CULTURE OF CELLS FROM ZEBRAFISH (*BRACHYDANIO RERIO*) EMBRYO AND ADULT TISSUES

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The zebrafish is a popular model for studies of vertebrate development and toxicology. However, in vitro approaches with this organism have not been fully exploited because cell culture systems have been unavailable. We developed methods for the culture of cells from blastula-stage diploid and haploid zebrafish embryos, as well as cells from the caudal and pelvic fin, gill, liver, and viscera of adult fish. The haploid embryo-derived cells differentiated in culture to a pigmented phenotype and expressed, upon exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin, a protein that was immunologically and functionally similar to rainbow trout cytochrome P450IA1. Zebrafish cultures were grown in a complex basal nutrient medium supplemented with insulin, trout embryo extract, and low concentrations of trout and fetal bovine serum; they could not be maintained in conventional culture medium containing a high concentration of mammalian serum. Using calcium phosphate-mediated transfection, a plasmid constructed for use in mammalian cells was introduced into zebrafish embryo cell cultures and expressed in a stable manner. These results indicated that the transfection procedures utilized in mammalian systems can also be applied to zebrafish cell cultures, providing a means for in vitro alteration of the genotype and phenotype of the cells.

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2. Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; EROD, 7-ethyoxyresorufin; HDPDS, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EDTA, ethylanediaminetetraacetic acid; FBS, fetal bovine serum; LDF, limit dilution factor; DMSO, dimethyl sulfoxide; ES, embryonal stem; PAH, polycylic aromatic hydrocarbons; ZG, zebrafish gill; ZBF, zebrafish pelvic fin; ZV, Zebrafish viscera; ZCF, zebrafish caudal fin; ZEM, diploid blastula-derived.

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INTRODUCTION

Fish are popular experimental organisms for studies of toxicology; it is economical to maintain sizable populations for large-scale exposures and many species display acute sensitivities to various xenobiotics (Schultz and Schultz, 1982a, 1982b; Babich and Borenfreund, 1991). Often such studies are conducted on young rainbow trout and other salmonids that are only available on a seasonal basis and require a year or more to become sexually mature (Hendricks et al., 1984; Bailey et al., 1984; Collodi et al., 1984). Recent attention has focused on the use of small aquarium species such as the medaka and zebrafish which grow rapidly, reach sexual maturity in approximately three months, and are tolerant of temperature and salinity (Hatanaka et al., 1982: Egami et al., 1981; Aoki et al., 1977; Bresch 1991; Bresch et al., 1990; Babich and Borenfreund, 1991).

Rapidly developing zebrafish embryos can be obtained at little expense and at any time of the year for laboratory investigations. Methods exist for producing homozygous embryos and clonal lines of fish, which allows for the identification of individual fish that possess nonlethal recessive mutations and thus allow for the maintenance of the mutation within a genetically homogeneous population (Streisinger et al., 1981). Methods also have been developed for derivation of transgenic zebrafish (Stuart et al., 1988, 1990). Approaches involving the genetic manipulation of zebrafish could lead to the derivation of strains highly sensitive to non-genotoxic carcinogens or environmental tumor promoters that would be useful for chemical screening.

Although *in vivo* studies of the effects of xenobiotics on various stages of the zebrafish life cycle (Bresch, 1991; Bresch et al., 1990; Nagel, et al., 1991; Dave and Xiu, 1991) have been informative, virtually no work has been directed to *in vitro* cell culture approaches to the study of zebrafish toxicology. This is probably the case because multipassage culture of zebrafish cell lines under conventional conditions with mammalian sera as a primary source of growth-stimulating factors has not been successful (Bols and Lee, 1991). As a result, little is known about the biochemical parameters that influence zebrafish embryonal cell growth and differentiation.

We and others have observed that some embryo-derived mammalian cells are growth-inhibited by mammalian sera (Loo et al., 1987, 1989; Rawson et al., 1991; Shirahata et al., 1990). Previously, we reported mitogenic activity in trout embryo extract that promotes growth of established fish cell lines and primary salmonid embryo cell cultures in medium containing little or no serum (Collodi and Barnes, 1990). In this paper we describe application of this approach to the culture of zebrafish cells. We report conditions supporting the long-term growth of cells derived from blastula-stage zebrafish embryos as well as in the culture of cells from zebrafish fin, liver, viscera, and gill.

We have also taken advantage of a useful aspect of the zebrafish system to produce cell cultures from haploid embryos. Fertilization of zebrafish eggs with UV-inactivated sperm

allows the production of haploid zebrafish embryos that undergo the early stages of development (Streisinger et al., 1981), well past the stages from which we could derive cell cultures. Haploid embryonal cell cultures may facilitate studies involving the inactivation of targeted genes. Clones could be derived that are specifically altered in a predetermined gene through targeted inactivation schemes and selections, providing useful lines for cell culture experiments (Kaufman et al., 1983; Ishino et al., 1990).

Mammalian embryonal cell cultures, such as 10T1/2 cells, have been used as a model to study the expression of genes coding for xenobiotic-metabolizing enzymes such as cytochrome P450 (Pottenger and Jefcoate, 1990). In this report we provide evidence that exposure of zebrafish embryo cell cultures to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in the expression of a new microsomal protein that was recognized by anti-rainbow trout P4540IA1 IgG. The TCDD-treated cells also displayed TCDD-inducible 7-ethoxyresorufin O-deethylase (EROD) activity; an activity mediated by cytochrome P450IA1 and P450IA2 enzymes (Kelley et al., 1987). Our results suggest that zebrafish embryo-derived cultures may provide a means for *in vitro* toxicological studies of xenobiotics activated by inducible polycyclic aromatic hydrocarbon-metabolizing enzymes.

MATERIALS AND METHODS

Cell Culture

Synchronously developing zebrafish blastula-stage embryos for the initiation of cultures (diploid blastula-derived ZEM cultures and haploid blastula-derived ZEMH cultures) were obtained by *in vitro* fertilization. Zebrafish are photoperiodic in their breeding; fish were maintained on a 14 hr light/10 hr dark cycle; eggs and sperm were collected within 90 min after fish were exposed to light. Fish were anesthetized with 3-amino benzoic acid ethylester and sperm from 2 to 3 males collected in a capillary tube and stored on ice in approximately 50 ul of Hank's solution. Sperm remain viable for up to 90 min. Eggs were collected from a gravid female (approximately 100 eggs/fish) by gently pressing on the ventral side of the fish. Eggs were mixed with sperm in a small petri dish and fertilization was initiated by the addition of 1 ml water to dilute the Hank's solution and activate the sperm. After 2 min, additional water was added to the fertilized eggs and embryos were allowed to develop undisturbed at 26°C (Streisinger et al., 1981).

