Production of interspecies chimeras by transplanting rosy barb (Puntius conchonius) embryonic cells to zebrafish (Danio rerio) embryos

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Establishment of a cell-mediated gene transfer system has potential as a new breeding technique for commercially valuable fishes. As an important step toward developing an inter-species chimera, cells from blastula-stage embryos of rosy barb (Puntius conchonius) were transplanted into zebrafish (Danio rerio) blastula-stage embryos to observe the development of the recipient. From the total of 473 transplants obtained only a fraction of 13 chimeras appeared perfectly normal after one month. Over two in normal 13 chimeras showed some characters from the donor cells with scarce pigmentation. This is the first successful inter-species study on zebrafish by using blastula cell transplants from rosy barb both belonging to the same family cyprinidae.

Keywords: Zebrafish, Rosy barb, Blastula cells, Chimera.

With the increased popularity of zebrafish (Danio rerio) for mutagenesis studies, efficient methods for performing insertional mutagenesis are needed. The pioneering work on germ-line chimeras with the use of the mouse embryonic stem cells (ES) had a profound influence on pure and applied research. With the use of ES cells that differ genetically in pigmentation from the recipients, chimeras with contributions from the ES cell can be recognized visually. The ability to make such germ-line chimeras provides the basis for performing targeted and promoter trap mutagenesis in mice. Establishment of cell-mediated gene transfer in teleosts is of value for basic research, such as in understanding gene function, and in applied fields, such as aquaculture. If transplanted stem cells could contribute to the germ cell lineage of a chimera, the chimera, referred to as a germ-line chimera, will produce progeny having the modified genome derived from the transplanted stem cells. Establishment of a cell-mediated gene transfer system has potential as a new breeding technique for commercially valuable fishes. Even though the zebrafish possesses many characteristics that make it a valuable model for genetic studies of vertebrate development, one deficiency of this model system is the absence of methods for cell-mediated gene transfer. Transgenic animals possessing a mutated copy of the targeted gene are generated when the transplanted cells contribute to the germ-line of a chimera embryo. In fish one crucial question concerns the possibility and/or efficacy of producing chimeras from cultured fish ES cells is the physiological compatibility between donor cells and host embryos.

Success in transplanting undifferentiated cells from one zebrafish embryo to another have been reported. Lin et al. have also performed similar experiments in zebrafish using donor cells from genetically pigmented embryos and albino recipients. Blastula cells possess the ability to contribute to multiple tissues (including the germ line) after their introduction into a host embryo. In medaka, cell transplantation techniques to produce germine chimeras have already been developed using isolated early blastomeres as donor cells. As an important step toward developing an interspecies chimera, it was thought to determine whether cells from blastula-stage embryos of rosy barb (Puntius conchonius) could be transplanted into zebrafish (Danio rerio) blastula-stage embryos and participate in forming the germ-line of the recipient.

In this study communication preliminary results on the production of the interspecies chimera between...
zebrafish (*D. rerio*) and rosy barb (*P. conchonius*) are reported.

**Materials and Methods**

*Fish and breeding—Zebrafish* (*D. rerio*) and rosy barb (*P. conchonius*) both belong to the same family Cyprinidae. Wild type zebra fish (*D. rerio*) were procured from a local (Qingdao, China) ornamental fish dealer, and maintained in the laboratory at 27±1°C at a density of one fish per litre. Rosy barb (*P. conchonius*) were also procured from a local ornamental fish dealer and maintained in the laboratory at 22±1°C. The fish were fed *ad libitum* with ornamental fish pellet twice a day. Water exchanges and tank cleaning were conducted as required. The mature fish were used repeatedly for experiments.

During late evening matured female and male zebra fish and rosy barb were placed in ‘breeding traps’ in 10 litre tank to prevent them from eating the eggs laid. The ratio of gravid female to male was 1:1. The fish usually laid eggs in the early morning next day. The naturally spawned and fertilized eggs were collected from the tank by siphoning, washed and transferred into petridishes containing egg water (0.03% instant ocean salt mix)²⁰. The eggs were checked under a stereo-microscope and only the cleaving eggs were used.

