Production of zebrafish germ-line chimeras from embryo cell cultures

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Edited by Michael S. Levine, University of California, Berkeley, CA, and approved December 14, 2000 (received for review September 19, 2000)

Although 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 and targeted gene inactivation. In mice, embryonic stem cell cultures are routinely used for gene transfer and provide the advantage of *in vitro* selection for rare events such as homologous recombination and targeted mutation. Transgenic animals possessing a mutated copy of the targeted gene are generated when the selected cells contribute to the germ line of a chimeric embryo. Although zebrafish embryo cell cultures that exhibit characteristics of embryonic stem cells have been described, successful contribution of the cells to the germ-cell lineage of a host embryo has not been reported. In this study, we demonstrate that short-term zebrafish embryo cell cultures maintained in the presence of cells from a rainbow trout spleen cell line (RTS34st) are able to produce germ-line chimeras when introduced into a host embryo. Messenger RNA encoding the primordial germ-cell marker, *vasa*, was present for more than 30 days in embryo cells cocultured with RTS34st cells or their conditioned medium and disappeared by 5 days in the absence of the spleen cells. The RTS34st cells also inhibited melanocyte and neuronal cell differentiation in the embryo cell cultures. These results suggest that the RTS34st splenic–stromal cell line will be a valuable tool in the development of a cell-based gene transfer approach to targeted gene inactivation in zebrafish.

The zebrafish is a popular model for studies of vertebrate development, possessing many favorable characteristics including a short generation time, external fertilization, and optically clear embryos that are suited for manipulations involving DNA transfer, cell labeling, and transplantation (1, 2). Also, methods have been established for performing large-scale mutagenesis screens in zebrafish to identify developmentally important genes (3–5). Despite these advantages, one deficiency of the zebrafish model is the absence of methods for targeted gene inactivation. In mice, the use of pluripotent embryonic stem (ES) cell cultures for the production of knockout mutants has provided a powerful approach to the study of gene function during embryogenesis (6, 7). Cultured ES cells possess the ability to contribute to multiple tissues (including the germ line) after their introduction into a host embryo (8, 9). By using this strategy, gene inactivation is accomplished in the cell cultures by targeted insertion of foreign DNA into the coding region of the gene by homologous recombination (10). ES cells that have undergone the targeting event are selected and grown in culture, and the genetic alteration is transferred to the germ line of a host embryo when the cultured cells are transplanted into the embryo, where they then contribute to the germ-cell lineage (11).

To use this cell-mediated strategy for the production of knockout mutants in other species, it is necessary to develop methods for the culture of cells from each species that possess the ability to produce functional germ cells *in vivo*. Germ-line contribution can be accomplished by using cultured pluripotent ES cells or primordial germ cells (PGC), the embryonic germ-cell precursor (12, 13). Although much effort has been devoted to the development of ES cell and PGC lines from several species, cultures that are able to contribute to the germ-cell lineage of a host embryo have been successfully reported only for mice and chickens (9, 14–16).

Development of a cell-mediated approach to targeted gene inactivation for the zebrafish would greatly enhance the value of this organism as a model for the genetic analysis of vertebrate development. To make this technology available, our laboratory and others have been working to develop zebrafish embryo cell cultures that will be suitable for gene transfer experiments. Previously, cultures were established that exhibit *in vitro* characteristics of pluripotent ES cells, including an ES-like morphology, alkaline phosphatase activity, and the ability to differentiate into multiple cell types (17–19). Despite their possessing ES-like properties, successful utilization of these cultures for the production of germ-line chimeras has not been reported. To address this problem, we have evaluated alternative culture conditions, including the use of feeder cells, to establish methods for the *in vitro* propagation of zebrafish embryo cells that are able to contribute to the germ-cell lineage of a host embryo. In this study, data are presented demonstrating that short-term cell cultures derived from gastrula-stage zebrafish embryos (cocultured with fish splenic–stromal cells) are able to generate germ-line chimeras after their introduction into a host embryo. The contribution of the cultured cells to the host germ line is demonstrated by the inheritance in the F1 and F2 fish of transgenic sequences and a pigmentation pattern donated by the cultured cells. Embryo cell cultures maintained on a feeder layer of spleen cells or in spleen cell-conditioned medium continue to express the primordial germ-cell marker, *vasa* (20–22), for more than 30 days, during which differentiation is inhibited.

