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High Frequency of Thyroid Tumor Induction by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in the Hermaphroditic Fish *Rivulus marmoratus*

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In the self-fertilizing hermaphroditic fish, *Rivulus marmoratus*, the susceptibility to tumor induction by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was evaluated. Seven-day-old fish larvae were exposed for 2 h to MNNG at concentrations ranging from 5 to 25 ppm in a static water bath. The exposed fish were observed at 2 and 4 months after carcinogen treatment to assess tumor development. Within 4 months after 25 ppm MNNG exposure, nearly all fish developed thyroid tumors. The tumor incidences were dose- and time-dependent, and the latent period of tumor induction was less than 2 months. Most induced neoplasms were papillary carcinomas similar histologically to those of rodents and humans, and the tumors were serially transplantable to other fish of the same species. These results demonstrate that rivulus could be useful as a model of thyroid carcinogenesis.

Key words: *Rivulus marmoratus* — Hermaphroditic fish — Thyroid tumor — MNNG

Studies of chemical carcinogenesis require sensitive animal models for tumor induction, but the rodent systems commonly used often have problems of limited sensitivity to chemicals and long latent periods before tumors develop.[1,2] This is so in the case of thyroid tumors.[3] It has been reported that the most effective protocol currently available for thyroid tumor induction is multiple exposures to chemical carcinogen in combination with lifelong goitrogen treatment in rats.[3,4] Even in this model, development of recognizable neoplasms needs more than 6 months. Several lines of oncogene transgenic mice for tumor induction have recently been produced, but no strain is yet available for thyroid tumors.[5]

The small rivulid fish *Rivulus marmoratus* (synonym: *R. ocellatus marmoratus*) inhabiting brackish water throughout the Caribbean is the only known vertebrate that naturally exhibits functional self-fertilizing hermaphroditism.[6] Consequently, a given population originated from the same progenitor of this species is genetically homogeneous, resulting from an essentially monoecious breeding system.[7,8] Previous studies have shown that this fish is a useful species for *in vivo* hepatocarcinogenesis,[9–12] teratogenicity[13] and DNA repair[14] studies due to the clonal nature of its reproduction and its low cost. To evaluate whether this species has a more general usefulness, the sensitivity of this fish to the chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG[4]), was tested. We found a very high frequency of transplantable thyroid tumor induction by low doses of this carcinogen with short latency.

MATERIALS AND METHODS

Fish Rivulus was bred and reared in our aquariums as described previously.[10] Our fish stock has originated from a single individual obtained from Zoologisches Institut und Zoologisches Museum, Universität Hamburg, in August 1981. Fish were housed in groups in a separated 80-liter glass tank containing 60 liters of 30% synthetic seawater made from tap water with Marine Mix Sea Salts (Korea Marine Salts Producing Co., Sorae, Korea). Because hyperplasia may occur in the thyroid of laboratory-raised fish in response to a low iodine concentration in the water,[15,16] iodine (50 μg/liter) was added to the water in amounts sufficient to prevent goiter development. Fish were kept in an air-conditioned room at 25 ± 1°C and with 14-h light cycle daily. Young fish (less than 1 month old) were fed brine shrimp (*Artemia salina*) nauplii (eggs from Metaframe, Calif.), and adult fish were given chicken liver and *Drosophila*.

Carcinogen MNNG (CAS 70-25-7) was purchased from Sigma Chemical Co., St. Louis, Mo. To minimize intergroup variability due to changes of carcinogen concentration, stocks of MNNG (50 mg/ml) were freshly prepared by dissolving the carcinogen in 5 ml of dimethyl sulfoxide (DMSO) at pH 5.0, because MNNG is relatively stable at low pH in aqueous solution.[17,18] *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer is widely used in cell and tissue culture

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[4] Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

medium and does not have carcinogenic or co-carcinogenic activity. In addition, MNNG is fairly stable in HEPES-buffered salt solution.[19] Thus, carcinogen stock solution was finally added into 10 m*M* HEPES-buffered (pH 7.0) 30% synthetic seawater to make appropriate carcinogen concentrations.

