Instituto Nacional de Investigación y Tecnología Agraria y Alimentación (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2008 6(4), 610-614
ISSN: 1695-971-X

Short communication. Evaluation of presumptive caudal fin blastema cells as candidate donors in intraspecies zebrafish (*Danio rerio*) chimaeras

M. Pérez-Camps[1]*, M. Francisco Simao[1,2], J. Cardona Costa[1] and F. García-Ximénez[1]

[1] *Laboratorio de Reproducción y Biotecnología Animal. Universidad Politécnica de Valencia (LARB-UPV). Camino de Vera, 14. 46071 Valencia. Spain.*

[2] *Faculty of Agriculture Sciences - Agostinho Neto University – Angola.*

Abstract

The blastema is a regenerative tissue with remarkable pluripotency. The aim of this work done on zebrafish (*Danio rerio*) was to define technical procedures required for obtaining and integrating blastema cells into embryos at the mid blastula transition stage (MBT) and the effect on survival, as well as the capacity to produce pigmented chimaeras. Wild type blastema cells were injected into gold type MBT embryos (E). Wild MBT blastomere cells were also injected into gold type MBT embryos as a control (C_1). A second control group, C_2, was not subjected to any manipulation. Survival was evaluated at 24, 48 and 72 h after performing the chimaerism, and the rate of adult chimaeras evaluated. The results showed significant differences in embryo survival between the E and C_1 groups in embryo survival at 24 and 48 h postchimaerism (24 h: E-83.49% *vs* C_1-54.8%, $p < 0.05$; 48 h: E-98.83% *vs* C_1-85.13%, $p < 0.05$). There was no significant difference, at any time, between E and C_2. The results at 72 h for E and the controls (E-89.41%; C_1-84.12% and C_2-92.55%) indicate that insertion of blastema cells does not have a negative effect on embryo development. The results in adults (E: 0 chimaeras from 7 specimens; C_1: 5 chimaeras from 17 specimens) suggest that the dedifferentiation grade of the blastema cells may not be enough to generate germ-line chimaeras, but their condition of potentially dedifferentiating cells may be an advantage when using them as donor nuclei in somatic cloning by nuclear transplant.

Additional key words: biodiversity, chimaerism, embryo, zebrafish.

Resumen

Evaluación de las células de la blastema de aleta caudal como donantes en quimeras intraespecíficas de pez cebra

El blastema es un tejido regenerativo con una pluripotencia remarcable. El objetivo del presente trabajo realizado en pez cebra *(Danio rerio)* es definir los procedimientos técnicos requeridos para la obtención e integración de células de la blastema en embriones en estadio MBT y su efecto en la supervivencia, así como también la capacidad de dar lugar a quimeras pigmentadas. Se inyectaron células de blastema de la estirpe silvestre en embriones gold en estadio MBT (E) y como control se inyectaron blastómeras silvestres MBT en embriones gold MBT (C_1). El segundo grupo control (C_2), no fue sometido a manipulación. Se evaluó la tasa de supervivencia a 24, 48 y 72 h post-quimerismo y la tasa de quimeras adultas. Los resultados mostraron diferencias significativas en la supervivencia embrionaria a las 24 y 48 h entre el grupo E y el C_1 (24 h: E-83,49% *vs* C_1-54,8%, $p<0,05$; 48 h: E-98,83% *vs* C_1-85,13%, $p<0,05$). No se detectaron diferencias significativas entre los grupos E y C_2 en ningún momento. Los resultados obtenidos a las 72 h, tanto en E como en los controles (E-89,41%; C_1-84,12% y C_2-92,55%) sugieren que la inserción de células de blastema no causa efectos negativos en el desarrollo del embrión. Los resultados obtenidos en adultos (E: 0 quimeras de 7 especímenes; C_1: 5 quimeras de 17 especímenes) apuntan a que el grado de diferenciación de las células de la blastema puede que no sea suficiente para generar quimeras en la línea germinal, pero su condición de células pluripotentes puede suponer una ventaja si son usadas como donantes de núcleos en clonación somática por trasplante nuclear.

Palabras clave adicionales: biodiversidad, embrión, pez cebra, quimerismo.

* Corresponding author: mipecam@dca.upv.es
Received: 03-12-07. Accepted: 26-08-08.

Abbreviations used: ESC (embryo stem cell), HBSS (Hanks buffered salt solution), H10 (HBSS diluted 10%), MBT (mid blastula transition), PGC (primordial germ cell).

