Transplantable Tumor Lines Generated in Clonal Zebrafish

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Abstract
Transplantable zebrafish tumors are a novel and very promising model in cancer research. However, further progress in this field has been contained by a lack of true inbred lines in zebrafish. To overcome this problem, we generated two lines of homozygous diploid clonal zebrafish lines (i.e., CB1 and CW1), which allowed us to carry out transplantation of any tissue, including tumors, from one fish to another within a line without rejection of the graft. The primary tumors in CB1 fish were induced by N-nitrosodiethylamine (DEN). The histologic analysis of these tumors revealed different types of hepatocellular carcinomas, hepatoblastomas, hepatoma, cholangiocarcinoma, and pancreatic carcinoma. Four spontaneous acinar cell carcinomas of pancreas were also found in 10- to 18-month-old CB1 fish. Small pieces of tissue or cell suspensions of either DEN-induced or spontaneous tumors were serially transplanted into the peritoneal cavity of syngeneic fish at different stages of development from 5-day-old larvae to adult fish. The development of grossly visible tumors occurred from 2 weeks to 3 months after tumor grafting and grew either as solitary smooth nodules or as an amorphous jelly-like mass infiltrating abdominal organs. The majority of tumors were also successfully transplanted to isogeneic (F1 generation from crossing CB1 x CW1) fish. At the present time, 19 transplantable zebrafish tumor lines have been generated and maintained for as long as 3 to 25 passages. This model provides a novel tool for studying experimental tumor biology and treatment. However, long and variable latent periods of tumor progression, asynchronous growth, and <100% tumor incidence rate make use of these currently available tumor models for development and screening of new antitumor drugs somewhat problematic. In the meantime, other standard mammalian tumor models have not been used in zebrafish until now. In particular, serially transplanted tumors have been used in investigation of tumor biology and experimental tumor therapy in rodents for >100 years (10). Generation of transplanted tumor lines in zebrafish might become an inexpensive alternative to this model in rodents. Furthermore, transplanted tumor lines would be an indispensable supplement for already established models of tumor development in zebrafish and as a source of transformed cell lines maintained ex vivo. The ability to transplant tumors from one fish to another was shown in the viviparous fish Poecillipsis lucida (11), which is considered to be a naturally inbred strain (12). Generation of a true inbred zebrafish line that would permit unconstrained transplantation of tissues between fish at present remains a difficult task (13). In this study, we proposed to generate transplantable tumors using clonal homozygous lines of zebrafish established by a procedure described elsewhere (14). Each such a line consisted of fish that were exact genetic copies of each other. This makes it possible to carry out transplantation of any tissue, including tumors, from one fish to another within a line without rejection of the graft. Here, we present data showing the successful generation of transplantable tumors in clonal zebrafish.

Material and Methods

Animals. Zebrafish of the line brass were purchased in a local pet shop. The wild-type (AB) zebrafish were obtained from Dr. E. Weinberg (University of Pennsylvania, Philadelphia, PA) and maintained in the laboratory for >8 years. The golden strain of zebrafish was obtained from Dr. H.G. Frohnhoefer (MPG for Developmental Biology, Germany). During the entire study, fish were maintained in 20-L acrylic tanks connected to a closed water recirculation system with 50 to 60 fish in each. Fish were maintained in standard conditions (15) briefly: 14/10-hour light/dark cycle, T = 26 ± 1°C. Tetramin (Tetra, Melle, Germany) and Ovo-vit (QXL, Poznan, Poland) were used as a basal diet supplemented with nauplii Artemia salina.

Generation of clonal homozygous zebrafish. Clones of homozygous zebrafish CB1 and CW1 derived from brass and AB strains of zebrafish, respectively, were obtained by heat shock procedure as described previously (14) with some modifications. Briefly, eggs from female AB and brass strains of zebrafish were fertilized by UV-inactivated sperm from male zebrafish of golden and AB strains, respectively. After 13-minute incubation at 28.5°C eggs underwent a 2-minute heat shock at 41.4°C to block the first cleavage. This procedure led to the development of a homozygous diploid fish. Eggs obtained from a raised to maturity homozygous female fish of either AB or brass strain underwent the second round of UV-sperm fertilization/heat shock procedure exactly as described above. The offspring obtained from each homozygous female were genetic copies of each other (i.e., clones of
homezygous fish). The further maintenance of homozygous strains was carried out by crossing fish within each clone. Mating of the fish that belonged to different homozygous clones led to the generation of an isogenic fish strain consisting of genetically identical but not homozygous fish.

