Zygote **20** (August), pp. 277–280. © Cambridge University Press 2011
doi:10.1017/S0967199411000104 First Published Online 24 March 2011

Somatic cells derived from haploid larvae are feasible as donors for nuclear transplant in zebrafish. Preliminary results

J. Cardona-Costa[1,3], *M. Pérez-Camps*[2,3] *and F. García-Ximénez*[2]
Laboratory of Animal Reproduction and Biotechnology (LARB-UPV), Universidad Politécnica de Valencia, Valencia, Spain

Date submitted: 19.08.10. Date accepted: 17.01.11

Summary

Somatic cells derived from zebrafish haploid larval (both androgenetic and gynogenetic) cultures were used as donors for nuclear transplant into non-enucleeated oocytes. Nuclei were transplanted either before or simultaneously with oocyte activation in the central region and in the incipient animal pole, respectively. Against expected results, 20% of transplanted embryos during oocyte activation using cells of gynogenetic origin reached the 100% epiboly stage, even two survived for up to 5 days, whereas no development was observed when cells from androgenetic origin were used. Results derived from this work open a novel possibility of studying somatic cell reprogramming and imprinting phenomena in zebrafish.

Keywords: Cell culture, Haploid, Nuclear transplant, Vertebrate, Zebrafish

Introduction

When a somatic diploid nucleus is transplanted into an enucleated oocyte, the successful development is conditioned by the nucleus reprogramming degree, in most cases showing an incomplete reprogramming (Latham, 2005; Ng and Gurdon, 2005, 2008). However, it has recently been observed that developmental rates of reconstructed embryos by nuclear transplant (NT) can be improved, in medaka fish, using non-enucleated diploidized oocytes as recipients as the more stable ploidy condition reduces the formation of haploid cells in reconstructed embryos (Bubenshchikova *et al.*, 2007). However, cell mosaicism is still observed (Kaftanovskaya *et al.*, 2005; Bubenshchikova *et al.*, 2007).

Zebrafish haploid larvae (both gynogenotes and androgenotes) can survive for up to 5 days (morphogenesis already completed; Nüsslein-Volhard & Dahm, 2002). This fact allowed us to derive cell cultures *in vitro* from these larvae (unpublished results). If these somatic cultured cells were transplanted into non-enucleated oocytes, the diploid condition would be reconstructed and, possibly, avoid any possible mosaicism in reconstructed embryos.

So, the aim of the present work was to test the developmental ability of reconstructed embryos using cells derived from both androgenetic and gynogenetic haploid larval cultures as donor nuclei.

Materials and methods

Gold zebrafish strain was used in all experiments.

Recipient eggs

Eggs were obtained by gentle extrusion of the ovary (Westerfield, 2007) and kept inactivated in egg medium (Hanks' saline medium supplemented with 1.5% (v/v) of BSA and 0.1 g of NaCl/100 ml of Hanks' saline; pH: 7.4; osmolarity: 310–320 mOsm) at 8 °C (Cardona-Costa *et al.*, 2009).

Haploid somatic larval cultures

Gynogenetic
Sperm was recovered from the genital male pore in individual glass microcapillaries (Westerfield, 2007)

[1]All correspondence to: J. Cardona-Costa. Laboratory of Animal Reproduction and Biotechnology (LARB-UPV), Universidad Politécnica de Valencia, Camino de Vera 14, 46022 Valencia, Spain. Tel: +34 96 3879433. Fax: +34 96 3877439. e-mail: cardona_costa_j@hotmail.com
[2]Laboratory of Animal Reproduction and Biotechnology (LARB-UPV), Universidad Politécnica de Valencia, Camino de Vera 14, 46022 Valencia, Spain.
[3]Both authors contributed equally to this work.

https://doi.org/10.1017/S0967199411000104 Published online by Cambridge University Press

and kept in egg medium at 8 °C (Cardona-Costa *et al.*, 2009). A sample of 100 μl of the sperm diluted in Hanks' balanced solution (Cardona-Costa *et al.*, 2009) was irradiated using a UV germicide lamp (General Electric, 30 W). Irradiation was carried out at 62 cm of focus-object distance, applying a radiation dose of 0.529 mW/cm^2, measured with a USB 4000 (Miniature Fiber Optic Spectrometer; Ocean Optics Inc. First In Photonics,). A vortex (MS1; IKA-Works Inc.) at 200 rpm was also used with the aim of homogenising the irradiation area during UV exposure (Francisco-Simão *et al.*, 2010). The sperm irradiation time was 1 min.

