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SUPPLEMENTARY MATERIAL

corresponding to:

Use of soluble sperm extract to improve cloning efficiency in zebrafish

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Supplementary Protocols

1. Preparation of soluble sperm extract

Sperm used for SSE derivation and gynogenetic activation passed quality control and genomic inactivation parameters. We use GFP-positive males for SSE preparation. Milt was collected and quality tested prior to be used only if it fulfilled the following criteria: (1) pooled milt had to contain 90 percent or more progressively motile spermatozoa; (2) sperm concentration should equal or exceed $1x10^6$ cells/ml; and, (3) when used for IVF, the milt had to produce fertilized embryos that develop at a rate of at least 90 percent at 24 hpf (Hagedorn and Carter, 2011). We also tested for complete genomic inactivation by performing IVF with ultraviolet-irradiated sperm (UV-sperm). The absence of sperm-derived transgene expression in all of the activated embryos was considered as successful sperm's DNA inactivation. We lysed the sperm by sonication (Fig. S4) and tested it again for its ability to fertilize, i.e., their use in IVF produced no viable embryos. The SSE used in this study came from activated sperm. We added hypotonic extract buffer (extract buffer-1) immediately after sperm collection to activate the sperm before SSE isolation.

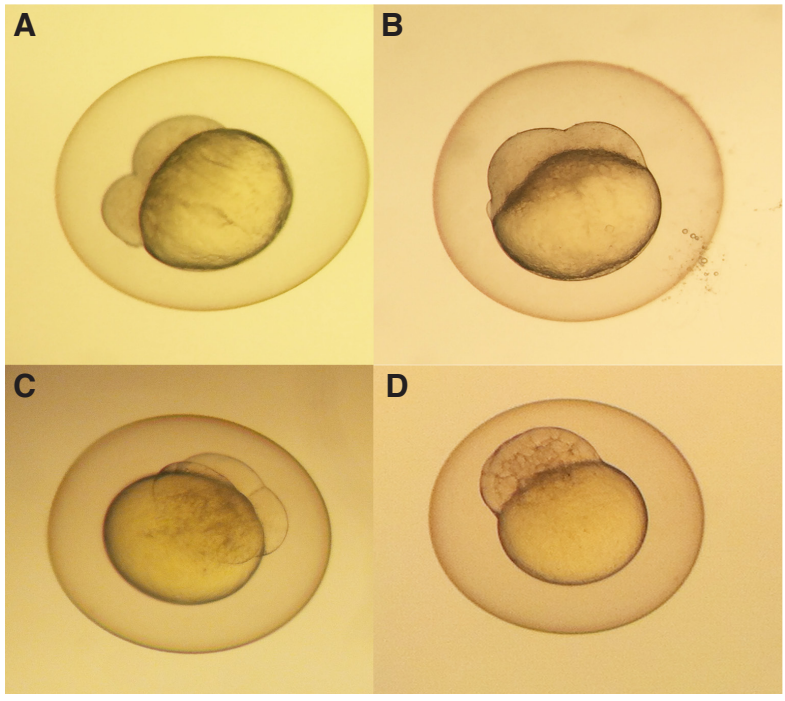
Extract buffers were formulated based on the previous study (Sharma and Kinsey, 2008). Extract buffer-1 was composed of: 1 mM glutathione; 10 nM EGTA; and 0.5 percent [v/v] protease inhibitor cocktail [catalog no. P1860, Sigma Aldrich] in double-distilled water). Extract buffer-2 was composed of: 1.5 M KCl; 30 mM NaCl; 0.1 M KH_2PO_4; and 0.8 M sucrose in double-distilled water. Extrcat-2 was added to the lysate in a ratio of 1: 9 (extract buffer-2: sperm lysate). Sonication protocol was conducted in five fifteen-second cycles at 20 kHz separated by one-minute resting intervals (Fig. S4). Trypan blue staining (Sigma Aldrich) was used to assess the breaking of sperm heads (Fig. S4 A-C) before harvesting the extract by centrifugation. Parthenogenesis experiments used a SSE batch containing 0.68 mg/ml of protein concentration. SCNT experiments used SSE batches containing 0.2 mg/ml and 1 mg/ml of protein concentrations.