The cell type used to carry out a biodiversity preservation strategy is important. Until now, current cryopreservation techniques have not enabled the preservation of embryos from species with meroblastic development, such as fish. However, it is possible to store embryonic cells (Cardona-Costa and García-Ximénez, 2007) which can give rise to germ-line chimaeras (Lin *et al*., 1992), although this involves destroying the donor embryo of the lineage to be preserved.

During caudal fin regeneration in fish, a regenerative tissue appears called blastema that grows atop each fin ray, between the wound epidermis and the amputation surface of the stump, which can regenerate the fin structure (Poss *et al.*, 2003). It is not clear whether blastema formation involves cellular dedifferentiation, the activation of quiescent cells, or both processes, but its pluripotency is remarkable (Akimenko *et al.*, 2003; Poss *et al.*, 2003).

If blastema cells are pluripotent embryo stem cells (ESCs), they would be ideal candidates for use as donor cells in germ-line chimaerism with the aim of preserving biodiversity of these animal species, as these undifferentiated cells can be obtained from adult specimens without causing any permanent damages to them. For this reason, it would be interesting to determine the potency of blastema cells and to investigate if it is possible to integrate them in the germ-line of chimera specimens, which has not been reported to date.

The aim of this work on zebrafish (*Danio rerio*), was to test the capacity of blastema cells to colonize and integrate into chimaeras, defining the technical procedures required and the effect on embryo and larval survival, as well as the capacity to produce pigmented chimaeras.

Adult zebrafish kept in a 6:3 proportion (females: males), and fed granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simao *et al.*, 2007), where used to obtain dechorionated embryos at the mid-blastula transition (MBT) stage (1,000 cells), the optimal stage to carry out chimaerism (Lin *et al.*, 1992). All chemicals and the culture media were from Sigma-Aldrich.

After washing with tap water on nylon mesh, embryos were selected under a microscope and washed again with tap water. Embryos in a suitable development stage and perfectly clean were kept in dechlorinated and decalcified tap water (Westerfield, 2003). No bleach treatment was applied, but sterilized media and materials (pipettes) and aseptic conditions were used.

Dechorionation was carried out by pronase treatment (1.5 mg mL^{-1} in H10), being H10 Hanks buffered salt solution (HBSS) diluted 10% in distilled water, v/v) followed by immersion twice in H10 with an osmolarity of 35 mOsm. Damaged embryos were discarded and only intact embryos were used in the experiments.

To remove the yolk, sterile hypodermic needles were used to puncture the yolk sac, and the embryo was then placed on a liquid surface where the yolk was removed immediately from the embryo by surface tension. The intact blastoderms were placed in HBSS without Ca^{2+} or Mg^{2+}. The blastoderm was disaggregated into blastomeres using a stretch and fire polished Pasteur pipette with an approximately inner diameter of 1/5 the size of a zebrafish blastoderm.

The blastema cells were from adult wild type zebrafish. Animals were anaesthetized with a clove oil solution (100 µL in conventional tap water; Grush *et al.*, 2004) and the caudal fins were amputated. After 4 days at 28.5ºC these fish were again anaesthetized and the distal region, where blastema was growing, was cut and kept in H10. The tissue was cleaned with a 0.2% bleach solution for 2 min, then washed twice in H10 and finally incubated for 30 min in HBSS without Ca^{2+} or Mg^{2+}. After this, the blastema region was isolated from the rest of the tissue using a scalpel, under a microscope. To dissociate blastema cells, three 100 µL drops of different incubation media were placed on a Petri dish, one of Trypsin-EDTA and two of L-15 media with 20% bovine foetal serum. The three drops were covered with mineral oil. The whole blastema was first incubated in the Trypsin-EDTA drop for 5 min and the cells were dissociated using a pulled Pasteur pipette. Treatment was stopped adding 10 µL of serum to the drop, next cells were washed twice in L-15 medium by transferring them to the other two drops, and finally cell viability was checked using 0.4% trypan blue.

Around 50-100 presumptive blastema cells were injected per recipient embryo into the marginal zone, where primordial germ cells (PGCs) are localized in MBT embryos The micromanipulation was carried out in H10 medium. Both blastema and MBT cells, were kept in HBSS without Ca^{2+} or Mg^{2+}. The outer diameter of the injection micropipette was 30 µm for blastema cells and 50 µm for MBT cells. Micromanipulation was carried out using a Leitz micromanipulator inverted microscope (Nikon ECLIPSE TE200). After performing the chimaerism, survival of remaining cells was tested using the trypan blue test.

