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# Ethylnitrosourea Induces Neoplasia in Zebrafish (*Danio rerio*)

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**SUMMARY:** The zebrafish (*Danio rerio*) has been successfully used to discover hundreds of genes involved in development and organogenesis. To address the potential of zebrafish as a cancer model, it is important to determine the susceptibility of zebrafish to tumors. Germ line mutations are most commonly induced for zebrafish mutant screens by exposing adult male zebrafish to the alkylating agent, ethylnitrosourea (ENU). To determine whether ENU induces tumors, we compared the incidence of tumors in ENU-treated fish with untreated controls. Interestingly, 18 of 18 (100%) fish mutagenized with either 2.5 or 3.0 mM ENU developed epidermal papillomas, which numbered 1 to 22 per fish, within 1 year of treatment. The induced epidermal lesions included epidermal hyperplasia, flat papillomas (0.2 to 1.2 mm), and pedunculated papillomas (1.2 to 8 mm in greatest dimension), but no skin cancers. Angiogenesis was evident in papillomas larger than approximately 1 mm. All but two papillomas contained the three cell types (keratinocytes, club, and mucous cells) of normal zebrafish epidermis; histologic variants lacked either club cells or mucous cells. Two cavernous hemangiomas and a single malignant peripheral nerve sheath tumor were also found in the treated fish. None of five untreated controls developed tumors. These studies establish the feasibility of the zebrafish as an experimental model for the study of skin tumors. (*Lab Invest 2000, 80:379–385*).

The zebrafish (*Danio rerio*), also known as zebra danio (Robins et al, 1991), was introduced as a model for carcinogen studies at the National Cancer Institute by Mearl Stanton (1965). More recently, the zebrafish has also become an important model system for the study of biological problems relating to human disease, including development and organogenesis (Driever and Fishman, 1996; Eisen, 1996; Zon, 1999). To address the feasibility of the zebrafish as a cancer model, it is useful to establish the sensitivity of zebrafish to chemical carcinogens. Ethylnitrosourea (ENU) is a direct-acting alkylating agent (reviewed by Shibuya and Morimoto, 1993) that is well known to cause tumors in a variety of animal models including the Eker rat (Kobayashi et al, 1997), the Sprague-Dawley rat (Koestner et al, 1971), and *Xiphophorus* (platyfish/swordtail fish) (Schartl et al, 1997). We therefore reasoned that tumors may arise in males exposed to ENU in a commonly-used germ line mutagenesis protocol (Mullins et al, 1994; Solnica-Krezel et al, 1994). Here, we report that, within 1 year of treatment, ENU induced an unexpectedly high frequency of papillomas in adult male zebrafish.

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## Results and Discussion

To prepare for two mutant screens in our laboratory (J. Moore, G. Tsao-Wu, and K. Cheng, unpublished observations), adult male zebrafish were treated with three 1-hour immersions in 2.5 or 3 mM ENU every third day (Mullins et al, 1994). The frequency of ENU-induced germ line mutations was measured by crossing the treated males by untreated females homozygous for *golden*. This recessive mutation causes a delayed and decreased pigmentation in retinal pigmented epithelium and melanocytes (Streisinger et al, 1986). We used *golden* as a marker, because ENU-induced mutations in *golden* occur at a rate between that of two other marker genes, *albino* and *sparse* (Solnica-Krezel et al, 1994). The mutation rates caused by 0, 2.5, and 3.0 mM ENU treatments were 0% (0 *golden* embryos of 1460 embryos scored), 0.11% (2 *golden* embryos of 1818), and 0.26% (5 *golden* embryos of 1905), respectively. These rates are approximately double that reported by other laboratories (Mullins et al, 1994; Solnica-Krezel et al, 1994). The difference may be potentially attributed to lot-to-lot variability of ENU or the shorter time elapsed between the beginning of the ENU dissolution procedure and dosing in our experiments (1 hour maximum).

Ten to twelve months after exposure, all 18 (100%) of the ENU-treated adult male fish (Fish F-W) developed epidermal papillomas; two also developed other tumors (Figs. 1 to 3). None of five control fish (Fish A to E) developed tumors of any type. The average number of papillomas per fish was 2 to 3 (range 1 to 7) for the 2.5 mM ENU-treated group (Fish F to M) ($n = 8$), and 6 (range 1 to 22) for the 3.0 mM group (Fish N to W) ($n = 10$) (Fig. 3). These data suggest that the number of papillomas per fish may be dose-dependent. The 100% incidence of papillomas in ENU-treated fish and absence of papillomas in controls indicate that ENU induced the papillomas.