2. Gynogenesis and ploidy manipulation

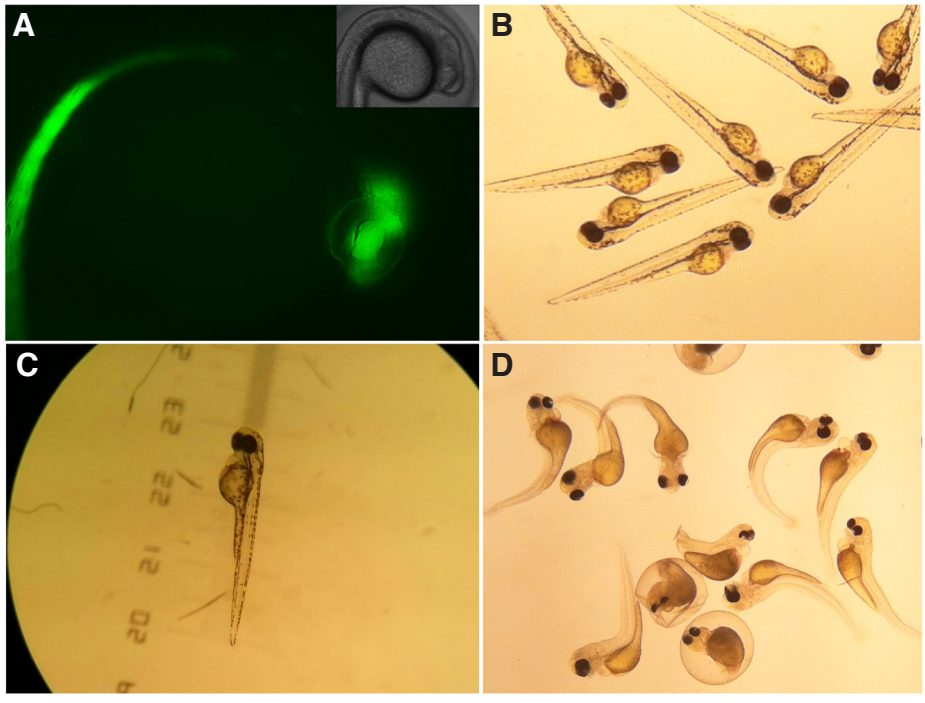
The protocol of ploidy manipulation called as heat shock was previously described (Mizgirev and Revskoy, 2010). We set one thermocycler at 28.5°C and another at 41.4°C. After the insemination, eggs fertilized with UV-sperm were maintained at 28.5°C; for 13 minutes postfertilization (mpf), and subsequently transferred to the 41.4°C thermocycler for two minutes and switched back into the 28.5°C maintenance chamber. Gynogenetic diploid embryos developed normally (Fig. S2C) and were morphologically comparable to WT embryos fertilized by untreated sperm (Fig. S2B). Fertilizing WT eggs with UV-sperm and leaving out the heat shock step produced gynogenetic haploid embryos, which showed abnormal characteristics, such as deformed heads, pericardial enlargement, and short trunks (Fig. S2D). We established the normal range for the head-to-tail length of embryos at 48 hours postfertilization (hpf) for both haploid and WT embryos to set a cut-off point to indicate a haploid's character. The body length of the haploid embryos (n=150) at 48 hpf was 2.62±1.18 mm — significantly shorter ($P<0.05$, per the independent T-test) than the WT embryos (n=170), which reached 3.05±0.34 mm.