One experimental and two control groups were established. In the experimental group (E) wild type blastema cells were injected into gold type MBT embryos. To evaluate the effect of the cell type on embryo and larval survival, wild type embryonic cells at the MBT stage were also injected into gold type MBT embryos (first control group, C_1). The aim was to evaluate the technical efficiency and the effect of chimaerism on survival, the second control group (C_2) was not subjected to any manipulation and embryos were kept in H10 for the same period of time as those that were micromanipulated. At least 100 embryos were manipulated, both in the experimental group (E) and in the control groups in the different sessions.

Survival was evaluated at 24, 48 and 72 h after performing the chimaerism, and the rate of adult chimaeras was evaluated after about three months.

Results were analysed using the c^2 test (Statgraphics Plus 4.0). When a single degree of freedom was involved, the Yates correction for continuity was performed.

During growth and elongation of the blastema, this transitory structure generates a cell proliferating-differentiation balance (Nechiporuk and Keating, 2002). This characteristic is important when selecting donor cells for transplanting because the regenerative outgrowth has different kinetic, morphological and molecular qualities in the days after amputation. In this work, the blastema used in our chimaerism experiments was recovered at 4 d post-amputation. At this stage, the transition between blastema formation and blastema outgrowth begins and the blastema precursor cells have just undergone the dedifferentiation process and have a high proliferation rate (Poleo *et al*., 2001), so most cells retrieved will be undifferentiated.

In our results, cells from the blastema differed in their morphology to cells from a non- amputated fin with regard to size and lobopodia signs (Harvey, 1983), typical movements of undifferentiating cells. The blastema cells tolerated both manipulation and treatments (bleach, trypsin, etc.) to isolate them, as shown by the viability tests after each session of chimaerism.

With regard to embryo viability, survival of embryos that supported the manipulation process, were evaluated at 24, 48 and 72 h after performing the chimaerism (103 from 134 in E; 135 from 202 in the control group 1, C_1). The technical efficiency of embryos that survived manipulation was 76.86% in blastema cells and 66.83% in blastomeres at the MBT stage. Embryo survival is shown in Table 1. Statistical analysis of the results showed significant differences ($p > 0.05$) between the E and C_1 groups in embryo survival at 24 and 48 h postchimaerism. This means that insertion of blastema cells involves less immediate damage on embryo survival than insertion of blastomeres at the MBT stage. The cause of the increase in deaths at 24 h, in the control group C_1 may have been due to pipette size (30 µm for blastema cells *vs* 50 µm for MBT cells) because mechanical damage caused by the injection process are higher as micropipette size increases. There were also significant differences ($p < 0.05$) between the two control groups, C_1 and C_2, at 24 and 48 h. However, at no time was there a significant difference between group E and group C_2. Another factor to take into account in embryo viability is the number of cells injected. Although blastema cells are larger than somatic cells (fibroblasts), they are much smaller than blastomere cells (Figure 1). This means that mechanical damage caused by injection of MBT blastomeres is greater than the damage caused by injection of the same number of blastema cells. The survival at 72 h, in E and the controls (C_1 and C_2) (Table 1) supports the

Table 1. Embryo survival at different stages

	Experimental group (E)[1]	**Control group (C_1)**[2]	**Control group (C_2)**[3]
Initial number	103	135	104
24 h post-chimaerism	86/103 (83.4%)[a]	74/135 (54.8%)[b]	96/104 (92.3%)[a]
48 h post-chimaerism	85/86 (98.8%)[a]	63/74 (85.1%)[b]	94/96 (97.9%)[a]
72 h post-chimaerism	76/85 (89.4%)	53/63 (84.1%)	87/94 (92.6%)
Final	76/103 (73.8%)[a]	53/135 (39.3%)[b]	87/104 (83.7%)[a]

Columns with different superscripts are statistically different (Statgraphics 4.0). [1] Experimental group (E): wild type blastema cells injected into gold type MBT embryos. [2] Control group 1 (C_1): wild type MBT cells injected into gold type MBT embryos. [3] Control group 2 (C_2): embryos not subjected to manipulation.