*Micropinjection and cell transplantation—Embryos* were collected and only the donor embryos (rosy barb) were dechorinated in Holtfrerers solution (0.35 g NaCl, 0.005 g KCl, 0.01 g CaCl₂ and 0.02 g NaHCO₃ in 100 ml distilled water). Cell transplants were performed on zebrafish embryos at the mid to late blastula stage (about 1000-2000 cells). The developing blastula cells of rosy barb were cut apart from the yolk sac with a glass needle and loaded by suction in the micropipette and then injected into the blastocoel of a recipient (zebrafish) embryo, without damage to the yolk cell. Usually, 50 to 100 cells were transplanted into a single zebrafish embryo under a dissecting microscope. To elucidate mechanisms of injected blastula cells in the morphogenetic movements at the cellular level the donor cells were stained overall with Nile blue sulphate solution²¹ and the stained cells were injected into the recipients (zebrafish).

*Larval culture—Hatched larvae were transferred to 10 litre glass tanks containing fresh water and cultured according to standard procedure²⁰. The larvae were first fed with paramecia two days after hatching, and 15 days later, they were fed with artemia nauplii. From day 45 post hatching the fish were fed with ornamental fish pellet.

*Chromosome preparations—Metaphases from zebrafish and rosy barb were prepared according to the method described by Kligerman and Bloom²². Tissues were prepared from 20 day old fish.

*PCR assay—DNA templates were isolated from zebrafish, rosy barb and chimeric zebrafish according to the method of Sambrook et al²³. Twelve arbitrary primers (Operon, USA) were tested and only six primers, (OPA-07, OPA-08, OPA-09, OPA-10, OPA-13 and OPA-15) which gave reproducible amplification fragments, were used. Their nucleotide sequences are given in Table 1. Amplification was performed in 50 μl reaction volume containing 10× taq reaction buffer, 2 mM/l MgCl₂, 0.1 mM/l of each dNTP, 8 μM/l/primer, 40 ng template DNA, and 1 unit of Taq DNA polymerase (Sangon, Canada) in GeneAmpPCR system 2400 (Perkin-Elmer, U.S.). Lambda DNA marker (Promega, Madison, WI, USA) (Eco RI +Hind III digest) was included as molecular weight marker. The PCR protocol consisted of an initial denaturation step of 2 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 37°C for 45 sec, extension at 72°C for 2 min, and a final extension at 72°C for 10 min followed by holding at 4°C continuously. A negative control, containing all reaction ingredients except for template DNA, was included for each primer. All amplifications were done in triplicate and on different days. Amplification products were analysed by electrophoresis with 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and photographed. The RAPD banding patterns between normal zebrafish, rosy barb and chimeric zebrafish were compared.

*Results*

*Production of chimeras—Chimerism is a rapid way of producing animals with new genetic traits. However before chimerism can be applied to a wider*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>OPA-07</td>
<td>5'-GMAACCGGTTG-3'</td>
</tr>
<tr>
<td>2</td>
<td>OPA-08</td>
<td>5'-GTGACGTAGG-3'</td>
</tr>
<tr>
<td>3</td>
<td>OPA-09</td>
<td>5'-GGTAAAGCCT-3'</td>
</tr>
<tr>
<td>4</td>
<td>OPA-10</td>
<td>5'-GTGATCGCAG-3'</td>
</tr>
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<td>5</td>
<td>OPA-13</td>
<td>5'-CAGCACCCAC-3'</td>
</tr>
<tr>
<td>6</td>
<td>OPA-15</td>
<td>5'-TCCGAACCC-3'</td>
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range of fish species, the use of species-specific developmental studies is pivotal for designing gene constructs. In this context zebrafish (D. rerio) and rosy barb (P. conchonius) belonging to the same Cyprinidae family have been used to produce interspecies chimeric fish. The advantage of using rosy barb for combination with zebrafish was the chromosome number which in both the species were 2n = 50 (Fig. 2 A and B).

The plan of the experiment for generating chimeras is outlined in Fig. 1. Embryos were collected almost simultaneously from rosy barb and zebrafish and blastula cell transplants were performed at mid to late blastula stage with the help of micropipette. Survival rates for chimeras were variable on different days of transplantation. Altogether 473 transplants were performed on 6 different days; 36 operated embryos survived and appeared essentially normal at four days of age and 13 survived to adulthood (Table 2). The chimera formation rate and survival rates varied among the different groups of injected zebrafish embryos. The highest per cent of surviving chimeras obtained was 11%. Abnormality rates were in proportion to the occurrence of normal developed chimera embryos.