Blastula-stage embryos were harvested 2 to 3 hr after fertilization and rinsed three times in the same basal nutrient medium used for cell culture (limit dilution factor medium [LDF], described below) and then rinsed once in a 0.5% bleach solution. Following three additional rinses in nutrient medium, the embryos were dechorionated in trypsin solution (0.2%, wt/vol, trypsin/1mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline, for approximately 10 min and the cells were dissociated by pipeting the embryos gently until a suspension of small cell aggregrates was obtained. The cell aggregates were then collected by centrifugation and plated in 96-well tissue culture dishes (Costar). Cells pooled from 3 to 5 embryos were added to each well.

For generation of haploid blastula-derived cell cultures, sperm was UV-irradiated (29 cm from a 43 cm long Sylvania germicidal tube) 2 min before fertilizing the eggs (Streisinger et al, 1981), followed by the cell culture procedures described above. Eighty percent of the resulting embryos develop normally through gastrulation; all die before hatching.

Cells were grown in LDF medium (50% Leibovitz's L-15, 35% Dulbecco's modified Eagle's and 15% Ham's F12 media) (Gibco) supplemented with sodium bicarbonate (0.15 mg/ml), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2), penicillin (200 international units/ml), streptomycin sulfate (200 ug/ml), ampicillin (25 ug/ml), bovine insulin (10 ug/ml; Sigma), trout embryo extract (25 ug/ml), trout serum (0.1%), and fetal bovine serum (FBS) (1%) (Gibco). After approximately 7 days the cells were trypsinized and passaged into a 24-well tissue culture plate and eventually expanded into 25 cm^2 flasks. Medium was changed once each week.

Primary cultures from adult tissues (zebrafish gill, pelvic fin, caudal fin, and viscera) were derived in the same medium by washing the dissected tissues 3 times in nutrient medium, mincing, and digesting the minceate in trypsin while pipeting to dissociate the cells. Fish were approximately one year old. The dissociated cells and cell aggregates were plated into 25 cm^2 tissue culture flasks and the medium was changed once a week. When a confluent monolayer had formed (approximately 2 weeks) the cells were trypsinized and passaged.

Cell Transfections

The pSV2-neo plasmid (Southern and Berg, 1982) was obtained from the American Type Culture Collection. For transfections, 25 ug of plasmid DNA in 0.5 ml to 0.25 M calcium chloride was added dropwise with constant mixing to 0.5 ml HEPES-buffered saline (250 mM HEPES, pH 7.0) containing 1.8 mM sodium phosphate. The resulting precipitate-containing suspension was incubated 30 min at room temperature, and the suspension sheared twice through a 25 gauge needle. The suspension was then added directly to a flask containing approximately 10^6 cells that had undergone a medium change approximately 4 hr previously. Medium was changed to remove the precipitate 6 hr after its addition. Cells were allowed to grow 7 days before addition of the antibiotic G418 (Geneticin) at a concentration of 500 ug/ml. Colonies obtained from the selection were isolated and expanded.

Preparation of Trout Embryo Extract

Approximately 100 Shasta strain trout embryos (21-day old, developed at 10°C and stored frozen at 80°C) were thawed and pooled in 5-10 ml of basal nutrient medium. The embryos were homogenized 3 times for 15 sec each time on ice with a Tissuemizer cell homogenizer (Tekmar). The resulting homogenate was centrifuged for 5 min (15,000 x g) and the supernatant collected, avoiding the layer of lipid which formed on the top of the supernatant. After diluting (1:5) with basal nutrient medium, the supernatant was filter sterilized through a 0.4 um prefilter followed by a 0.2 um filter. Protein concentrations were determined by the method of Bradford (1976).

Preparation of Fish Serum

Fish serum was prepared from the blood of Shasta strain rainbow trout. After removing the cells by centrifugation, the plasma was allowed to clot at 4°C for 16 hr. Serum was separated from the clotted protein by centrifugation and sterilized by filtration (0.2) um filter). The serum was incubated at 56°C for 20 min before use in cell culture.

Growth Assays

Growth assays were conducted at 26°C in duplicate in 6-well (35-mm diameter) tissue culture plates (Falcon). Medium was changed every 5 days during the assay and the cells were grown in ambient air. Each plate was wrapped in Parafilm to prevent evaporation. For each assay, cells were plated at 5 x 10^4/well in the appropriate medium and at the designated time a suspension of trypsinized cells in PBS was counted using a Coulter particle counter.

Karyotype Analysis

Rapidly growing cultures were incubated in colcemid (0.2 ug/ml) for 9 hr. Cells were then trypsinized, centrifuged, and the pellet gently resuspended in 0.4% KC1 and incubated at room temperature (20 min). Following incubation, 1 ml fixative was added to halt the action of the hypotonic KC1. The cells were then fixed and washed 3 times in methanol/acetic acid (3:1). Chromosome spreads were prepared and stained in 3% Giemsa solution prepared in Sorenson's buffer, pH 6.8, and karotype analysis was performed on approximately 50 metaphases (Ernst et al., 1991). This procedure is derived from that used for mammalian cell cultures, and optimization for zebrafish cells (e.g., incubation time with hypotonic solution, KC1 concentration) has not yet been addressed.

Exposure to TCDD In Vitro and Evaluation of Enzyme Induction

Confluent haploid zebrafish embryo-derived (ZEMH) cells grown in 175-cm^2 culture flasks (approximately 10^7 cells/flask) were exposed to culture medium containing dimethyl sulfonide (DMSO) (control) or TCDD (10 nM) in DMSO. The final concentration of DMSO was 0.01%. After incubation at 26°C for 48 hr, the cells were scraped and collected by centrifugation. The pelleted cells were resuspended in 0.1 M Tris acetate buffer, pH 7.4 containing 0.1 M potassium chloride, 1 mM EDTA and 0.1 mM PMSF (alpha tolunesulfonylflouride). The cells were disrupted by sonication and then homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 30 min and the resulting supernatant was recentrifuged at 105,000 g for 90 min. The microsomal pellet was resuspended in 10 mM Tris acetate buffer, pH 7.5, containing 20% glycerol and 0.1 mM EDTA.