Experimental protocol Duplicate lots of 100–200 fish larvae (7 days old) were exposed to the above water containing MNNG or the control water containing the solvent (DMSO) instead of MNNG at 25±1°C for 2 h. Loading density was 100 fish/liter. After treatment, each group of larvae was washed several times with 30% synthetic seawater and placed in a separated 80-liter glass tank filled with 60 liters of 30% synthetic seawater supplemented with iodine at 25±1°C until termination of the experiment. Sea salts from the same batch were used throughout the experiments to exclude possible effects of differences in salts on water quality. They were fed the same diet mentioned earlier. The carcinogen-contaminated water was carefully collected, alkalized with NaOH to decompose carcinogens,[17, 18] and discarded after neutralization. The carcinogen was handled under the guidelines for laboratory safety precautions set forth by Montesano *et al.*[20]

Sampling At 2 and 4 months after carcinogen treatment, the surviving fish were sampled to determine tumor development — in particular, thyroid tumor incidence. Weight gain and growth in total length were also recorded at the time of sampling. Fish were starved for 1 day prior to being killed and anesthetized with tricaine methanesulfonate. The body and thyroid glands were carefully examined externally under a stereomicroscope, and then the entire fish was fixed in 10% buffered formalin for subsequent histological determination.

Histology Because the size of the specimens was small enough for all tissues to be examined, every fixed whole fish were embedded in paraffin, and serially sectioned from the mouth to the anus in transverse planes to investigate the histology. Sections were stained with hematoxylin and eosin. The histological classification of thyroid tumors was based on criteria applying to laboratory animals[21] and humans.[22]

Tumor transplantation The anterior eye chambers were chosen as the transplantation site since graft growth could easily be observed without killing the hosts and the damage to the eyes during operation readily heals even in the aquatic condition. A total of 29 macroscopically visible tumors were cut into small pieces about 0.1 mm in diameter and grafted into one of the anterior eye chambers of recipient fish (6 months old), and the tissues from 10 normal thyroids were simultaneously transplanted into the contralateral eye of the same fish as control grafts using the method of Hyodo-Taguchi and Matsudaira.[23] Growth of the grafted tumor was observed every week under a dissecting microscope to determine the transplantability of the tumors. Successful engraftment of tumor tissues was confirmed by histological examination of vascularization. Serial transplantation was also performed with tumors from 15 primary transplantations.

Statistical analysis The significance of quantitative data was analyzed by using Student's *t* test. Differences were considered significant at *P* levels less than 0.05.

RESULTS

Growth The body weight gain and growth in total length of the control and MNNG-treated fish are shown in Table I. The fish exposed to MNNG showed significantly less body weight gain and growth than the corresponding control groups. The growth retardation was dose-related.

Tumor incidence and mortality MNNG produced thyroid tumors in the rivulus (Fig. 1). The incidence of thyroid tumors is summarized in Table II. Following a short-term (2 h) exposure to a low dose of MNNG (25 ppm), nearly all fish developed tumors within 4 months, whereas none of the control fish did. Furthermore, within 2 months more than 50% of the fish treated with 25 ppm MNNG developed thyroid tumors. Therefore, the latent period was less than 2 months. As shown in Table II, there was a dose- and time-dependent increase of tumor incidence. Although complete serial sectioning of tissues was attempted, no tumorous changes were observed in tissues other than thyroid.

Table I. Average Body Weight and Total Length

Treatment	Body weight, mg±SD (No. of fish)		Total length, mm±SD (No. of fish)	
	2-month sample	4-month sample	2-month sample	4-month sample
Control	147±74 (80)[a]	206±17 (96)[a]	24.7±8.5 (80)[a]	25.9±1.3 (96)[a]
5 ppm MNNG	113±86 (80)	195±97 (77)	21.4±1.4 (80)[b]	24.6±5.1 (77)[b]
15 ppm MNNG	106±78 (80)[b]	157±26 (106)[b]	21.2±1.3 (80)[b]	24.4±2.4 (106)[b]
25 ppm MNNG	104±19 (80)[b]	142±56 (82)[b]	20.1±1.3 (80)[c]	22.5±2.2 (82)[c]

a), *b*), *c*) Values with different subscript letters were significantly different from those with the preceding letter at $P<0.05$.