Androgenetic

Eggs were collected as described in 'Recipient eggs'. Then, they were UV irradiated by the same procedure as used with sperm, but without shaking, the irradiation time being 12 min.

In vitro fertilization of non-irradiated oocytes with irradiated sperm and vice-versa was carried out as Westerfield (2007) described. Briefly, non-activated eggs and sperm were mixed in a 35 mm Petri-dish and both gametes activated by the addition of a 1 ml of system water. After 2–3 min, the dish was filled with system water to achieve well developing embryos. Further embryo culture was done at 28.5 °C. Larvae at 24 h stage that showed the 'haploid syndrome' (Westerfield, 2007) were selected for *in vitro* culture.

Haploid larvae were mechanically dechorionated, disinfected with 0.2% v/v bleach/system water and washed in Hanks' buffered salt solution. Both zebrafish androgenetic and gynogenetic larvae were cultured separately in Leibovitz medium supplemented with 20% of FBS and 0.036 g/100 ml of glutamine (L15-FBS) at 28.5°C (Westerfield, 2007).

Somatic cell nuclear transplant

Somatic cells used as nuclear donors came from primary cultures derived from both gynogenetic and androgenetic larvae.

The two somatic cell nuclear transplant techniques, as well as the equipment employed were described by Pérez-Camps *et al.* (2010a).

NT before egg activation

The somatic cell nucleus was inserted in 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. Non-activated eggs were activated by fresh water after their NT.

NT during egg activation

The donor nucleus was injected in the incipient animal pole, just at the position where zygote nucleus was being constituted. The handling drop was composed of fresh water so that the eggs became the activation and the micromanipulation area was not cooled (room temperature).

Experimental design

Experiment 1

Primary cell cultures derived from haploid androgenetic larvae were used as nuclear donors. Two differenced groups were established depending on the nuclear transplant methodology: NT before egg activation and NT during egg activation.

Experiment 2

Due to the extremely 'bad' results obtained when cell donor nuclei were from androgenetic larvae (Experiment 1) and as zebrafish gynogenetic diploid specimens reach adult stages and become fertile (Nüsslein-Volhard & Dahm, 2002), in Experiment 2, cells derived from primary haploid gynogenetic larvae cultures were used as nuclear donors. Two differentiated groups were also established depending on the nuclear transplant methodology: 'NT before egg activation' and 'NT during egg activation'.

Assessments

Survival rates from experimental groups were evaluated at mid blastula transition stage (MBT), 100% epiboly, 24 h and 5 days. To assess egg quality of recipient eggs, fertilizability control groups were immediately carried out after extracting eggs, whatever the time and method of transplant carried out in each experiment. Nuclear transplant sessions were considered only when the initial fertilization rates were higher than 70%.

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

Experiment 1

From the six NT sessions (both before and during egg activation) using cell cultures derived from androgenetic haploid larvae, no reconstructed embryos reached upper developmental stages to MBT; in fact, only two out of 15 embryos reached this stage.

In the light of results obtained from these experimental sessions, the heteroparental reconstruction was aborted.

https://doi.org/10.1017/S0967199411000104 Published online by Cambridge University Press

Table 1 Survival rates of reconstructed embryos derived from the transplant of somatic cell nuclei derived from haploid gynogenetic larvae of 24 h stage into metaphase II eggs or early zygotes in zebrafish

	NT before egg activation% (n)	NT during egg activation% (n)
MBT*	15^{x} (8/54)	31^{y} (16/51)
100% EPIBOLY	0^{a} (0/54)	20^{b} (10/51)

$^{a,\,b,\,x,\,y}$Data in columns with different superscripts are statistically different ($p < 0.05$).
*Data statistically differ at 10% ($p < 0.1$).