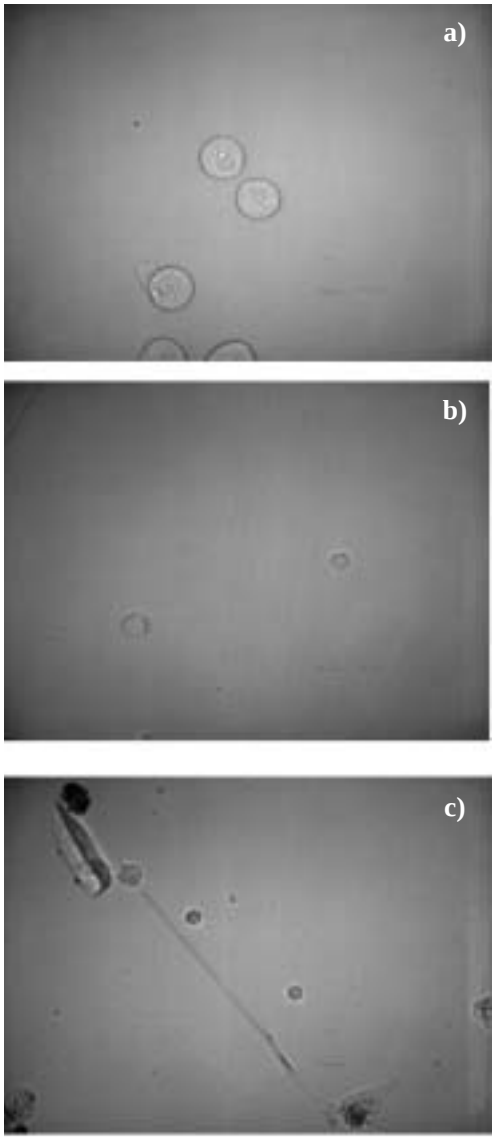

Figure 1. a). Embryonic cells (200x). Blastomeres obtained from embryos at MBT stage (1000-cells). b). Blastema cells (200x). Cells obtained 4 d after caudal fin amputation. c). Somatic cells (200x). Fibroblasts obtained from primary caudal fin cultures**.**

previous conclusion that mechanical damage in the manipulation process causes the differences observed. Thus, although blastema cells have molecular qualities and expression patterns that differ from the blastomeres at the MBT stage, embryo survival is higher in the former. It is worth noting that zebrafish embryos generates a favourable microenvironment for keeping somatic cells in an undifferentiated state and human metastatic cells can survive, divide, and even migrate and integrate into zebrafish embryos (Lee *et al*., 2005; Piliszek *et al*., 2007).

The results of this work suggest that insertion of blastema cells does not have a negative effect on embryo development for at least three days post fertilization.

Although the process of cell dedifferentiation explains the formation of the blastema after a limb lesion in aquatic urodele amphibians (Straube *et al.*, 2004**;** Brockes and Kumar, 2005; Straube and Tanaka, 2006), in teleost fishes there are no molecular markers available to prove the cellular reprogramming before forming the blastema. However, several cytological (Becerra *et al*., 1996) and cell signalling studies (Santamaría *et al*., 1996**;** Poleo *et al*., 2001; Nechiporuk and Keating, 2002; Santos-Ruiz *et al.*, 2002) suggest that dedifferentiation of mesenchymal cells would be a possible mechanism of blastema formation. If this were true, their integration into a chimera specimen would be possible, even in the germ line.

Somatic chimaerism begins to show at 48 h postfecundation, because this is the stage at which the first melanocytes differentiate (Rawls *et al*., 2001). As skin pigmentation chimaerism (Figure 2) is a good marker for detecting germ-line contributions from transplanted cells in zebrafish (Lin *et al*., 1992), the results from adults in this work (E: 0 chimaeras from 7 specimens; C_1: 5 chimaeras from 17 specimens; data not shown) suggest that blastema cells are not totipotent, which precludes their use as donor cells in strategies of biodiversity preservation via chimaerism. Although the dedifferentiation grade of the blastema cells may not be enough to generate germ-line chimaeras, their condition of potentially dedifferentiating cells may be an advantage

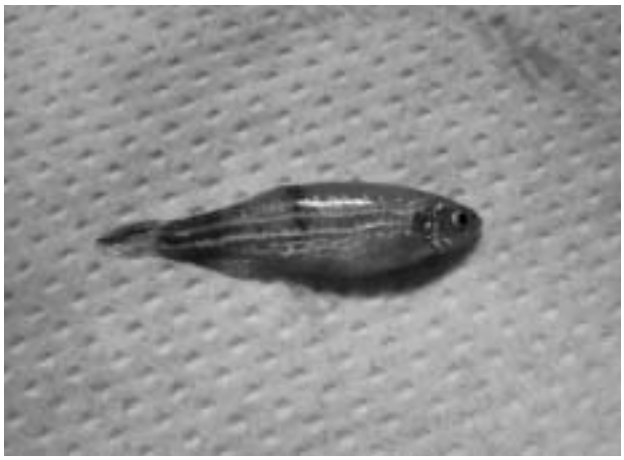

Figure 2. Somatic chimaerism in zebrafish.

when using them as donor nuclei in somatic cloning by nuclear transplant, increasing technical efficiency in fish.

