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Definition of three somatic adult cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization

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Summary

Zebrafish somatic nuclear transplant has only been attempted using preactivated eggs. In this work, three methods to carry out the nuclear transplant using adult cells before, during and after the egg activation/fertilization were developed in zebrafish with the aim to be used in reprogramming studies. The donor nucleus from somatic adult cells was inserted: (method A) in the central region of the egg and subsequently fertilized; (method B) in the incipient animal pole at the same time that the egg was fertilized; and (method C) in the completely defined animal pole after fertilization. Larval and adult specimens were obtained using the three methods. Technical aspects related to temperature conditions, media required, egg activation/fertilization, post-ovulatory time of the transplant, egg aging, place of the donor nucleus injection in each methodology are presented. In conclusion, the technical approach developed in this work can be used in reprogramming studies.

Keywords: Egg activation, Fertilization, Microinjection, Nuclear transplant, Zebrafish

Introduction

After 1952, when Briggs and King obtained normal hatched tadpoles by blastomere nuclear transfer, nuclear transplant technology began to be developed for reprogramming studies (Kikyo *et al.*, 2000; Wade & Kikyo, 2002; Li, 2002; Giraldo *et al.*, 2008). Mammalian cloning by nuclear transfer has been successfully achieved in several species (Wilmut *et al.*, 1997; Wakayama *et al.*, 1998; Byrne *et al.*, 2007; French *et al.*, 2008) with varied somatic cell types as donors (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Wakayama *et al.*, 1998; Shiga *et al.*, 1999).

Although fish cloning is less developed, several recent works using medaka (*Oryzias latipes*) have been reported, in which both blastomeres (Bubenshchikova *et al.*, 2005) and somatic larval and adult cells (Bubenshchikova *et al.*, 2005, 2007; Kaftanovskaya *et al.*, 2007) were used as donors, and non-enucleated and activated eggs were used as recipients in all cases. In this species, embryonic nuclear transplants using functionally enucleated and non-activated eggs have been achieved only with blastomeres as nuclei donors (Wakamatsu *et al.*, 2001).

In contrast, in zebrafish (*Danio rerio*), the first successful embryonic (10–15 somites) somatic cloning by nuclear transplant with mechanically enucleated and previously activated eggs was described by Huang and colleagues in 2003. Since this work and to date, to our knowledge, no additional improvement in nuclear transplant techniques in zebrafish has been published by these or any other authors.

To date, due to technical (Nüsslein-Volhard & Dahm, 2002) or biological (Westerfield, 2003) limitations, fish somatic nuclear transplant in these two laboratory species with somatic embryonic (10–15 somites; Huang *et al.*, 2003) or adult cells (Bubenshchikova *et al.*,

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2007) has only been attempted using preactivated eggs as recipients. These limitations have hindered the study of the reprogramming effects of cytoplasmic factors characteristic of the metaphase II status in the oocyte, the effects of the synchrony degree between activation and nuclear transplant, egg aging or the previous donor cell reprogramming treatments. This situation is not the case in mammals, in which these reprogramming factors have been studied because somatic adult nuclear transplant has been more easily carried out before, at the same time and after oocyte activation (Cambell *et al.*, 1996; Wilmut *et al.*, 1997).

As the zebrafish model is a powerful genetic and developmental system in which the genome has already been sequenced, the aim of this work was to develop three methods to enable nuclear transplant to be carried out using adult cells prior, simultaneously with or following egg activation/fertilization in zebrafish to be used in reprogramming studies.

Material and methods

Care and maintenance of zebrafish colony

Two zebrafish (*Danio rerio*) colonies (wild and gold strains) were established in our laboratory from specimens purchased in a specialized establishment and kept in closed reproduction for five years. Adult zebrafish were kept in 20 litre tanks in a 2:1 ratio (females/males) and fed on granular food supplemented with recently defrosted hen egg yolks and shrimp meat (Simao *et al.*, 2007). The light cycle was regulated at 14 h light/10 h dark.