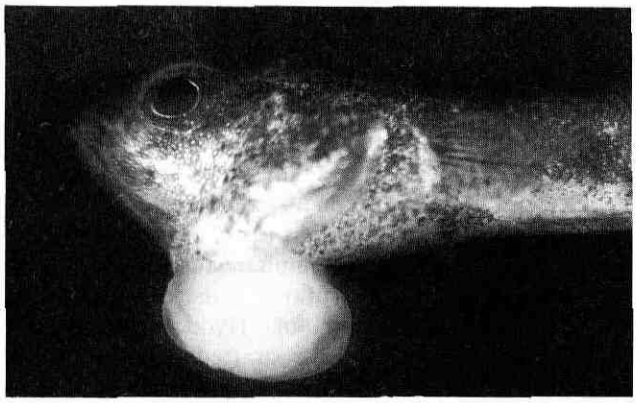

Fig. 1. Rivulus with a large thyroid tumor from the 4-month sample of 15 ppm MNNG-treated group.

Mortality was increased relative to the controls in a dose-related manner, being significant for all MNNG doses. Approximately 71% of dead fish (n=234) recovered between scheduled samplings from carcinogen-treated groups had visible thyroid tumors when they were examined grossly. In contrast, no tumor was observed among a number of dead fish from control groups. Thus, thyroid tumor was a main cause of the high mortality in the MNNG-treated groups.

Gross observations and histology of tumors Normal thyroid gland of adult rivulus is a subspherical organ which is located in the anterior midline just caudal of the lower jaw, and its size is approximately 1–2 mm in diameter. The follicles lining the simple cuboidal epithelium contain colloid and closely resemble those of higher vertebrates (Fig. 2A). Exuberant growth of thyroid tumors was readily apparent 2 months after carcinogen treatment. The extent of the growth was clearly seen with a low-power stereomicroscope after opening the operculum. When 100 visible thyroid tumor nodules more than 0.1 mm in diameter under the dissecting microscope were examined histologically, 98 were confirmed to be thyroid tumors. Thus, gross observation has 98% confidence for tumor diagnosis. By the 4th month, neoplasms usually protruded beyond the gill chamber (Fig. 1), where they interfered with the opercular respiratory movements.

Histopathological classification of tumors is given in Table III. Most tumors (87.5%) were typical papillary carcinoma (Fig. 2B), and were very similar histopathologically to those observed in rodents[3, 20] and humans.[21] Carcinomas varied considerably in size and were usually not encapsulated by scirrhous fibrosis. Tumor cells arranged in irregular papillary infoldings. Follicular carcinomas (Fig. 2C) were also observed, but their frequencies were low (27.2%). Some of this type infiltrated into the adjacent region of thyroid. They are almost anaplastic and rudimentary, or varying sizes of follicles were present. Papillary and follicular carcinomas sometimes showed mixed patterns (18.7%). They were present in the same tumor nodules in some cases. Adenomas (4%) were usually small and the follicular epithelium presented a distinct papillary pattern which might project into a cystic lumen (Fig. 2D). The metastasis of thyroid tumors were not detected in this study. Other pathologic changes observed in the carcinogen-treated groups included sarcomas developed near the thyroids. Their incidence was very low (2.0%). It could, however, not be assessed whether these tumors had originated from tissue of the thyroid gland or not.

Table II. Thyroid Tumor Incidence in Rivulus

Treatment	Effective No. of fish	Tumor incidence (% mean ± SD)				Cumulative mortality (% mean ± SD)	
		2-month sample		4-month sample			
Control							
Tank A	86	0/40	(0.0 ± 0.0)[a]	0/46	(0.0 ± 0.0)[a]	14/100	(12.0 ± 2.8)[a]
Tank B	90	0/40		0/50		10/100	
5 ppm MNNG							
Tank A	77	2/40	(3.8 ± 1.8)[a]	4/37	(11.7 ± 1.2)[b]	23/100	(21.5 ± 2.1)[b]
Tank B	80	1/40		5/40		20/100	
15 ppm MNNG							
Tank A	89	2/40	(16.3 ± 15.9)	29/49	(63.0 ± 5.3)[c]	61/150	(38.0 ± 3.8)[c]
Tank B	97	11/40		38/57		53/150	
25 ppm MNNG							
Tank A	80	27/40	(63.8 ± 5.3)[b]	39/40	(98.8 ± 1.8)[d]	120/200	(59.5 ± 0.7)[d]
Tank B	82	24/40		42/42		118/200	

a), *b*), *c*), *d*) Values with different subscript letters were significantly different from those with the proceding letter at $P < 0.05$.