Materials and Methods

Zebrafish Strains. Zebrafish of the transgenic B7–43 strain possess melanocyte pigmentation on their bodies and are homozygous for the bacterial neomycin phosphotransferase gene (*neo*; ref. 23). Zebrafish from the GASSI strain are homozygous for the b2 allele at the brass locus and therefore lack heavy melanocyte pigmentation on their bodies (24).

Cell Culture. RTS34st cells were grown in L-15 medium (Sigma) supplemented with 30% FBS (vol/vol; Harlan Laboratories, Indianapolis, IN; ref. 25). Conditioned medium was collected from confluent cultures every 3 days, sterile filtered, and stored at 4°C for a maximum of 1 week before use. Embryos used for cell culture were obtained from the B7–43 line of zebrafish. To initiate embryo cell cultures, approximately 50 germ-ring stage embryos (6 h after fertilization) were washed in bleach solution (0.5%), rinsed several times in culture medium, and dechorion-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ES, embryonic stem; PGC, primordial germ cell; RTS34st, rainbow trout spleen cells; LIF, leukemia-inhibitory factor; *vasa*, primordial germ-cell marker.

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ated by pronase treatment (0.5 mg/ml pronase in Hanks' solution; GIBCO). The embryos were dissociated in trypsin/EDTA solution (0.2% trypsin/1 mM EDTA in PBS) with gentle pipeting, and the cells were collected by centrifugation. The pellet was resuspended in LDF medium (50% Leibovitz L-15/35% Dulbecco's modified Eagle's/15% Ham's F12 media; ref. 26) and the suspension of individual cells was added either to a 25-cm^2 flask or to each well of an 8-well chamber slide (Nunc). For experiments involving a feeder layer, the embryo cells were added to a confluent monolayer of RTS34st cells. In some experiments, RTS34st cell-conditioned medium (25% final volume) was added in place of a feeder layer. After the embryo cells attached to the culture surface (30 min), the medium was supplemented with bovine basic fibroblast growth factor (50 ng/ml; R & D Systems), murine epidermal growth factor (50 ng/ml; GIBCO), bovine insulin (10 μg/ml; Sigma), sodium selenite (10 nM), trout embryo extract (50 μg protein/ml), trout serum (0.4%), and FBS (5% vol/vol). Trout embryo extract and trout serum were prepared as previously described (27). Cultures used for transplantation into host embryos also contained human recombinant stem cell factor (50 ng/ml; GIBCO/BRL) and leukemia inhibitory factor (LIF; 5 ng/ml; Roche Molecular Biochemicals).

At various times after culture initiation, zebrafish embryo cells were harvested and microinjected into recipient embryos or examined for the presence of *vasa* mRNA. Cultures to be used for microinjection were initiated on RTS34st feeder layers as described above. To harvest the cells, EDTA solution (0.25 mM EDTA in PBS) was added to the culture, causing the embryo cells to dissociate from the feeder layer. The embryo cells were collected, leaving the RTS34st cells behind, and dissociated to single cells with gentle pipeting. After being washed one time, the cells were suspended in LDF medium and loaded into a needle formed from a drawn-out Pasteur pipet.

Embryo Microinjection. Recipient embryos used for cell transplantation experiments were obtained from the GASSI line of fish that lack heavy melanocyte pigmentation on the body. Blastula-stage embryos were dechorionated with pronase, rinsed in embryo medium (28), and placed in a depression made in agarose in a 60-mm Petri dish. Approximately 50 to 200 cells were delivered into the cell mass of each recipient blastula by using a dissecting microscope and a hand-held Pipet-Aid (Sigma; ref. 29). After injection (1 h), the embryos were transferred into a Petri dish containing embryo medium; after 7 days, they were placed in a recirculating incubator system. After an additional 7 days, the embryos were transferred to a 2.5-gallon tank and reared to sexual maturity (approximately 3 months). To test for germ-line chimerism, individual fish developed from injected embryos were bred to GASSI fish and the F1 individuals were examined for the presence of *neo* or melanocyte pigmentation as described below.