3. Soluble sperm extract microinjections

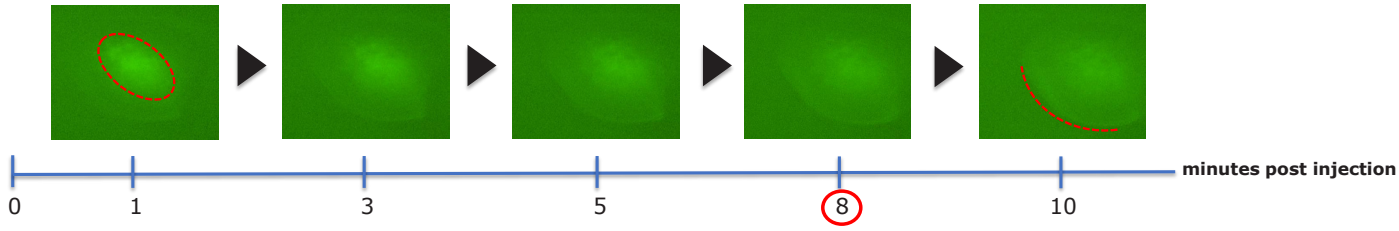
The first microinjection approach was similar to the SCNT process. The manipulation instruments comprise of an injecting needle, a holder pipette, and a supporting pipette (Siripattarapravat *et al.*, 2016). Micromanipulation was performed on an inverted microscope equipped with microinjectors. The manipulation dish contained drops of 5 percent (w/v) of polyvinylpyrrolidone (PVP) in CSOF and drops of SSE separately. The volume injected was calibrated by measuring the radius of an injected drop and calculating its spherical volume. The second approach was conducted by an electronic microinjector. We prepared an injection needle with an inner diameter of eight to ten μm and a thirty-degree beveled angle. The manipulation dish was coated with 1.5 percent agarose gel in Hank's balanced salts solution (HBSS, cat. no. H1387, Sigma). We molded the gel to create a small slit for use as an injection venue. Microinjection was made at the center of the eggs under the navigation of an automatic direction controller. We pre-evaluated the time it would take for the extract to diffuse through the yolk from the injection site to the cytoplasmic rim by microinjecting 0.1 mM fluorescent dextran (10,000 MW) diluted in SSE buffer (cat. no. D1821, Life Technologies). Time-lapse fluorescent imaging had demonstrated that the fluorescent dye in the SSE buffer required eight minutes to travel from the center to the periphery (Fig. 6). A stage micrometer calibration slide (cat. no. MR095, AmScope) was used to calibrate the volume of injection.



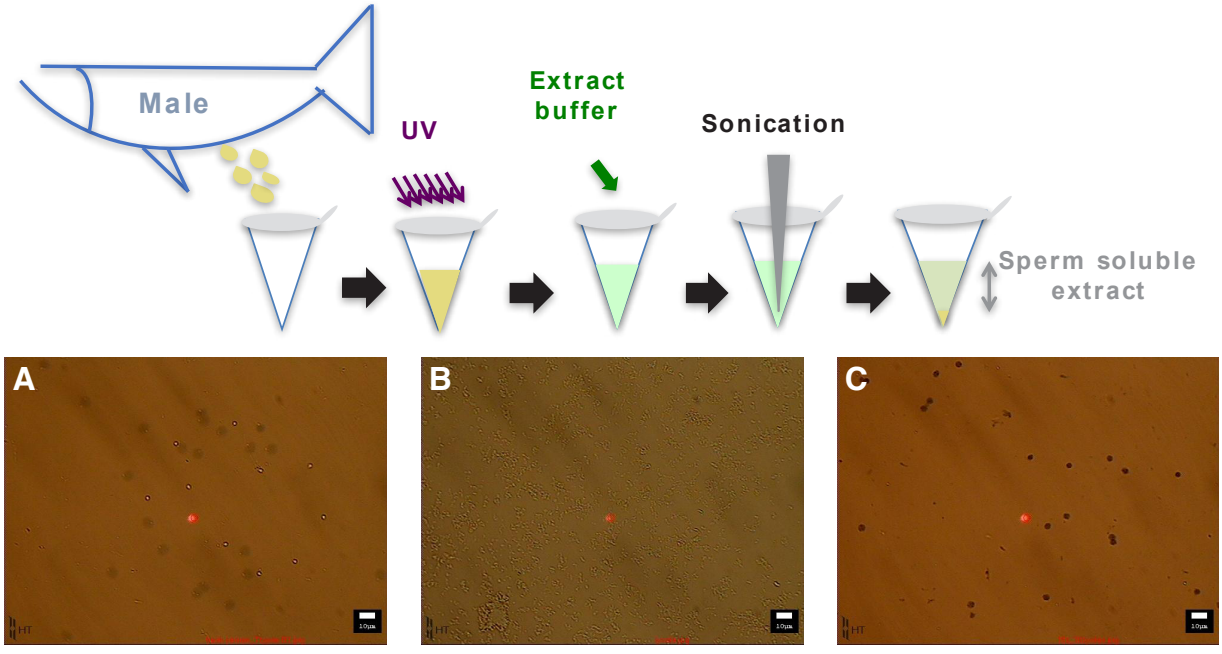
Supplementary Fig. 1. Morphological appearances of SSE-induced parthenogenetic embryo and the spontaneously activated egg. *SSE-induced parthenogenetic embryos at 2-cell stage (40 minutes post activation, mpa,* **(A)**, *4-cell stag (60 mpa)* **(C)**, *and 128-cell stage (135 mpa)* **(D)**. *At 40 mpa, spontaneously activated egg showed an asymmetrical two-cell-like structure at the animal pole that had no line of the cleavage furrow* **(B)**.