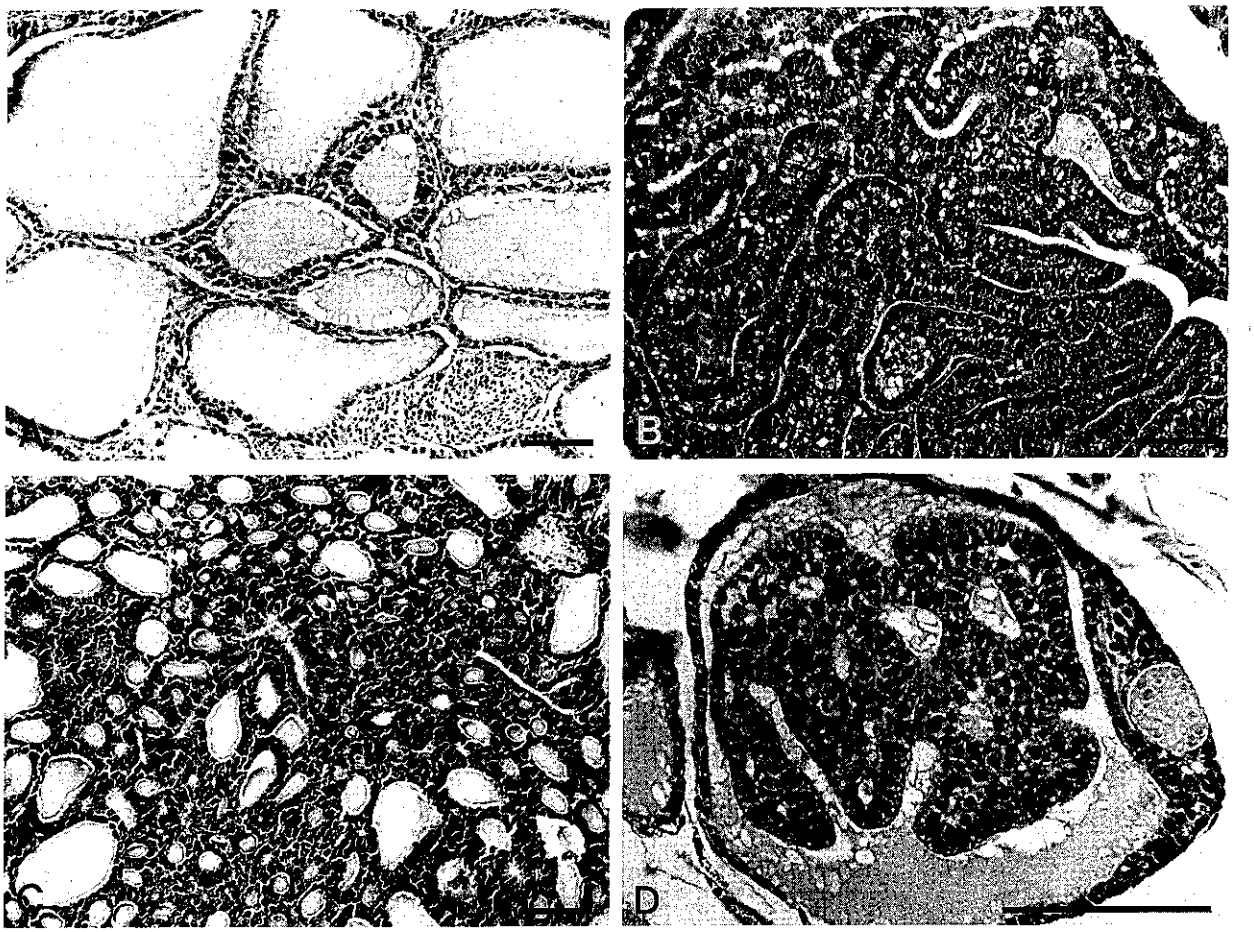

Fig. 2. Histology of control thyroid of 4-month-old rivulus (A). Papillary carcinoma from the 4-month sample of 15-ppm MNNG-treated fish (B). Follicular carcinoma from the 4-month sample of 25 ppm MNNG-treated fish (C) and papillary adenoma from the 2-month sample of 15 ppm MNNG-treated fish (D). H & E. Bars, 50 μm.