Microsomes were subjected to SDS-polyacrylamide gel electrophoresis and fractionated proteins were transferred to nitrocellulose by electroblotting. Filters were exposed to primary antibody (antitrout P450IA1 IgG) and goat antirabbit IgG was conjugated with horseradish peroxidase with detection by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL). EROD activity of microsomes was measured according to the method of Prough et al., 1978. Microsomal protein was determined by the method of Lowry et al., 1951.

RESULTS

Derivation and Characteristics of Zebrafish Cll Cultures

We have derived zebrafish cultures from diploid blastula (ZEM), haploid blastula (ZEMH) and from adult tissues: gill, pelvic fin, caudal fin, and viscera. The diploid blastula-derived cell line (ZEM) has been cultured continuously for more than 15 passages (approximately 40 population doublings) in LDF basal nutrient medium supplemented with sodium bicarbonate, insulin, trout embryo extract, trout serum and FBS. The doubling time of the embryo cells under these conditions was approximately 96 hrs. Growth of the embryo cells in LDF was superior to that achieved with several other basal nutrient media alone and in combination (Table 1).

TABLE 1. Growth of ZEM Cells in Basal Nutrient Medium Formulations and Mixtures

MEDIUM	CELLS/WELL
Dulbecco's modified Eagle's medium (DME)	7.6×10^4
Ham's F12 and DME (50:50 mixture)	2.1×10^4
Ham's F12 and DME (70:30 mixture)	4.8×10^4
Ham's F12 and DME (30:70 mixture)	2.0×10^5
Liebovitz' L-15 and DME (50:50 mixture)	5.2×10^5
Liebovitz's L-15, Ham's F12 and DME (10:7:3 mixture)	3.2×10^5
Liebovitz's L-15, DME and Ham's F12 (50:35:15 mixture)	7.7×10^5
Enriched RPMI-DME-F12 (50:25:25)	6.7×10^4

Cells were plated (5×10^4 per well) in 6-well (35-mm diameter) tissue culture plates as described in "Materials and Methods". A suspension of trypsinized cells in phosphate-buffered saline was counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. Each medium or mixture was supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%) and trout embryo extract (25 ug/ml). Enriched RPMI-DME-F12 mixture was obtained from Kyokuto Pharmaceutical, Tokyo.

Cell lines with similar growth rates were derived from caudal and pelvic fin, gill, and viscera (Figures 1 and 2). Cultures were also derived from adult zebrafish liver (Figure 2); these cells have been propagated in culture for approximately 5 population doublings. The growth rate was slow (doubling time greater than 4 days), but a high percentage of the cells comprising the culture were capable of proliferation *in vitro*. Fin-derived cells were fibroblastic in appearance, while liver-derived cultures were primarily epithelial. Viscera-derived cell cultures, initiated from liver-associated tissue that had been dissected free, appeared fibroblastic (not shown).

All of the cell lines required a low concentration (1-3%) of FBS for optimal growth; higher concentrations of FBS inhibited both ZEM and viscera cell growth, but not the growth of the fin-derived cell lines (Figure 3). For short-term culture of ZEM cells, beta-mercaptoethanol (10^{-4} M) could replace FBS, an advantage when a more defined medium is useful. All 5 cell lines exhibited a strong dependence for growth on a low concentration of trout serum (Figure 4). As little as 0.1% allowed optimal growth of ZEM Cells and, as with FBS, the cells were growth-inhibited by higher concentrations of trout serum. The viscera- and fin-derived cell lines were not inhibited by higher trout serum concentrations.

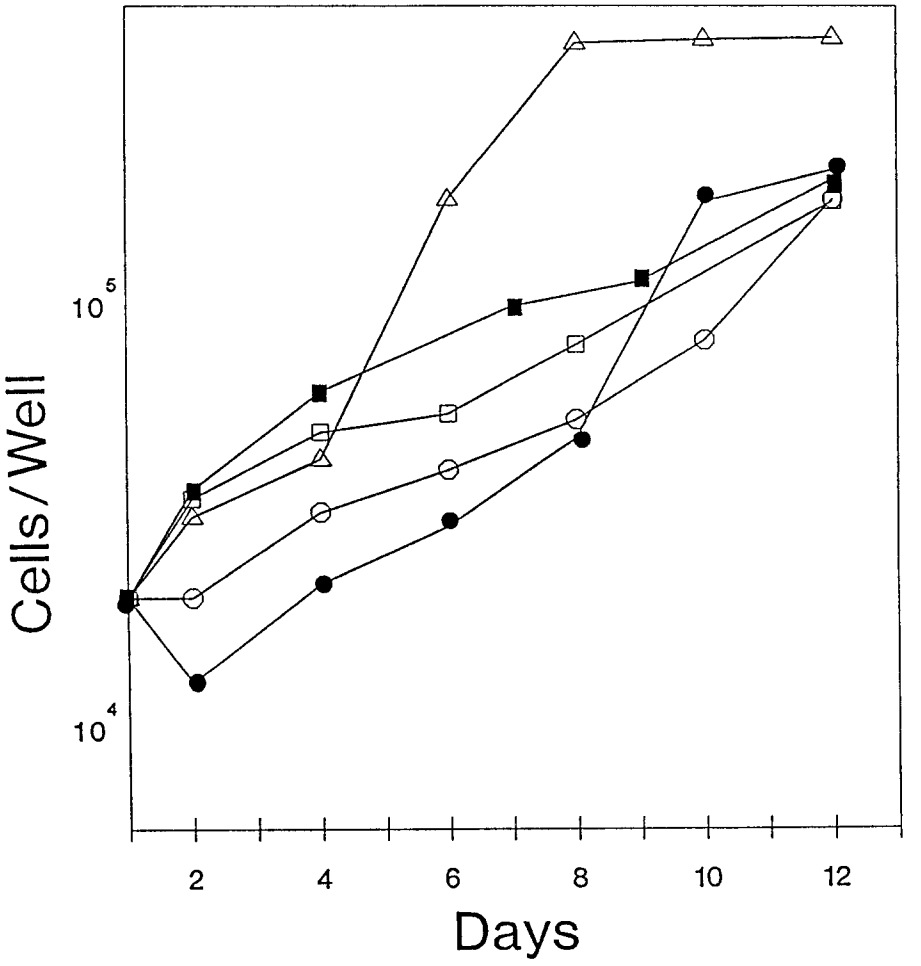

FIGURE 1. Zebrafish cell growth in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum 0.4%), and trout embryo extract (25 ug/ml). Cells were plated as described in Table 1 and counted on the days indicated. Average variation of single determinations from the mean was less than 10%. ZG (Δ); ZPF (●); ZCF (○); ZV (■); ZEM (❑).