Morphological observations in chimeras—After 14 hr post fertilization when embryos developed to 100%

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Table 2—Frequency of germ-line chimerism

<table>
<thead>
<tr>
<th>Groups of injected zebrafish embryos</th>
<th>Total number of transplanted embryos</th>
<th>14 (hr)</th>
<th>24 (hr)</th>
<th>48 (hr)</th>
<th>96 (hr)</th>
<th>Abnormal embryos</th>
<th>Normal embryos</th>
<th>Adult</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>61</td>
<td>40</td>
<td>26</td>
<td>17</td>
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<td>8</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>56</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>Nil</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>61</td>
<td>15</td>
<td>10</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>56</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>2</td>
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<td>61</td>
<td>23</td>
<td>10</td>
<td>7</td>
<td>20</td>
<td>22</td>
<td>13</td>
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</table>
epiboly it was observed that the transplanted embryos became oblong in shape (Fig. 3). To ascertain mechanisms of morphogenetic movements of donor cells at the cellular level, the behavior of Nile blue stained donor cells in chimeric embryos was traced (data not shown). It was observed that the donor cells were well distributed throughout the blastoderm and the shape of the embryo became oval after 12hr of development. Even though results of stained cells have been confounded by the lack of specificity as the diffusing dye stained underlying cells of the embryo, it seems clear that the ultimate distribution of stained cells are truly the result from morphogenetic movements undergone by the originally stained cells. Notably the donor cells were observed in the ventral part of the developing embryo and notochord were often mislocated around the trunk or on the surface of the yolk. In rare cases they were found in tail somites (Fig. 3D). This can also be clearly noticed from the abnormally developed embryos (Fig. 3D,E,F), which hatched and developed to swim up fry (5-6 days).

The abnormality in embryos was observed after 24hr post fertilization with notochord bend upwards (Fig. 3D), notochord abnormality (Fig. 3E) and puffer

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Fig. 3—Micrographs of blastula cell transplanted zebrafish embryos and larvae. [A. normal gastrula (12 hr); B. transplanted gastrula (12 hr); C. normal larva (50 hr); D. transplanted larva with tail bend (50 hr); E. notochord abnormal (50 h); F. puffer shaped larva (50 hr); G. normal larva (56hr) and H. scarce pigmentation (56 hr).]
shaped embryo (Fig. 3F). By 56 hr post fertilization, when pigment was clearly visible in the eyes and on the body of wild-type fish (Fig. 3G), scarce pigmentation was observed in chimeras (Fig. 3H). Some larvae with variegated pigmentation survived till adulthood, whereas some survived for 10-15 days only. The reasons for the death of larvae after 15 days are not known. In wild-type zebrafish dark horizontal stripes alternate with light stripes. The transplanted zebrafish of one and half month old did not have dark stripes, rather they clearly show colorless horizontal stripes. The morphological observations thus reveal germ-line transfer of cells in the transplanted embryos.

The results obtained using inter species combination are encouraging. Based on this technique

tests of these methods using a wider range of organisms may provide more information on their suitability as a routine method for chimera production.

Selection of primers—Six primers viz., OPA-07, OPA-08, OPA-09, OPA-10, OPA-13, and OPA-15 were selected from 12 primers. These primers were found to give best and reproducible results.

RAPD-PCR analysis—Zebrafish, rosy barb and chimera zebrafish were analysed by RAPD-PCR. The characteristic fingerprint patterns obtained with six primers are shown in Fig. 4. Though RAPD results were obtained from all 12 primers used, these six primers gave reproducible and consistent results and hence they were chosen for data analysis. The position of bands generated by primers OPA-07 and OPA-08 are shown in Fig. 4A, by primers OPA-09, and OPA-10 are shown in Fig. 4B and by primers OPA-13 and OPA-15 are shown in Fig. 4C. The RAPD-PCR analysis thus successfully differentiated between zebrafish and chimera zebrafish.