Embryo Analysis. PCR analysis was performed on a group of the embryos at various times after injection and on embryos from the F1 generation. DNA extraction was performed by using the QIAamp DNA Mini Extraction kit (Qiagen, Chatsworth, CA), and *neo* sequences were detected with the following primers: forward, 5′-GGATGATC TGGACGAAGAGC-3′ and reverse, 5′-GAAATCTC GTGATGGCAGGT-3′. The PCR reaction mixture contained genomic DNA (100 ng), Tris·HCl (10 mM, pH 8.3), KCl (50 mM), dNTPs (200 μM), and each primer (1 μM). Typical amplification conditions consisted of 35 cycles of 1 min each at 94°C, 62°C, and 72°C. The 392-bp amplification product was visualized by agarose-gel electrophoresis and ethidium-bromide staining. Product identity was confirmed by sequencing. Southern blot analysis was conducted on genomic DNA isolated from embryos, adult fish, and cultured cells (30). DNA (35 μg) was digested with *Eco*RV, fractionated by agarose-gel electrophoresis, and transferred to a nylon membrane (Schleicher & Schuell). Hybridizations were conducted at 65°C in a solution containing SSC (6×; 1 × SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), Denhardt's solution (6×), SDS 0.1%, and yeast RNA (0.1 mg/ml) by using a ^{32}P-labeled probe generated by PCR amplification of *neo* sequences from the pBK-RSV plasmid (Stratagene). The probe was purified by using ProbeQuant G-50 micro columns (Amersham Pharmacia), and 2×10^6 cpm/ml was added to each hybridization. After hybridization, the membranes were subjected to high-stringency washes (0.1 × SSC and 0.1% SDS).

Detection of *vasa* mRNA. For reverse transcription–PCR analysis of embryo cell cultures, total RNA extraction and cDNA synthesis were performed by using the QuickPrep RNA Extraction kit (Amersham Pharmacia) and Smart cDNA Synthesis kit (CLONTECH), respectively. PCR was conducted by using the following primers: 5′-TGTGGACGTGAGTGGCAGC-AATC-3′ and 5′-CTAGATAGCGCACTTTACTCAGG-3′ with 35 cycles at 94°C (30 s), 64°C (1 min), and at 72°C (1 min); the 505-bp product was visualized by agarose-gel electrophoresis. Product identity was confirmed by sequencing. For *in situ* hybridization, embryo cells cultured on a glass chamber slide (Nunc) were fixed (4% formaldehyde, 5% acetic acid in 0.9% NaCl), dehydrated in a xylene series, rehydrated, treated with pepsin (0.1% pepsin, 10 min at 37°C), and postfixed (1% formaldehyde in PBS) before hybridization (16 h at 42°C; ref. 31). After being washed (at 50°C), the cells were cleared with glycerol, the number of stained colonies was determined, and the diameter of each colony was measured at the widest point. The probe was prepared by *in vitro* transcription (MAXIscript; Ambion, Austin, TX) by using a 0.5-kb fragment of *vasa* cDNA as a template (nucleotides 779-1284, GenBank accession number AB005147; ref. 22).

Results

Zebrafish cells obtained from germ-ring stage embryos were cocultured on a monolayer of the RTS34st cell line (ref. 25; Fig. 1*A*). In these conditions, the embryo cells began to aggregate within 6 h to form homogeneous clusters of tightly adherent cells distributed throughout the culture. In the presence of the feeder layer, the embryo cell aggregates continued to increase in size for more than 20 days without exhibiting morphological characteristics of differentiation (Fig. 1*B*). In contrast, embryo cells maintained in the same conditions without the RTS34st cells formed aggregates that exhibited characteristics of differentiated melanocytes and neuronal cells after 5 days in culture. Groups of pigmented melanocytes were apparent throughout the cultures and the neurites extended from the cell aggregates, making contact with neighboring cells (Fig. 1 *C* and *D*). Previous studies have shown that the neurites possess synaptic vessicles and growth cone morphology, and their appearance coincides with both elevated levels of acetylcholinesterase enzyme activity and the expression of neuron and astrocyte-specific marker proteins in the culture (32). Addition of RTS34st cell-conditioned medium to the embryo cell cultures delayed the appearance of the differentiated cell types until approximately day 25 (Fig. 1*E*).