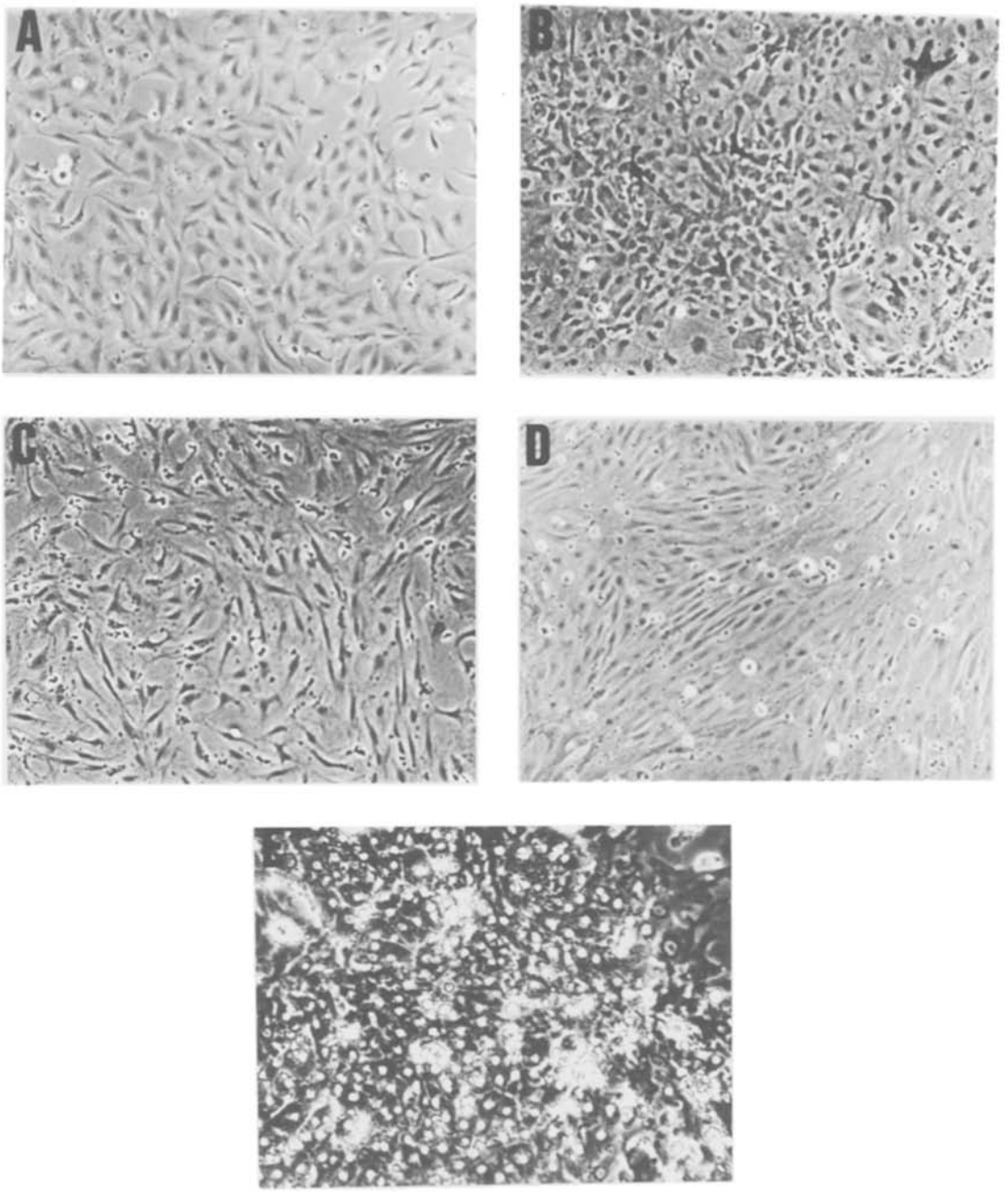

FIGURE 2. Photomicrographs of zebrafish cells grown in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%), and trout embryo extract (25 ug/ml): A, ZEM; B, ZEMH; C, ZPF; D, ZCF; E, liver. All are 100X magnification except E (200X).