Experiment 2

Regarding the developmental rates of reconstructed embryos, when donor cells were derived from gynogenetic haploid larval cultures, significant differences were observed at the MBT stage ($p = 0.074$; <0.1) in favour of the NT performed 'during egg activation' (Table 1). These differences became more evident ($p<0.05$) at the 100% epiboly stage. In this group, 20% of reconstructed embryos developed and reached the 100% epiboly stage, although embryos transplanted before activation stopped their development at MBT stage (Table 1). Five of the surviving embryos at 100% epiboly continued developing to 24 h (26 somites stage) but only two survived for up to 5 days. In this case, these larvae showed morphologic anomalies, although they presented a heartbeat.

Discussion

Survival and developmental degree rates obtained from the transplant of nuclei derived from haploid gynogenetic larval cultures were, unexpectedly, much higher than those transplants from androgenetic cultures, also being higher than those results obtained by the transplantation of diploid somatic nuclei into non-enucleated oocytes (Pérez-Camps *et al.*, 2010b). These results could be explained in different ways: (i) They could be due to a possible gametic imprinting. However, on the one hand, in fish, diploids of both gynogenetic and androgenetic condition are viable (Nüsslein-Volhard & Dahm, 2002), and on the other, although this homoparental condition is possible, the expected results pointed to reaching better efficiencies in reconstructing the heteroparental condition. (ii) The culture conditions may also explain the present results. Haploid androgenetic larval cultures showed lower growth rates than haploid gynogenetic (non-published results), which could be an indication of the need for differential culture requirements, possibly due to the existence of a genomic imprinting (Tsalavouta *et al.*, 2009; Jiang *et al.*, 2010). (iii) Finally, this difference could be due to the process of obtaining androgenetic larvae. The oocyte UV irradiation method may cause cellular damage which could result in a cell penalization from the outset in haploid androgenetic cultures (Nüsslein-Volhard & Dahm, 2002), which may imply lower *in vitro* growth rates and lower NT efficiencies.

The NT of nuclei from cells derived from haploid gynogenotes larvae during egg activation by water overcome the MBT developmental barrier, in contrast to that observed when transplants were done before oocyte activation by water. Given that the MBT is the stage at which the zygote transcription begins in zebrafish (Kane & Kimmel, 1993; Korz, 2009), the higher developmental stages reached by NT reconstructed embryos (100% epiboly, 24 h and even until 5 days) suggest a complete oocyte activation. However, the reprogramming or integrating degree of transplanted nuclei was not enough to achieve an adult development.

On the other hand, the characterization of recessive mutations with haploid cells has been known for many years in yeast (Botstein & Fink, 1988). Application of this cell ploidy condition is of early use in vertebrate cells, mainly because of the effectiveness in obtaining and culturing these cells has been highly difficult (Yi *et al.*, 2009). To our knowledge, one human near-haploid cell line has been derived from carcinogenetic haematopoietic origin to investigate the host/pathogen interaction (Carette *et al.*, 2009) and one haploid ESCs line was established in medaka fish (Yi *et al.*, 2009). In this way, zebrafish cell cultures from haploid larvae used in this work could open the possibility to carry out genetic screening of somatic cells of non-tumoural origin in the widely used zebrafish model system. These haploid cell cultures could enable the direct genetic analyses of recessive phenotypes (Yi *et al.*, 2009), and mutagenesis-based genetic approaches, which should also allow the generation of null mutants for most non-essential genes in vertebrate cells (Carette *et al.*, 2009).

Author disclosure statement

The authors declare that no conflicting financial interests exist.

Acknowledgements

We would like to thank Mr Javier Rubio Rubio for technical support. The authors also thank Mr Neil Macowan for revising the English of this manuscript.

https://doi.org/10.1017/S0967199411000104 Published online by Cambridge University Press

References

Botstein, D. & Fink, G.R. (1988). Yeast: an experimental organism for modern biology. *Science* **240**, 1439–43.