To determine whether the embryo cell cultures exhibited characteristics of PGCs, the cultures were evaluated by reverse transcription–PCR for the expression of the PGC marker gene, *vasa* (20–22). *vasa* mRNA was detected in embryo cell cultures maintained on the RTS34st feeder layers for at least 25 days (Fig. 2*A*, lanes e–g). The *vasa* mRNA was also detected in 25-day-old cultures grown in the presence of RTS34st cell-conditioned medium in place of the feeder cells (Fig. 2*A*, lane d). Because the cultures maintained in the conditioned medium began to exhibit characteristics of differentiation around day 25,

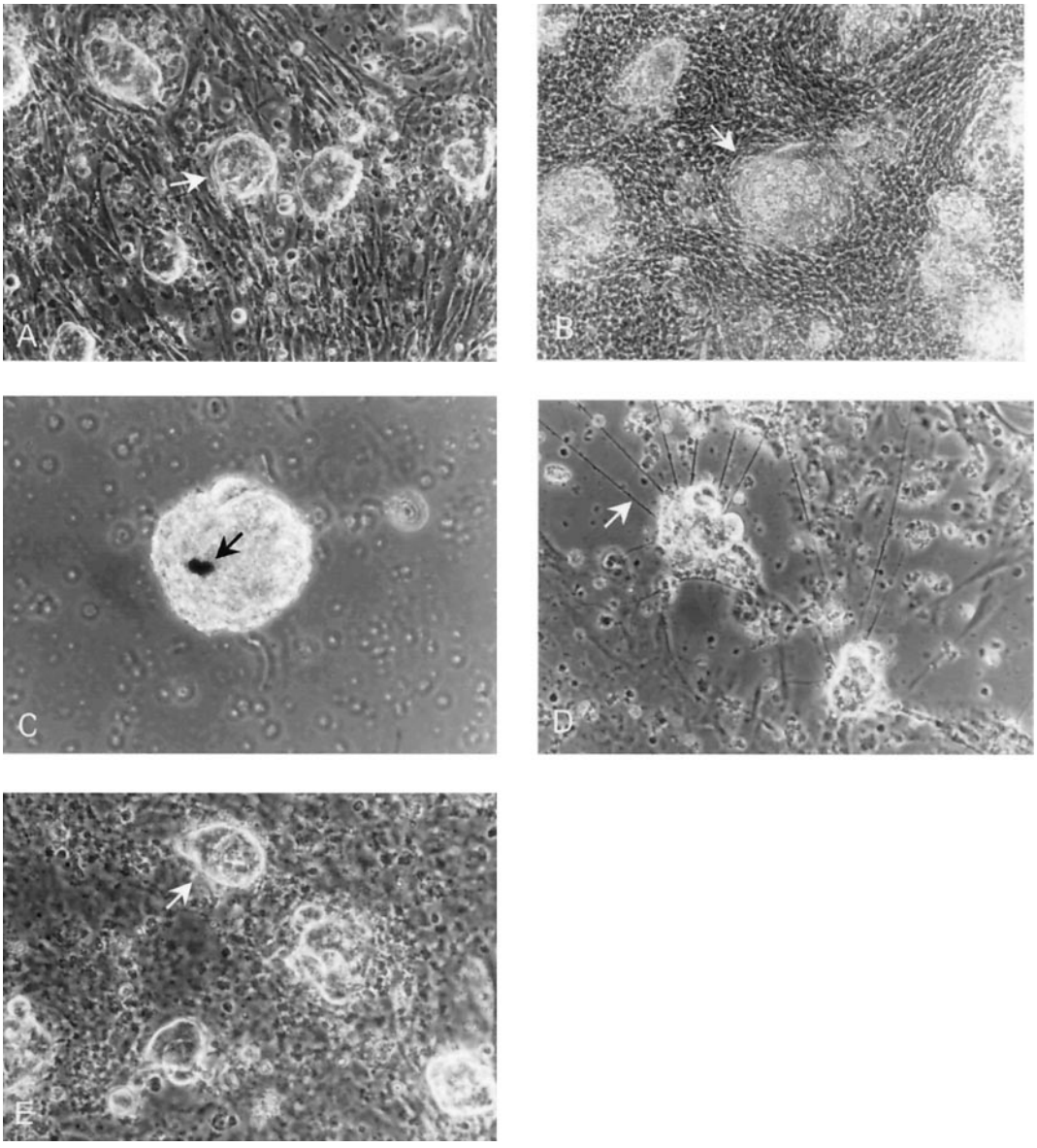

Fig. 1. Phase contrast photomicrographs of zebrafish embryo cell cultures. (*a*) Culture (24 h) of embryo cell aggregates (arrow) on a feeder layer of RTS34st cells. (*b*) Culture (20 day old) of embryo cell aggregates (arrow) grown on an RTS34st feeder layer. (*c*) Culture (15 day old) maintained in the absence of feeder cells showing the presence of melanocytes (arrow). (*d*) Culture (10 day old) maintained in the absence of feeder cells showing the presence of neurites (arrow), indicating that neural cell differentiation has occurred. The neurites begin to appear in the culture on approximately day 5. (e) Culture (25 day old) of embryo cells in RTS34st cell-conditioned medium without a feeder layer, illustrating embryo cell aggregates (arrow) on a monolayer of embryo fibroblasts.

one of the cultures was passaged on day 24 by partially dissociating the embryo cell aggregates with trypsin and reseeding the small aggregates onto a feeder layer of RTS34st cells. Eight days after passage, the cell aggregates had increased in size without exhibiting signs of differentiation and continued to possess *vasa* mRNA (Fig. 2*A*, lane h). Removal of human LIF and stem cell factor from the culture medium did not affect the appearance or level of *vasa* mRNA when RTS34st cell-conditioned medium or feeder cells were present. In the absence of the feeder layer or cell-conditioned medium, *vasa* mRNA disappeared from the embryo cell cultures after 5 days (Fig. 2, lanes i–k). In the presence of cell-conditioned medium, the cultures produced a monolayer of fibroblastic cells along with the homogeneous cell aggregates that were distributed throughout the culture (Fig. 1*E*). *In situ* staining for *vasa* mRNA demonstrated that the *vasa*-positive