**Tumor induction.** Sixty-five 2.5-month-old fish from the clonal CB1 line were used for tumor induction by continuous immersion in N-nitrosodiethyamine (DEN) solution (100 ppm) during the 8 weeks. During DEN, exposure fish were maintained in 20-L acrylic tanks equipped with a mechanical filter and an automatic heater. The filters were cleaned twice weekly. The complete exchange of water containing the carcinogen was done every 2 weeks. The water temperature was maintained at 26 ± 0.5°C. Following completion of DEN exposure the fish were transferred to a 20-L system tank and remained under observation for up to 9 months.

**Tumor transplantation.** The tumor tissue for further grafting was obtained from fish with grossly visible neoplasms located in the abdomen. These fish were euthanized by 0.015% tricaine (Sigma, Munich, Germany) solution and were opened ventrally with iridectomy scissors. Tumors were gently separated from surrounding tissues and placed into cold (4°C) Ringer solution with antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin) for 2 to 30 minutes (i.e., until transplantation). In the meantime, 2.5- to 5-month-old syngeneic recipients were placed in 0.015% tricaine solution in groups of three to six fish. Immediately after reaching immobility, the fish were transferred individually onto a wet sponge for further transplantation of tumor tissue. I.p. transplantation of tumor tissue was carried out by a small punch using a thick syringe needle (Sigma, Munich, Germany) and then was further cut with scissors. Recipient larvae were transferred onto a wet filter paper in groups with a filament and made with a vertical puller. Recipient larvae were placed in a 0.5-mL cryopreservation tube (Sarstedt, Nuembrecht, Germany) containing 25 μL of cold (4°C) PBS and filtered through 40-μm mesh (Becton Dickinson Labware, Mountain View, CA). After a short centrifugation, the pellets consisting of single cells and cell clusters (5-20 cells) was resuspended in 25 μL of PBS and kept on ice until transplantation. Using a flexible syringe needle “MicroFIl” (WPI, Inc., Tonasket, WA), ~5 μL cell suspension was placed into a glass micropipette with an external diameter at the tip of 30 μm without a filament and made with a vertical puller. Recipient larvae were placed into 0.015% tricaine solution until they became immobile. Then, the larvae were transferred onto a wet filter paper in groups of 25 animals. After i.p. injection of ~50 to 100 mL (depending on recipient size) of cell suspension containing 2.5 to 3 × 10^6 cells per 10 μL, all the larvae were returned to clean tap water. The animals in the control group were injected with the same volume of Ringer solution.

**Cryopreservation.** Large tumors nodules that underwent 5 to 20 passages were extracted from fish abdomens and cut into 2 to 3 parts (~5 × 5 × 5 mm). Each part was placed in a 0.5-mL cryopreservation tube (Sarstedt, Nuembrecht, Germany) containing 400 μL DMEM supplemented with 20% FCS and 10% DMSO (Sigma, Munich, Germany) and then was further cut with scissors into pieces of about 1 × 1 mm. The tubes were kept at 4°C for 30 minutes followed by overnight storage at −80°C and then transferred to liquid nitrogen for a long-term storage. Thawing was carried out by a quick transfer of the tubes from liquid nitrogen to a water bath at 30°C to 32°C followed by a replacement of cryopreservation medium with DMEM without FCS and DMSO. After thawing, samples were kept at 4°C until transplantation as described above.

**Histology.** After 24 to 72 hours of fixation in 4% solution of neutral paraformaldehyde, all primary and transplanted tumors together with adjacent abdominal organs underwent standard histologic processing. Tissue slices (3-4 μm) were stained by H&E or, if necessary, with other staining methods. Photographs of the slides were obtained by Nikon Coolpix 4500 digital camera, mounted with an optical adapter on the microscope BIOLAM-I (LOMO, St. Petersburg, Russia).