Non-activated eggs and sperm collection

Eggs were collected after evaluation of the sexual behavior of both gold strain males and females at dawn. Only females that manifested this sexual behaviour were anesthetized in an oil clove solution (100 µl in 1 l of dechlorinated and decalcified water: system water) for a few minutes and the eggs were obtained by gentle extrusion of the ovary. It is important to prevent eggs coming into contact with fresh water, because they activate immediately. Only good eggs (yellow and translucent colours) were kept in Hanks' buffered salt solution (H10) supplemented with 1.5% (v/v) of bovine serum albumin (BSA) and 0.1 g of NaCl/100 ml of Hanks' medium (egg medium; pH: 7.4; osmolarity: 310–320 mOsm) at 8 °C until their use (1 h and 30 min as maximum time).

The gold zebrafish males that showed reproductive behaviour were also anesthetized as described before. The abdominal region was gently pressed while the sperm was being recovered from the genital pore into individual glass microcapillaries (1 × 90 mm Narishige Scientific Instrument Laboratory). A pool from 2–3 different males (0.5–2 µl/male) was diluted in 200 µl egg medium, which can also keep the sperm in a non-activated status, and then the dilution was stored at 8 °C until use.

In vitro fertilization

In zebrafish, the eggs quickly lose their post-ovulatory ability to be fertilized (90 min). Moreover, the time between complete egg activation and *in vivo* fertilization is extremely short (seconds) in zebrafish (Nüsslein-Volhard & Dahm, 2002). Non-activated eggs and sperm were mixed in egg medium and stored at 8 °C until fertilization, for all nuclear transplant methods (see Experimental design). To activate both gametes, 1 ml of system water at room temperature was added to the egg–sperm mixture. After 2–3 min, the time required for fertilization in zebrafish, the 35 mm Petri dish (Corning) was fully filled with the water system for achieving well developed embryos. Further culture was done at 28 °C.

Primary culture and somatic cell collection

Somatic cells used as nuclei donor came from wild zebrafish caudal fin primary cultures. The tissue was obtained by caudal fin amputation of adult specimens after they had been anesthetized in clove oil solution. The tissue was cleaned with a 0.2% bleach solution for 2 min, then washed twice in 10% Hanks' buffered salt solution (H10) and then each tissue fragments were plated individually into a 35 mm Petri dish (Corning). Next, the tissue was incubated in Leibovitz medium supplemented with 20% of fetal bovine serum (FBS) and 0.036 g/l of glutamine (L15–FBS) at 28.5 °C (Westerfield, 2003).

Before use, donor cells were incubated in Hanks' buffered salt solution without Ca^{2+} and Mg^{2+} at room temperature for 30 min before performing the nuclear transplant. No additional detachment treatment was realized. Once the cells had come off the substrate, L15–FBS was added and the cell dish was preserved at 5 °C throughout the daily experimental session.

Somatic cell nuclear transplant equipment

The nuclear transplant was performed using a Nikon inverted microscope equipped with two Leitz micromanipulators. During the manipulation process, the non-dechorionated eggs were held with a 260 µm outer diameter holding pipette and the cells were picked, lysed and injected into the eggs by means of a 10–12 µm inner diameter microinjection pipette. The microinjection pipette was fire polished, beveled and sharpened.

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To perform the nuclear transplant, two separated drops were deposited in a Petri dish (Corning) (90 mm) and covered by mineral oil. One of them contained the donor somatic cells and was in L-15–FBS medium (300 mOsm). The other drop was the handling drop, which means the place where the nuclear transplant was performed, so the medium differed depending upon the nuclear transplant method tested in each case (see below).

The donor cell was picked up and lysed by aspiration with the injection micropipette before injection. The exact place where the cellular content was to be deposited was dependent on the nuclear transplant method tested in each case (see below).

Nuclear transplant was performed at different temperatures depending on the nuclear transplant method (see below).

Nuclear transplant methods

In order to carry out nuclear transplant whatever the status of the egg activation, three nuclear transplant methods were developed in which the somatic nuclear transplant was performed prior, simultaneous or posterior to the egg activation by the spermatozoa. As the aim of this work was to establish these methods technically and they were independently performed, no comparison of their technical efficiencies was made.