cells were present within the aggregates (Fig. 3). The number and size of *vasa*-positive colonies increased over an 8-day culture period, indicating that the cells were proliferating. From day 4 to day 8, the number of *vasa*-positive colonies detected by *in situ* hybridization more than doubled (from 20 to 47), and the average size of the positive colony increased 25%.

As the spleen cells influenced the differentiation of the embryo cells in culture and promoted the survival of *vasa*-positive cells, the effect of the RTS34st feeder layers on the embryo cells' ability to contribute to the germ-cell lineage of a host embryo was evaluated. Cell cultures, derived from the B7–43 transgenic line of zebrafish that possesses melanocyte pigmentation on the body and carries the bacterial gene, *neo*, were introduced by microinjection into host embryos obtained from the nontransgenic GASSI strain of zebrafish that lacks melanocyte body pigmentation (23, 24). Four groups of embryos were injected on separate days and the survival rate varied from 10% to 50%. PCR analysis of a group of embryos killed 3 weeks after each injection revealed that approximately 40% of the fish

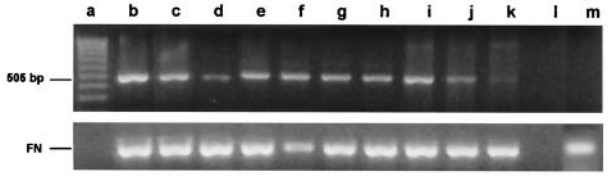

Fig. 2. Reverse transcription–PCR analysis of *vasa* mRNA in zebrafish embryo cell cultures. cDNA was synthesized from total RNA obtained from embryo cell cultures. PCR amplification was performed with *vasa*-specific primers designed to generate a 505-bp product. Product identity was confirmed by sequencing. Lane a, MW markers; lanes b, c, and d, embryo cells maintained for 5, 15, and 25 days, respectively, in RTS34st cell-conditioned medium; lanes e, f, and g, embryo cells maintained for 5, 15, and 25 days, respectively, on an RTS34st feeder layer; lane h, embryo cells maintained at first for 24 days in RTS34st cell-conditioned medium, and then (after passaging) for 8 days on an RTS34st feeder layer; lanes i, j, and k, embryo cell cultures maintained for 1, 3, and 5 days, respectively, in the absence of RTS34st feeder cells or cell-conditioned medium; lane l, negative control (no template); lane m, RTS34st cells cultured in the absence of zebrafish embryo cells. Primers that amplify fibronectin cDNA were used to control for equal amounts of sample in each lane.

carried the *neo* gene, but only two individuals were identified that possessed melanocyte pigmentation on their bodies.