**The mean survival time tumor-bearing fish.** The mean survival time of tumor-bearing fish was assessed in two most fast growing tumor lines zt23 and s1 implanted i.p. to syngeneic 3.5-month-old fish or to 12-day-old syngeneic larvae (zt23 only). Each experimental and control group consisted of either 10 adult females or 29 larvae. During the observation, each group of fish was kept in a separate 20-L system tanks (for adults) or in 400-mL beakers (for larvae) at 26°C and fed a standard diet or nauplii Artemia, respectively. The assessment of survival time continued until the death of the last fish in each experimental group.

**Results**

Treatment of zebrafish with DEN resulted in 35 primary tumors localized in the abdominal area and reaching from 3 to 10 mm in diameter. The grossly visible tumors began to emerge in fish 3 to 7 months after the beginning of exposure to DEN. Four spontaneous tumors diagnosed as acinar cell carcinomas of the pancreas were detected in 10.5- to 18-month-old female CB1 fish (Fig. 1G).

Of 29 transplanted DEN-induced and spontaneous tumors, 19 underwent from 3 to 25 consecutive passages in syngeneic fish. The other tumors were lost as a result of the recipient’s death or because of a tumor growth arrest after several passages. In the majority of cases, i.p. or i.m. transplanted tumors showed almost synchronous growth in fish inoculated simultaneously. The efficacy of tumor transplantation to both sites was close to 100% as would be expected in syngeneic recipients. The main features of the transplanted tumor lines that are currently being maintained in the lab are shown in Table 1. The majority of tumors were characterized by a moderate growth rate and required further passage approximately every 1 to 2 months. The others tumor lines remained either slow growing during the entire period of observation (zt10, zt12, zt16, zt20, and s3) or, by contrast, had a very high growth rate and required transplantation every 2 to 4 weeks (s1, zt23, and zt34). It is noteworthy that invasiveness also varied among different transplanted tumor lines. In particular, tumor lines zt23 and s1 seemed to be the most invasive. Macroscopically, these tumors were composed of an amorphous jelly-like mass of milky-white (s1) or yellowish (zt23) color, filling essentially the entire abdominal cavity (Fig. 1D). The liver, ovaries, and abdominal wall were the most common sites of invasion of these tumors. Less invasive tumors typically grew as solitary pink or yellowish solid smooth nodules up to 1 cm in diameter, well separated from surrounding abdominal organs (Fig. 1E). These nodules were usually connected to the host vascular system by a
blood vessels stalk. The majority of these tumors also showed invasive growth. However, in this case, the signs of invasion into surrounding organs could be determined after a microscopic assessment only (Fig. 2F). “Minimal deviation” hepatoma (zt20) grew as a relatively benign noninvasive tumor, forming structures grossly similar to those of normal liver with regard to shape, color, and consistency (Fig. 1F).

During multiple serial transplantations, all tumor lines retained a close similarity to the histologic structure of the primary tumors. Most of the DEN-induced tumor lines were diagnosed as a typical hepatocellular (zt8, zt9, zt12, zt15, zt16, zt18, zt28, zt29, and zt34; Fig. 2C) and cholangiocellular (zt10; Fig. 2D) carcinomas. The morphology of these types of tumors has been described in detail for various fish species, including zebrafish (7, 16). Hepatoblastomas (zt2, zt6, and zt21) were typically composed of undifferentiated embryonal-like cells, sometimes in combination with hepatocarcinoma cell areas that often have an arrangement suggestive of a rosette-like formation. The histology of these neoplasms resembled tumors observed in Fundulus heteroclitus from a creosote-contaminated area (17). The “minimal deviation” hepatoma zt20 was composed of essentially normal hepatocytes with round uniniform nuclei and vacuolated cytoplasm (Fig. 2F).

