to the cytoplasmic rim of an egg at a more specific time. To determine the time it took the injected fluid reach the cytoplasmic rim from the injection site, we evaluated diffusion of a fluorescent dye. At 8 minutes post-injection, the fluorescent dye was visible at the cytoplasmic rim (Fig. S3). So that, we microinjected SSE through the chorion of non-activated eggs at seven minutes after donor cell transplantation. This allowed us to mimic the natural events taking place during fertilization, since the diffusing extract reached the region where fertilization typically begins simultaneously with spontaneous activation that occurred right after the 15-minute incubation period ended. We performed two replications (n=60) of the SSE-diffusion approach, using two control groups: a conventional SCNT (n=19) and a sham injection of SSE extraction buffer (n=21). We also repeated five replications of SSE-micropyle (n=114; control n= 87) microinjections using the same batch of SSE.

The SSE-diffusion technique yielded cloned embryos with a higher developmental ratio during the cleavage-to-blastula period (53 to 62%) than the two controls (5 to 33%). By the time zygotic genome activation occurred (during the high-to-sphere stages), the percentage of live clones in the treatment group remained at 40 (n=24) and dropped drastically when the embryos entered the gastrula period (15%, n=9). Clones from the SSE-diffusion group survived through the segmentation period, and 6.7% (n=4) reached one day old. Both control groups had lower proportions of live clones; eventually, all of them ceased development late in the segmentation period, at the 70 percent-epiboly stage (Table 3 – step C/SSE-diffusion). The SSE-diffusion group clones had significantly higher cumulative survival times than the control clones (P<0.01), with no significant observable difference (P=0.32) among controls (Fig. 2A).

The SSE-micropyle group had a development percentage comparable to the experiment of SSE-high (Table 3 - step C/SSE-micropyle and step B/SSE-high). Cloned embryos in the SSE-micropyle group developed steadily during the cleavage-to-blastula period (32 to 45 %), at which point the percentage of live clones abruptly fell (to 14 %) at the midblastula period. Both groups had similar outcomes after using the same concentration of SSE and following the same delivery method — differing only in the batches of SSE — suggesting that in our hands, the results are repeatable. While the SSE-micropyle method produced, overall, a higher percentage of live clones than the control (Table 3 - step C/SSE-micropyle), survival analyses showed no significant difference in their cumulative survival times (P=0.17) (Fig. 2B).

Discussion

Previous works have shown that eggs activated using UV-sperm go through organogenesis even without diploidization, however if activation is done with hypotonic water only, eggs cannot divide properly (Walker *et al*., 2009). Gynogenesis highlights the impact of non-DNA components in the sperm – such as ligands that can bind to the surface receptor of eggs (Kashir *et al*., 2013) and another set of centrosomes – that play a role during development (Yabe *et al*., 2007). While SSE-induced parthenogenesis proved far less efficient than gynogenesis it was capable of overcoming the abortive cleavage that commonly occurred in spontaneously activated eggs (Table 2).

We used activated sperm to produce SSE based on an earlier study showing that activated sperm produces more embryos than inactivated sperm during ICSI (Poleo *et al*., 2001). Our SSE protein concentrations varied among batches, however we normalized them using a centrifugal evaporator. Based on our power analysis

the maximum dilution we could use for each batch of SSE was one mg/ml. Doing so provided consistent results between the SSE-high and SSE-micropyle treatment groups, minimizing – or eliminating – potential differences between SSE batches. Microinjecting SSE at a protein concentration of 1 mg/ml into reconstructed eggs through their micropyles was technically feasible however, we found that this method did not improve the cumulative survival time of the eggs (Fig. 1B and 2B). We hypothesized that the reason had to do with the timing of SSE injection. According with our reported SCNT protocol, once a cell is delivered into the enucleated oocyte, the reconstructed egg must be placed for 15 minutes in CSOF and then moved to water for activation. If SSE and water are both used for activation, it should be done simultaneously. We then revised our first method of delivering SSE along with the somatic cell nucleus (SSE-micropyle) and tested a new approach – SSE diffusion – in which the nucleus is injected through the micropyle first, and 7 minutes later, SSE is injected through the chorion, 8 minutes later the reconstructed eggs are placed in hypotonic water. The SSE-diffusion method increased the efficiency of cloned embryos when compared to SSE-micropyle. We speculate this is due to a more synchronized intracellular calcium.

Previous studies have shown that sperm egg/oocyte activation is mediated by calcium, which the sperm cytosolic fraction alone has proven to play a role in zygotic development by inducing repetitive waves of calcium in rabbit oocytes (Fissore *et al.*, 1993) and triggering a single peak of calcium in zebrafish eggs (Sharma and Kinsey, 2008). Microinjection of sperm extract can support parthenogenetic development of horse oocytes (Bedford *et al.*, 2003). Our results show that SSE improves early embryonic development of parthenogenetic and cloned embryos, however the precise mechanism whereby SSE induces the calcium-releasing cascade in zebrafish remains to be elucidated. We speculate that injections of SSE may have contributed to proper centrosome duplication during the first mitosis (Matsumoto and Maller, 2002) and thereby facilitated parthenogenetic development and improved the cumulative survival of cloned zebrafish.

Phospholipase C zeta (PLCδ) isoform is the molecule responsible for the egg-activation activity observed in soluble sperm extract of mammals (Ross *et al.*, 2008). It acts on the inositol-triphosphate (IP_3) mediated calcium releasing pathway. In ascidians, PLC gamma (PLCγ) triggers calcium release in eggs; Fyn kinase mediates its release (Runft and Jaffe, 2000). During zebrafish fertilization, Fyn kinase acts as an upstream activator of PLC gamma as well; becoming active within 30 seconds after fertilization (Wu and Kinsey, 2000). Spontaneous activation occurs without any Fyn kinase intervention. Whether SSE participates in the phosphorylation of Fyn kinase, remains to be determined

We have also optimized the delivery method for SSE using a picoinjector (FemtoJet) allowing us to precisely control the injected volume. Using this technique, we found that the tasks of somatic cell- and SSE injection can be performed by two different people, speeding up the process and facilitating control of injection timing.

In conclusion, we found that in zebrafish, injections of soluble sperm extract followed by exposure to hypotonic water can induce parthenogenetic development of unfertilized eggs, reaching stages of embryonic development beyond what can be obtained using hypotonic water only. Additionally, microinjecting SSE in SCNT reconstructed eggs improves cloning efficiency. A critical issue for injection of SSE is timing, and amount of SSE injected. Further experiments are required to further develop this protocol, with the goal of imitating the events trigger by the sperm at fertilization. Although this study did not determine the mechanism by which SSE acts on its active target(s), numerous studies have found evidence for its association with the release of calcium. Future work should focus on the connection of SSE with Fyn kinase and phospholipase C. The isolation of the active molecule(s) present in zebrafish SSE along with dosage optimization and timing of microinjections hold great promise to improve the efficiency of SCNT.

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