A total of 99 embryos that were injected with cells cultured on the RTS34st feeder layers survived to sexual maturity; four of these fish were found to be germ-line chimeras (Table 1). To identify the chimeras, each of the survivors was bred with a GASSI mate and the F1 individuals resulting from the cross were examined for the presence of *neo* and melanocyte pigmentation. Four fish, obtained from two different groups of injected embryos, produced *neo*-positive (Fig. 4), pigmented (Fig. 5 and Table 1) F1 fish. Contribution of the cultured cells to the germ line in each of the four founder chimeras was variable, ranging from 1.2% to 8.2% of the F1 individuals who were *neo* positive and possessed pigmentation derived from the cultured cells (Table 1). The fish exhibiting the highest degree of germ-line chimerism (8.2%) was a female; the second highest individual

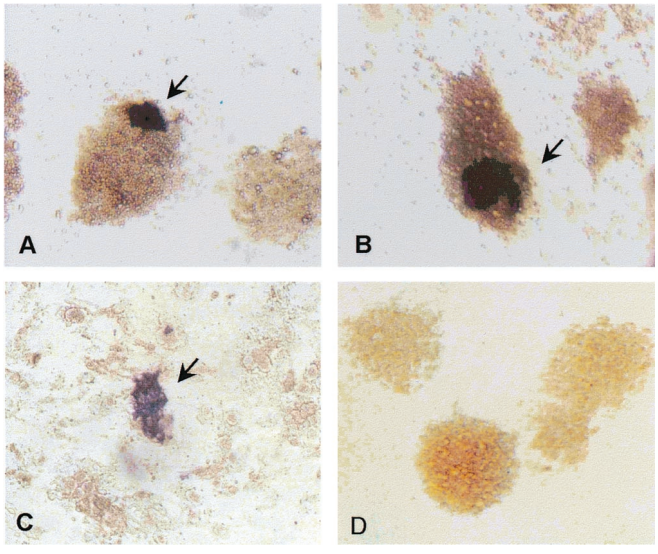

Fig. 3. Distribution of *vasa*-positive embryo cells. Cultures maintained for 3 days (*a*) and 8 days (*b*) in RTS34st cell-conditioned medium or 3 days on RTS34st feeder cells (*c*) were examined by *in situ* hybridization by using a *vasa*-specific antisense probe. The control culture (*d*) was grown for 8 days in conditioned medium and hybridized with sense probe. [Magnification = ×200 (*a*, *b*, and *d*), and = ×100 (*c*).]

Table 1. Frequency of germ-line chimerism

Group of injected embryos*	Survivors†	Germ-line chimeric‡	F1 transmission§	F2 frequency¶
1	49	1	19/342	198/267
		1	1/82	—
2	16	0	0	—
3	26	1	1/81	—
		1	8/97	93/126
4	8	0	0	—
Total	99	4	29	—

*Each group of embryos was injected with cultured cells on different days.
†Injected embryos that survived to sexual maturity.
‡Survivors were bred with GASSI fish and germ-line chimeras were identified by the production of *neo*$^+$ pigmented F1 fish.
§Number of *neo*$^+$ pigmented F1 fish produced by each germ-line chimera/total number of embryos produced.
¶Number of *neo*$^+$ pigmented F2 fish produced by breeding positive sibling fish from the F1 generation/total number of embryos produced.