Bubenshchikova, E., Kaftanovskaya, E., Motosugi, N., Fujimoto, T., Arai, K., Kinoshita, M., Hashimoto, H., Ozato, K. &Wakamatsu, Y. (2007). Diploidized eggs reprogram adult somatic cell nuclei to pluripotency in nuclear transfer in medaka fish (*Oryzias latipes*). *Dev. Growth Differ.* **49**, 699–709.

Cardona-Costa, J., Pérez-Camps, M., García-Ximénez, F. & Espinós, F.J. (2009). Effect of gametes aging on their activation and fertilizability in zebrafish (*Danio rerio*). *Zebrafish* **6**, 93–5.

Carette, J.E., Guimaraes, C.P., Varadarajan, M., Park, A.S., Wuethrich, I., Godarova, A., Kotecki, M., Cochran, B.H., Spooner, E., Ploegh., H.L. & Brummelkamp., T.R. (2009). Haploid genetic screens in human cells identify host factors used by pathogens. *Science* **326**, 1231–5.

Francisco-Simão, M., Cardona-Costa, J., Perez Camps, M. & García-Ximénez, F. (2010). Ultraviolet radiation dose to be applied in recipient zebrafish embryos for germ-line chimaerism is strain dependent. *Reprod. Domest. Anim.* **45**, 1098–1103.

Jiang, X., Yu, Y., Yang, H.W., Agar, N.Y., Frado, L. & Johnson, M.D. (2010). The imprinted gene PEG3 inhibits Wnt signaling and regulates glioma growth. *J. Biol. Chem.* **285**, 8472–80.

Kaftanovskaya, E., Motosugi, N., Kinoshita, M., Ozato, K. & Wakamatsu, Y. (2007). Ploidy mosaicism in well-developed nuclear transplants produced by transfer of adult somatic cell nuclei to nonenucleated eggs of medaka (*Oryzias latipes*). *Dev. Growth Differ*. **49**, 691–8.

Kane, D.A. & Kimmel, C.B. (1993). The zebrafish midblastula transition. *Development* **119**, 447–56.

Korzh, V. (2009). Before maternal-zygotic transition. . .there was morphogenetic function of nuclei. *Zebrafish* **6**, 295–302.

Latham, K.E. (2005). Early and delayed aspects of nuclear reprogramming during cloning. *Biol. Cell* **97**, 119–32.

Ng, R.K. & Gurdon, J.B. (2005). Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer. *Proc. Natl. Acad .Sci. USA* **102**, 1957–62.

Ng, R.K. & Gurdon, J.B. (2008) Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. *Nat. Cell Biol*. **10**, 102–9.

Nüsslein-Volhard, C. & Dahm, R. (2002) *Zebrafish: A Practical Approach* pp. 164–167. England: Oxford University Press.

Pérez-Camps, M., Cardona-Costa, J., Francisco-Simao, M. & García-Ximénez, F. (2010a). Definition of three somatic adult cell nuclear transplant methods in zebrafish (*Danio rerio*): before, during and after egg activation by sperm fertilization. *Zygote* **18**, 33–9.

Pérez-Camps, M., Cardona-Costa, J. & García-Ximénez, F. (2010b) Transplantation of adult fibroblast nuclei into the central region of metaphase II eggs resulted in mid blastula transition embryos. *Zebrafish* **7**, 215–8.

Tsalavouta, M., Astudillo, O., Byrnes, L. & Nolan, C.M. (2009) Regulation of expression of zebrafish (*Danio rerio*) insulin-like growth factor 2 receptor: implications for evolution at the IGF2R locus. *Evol. Dev*. **11**, 546–558.

Yi, M., Hong, N. & Hong, Y. (2009) Generation of medaka fish haploid embryonic stem cells. *Science* **326**, 430–3.

Westerfield, M. (2007) *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish* (Danio rerio). 5th edn. Eugene, OR, USA: Univ. of Oregon Press.

https://doi.org/10.1017/S0967199411000104 Published online by Cambridge University Press