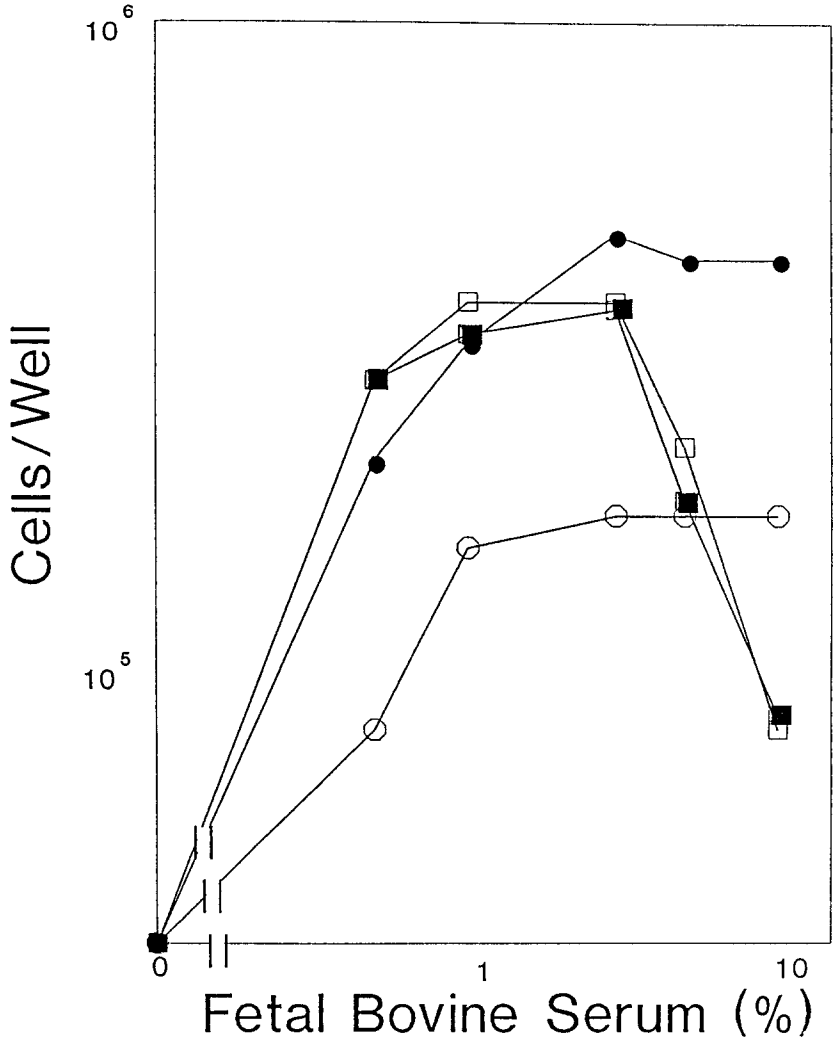

FIGURE 3. Effect of FBS on zebrafish cell growth. Cells were plated in LDF medium supplemented with insulin (10 ug/ml), trout serum (0.4%), trout embryo extract (25 ug/ml) and FBS (0 to 10%) as described in Table 1 and counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. ZEM (❑); ZV (■); ZCF (○); ZPF (●).

Trout embryo extract and insulin were necessary to establish all of the cell lines in culture; without these factors cultures initiated from primary tissue stopped growing after a few days. After the cultures had been propagated for 15 or more population doublings *in vitro*, all but one of the cell types required only FBS and trout serum as supplements for optimal cell growth in a short-term experiment (Figure 5). Only caudal fin cells continued to acutely require supplementation with embryo extract for optimal growth under these conditions, although several of the cell types exhibited a continued requirement for the trout embryo-derived extracts for optimal growth in longer-term multipassage experiments.

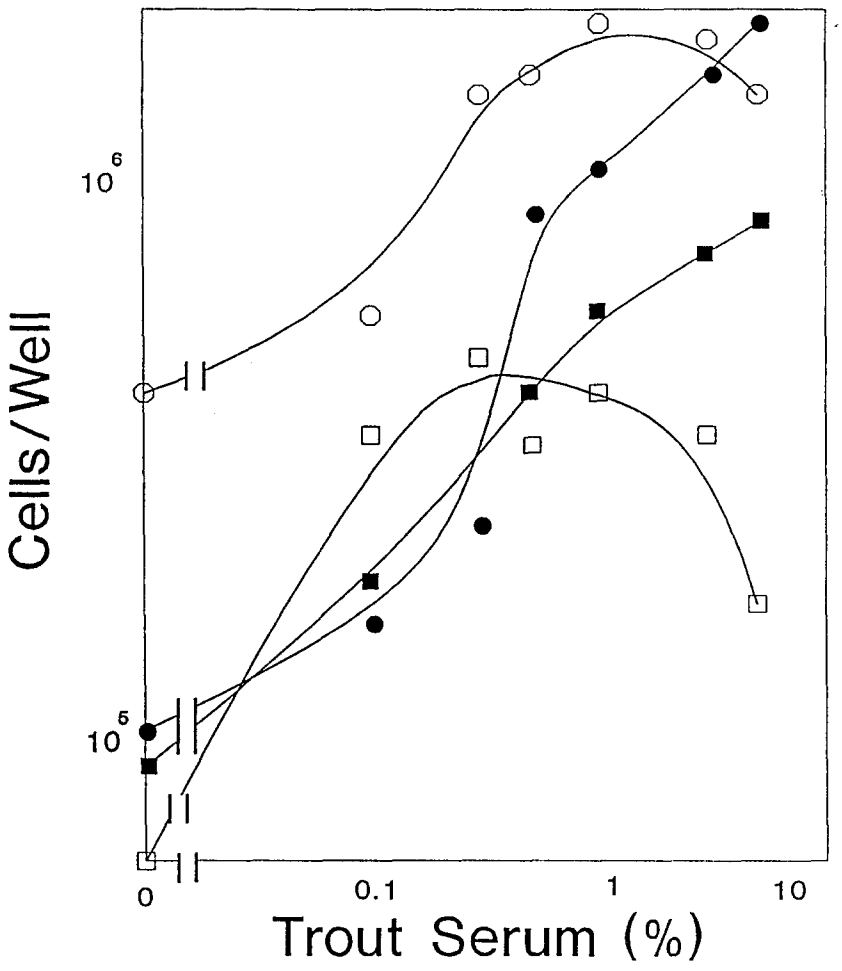

FIGURE 4. Effect of trout serum on zebrafish cell growth. Cells were plated in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout embryo extract (25 ug/ml), and trout serum (0 to 10% as described in Table 1 and counted 12 days after plating. Average variation of single determinations from the mean was less than ten percent. ZEM (❑); ZV (■); ZCF (○); ZPF (●),

The basis for difference in trout embryo extract requirements for the two fin-derived cultures (caudal and pelvic) is not clear. Caudal fin cells were grown in culture for a similar number of generations as pelvic fin cells at the time of the experiment, indicating that the difference in response is not the result of a difference in general adaptation to *in vitro* conditions. The difference in behavior *in vitro* may reflect a difference in available tissue source at the time of preparation of the cells. Material for derivation of the fin cell cultures contained both ray and interray tissue, and these cultures were initiated from the mixture of cell types present in these areas.