Method A: nuclear transplant prior to egg activation/fertilization

The somatic cell nucleus was inserted into the central region of the egg. To prevent egg activation, the transplant was performed in a handling drop composed of egg medium and the micromanipulation area was cooled down to 8 °C. This temperature around the handling zone was reached by cooling air cooled with liquid N_2. Then, transplanted eggs were individually *in vitro* fertilized and cultured at 28.5 °C in the system water (Westerfield, 2003).

Method B: nuclear transplant simultaneously with egg activation/fertilization

In this case, previously mixed non-activated eggs and sperm were kept at 8 °C and deposited individually in the handling drop that contained the system water, so that gametes were activated and fertilized. The micromanipulation area was not cooled (room temperature). The donor nucleus was injected in the incipient animal pole, just where the zygote nucleus was found (Wolenski & Hart, 1987). The reconstructed embryos were incubated under the same conditions described previously.

Method C: nuclear transplant following egg activation/fertilization

In order to carry out nuclear transplant after fertilization, eggs and sperm were previously mixed and activated, then fertilized at room temperature as described in the second technique but, in this case, nuclear transplant was realized a few minutes after fertilization, just the time required for visualizing the completely defined animal pole. After injecting the donor nucleus at the animal pole, reconstructed embryos were incubated under the same conditions as described above.

Experimental design

The three techniques tested (A, B and C) were not carried out simultaneously, but were developed and assessed independently. In the three cases, in order to evaluate how post-ovulatory aging affects reconstructed embryo survival, two batches consisting of 3–5 eggs each were transplanted sequentially and compared in each session (A1, A2; B1, B2; C1, C2). Overall, the length of each experimental session did not exceed 90 min in all cases, the maximum time for efficient egg fertilization (Nüsslein-Volhard & Dahm, 2002). In this way, A1, B1 and C1 were manipulated during the first 45 min and A2, B2 and C2 during the last 45 min. A non-manipulated control group was fertilized at the end of each experimental session, at 90 min (CA, CB and CC) to test the ability of the egg to be fertilized at this time.

Given that the aim of the present work was mainly technical, only the embryo and larval survival rates of reconstructed embryos from the three techniques (A, B and C) were evaluated at different developmental stages: at mid blastula transition (MBT) stage (2 h after nuclear transplant); at 50% epiboly stage (7 h after nuclear transplant); at 24 h post-nuclear transplant; at 48 h post-nuclear transplant; and at larval stage (5 days after nuclear transplant) (Westerfield, 2003). Moreover, at 24 h, 48 h and at 5 days post-nuclear transplant, normal and abnormal development was registered. In the non-manipulated control group, only the fertilization rate was evaluated by the survival rate at the MBT stage.

At least three replicates were done in all experimental groups. Results were analysed using the chi-squared test. When a single degree of freedom was involved, Yates' correction for continuity was performed.

Results and discussion

The technical aspects for three methodologies for zebrafish somatic nuclear transplant prior to, simultaneously with and following egg activation/fertilization have been established in our laboratory. All three were

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developed and evaluated using non-irradiated eggs activated/fertilized by non-irradiated spermatozoa. In this way, the effects on survival and further embryo and larval development can be attributed exclusively to the transplant methodology employed, because the background noise due to the exigency of the spermatozoa for egg activation in zebrafish (Lee *et al.*, 1999) and the developmental limitations caused by a haploid condition (Nüsslein-Volhard & Dahm, 2002) would not exist in this case. On this subject, it has to be taken into account that, in these fish species, the triploid or tetraploid condition that could occasionally be derived from the addition of a somatic nucleus to the resident zygote pronuclei does not affect the embryonic, and even larval, development in a relevant way (Diter *et al.*, 1988; Peruzzi & Chatain, 2003).

As a first general comment regarding the efficiency of the *in vivo* artificial collection of ovarian oocytes, the sexual behaviour synchrony showed by the separate fish colonies must be pointed out, in such a way that a large number of eggs could be collected in some sessions whereas no eggs might be obtained in others. Another relevant consideration concerns the fact that MBT nuclear transplant embryos were obtained in the great majority of the daily experimental sessions, whatever the transplant method used.