This tumor may represent the zebrafish analogue of “minimal” hepatomas described earlier in rats (18). The DEN-induced acinar cell carcinoma in pancreas (zt23) commonly grew as large fields consisting of separate strongly basophilic small cells infiltrating the gaps between adjacent organs (Fig. 1K). In some cases, we observed liver metastases after i.p. grafting of this tumor. The histology of these metastases was similar to the tumor that they were derived from (Fig. 2G). Spontaneous acinar cell carcinomas in pancreas (s1, s3, s10, and s11) grew as solid sheets formed by large polygonal cells with moderately polymorphic nuclei, with prominent nucleoli and basophilic cytoplasm that contained bright eosinophilic secretory granules (Fig. 2F). In some areas, the structure of these tumors strongly resembled normal pancreatic tissue in zebrafish (Fig. 2B). Histologic diagnoses of all tumor lines were validated by the Registry of Tumors in Lower Animals (Sterling, VA).

Signs of advanced stages of tumor growth, such as significant enlargement (often asymmetric) of the abdomen (Fig. 1A), development of ascit in the abdominal cavity, tumor penetration of the abdominal wall (Fig. 1C), cachexia, or various combinations of these symptoms, were as criteria for expediency of the next i.p. tumor passage in adult fish. By contrast, slow growing (zt10, zt12, zt16, and s3) or relatively benign (zt20) tumor lines underwent consecutive passages approximately every 2 to 3 months without strong dependency upon macroscopic signs of tumor progression. Tumor size was the main criteria for the next tumor passage after l.m. transplantation (Fig. 1B).

As expected, we failed to transplant several moderately and fast growing tumor lines (zt9, zt18, and zt23) to wild-type (AB) zebrafish. By contrast, transplantation of the majority of tumor lines to isogenic zebrafish was successful in all cases (Table 1; Fig. 1H). However, the growth rate of tumors transplanted to isogenic hosts was typically ~50% slower than that in their syngeneic counterparts.
The frequency of successful transplantation of tumor lines zt23, zt34, and s1 recovered after cryopreservation remain as high as 100%, 100%, and 33%, respectively. All tumors that developed after cryopreservation showed invasive growth and retained a histologic structure of the corresponding tumor lines.

The mean survival time of adult fish after i.p. tumor grafts was 25 ± 4.0 and 44.7 ± 6.2 days for tumor lines zt23 and s1, respectively (Fig. 3A). No fish death occurred in a control group during the entire period of observation. The mean survival time of 12-day larvae i.p. injected with zt23 tumor cells was 15 ± 4.2 days (Fig. 3B).

**Discussion**

Transplantable tumor lines remain one of the main models for investigation of different aspects of tumor biology in rodents (19). Various attempts have been made to establish transplantable tumor lines in lower vertebrates, including homologous epithelioma grafting into the anterior eye chamber in catfish (20). More recently, a possibility of transplantation of carcinogen-induced liver tumors to intact adult fish through more conventional routes was shown by M. Schultz and R. Schultz (11) using a naturally inbred stock of *Poeciliopsis licuda*. Considering a significant progress in developmental biology and genetics of zebrafish, this animal hold a great potential with regard to novel cancer research models. It has been shown that mMyc-induced leukemia (8), MycN-induced neuroendocrine tumors (9) and mutated BRAF V600E-induced melanoma (21) can successfully be transplanted to γ-irradiated zebrafish. In addition, zebrafish embryos were shown to be potentially suitable for human and mammalian tumor cells grafting (22, 23). However, further progress in generation of sustainable