Supplementary Fig. 2. Morphological differences of zebrafish embryos derived from different activation approaches. *Wild-type embryo expressed sperm-derived transgene (green fluorescent protein, GFP) at 24 hpf* **(A)** *(40X) and showed normal morphology at 48 hpf* **(B)**. *A heat-shocked embryo* **(C)** *had a normal morphology and head-to-tail length ≥3 mm. Embryos derived from UV-sperm* **(D)** *showed haploid characteristics including deformed head, spinal curvature, pericardial enlargement and short body length.*



Supplementary Fig. 3. The time-lapse fluorescent imaging of non-activated egg microinjected with 0.1 mM fluorescent dextran using SSE-diffusion approach. *Fluorescence microscopy imaging were carried out every 20 seconds. A series of photos demonstrates the propagation of fluorescent dye that took eight minutes to travel from the injection site (the center) to the periphery (dashed line).*



Supplementary Fig. 4. Soluble sperm extract preparation and trypan blue staining of sperm heads. *Sperm was collected from males; the pooled milt was irradiated with UV before subjected to sonication in extract buffer-1 (hypotonic buffer) and the soluble fraction was separated by centrifugation. Staining the sperm head before sonication showed the integrity of the membrane, which disallowed the dye to permeate* **(A)**. *Extract buffer-1 was formulated as a hypotonic solution, which facilitated the breaking of sperm head* **(B)**, *while sonicating in physiological buffer prevented the complete disruption of sperm cell membrane* **(C)**. *Scale bar is 10 μm.*

SUPPLEMENTARY TABLE 1

THE APPLICATION OF SSE IN PARTHENOGENESIS EXPERIMENTS AND CLONING EXPERIMENTS

Experiments	SSE batch number	Protein conc. (mg/ml)	Microinjection method	Drop size (microns)	Volume (nanoliters)	Amount of injected protein (nanograms)	Amount of injected protein divided by amount in one sperm	Amount of protein from one sperm (nanograms)
Parthenogenesis	#1	0.68	micropyle	7.2-7.5	1.56-1.76	1.06-1.19	1.14-1.28	0.93
SSE-low	#2	0.20	micropyle	7.2-7.5	1.56-1.76	0.31-0.35	0.34-0.38	0.91
SSE-high	#2	1.00	micropyle	7.2-7.5	1.56-1.76	1.56-1.76	1.71-1.93	0.91
SSE-micropyle	#1	1.07	micropyle	7.2-7.5	1.56-1.76	1.67-1.88	1.80-2.02	0.93
SSE-diffusion	#1	1.07	Injector	10	4.2	4.49	4.83	0.93