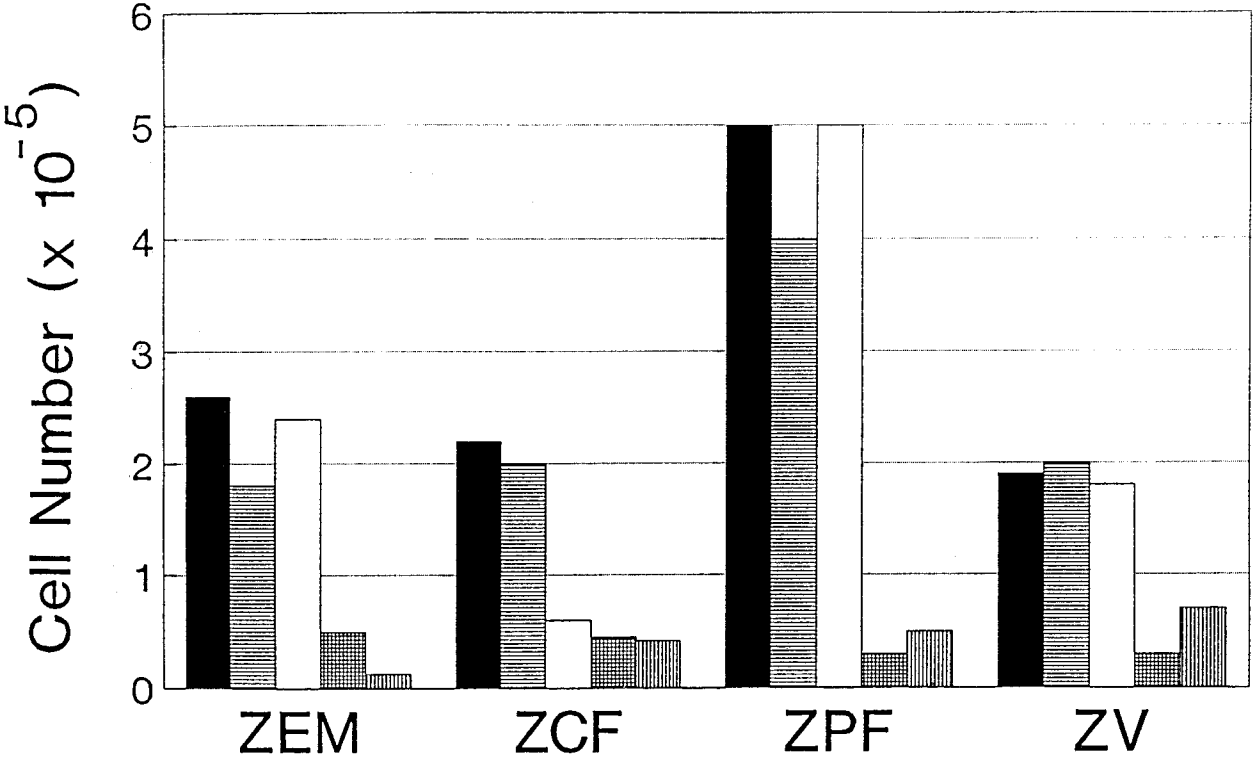

FIGURE 5. Effect of LDF medium supplements on zebra fish cell growth. Cells were plated in 6-well (35 mm-diameter) tissue culture plates as described in Table 1 in LDF medium supplemented with insulin (10 ug/ml), FBS (1%), trout serum (0.4%) and trout embryo extract (25 ug/ml) (■) or medium from which insulin (▤), trout embryo extract (❑), trout serum (▥), or FBS (⊞) was individually omitted. Cells were counted 12 days after plating. Average variation of single determination from the mean was less than ten percent.

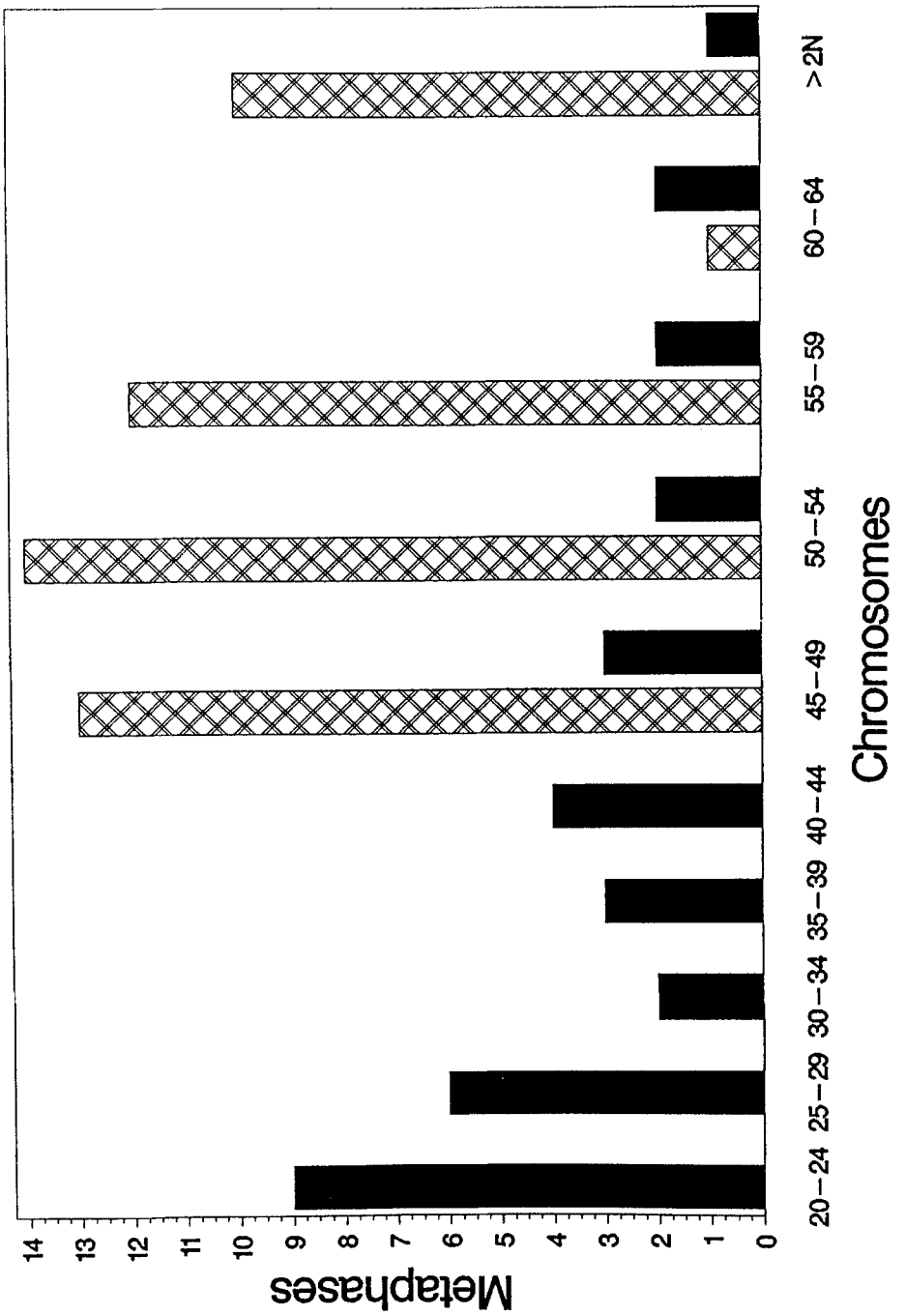

FIGURE 6. Karyotypic analysis of zebra fish cells. ZEM and ZEMH cells were grown and prepared for karyotyping as described in "Materials and Methods". The chromosome number distribution was obtained from 50 ZEM and 34 ZEMH metaphases. ZEM, crosshatched bars; ZEMH, solid bars.