Discussion

Using a combination of methods which include recombination DNA technique and the use of embryonic stem cells, researchers have been able to produce chimeric mice and fish. Cell transplantation between zebrafish embryos have been used15,14 to study the behavior of cells derived from developmentally important mutants. The experiments described here demonstrate that cells from mid-blastula stage of rosy barb (P. conchonius) can contribute to the germ-line after transplantation to recipient, zebrafish (D. rerio) embryos. The experiment also demonstrates that pigmentation is an excellent marker for detecting germ-line contributions from transplanted cells. Walker and Streisinger27 and Streisinger et al.28 have shown the usefulness of pigmentation as a marker in zebrafish for establishing conditions of γ-ray mutagenesis and for studying cell lineage in the eye. The ability to make germ-line chimeras in zebrafish suggests that it is possible to introduce genetically engineered cells into zebrafish embryos and to identify the offspring of these cells by pigmentation at 2 days of age15. An additional observation in these studies was abnormal development in the trunk, tail and ventral part of the chimeric embryo, a result that supports evidence that blastula cells of rosy barb contribute in insertional mutagenesis. The identification of developmental

Fig. 4—Band patterns of the RAPD products using the primers A7, A8, (Fig. 4A); A9, A10, (Fig. 4B); and A13, A15 (Fig. 4C). [(A-C) M: Marker; lane1-(zebrafish), 2- (rosy barb), 3-(chimera zebrafish), 4-(negative control), lane5-(zebrafish), 6-(rosy barb), 7-(chimera zebrafish), 8-(negative control)].
genes through insertional mutagenesis into zebrafish genome, may allow the isolation and cloning of developmental genes.

In the present experiments, the mid to late blastula cells were chosen for transplantations because we wanted donor cells to undergo transcription as midblastula stage embryos undergo several changes in cell behavior and significant levels of transcription\(^{29,30}\). Whether all the blastomeres that were transplanted have the potential of germ-line chimeras remains to be learned. The results of Nile blue stained donor cells could identify the mechanisms of morpogenic movements of donor cells at the cellular level (data not shown). The present experiments suggest that if donor (rosy barb) blastula cells enter the germ-line after transplantation into zebrafish recipients, then 50% of the offspring of such germ-line chimeras should have gene trap integrations and hence be heterozygous for insertion induced mutations. From the total of 473 transplants only a fraction of 13 chimeras appeared perfectly normal after one-month duration. A striking result was the appearance of pigmentation on the body of chimeras after 56 hr post fertilization and at 4 to 6 weeks of age. Over 2 in normal 13 chimeras showed germ-line contributions from the donor cells. The total number of chimera examined here is still small, but the preliminary frequency is encouraging.

Successful production of germline chimeras in zebrafish by using blastula cell transplantations from rosy barb both belonging to the same family cyprinidae with chromosome number 2n=50. Polymerase chain reaction (PCR) protocols are developed to compare intra and inter-specific differences in chimaera fish\(^{26}\). RAPD-PCR analysis could successfully differentiate between normal zebrafish and chimera zebrafish. The analysis by using six primers when compared with zebrafish, rosy barb and chimera zebrafish allowed us to directly correlate band differences.

In conclusion, we established a technique for developing inter-species chimera, which will be useful to isolate new phenotypes in fish and identify those appear interesting. The results reported here may prove to be one step toward this goal.

References

The number of germ-line chimera fish produced also varies with the laboratory and the species of fish ranging from about 1-2% to 40% in zebrafish\(^{15}\), none in medaka\(^{12}\), 4% in zebrafish\(^{26}\), and 0.3 to 14% in rainbow trout\(^{28}\). Successful production of germ-line chimeras in loach (*Misgurnus anguillicaudatus*) by using wild type and orange type as donor and recipient has also been reported\(^{34}\). Fadool *et al.*\(^{35}\), have demonstrated that the *mariner* element from *Drosophila mauritiana* can be used for generation of stable transgenic lines in zebrafish. Gene transfer by microinjection is a laborious and expensive method. Thus easier and more efficient method for gene transfer into large number of eggs by blastula cell transplantation have been sought, but with varying degree of success. However the efficiency of cell transplantation is dependent on the acceptance of species-specific cells for expression in chimera fish after transfer.

This is the first successful inter-species study on zebrafish by using blastula cell transplantations from rosy barb both belonging to the same family cyprinidae with chromosome number 2n=50. Polymerase chain reaction (PCR) protocols are developed to compare intra and inter-specific differences in chimaera fish\(^{26}\). RAPD-PCR analysis could successfully differentiate between normal zebrafish and chimera zebrafish. The analysis by using six primers when compared with zebrafish, rosy barb and chimera zebrafish allowed us to directly correlate band differences.

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