A technical advantage common to the three methodologies developed in our laboratory was the avoidance of previous dechorionation. In fish nuclear transplant, when the oocyte is activated but not enucleated, the donor nucleus is usually inserted into the perinuclear region of the oocyte, the closest as possible to the female nucleus, which is located in the cytoplasm subjacent to the plasma membrane under the micropyle (Amance & Iyengar, 1990). In various teleost species (such as medaka, catfish or tilapia), the animal pole position can easily be detected in preactivated oocytes because the micropyle can be visualized at this stage, although, unfortunately, this is not the case in zebrafish (Poleo *et al.*, 2001).

One possible way to obviate such biological difficulty in zebrafish was attempted in method A, in which the somatic cell nucleus was inserted into the central region of the egg. This point of transplant was chosen because, in terms of probability, the central region will be closer to the female nucleus, whatever its real localization. So, the donor nucleus will be more likely to migrate to the microvilli cluster in animal pole. This is the place where fertilization occurs (Wolenski & Hart, 1987) through the cytoplasmic flows together with the pronuclei at the time of the activation.

The earliest manipulated group (A1) reached significant higher survival rates compared with the most aged group (A2) both at epiboly and 24 h stage (Table 1). However, in more advanced stages (48 h and larval stage), the observed differences did not reach

Table 1 Method A: survival rates of nuclear transplant prior to the egg activation/fertilization.

Group	Transplanted eggs (n)	Embryos at MBT stage (n, %)	Embryos at epiboly stage (n, %)	Embryos at 24 h post-transplant			Embryos at 48 h post-transplant			Survival larvae (n, %)
				Normal (n, %)	Abnormal (n)	Total (n)	Normal (n, %)	Abnormal (n)	Total (n)	
A1	82	23 (28%)^{a}	18 (22%)^{a}	12 (15%)^{a}	2	14	8 (10%)^{a}	3	11	6 (7%)^{a}
A2	50	6 (12%)^{a}	3(6%)^{b}	1 (2%)^{b}	1	2	1 (2%)^{a}	1	2	0 (0%)^{a}
Total A	132	29 (22%)	21 (16%)	13 (10%)	3	16	9 (7%)	4	13	6 (5%)
Control CA	301	90 (30%)	–	–	–	–	–	–	–	

^{a,b}Rows with different superscripts are statistically different.

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significant levels, probably due to the low number of embryos that developed to these stages. The egg control group (CA) fertilized at the end of each experimental session showed MBT rates equal or higher than the two experimental timing groups, indicating the maintenance of the egg fertilization ability until the end of the transplant session. Anyway, the larval survival rate was 7% (A1:6 larvae from 82 manipulated) in the first group and 5% (6 larvae from 132 manipulated) taking into account the overall two groups.

It must be underlined that to cool down to 8 °C the temperature of the micromanipulation area was critical in maintaining the egg in a non-activated state during transplant. This initial strategy permits the impregnation of the donor nucleus in the reprogramming factors present in the egg at metaphase stage. In further experiments the effect of different times of donor nucleus impregnation before the activation will be tested.

In method B (Table 2), nuclear transplant and fertilization were performed at the same time, which meant transplanting the donor nucleus while the egg was activating. Egg activation and fertilization are both marked by elevation of the chorion and a dramatic reorganization of the yolk cytoplasm. In this way, the animal pole is segregated through the place where the female nucleus will be located (Wolenski & Hart, 1987). This approach enabled detection of the incipient animal pole in order to deposit the somatic nucleus in the female perinuclear region.

The survival rate differences between the first (B1) and second group (B2) did not reach significant levels in any assessment. However, it must be pointed out that these differences decreased over time and that the survival rates were finally similar at the 48 h stage (B1: 19% vs B2: 20%). In this case, the fertilization rate of the final control group (CB) was lower than the Total B, even than that found in the B2, which involved an obvious effect of the egg aging and a very slightly negative effect of the assayed nuclear transplant technique.