Table III. Histological Findings of Thyroid Tumors of Rivulus

No. of tumor-bearing fish examined	No. (%) of fish with			
	Papillary carcinoma	Follicular carcinoma	Mixed papillary-follicular carcinoma	Adenoma
224	196 (87.5)	61 (27.2)	42 (18.7)	9 (4.0)

Transplantability Twenty-seven out of 29 transplantations of thyroid tumors into the anterior eye chamber of sibling fish were successful. The graft attached within a few days, and by the end of 2–3 months the anterior chamber was filled with a soft, well-vascularized mass of tumor tissue (Fig. 3). All grafts of normal thyroids (n=10) degenerated and disappeared within 2 to 3 weeks. The newly developed tumor tissues from primary transplantations were grafted into the eye chamber of 15 fish. They grew progressively in 12 fish, and have been successfully passed for another four generations.

DISCUSSION

We have shown that thyroid tumors were induced with very high incidences after short-term exposure of the genetically homozygous hermaphroditic fish, *R. marmoratus*, to MNNG. In rodents thyroid tumors have been induced by various chemical carcinogens or ionizing radiation, either alone or in combination with various kinds of goitrogens.[3,4] The thyroid tumor development following exposure to carcinogens is, however, slow in rodent systems. Furthermore, carcinogen alone usually