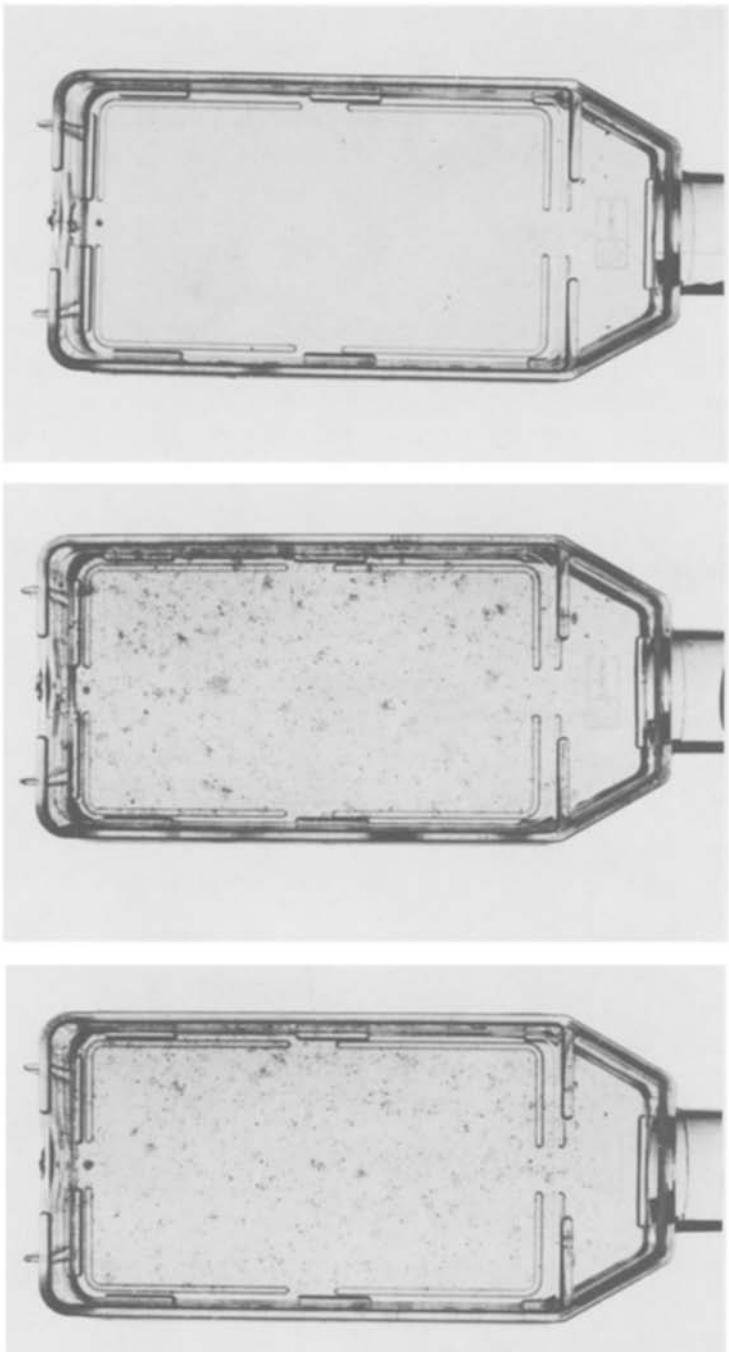

FIGURE 7. Growth of pSV2-neo transfected *ZEM* cells in G418. Following transfection and selection in G418 as described in "Materials and Methods", 10^4 cells were plated in 25 cm^2 tissue culture flasks and grown for 3 weeks in the presence or absence of 400 ug/ml G418. Colonies present in each flask were visualized by staining with crystal violet as described in "Materials and Methods". Nontransfected ZEM cells (top flask) and two pSV2-neo transfected clones are shown.

In addition to the ZEM cell line, a second embryo-derived cell line, ZEMH, was established from blastula-stage embryos developed from eggs fertilized with UV-inactivated sperm. Karyotype analysis of ZEMH cells revealed that after approximately 30 population doublings in culture, 79% of the population possessed a hypoploid chromosome number and 63% of these cells possessed the haploid chromosome number (Figure 6). The mode for the population was 24; the modal chromosome number for ZEM cells was 51. Diploid chromosome number for *Brachydanio rerio* is 50 (Endo and Ingalls, 1968). ZEMH cells were similar in appearance to ZEM cells, but were capable of differentiation in culture to a pigmented phenotype (Figure 2). These cells appear black in the light microscope and may be melanocytes, although other pigmented cells exist in the fish that also may appear black upon microscopic examination. Individual pigmented ZEMH cells appeared in dense cultures and represented less than 1% of the total cell population. Neither the haploid nor diploid embryo-derived cultures appeared transformed by morphological criteria associated with transformed mammalian cells in culture.

Transfection of ZEM cells

To demonstrate the potential of zebrafish cells in culture to express exogenous genes introduced into the cells by transfection, we isolated stable transfectants expressing the pSV2-neo plasmid containing the gene for bacterial aminoglycoside phosphotransferase under the influence of the simian virus 40 (SV40) early promoter and polyadenylation signal (Southern and Berg, 1982). This gene confers resistance to the antibiotic G418 and the plasmid construction is active in cell cultures derived from trout and salmon embryo and trout hepatoma (Helmrich et al., 1988). G418- resistant colonies appeared with a frequency of approximately one in 10^5 cells (Figure 7).

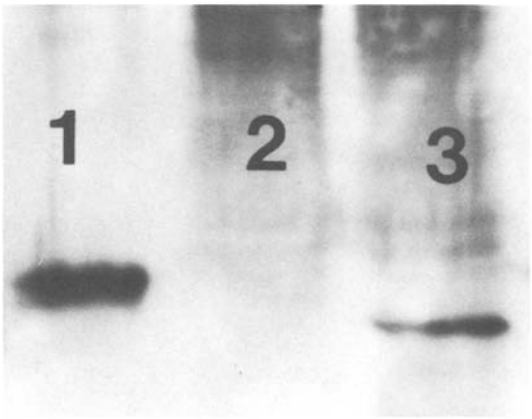

FIGURE 8. Immunoblot of liver microsomes from control and TCDD-treated ZEMH cells probed with anti-trout P450IA1 IgG. Procedures are described in Materials and Methods. Lane 1, purified trout P450IA1 (0.25 pmols); lane 2, control ZEMH (25 ug); lane 3, TCDD-treated ZEMH (25 ug).