| Table 1. Main features of transplantable tumor lines in zebrafish |
|---|---|---|---|---|---|---|
| No. | Tumor line | Tumor origin | No. passages* | Interval between passages days | Duration of tumor maintenance (mo) | Invasiveness | Growth in isogenic fish | RTLA no. | Histologic diagnosis of tumor lines |
| 1 | zt2 | DEN induced | 11 | 45-60 | 18.0 | +/− | Yes | 7606 | Hepatoblastoma and Hepatocellular Carcinoma |
| 2 | zt6 | DEN induced | 10 | 45-60 | 17.5 | +/− | Yes | 7607 | Hepatoblastoma and Hepatocellular Carcinoma |
| 3 | zt8 | DEN induced | 13 | 30-45 | 17.3 | +/− | Yes | 7609 | Hepatocellular Carcinoma |
| 4 | zt9 | DEN induced | 13 | 30-45 | 17.3 | +/− | Yes | 7610 | Hepatocellular Carcinoma |
| 5 | zt10 | DEN induced | 7 | 65-80 | 17.3 | +/− | Yes | 7611 | Cholangiocarcinoma |
| 6 | zt12 | DEN induced | 7 | 60-90 | 17.3 | +/− | Yes | 7612 | Poorly differentiated hepatocellular carcinoma |
| 7 | zt15 | DEN induced | 12 | 30-45 | 17.3 | +/− | Yes | 7613 | Hepatocellular carcinoma |
| 8 | zt16 | DEN induced | 8 | 50-75 | 17.0 | +/− | Yes | 7614 | Poorly differentiated hepatocellular carcinoma |
| 9 | zt18 | DEN induced | 13 | 40-50 | 16.7 | +/− | Yes | 7615 | Poorly differentiated hepatocellular carcinoma |
| 10 | zt20 | DEN induced | 8 | 45-75 | 16.5 | − | Yes | 7616 | “Minimal deviation” hepatoma |
| 11 | zt21 | DEN induced | 12 | 40-50 | 16.5 | +/− | Yes | 7617 | Hepatoblastoma |
| 12 | zt23 | DEN induced | 25 | 10-20 | 16.5 | + | Yes | 7618 | Pancreatic acinar cell carcinoma |
| 13 | zt28 | DEN induced | 11 | 45-60 | 16.3 | +/− | Yes | 7619 | Hepatocellular carcinoma |
| 14 | zt29 | DEN induced | 12 | 30-45 | 16.3 | +/− | Yes | 7620 | Hepatocellular carcinoma (with spindle cell pattern) |
| 15 | zt34 | DEN induced | 18 | 20-30 | 16.2 | +/− | Yes | 7621 | Poorly differentiated hepatocellular carcinoma |
| 16 | s1 | Spontaneous | 14 | 20-30 | 12.5 | + | Yes | 7605 | Pancreatic acinar cell carcinoma |
| 17 | s3 | Spontaneous | 5 | 50-75 | 10.0 | − | NT | Pancreatic acinar cell carcinoma |
| 18 | s10 | Spontaneous | 3 | 30-45 | 2.0 | − | NT | Pancreatic acinar cell carcinoma |
| 19 | s11 | Spontaneous | 3 | 30-45 | 1.5 | − | NT | Pancreatic acinar cell carcinoma |

Abbreviation: NT, not tested.

*Number of tumor passages up to date.

†Average interval between two consecutive tumor transplantations (recipient’s age ranged between 2.5 and 6 mos).

‡Total duration of the tumor line maintenance beginning from the first passage.

³(+), infiltrative growth; (+/−), invasive growth detected during histologic analysis only; (−), tumor invasion is not detected.

⁴Identification no. at the Registry of Tumors in Lower Animals.

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transplantable tumors in zebrafish has been contained because of the lack of a true inbred zebrafish strain as a result of lost of fertility during the inbreeding (24). To overcome this obstacle, we generated two clonal homozygous lines of zebrafish whose viability and fertility was comparable with that of wild-type fish. The lack genetic difference between individual fish within a clone enabled us to carry out successful multiple passages of DEN-induced and spontaneous tumors in zebrafish.

As expected, the majority of DEN-induced tumors in clonal zebrafish were liver-derived tumors. A carcinoma of pancreas was the only extrahepatic DEN-induced tumor. This finding was somewhat surprising because before this study, benign pancreatic tumors were described in zebrafish treated with N-methyl-N'-nitro-N-nitrosoguanidine (5) and 7,12-dimethylbenz(a)anthracene (6) but not with DEN (7). It is noteworthy that four spontaneous tumors diagnosed as acinar cell carcinomas of pancreas were detected in 10.5- to 18-month-old female CB1 zebrafish. Considering the fact that spontaneous tumors of pancreas occur very seldom in zebrafish (25), we suggest that CB1 fish might have genetic predisposition to development of pancreatic tumors (e.g., because of a possible loss of heterozygosity of tumor suppressor genes; ref. 26) as a result of homzygous nature of their genome. This hypothesis is supported by the recent reports on mutant lines of zebrafish that are highly susceptible to spontaneous tumors (27–29).