(5.5%) was a male. None of the founder germ-line chimeras exhibited melanocyte pigmentation. Germ-line contribution was not detected when embryo cell cultures were maintained in the absence of the spleen cell feeder layers. To examine the stability of germ-line transmission, F2 generations were produced by breeding pigmented F1 sibling fish and by determining the frequency of F2 pigmentation and *neo* inheritance. As predicted

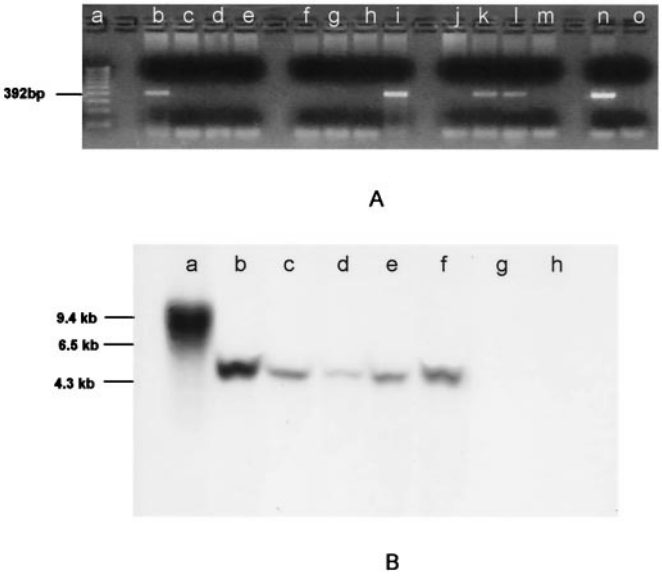

Fig. 4. PCR (*A*) and Southern blot analysis (*B*) of genomic DNA showing the presence of *neo* sequences. (*A*) Genomic DNA isolated from individual F1 fish (lanes b–m) produced from a single spawning of GASSI fish that were injected as embryos with cultured cells and bred with noninjected GASSI individuals. DNA was amplified with *neo*-specific primers designed to generate a 392-bp product. Product identity was confirmed by sequencing. *neo* sequences were detected in lanes b, i, k, and l. Lanes a, n, and o are molecular weight markers, positive control (*neo*-containing plasmid template) and negative control (no template), respectively. (*B*) Southern blot analysis of genomic DNA by using a *neo*-specific probe. The same integration pattern for *neo* sequences was observed in DNA isolated from B7–43 fish (lane b), cell cultures derived from B7–43 embryos (lane c), individual F1 fish (lanes d and e) obtained from a GASSI chimera that was injected at the blastula stage with cultured B7–43 embryo cells and bred with a noninjected GASSI fish, and an F2 individual (lane f) obtained by breeding positive F1 siblings. Lane a, DNA isolated from fish embryo cells transfected in culture with *neo*-containing plasmid showing a different integration pattern. Lanes g and h, DNA isolated from a GASSI fish and another nontransgenic line of zebrafish.