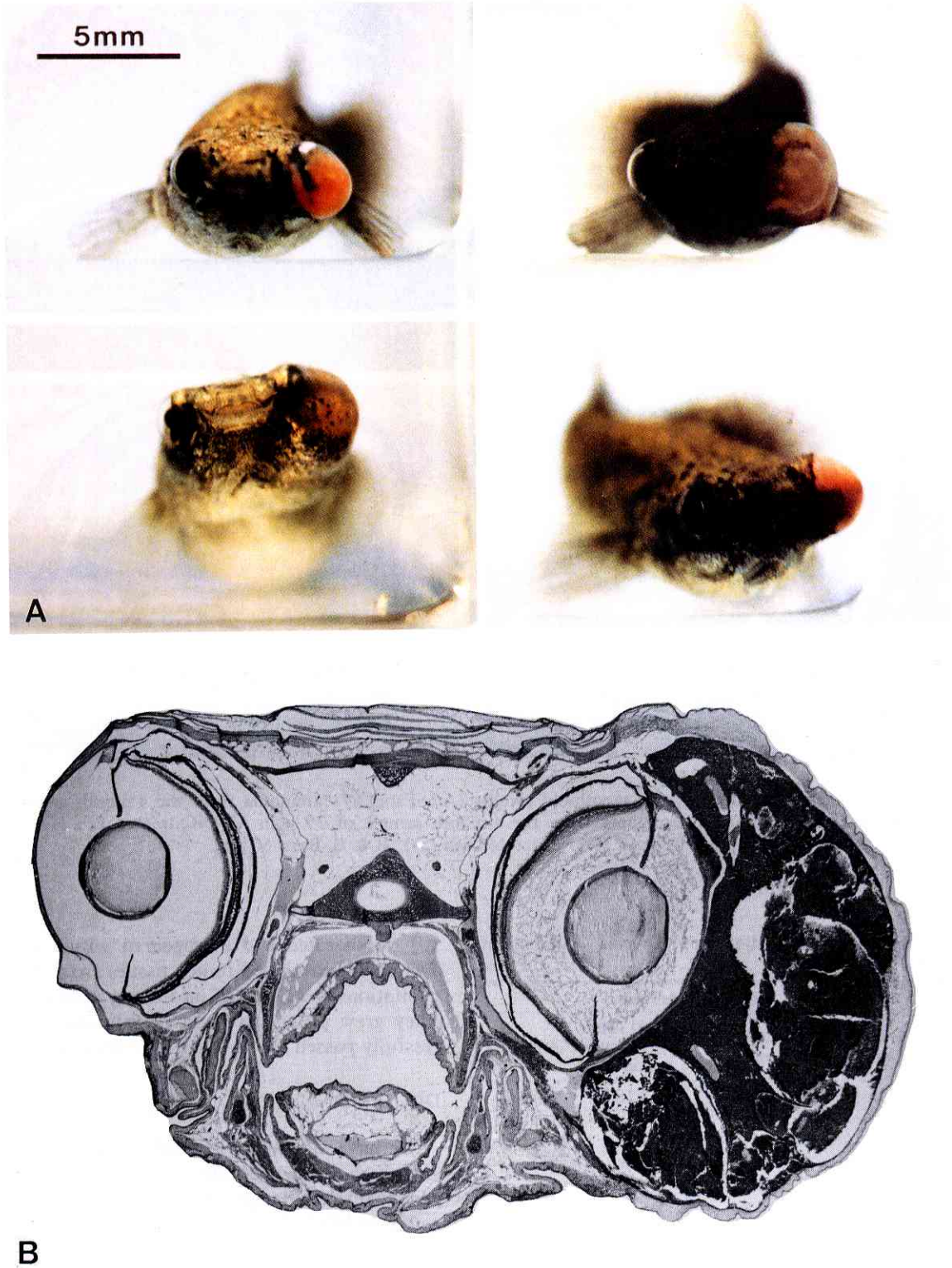

Fig. 3. Growth of the engrafted thyroid tumor tissue after transplantation into the anterior chamber of the eye. At 60 days after transplantation, the eye chambers was filled with well-grown tumor (A). Histologic section of eye with a tumor that developed after transplantation (B).

does not initiate tumors. High incidence of thyroid tumor induction in rodents is only predictable when multiple exposure to carcinogens is applied, followed by long-term goitrogen therapy. However, the rivulus thyroid is exceptionally sensitive to the action of carcinogen without goitrogen administration. Although high sensitivity of fish to chemical carcinogens and the short latent period of tumor induction have been reported for several species including rainbow trout (*Oncorhynchus mykiss;* synonym: *Salmo gairdneri*)[24] and medaka (*Oryzias latipes*),[23,25] thyroid tumor induction has not been reported in these fish models. Several cases of spontaneous or induced thyroid tumors were reported from aquarium and captive fish.[26–30] Most of these was goitrous hyperplasia caused by the extremely low levels of environmental iodine and potentially reversible by increasing the iodine availability. Since some cases could not be explained on the basis of iodine deficiency, contamination of the water with natural and man-made goitrogens may also be important.[31]

MNNG induces liver and kidney tumors in rainbow trout[32,33], or melanomas[23] and branchial blastomas[34] in the medaka. Thyroid tumors did not, however, develop in these species. The reasons for the different organotrophy and difference in susceptibility to the same carcinogen between fish species is unclear. In the case of rainbow trout, liver and kidney neoplasms developed from 9 months after MNNG treatment.[32,33] Thus liver and kidney of rivulus could also respond to MNNG if the follow-up time is extended beyond 4 months after exposure to low doses of this carcinogen, which did not induce high mortality before termination of the experiment.

In most teleost fish the thyroid gland is usually diffuse, follicles being scattered throughout the lower jaw region. This dispersed arrangement of follicles makes them difficult subjects for histological study. However, the thyroid glands of Elasmobranchii, Holocephali and a few species of teleosts have evolved into a compact organ.[15,35] Even though rivulus is one of the teleost fish, its thyroid remains compact with few exceptions. Thus, normal and tumorous thyroids of rivulus were easy to handle for histological and transplantation studies. Ectopic thyroid has been widely reported in several fish species as well as in mammals, including man.[36] The ectopic thyroids of rivulus are also found in the kidney, liver and intestine of fish older than 9 months.

The present experiments also showed that induced thyroid tumors were serially transplantable into the anterior chamber of the eye in other individuals of rivulus. This is the second success of serial transplantation of tumors in fish. The melanomas induced by MNNG in the medaka have been serially transplanted into highly inbred syngeneic fish.[23] Experimental systems for tumor transplantation are very useful for studies in tumor biology and clinical oncology. For this purpose, the nude mouse system is a model for some fish tumors[37] as well as for rodent tumors. The clonal nature of our fish eliminated histocompatibility barriers and encouraged us to transplant tumor tissues into other members of the same species. This could represent an additional advantage of this fish as model for carcinogenesis.

The present experiment demonstrates that rivulus is an advantageous species for studying chemical carcinogenesis of thyroid. Therefore, these fish may be useful as an animal model for the study of the molecular oncology of thyroid tumors. If oncogenes can be analyzed in this organism as in *Xiphophorus* fish,[38,39] important information on agent-specific oncogene activations resulting in thyroid cancers[40,41] may be obtainable.

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