The majority of DEN-induced and spontaneous tumors that emerged in CB1 zebrafish showed growth after grafting to both syngeneic and isogeneic but not to wild-type animals. These results indicate that immunogenicity of transplantable tumors in zebrafish is determined by their histocompatibility complex (30) and therefore is similar to that of mammalian tumors (31). Currently, of 19 transplantable tumors that we continue to maintain in the laboratory, 12 lines have undergone from 10 to 25 passages. These tumors retain their morphology and growth rate that are hallmarks of stable tumor lines. The tumor lines had significant differences in their growth rate and in other biological features just as occurs in the rodent model (32). Some transplantable tumors generated in this study underwent growth arrest after several consecutive passages (data not shown). In such cases, necropsy did not reveal visible tumor connection to the recipient’s vascular system. It can be suggested that such tumors lack an ability to promote angiogenesis playing a vital role in carcinogenesis (33). The growth rate of tumors derived from spontaneous (s1) and DEN-induced (zt23) carcinomas in pancreas was similar to that the most fast growing tumor lines in mammalians (34).

Figure 2. Histologic structure of normal and tumors tissue in zebrafish. Histologic structure of normal liver (A) and pancreas (B). C, moderately differentiated hepatocellular carcinoma zt34. D, cholangiocarcinoma zt10. E, ‘‘minimal’’ hepatoma zt20 displayed histologic structure that resembles with normal liver. F, spontaneous acinar cell carcinoma of pancreas s1 containing areas morphologically similar to normal pancreas. G, a metastasis of zt23 tumor within the lumen of a liver blood vessel (arrowhead). H, destruction of muscle tissue cause by invasion of zt23 tumor in a 23-day-old juvenile fish on 11 days after i.p. transplantation of the tumor cell suspension. Bar, 50 μm (A–F and H) and 250 μm (G).

Figure 3. Lifespan of tumor-bearing fish. A, i.p. transplantation of tumor zt23 (●) and s1 (▲) to 2.5-month-old fish. Mean lifespan: - 25 ± 4.0 and 45 ± 6.2 days in zt23 and s1 tumor-bearing fish, respectively. Control intact fish (○). B, i.p. zt23 tumor transplantation (●) to 12-day larvae. Mean lifespan: - 15 ± 4.6 days. Control intact fish (○).
chemotherapy and screening of antitumor drugs. For these applications, we propose to use the mean survival time of tumor-bearing adult fish or 5- to 14-day-old larvae grafted with tumor cells as a criterion of treatment efficacy. Further refinement of this technology could be achieved by, for example, injecting tumor cells constitutively expressing fluorescent tags (8, 35) into embryos and early larvae. The small size and transparent body of the developing zebrafish make this approach applicable to high-throughput screening assays based on a 96- or 24-microwell format (36). Moreover, some standard methods that were routinely used in rodents to assess tumor growth might be readily adapted to zebrafish. In particular, our preliminary experiments showed that the dynamics of body weight gain in tumor-bearing fish may also be used for quantitative assessment of tumor growth in zebrafish (data not shown). A direct measurement of the size of i.m. transplanted tumors may also be optimized for the zebrafish model by computer morphometry.

The successful transplantation of zt23, zt34, and s1 tumors recovered after cryopreservation in liquid nitrogen showed the possibility of long-term storage of zebrafish tumor lines. This method makes it unnecessary to maintain the tumor lines by routine serial transplantation in live fish and therefore would reduce labor and cost of maintenance of large number of tumor lines.

In conclusion, a comparison of transplanted tumor lines in zebrafish and in rodents shows a striking similarity in their biology. We were not able to detect any specific genetic feature of zebrafish tumors that had not been described earlier in mammals (19). This indicates a similarity if not an identity of the molecular, cellular, and immune mechanisms of tumor formation in fish and mammals. Therefore, the model proposed in this study might be successfully used in the same areas of experimental cancer research, where until now mammalian models were the experimental tool of choice. The advantages of using transplanted tumors in clonal zebrafish are not limited to significantly lower cost of fish maintenance. The availability of genetically identical subjects opens new opportunities for investigations in tumor immunology (37) and cancer genetics (38). In addition, unlike transplanted zebrafish tumors described elsewhere (8, 9, 21), our approach does not require sublethal irradiation of recipients to suppress host immunity and therefore makes our model suitable for much broader applications, including investigation of host-tumor interactions.

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