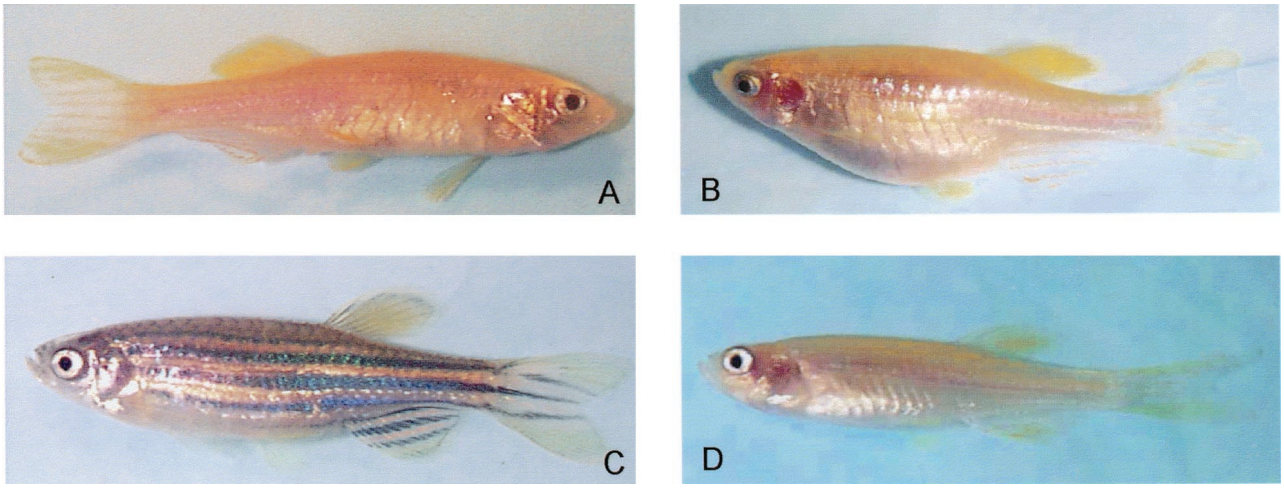

Fig. 5. Zebrafish phenotypes. (*a*) A chimeric zebrafish from a GASSI embryo that had been injected at the blastula stage with cultured cells derived from B7–43 embryos. Melanocyte pigmentation is absent on the body of the chimera. (*b*) GASSI fish that was bred with the chimera shown in *a* to produce (*c*) F1 individuals that exhibited a pigmentation pattern characteristic of B7–43 and (*d*) the nonpigmented GASSI phenotype.

from a cross involving two heterozygous F1 siblings, approximately 75% of the F2 fish were positive (Table 1).

Discussion

The results of this study indicate that factors released by RTS34st cells inhibit zebrafish embryo cell differentiation and enhance the survival of zebrafish PGCs in culture. Although suppression of melanocyte and neuronal cell differentiation was observed in embryo cell cultures maintained on either RTS34st feeder cells or in cell-conditioned medium, a greater inhibitory effect was found in the presence of the feeder cells. A similar situation has been reported for mouse ES cells cultured in the presence of embryonic fibroblasts compared with fibroblast-conditioned medium (33). Pluripotency of the ES cells was maintained more efficiently by the fibroblasts and was attributed to the presence of both the matrix-associated and the soluble forms of LIF in the cocultures, as opposed to only the soluble peptide in conditioned medium (33, 34). LIF and stem cell factor are both required for the *in vitro* survival and growth of mammalian PGCs (12), but the addition of mammalian LIF and stem cell factor to the zebrafish culture medium did not affect the persistence of *vasa* mRNA in the embryo cell cultures. Presumably the fish equivalents of these peptides may be among the factors supplied by the RTS34st cell line. It has been demonstrated (25) that RTS34st cells support the growth of trout macrophages in coculture, and that conditioned medium from RTS34st cells enhances [^{3}H]thymidine incorporation by trout peripheral blood and head kidney leukocytes, indicating that the spleen cells produce and release soluble growth factors into the medium.

This study reports zebrafish germ-line chimera production using cultured cells. Embryo cells were able to contribute to the host germ line if cultured in the presence of RTS34st cells. The frequency of germ-line chimeras was low (4%) compared with published frequencies for mouse ES cells (5%–14%; ref. 8), but this frequency is compensated for by the large number of fish embryos available for injection. Although the degree of germ-line chimerism in individual founder fish was also low, it was adequate for generating transgenic lines because relatively large numbers of embryos (30 to 100) are produced by a pair of fish at each spawning, and founder fish can be bred frequently (once per week; ref. 28). In future work, contribution to the host germ line may be enhanced by using irradiated recipient embryos (35).

Cell-mediated gene transfer may be used to target gene inactivation and generate zebrafish mutants. To accomplish this objective, longer-term cultures will have to be used to provide sufficient time for the introduction of the targeting vector and selection of colonies possessing the targeted insertion (10). This production of long-term cultures will be aided by the fact that RTS34st cell-conditioned medium suppressed zebrafish embryo cell differentiation and promoted the *in vitro* propagation of *vasa*-positive cells. The introduction of the targeting vector and the selection of cells that have undergone homologous recombination can be conducted in the absence of feeder cells using the conditioned medium. Isolated colonies can then be transferred back onto feeder layers and expanded for long-term culture.

In summary, a cell culture system has been developed for *in vitro* propagation of zebrafish embryo cells that are able to produce viable germ cells *in vivo* and may be useful in the future for introducing targeted mutations into fish.

We thank Dr. P. Gibbs for the GASSI and the B7–43 lines of zebrafish. This work was supported by grants to P.C. from the National SeaGrant Program (R/MBE-06–97) and the United States Department of Agriculture (#99–35205–8186).

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