Exposure of ZEMH Cells to TCDD

Immunoblotting (Western) blotting established that TCDD induced a microsomal protein in ZEMH cells that is recognized by anti-trout P450IA1 IgG. Figure 8 shows that microsomes derived from TCDD-treated ZEMH cells contained an immunoreactive band running slightly below that of purified trout P450IA1 (Mr=60,000), as with other fish species (Goksoyr et al., 1991). This band was not found in control (DMSO-treated) microsomes. The microsomes from TCDD-treated ZEMH cells also displayed low, but measurable EROD activity (12 pmol/min/mg protein), which was not exhibited by the control microsomes. Similar results were obtained when ZEM and liver-derived cultures were exposed to TCDD (Collodi et al., 1992b).

DISCUSSION

This paper describes the derivation and characterization of cell cultures initiated from blastula-stage zebrafish embryos (ZEM, ZEMH), and from several tissues of adult zebrafish. Our success in deriving the cultures was largely due to the use of a complex mix of basal nutrient media, use of serum concentrations lower than are generally used in cell culture, and the supplementation of trout embryo extract with growth promoting activity. ZEM cells are near-diploid and have been grown for approximately 40 population doublings. The ZEMH culture, initiated from haploid blastula-stage embryos, has been propagated for approximately 30 population doublings *in vitro* and exhibited a high proportion of haploid cells with the majority of the remaining cells exhibiting a hypoploid chromosome number.

The wide range in chromosome number in the ZEMH culture may be due to a gradual diploidization of the cell line as it is grown in culture. Spontaneous diploidization has been observed *in vitro* with haploid murine embryonal stem (ES) cells grown in culture and *in vivo* following transplantation of the haploid cells into a diploid embryo (Kaufman et al., 1983; Ishino et al., 1990). Alternatively, the range in chromosome number may reflect that the culture was derived from a pool of dissociated cells from several embryos possibly with different chromosome numbers. It should be possible to maintain a homogeneous population of haploid cells by routinely subcloning the cells or deriving cultures from individual haploid embryos.

All of the cultures required trout embryo extract and insulin in addition to FBS and trout serum during the initial passages in culture. Trout embryo extract promotes the serum-free growth of several established piscine cell lines, and its effect on the cells cannot be mimicked by purified mammalian growth factors (Collodi and Barnes, 1990). The partially purified mitogenic activity is heat stable and associates with two fractions of 25 and 60 kilo-daltons when analyzed by gel filtration chromatography or SDS polyacrylamide gel electrophoresis.

In most cases, cultures propagated for about 15 generations *in vitro* were able to grow in short-term experiments in medium supplemented with only FBS and trout serum. This change in phenotype may represent a metabolic adaptation of the cells to the culture conditions, or

may represent a selection during the culture period for a subpopulation of cells that were best suited to grow under the culture conditions. Such a subpopulation might be present initially in the cultures or might arise by genetic alteration upon proliferation *in vitro*.

In addition to the direct uses of zebrafish cell cultures in toxicology testing and mechanism studies and comparison with *in vivo* studies, zebrafish embryonal cell cultures represent a first step in developing the *in vitro* systems needed to conduct investigations of the genetic and biochemical parameters that influence embryonal cell growth and differentiation in this system. A potential application of this approach is the development of ES cell lines that can be utilized to produce chimeric embryos (Evans and Kaufman, 1981; Robertson et al., 1986).

The zebrafish blastula is comprised of indeterminant cells (Kimmel and Warga, 1986) that possess the potential to give rise to pluripotent cell lines, and when genetically manipulated, blastula-derived ES cells could be used as a vector to introduce foreign genes into the chimera, an alternative method of producing transgenic fish. This method provides an advantage over direct injection of DNA into the embryo because cells possessing the desired phenotype, such as the proper level of exogenous gene expression, can be selected *in vitro* before introduction into the embryo (Evans and Kaufman, 1981; Evans et al., 1983; Ishino et al., 1990; Nandi et al., 1988; Robertson et al., 1986; Collodi et al., 1992a).

Furthermore, the ability to grow fin cells from primary culture, demonstrated by the growth of the caudal and pelvic fins, enables one to screen for chimeras or carry out other genetic analyses using DNA derived from cultured cells initiated from fin tissue without sacrificing the embryo. Also, the ability to culture cells derived from liver, viscera, and gill makes possible an *in vitro* analysis of the tissue-specific expression of particular plasmid/promoter constructs used in producing the transgenic embryo and the tissue-specific expression of xenobiotic metabolizing enzymes (Helmrich et al., 1988; Bols and Lee, 1991; Pesonen et al., 1989; Pottenger and Jefcoate, 1990; Robertson et al., 1986; Stuart et al., 1988; Stuart et al., 1990; Collodi, et al., 1992a; Collodi et al., 1992b).

Another application of this approach to the production of transgenic animals is the use of homologous insertional mutation to produce ES cells deficient in specific gene products and thereby produce chimeric embryos exhibiting the same deficiency (Nandi et al., 1988). This application would be facilitated by the availability of a haploid ES cell line. We have demonstrated the feasibility of producing such a haploid cell line by initiating the ZEMH cell line from haploid embryos. ZEMH cells exhibit the ability to undergo differentiation in culture to a pigmented phenotype, making the cell line a potentially useful system for the *in vitro* study of cell differentiation.

The mammalian hypertetraploid cell line, C3H/10T1/2, developed from mouse embryos, has been shown to possess an intrinsic cytochrome P450 activity by its ability to metabolize polycyclic aromatic hydrocarbons (PAH) (Pottenger and Jefcoate, 1990). Pretreatment with

TCDD induces the PAH-metabolizing activity of these cells. In the present study, we demonstrate for the first time that fish cells (ZEMH), when exposed to TCDD in culture, were capable of expressing a new protein that was recognized by antitrout P450IA1 IgG in immunoblots. These TCDD-treated cells also displayed EROD activity which was not detected in the control cells. In an earlier study, TCDD was found to induce EROD activity in primary cultures of rainbow trout hepatocytes (Pesonen et al., 1989).

These findings suggest that TCDD induces the synthesis of a protein in ZEMH cells that is immunologically and functionally related to trout P450IA1. These zebrafish cultures may provide a unique model for toxicological studies involving the expression and regulation of PAH-metabolizing enzymes and their genes following exposure of the cells to environmental chemicals.

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