Acknowledgements

We would thank Mr. Javier Rubio Rubio for technical support in colony maintenance, microinstrument making and embryo micromanipulation. The authors also thank Mr. Neil Macowan for revising the English version of the manuscript.

References

AKIMENKO M., MARÍ-BEFFA M., BECERRA J., GÉRAUDIE J., 2003. Old questions, new tools, and some answers to the mystery of fin regeneration. Dev Dyn 226, 190-201. doi:10.1002/dvdy.10248.

BECERRA J., JUNQUEIRA L.C., BECHARA I.J., MONTES G.S., 1996. Regeneration of fin rays in teleosts: a histochemical. Radioautographic, and ultraestructural study. Arch Histol Cytol 59, 15-35. doi:10.1679/aohc.59.15.

BROCKES J.P., KUMAR A., 2005. Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310(5756), 1919-1923. doi:10.1126/science.1115200.

CARDONA-COSTA J., GARCÍA-XIMÉNEZ F., 2007. Vitrification of zebrafish embryo blastomeres in microvolumes. Cryoletters 28(4), 303-309.

GRUSH J., NOAKES D.L.G., MOCCIA R.D., 2004. The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). Zebrafish 1(1), 46-53. doi:10.1089/154585404774101671.

HARVEY B., 1983. Cooling of embryonic cells, isolated blastoderms, and intact embryos of the zebrafish *Brachydanio rerio* to -196ºC. Cryobiology 20, 440-447.

LEE L., SEFTOR E.A., BONDE G., CORNELL R.A., HENDIX M., 2005. The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in absence of tumor formation. Dev Dyn 233(4), 1560-1570. doi:10.1002/dvdy.20471.

LIN S., LONG W., CHEN J., HOPLINS N., 1992. Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. Proc Natl Acad Sci 89, 4519-4523. doi:10.1073/pnas.89.10.4519.

NECHIPORUK A., KEATING M.T., 2002. A proliferation gradient between proximal and *msxb*-expresing distal blastema directs zebrafish fin regeneration. Development 129, 2607-2617.

PILISZEK A., MODLINSKI J.A., PYSNIAK K., KARASIEWICZ J., 2007. Foetal fibroblast introduced to cleaving mouse embryos contribute to full-term development. Reproduction 133(1), 207-218. doi:10.1530/REP-06-0013.

POLEO G., BROWN C.W., LAFOREST L., AKIMENKO M.A., 2001. Cell proliferation and movement during early fin regeneration in zebrafish. Dev Dyn 221, 380-390. doi:10.1002/dvdy.1152.

POSS K., KEATING M., NECHIPORUK A., 2003. Tales of regeneration in zebrafish. Dev Dyn 226, 202-210. doi:10.1002/dvdy.10220.

RAWLS J.F., MELLGREEN E.M., JOHNSON S.L., 2001. How the zebrafish gets its stripes. Dev Biol 240, 301-314. doi:10.1006/dbio.2001.0418.

SANTAMARIA J.A., MARI-BEFFA M., SANTOS-RUIZ L., BECERRA J., 1996. Incorporation of brodeoxyuridine in regenerating fin tissue of the gold fish *Carassius auratus*. J Exp Zool 275, 300-307.

SANTOS-RUIZ L., SANTAMARIA J.A., RUIZ-SANCHEZ J., BECERRA J., 2002. Cell proliferation during blastema formation in the regenerating teleost fin. Dev Dyn 223(2), 262-72. doi:10.1002/dvdy.10055.

SIMAO M.F., PEREZ-CAMPS M., GARCÍA-XIMÉNEZ F., 2007. Short communication. Zebrafish embryo development can be reversibly arrested at the MBT stage by exposure to a water temperature of 16ºC. Span J Agric Res 5(2), 181-185.

STRAUBE W.L., TANAKA E.M., 2006. Reversibility of the differentiated state: regeneration in amphibians. Artif Organs 30(10), 743-55. doi:10.1111/j.1525-1594.2006.00296.x.

STRAUBE W.L., BROCKES J.P., DRECHSEL D.N., TANAKA E.M., 2004. Plasticity and reprogramming of differentiated cells in amphibian regeneration: partial purification of a serum factor that triggers cell cycle re-entry in differentiated muscle cells. Cloning Stem Cells 6(4), 333-44. doi:10.1089/clo.2004.6.333.

WESTERFIELD M., 2003. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed., Univ. of Oregon Press, Eugene, OR, USA.