When nuclear transplant was performed following egg fertilization, method C (Table 3), the variability in the time required for showing the perivitelline space as an activation signal should be pointed out. This factor represented a critical point due to the technical difficulty involved in fixing the egg with the holding pipette, because the egg rotated inside the chorion while this space was increasing. With activation, the micropyle can be more easily detected but, a few seconds after the fertilization, the chorion hardened and the micropyle sealed (Poleo *et al.*, 2001). This factor made it very difficult to insert the donor nucleus through this point even if the microinjection pipette was fire polished, beveled and sharpened, as in our case. A possibility to obviate

Table 2 Method B: survival rates of nuclear transplant simultaneously to the egg activation/fertilization.

Group	Transplanted eggs (n)	Embryos at MBT stage (n, %)	Embryos at epiboly stage (n, %)	Embryos at 24 h post-transplant (n)			Embryos at 48 h post-transplant			Survival larvae (n, %)
				Normal (n, %)	Abnormal (n)	Total (n)	Normal (n, %)	Abnormal (n)	Total (n)	
B1	59	32 (54%)^a	22 (37%)^a	14 (24%)^a	6	20	12 (20%)^a	5	17	11 (19%)^a
B2	54	19 (35%)^a	15 (28%)^a	11 (20%)^a	4	15	11 (22%)^a	1	12	11 (20%)^a
Total B	113	51 (45%)	37 (33%)	25 (22%)	10	35	23 (20%)	6	29	22 (19%)
Control CB	53	15 (28%)	–	–	–	–	–	–	–	

^{a,b}Rows with different superscripts are statistically different.

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Table 3 Method C: survival rates of nuclear transplant posterior to the egg activation/fertilization.

Group	Transplanted eggs (*n*)	Embryos at MBT stage (*n*, %)	Embryos at epiboly stage (*n*, %)	Embryos at 24 h post-transplant			Embryos at 48 h post-transplant			Survival larvae (*n*, %)
				Normal (*n*, %)	Abnormal (*n*)	Total (*n*)	Normal (*n*, %)	Abnormal (*n*)	Total (*n*)	
C1	49	26 (53%)^{a}	21 (43%)^{a}	16 (33%)^{a}	5	21	14 (29%)^{a}	3	17	14 (29%)^{a}
C2	22	9 (41%)^{a}	7 (32%)^{a}	4 (18%)^{a}	1	5	3 (14%)^{a}	1	4	3 (14%)^{a}
Total C	71	35 (49%)	28 (39%)	20 (28%)	6	26	17 (24%)	4	21	17 (24%)
Control CC	374	147(39%)	–	–	–	–	–	–	–	

^{a,b}Rows with different superscripts are statistically different.

this difficulty could be to dechorionate the egg after fertilization, but this technique is time consuming and the first cleavage is very early (minutes) in zebrafish. Moreover, the reduction of the temperature to arrest development before the MBT stage involves embryo lethality (Francisco-Simao *et al.*, 2007).

Regarding survival differences between both handling groups (C1 and C2), it should be emphasized that, as in technique B, the differences observed did not reach significant levels in any case. However, these differences were relevant, ranged from a differential of 10% at MBT stage up to 15 points at the larval stage. The explanation for not reaching significance level could be the low number of surviving embryos that developed to this stage, owing to the aforementioned technical difficulty. The survival rate of the control group (CC), compared with the C1 and C2 groups, showed again a considerable egg aging effect, which means that the time for performing the nuclear transplant in zebrafish must be shorter in order to avoid (or minimize) such a pronounced negative effect.

Adult specimens showed the gold phenotype. This fact does not discard a possible mosaicism or that the reconstructed embryos with the donor nucleus incorporated more efficiently did not reach adult stages. However, it has to be taken into account that the nuclear fate of the transplanted nuclei was not analysed because, as mentioned previously, the main aim of this work was to establish three nuclear transplant protocols in zebrafish by a technical approach. In this way, after the establishment of these three techniques presented, the nuclear fate will be studied, as well as its integration degree and form in the specimens.

In conclusion, the reasonable technical efficiencies achieved in the present work make the use of these three methods interesting for future reprogramming studies by nuclear transplant in this species.

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