Grossly, all but two papillomas were white, smooth, and glistening (Fig. 1, A and D). Microscopically, these papillomas were composed of a mixture of the three cell types of normal zebrafish epidermis: keratinocytes, and club and mucous cells. Club cells (*Schreckstoffzellen*, alarm substance cells) measure 15 to 20 mm, have pink cytoplasm that is frequently scalloped

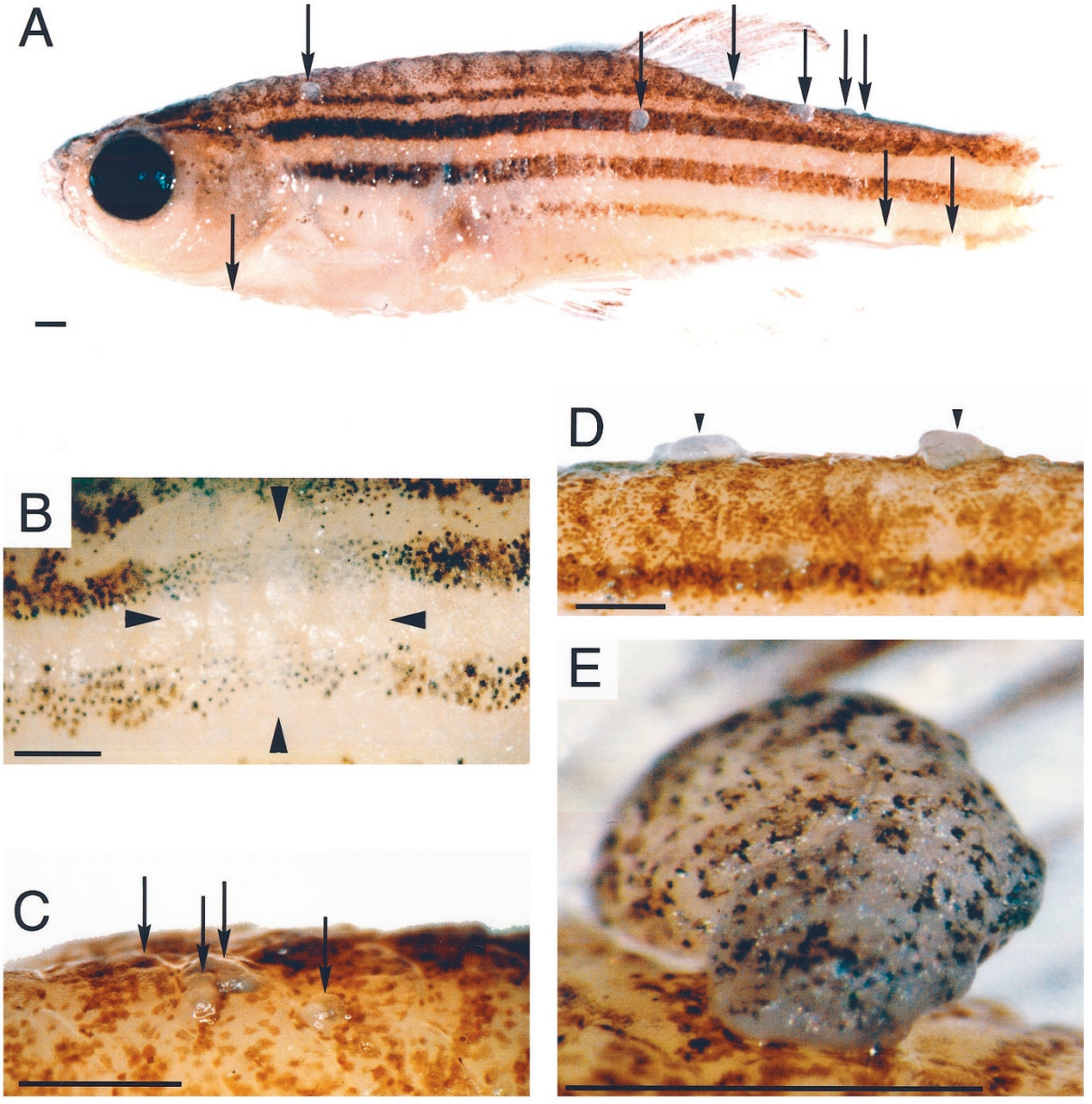


**Figure 1.**
Ethylnitrosourea (ENU)-induced hyperplasia and papillomas (gross). A, Multiple papillomas (*arrows*) in a fish with a total of 22 papillomas (Fish W). B, This white plaque of epidermal hyperplasia (between *arrowheads*) obscured the normal melanophore pattern of the zebrafish stripes (brown dot pattern, left and right) (Fish K). Epidermal hyperplasia obscured the pigment pattern, because melanophores are near the epidermal basement membrane, deep to the thickened epidermis. C, Early sessile papillomas (*arrows*) frequently occurred at or near the distal ends of scales (Fish I). D, Two sessile papillomas (*arrowheads*) on the dorsum, midline, of Fish O. E, A pigmemted pedunculated papilloma. This papilloma was 1.5 × 1.2 × 1 mm and had a narrower 0.4 × 0.3 × 0.1 mm stalk (Fish N). Cells with melanin pigment were present histologically (see Fig. 2F). Scale bars: 1 mm.

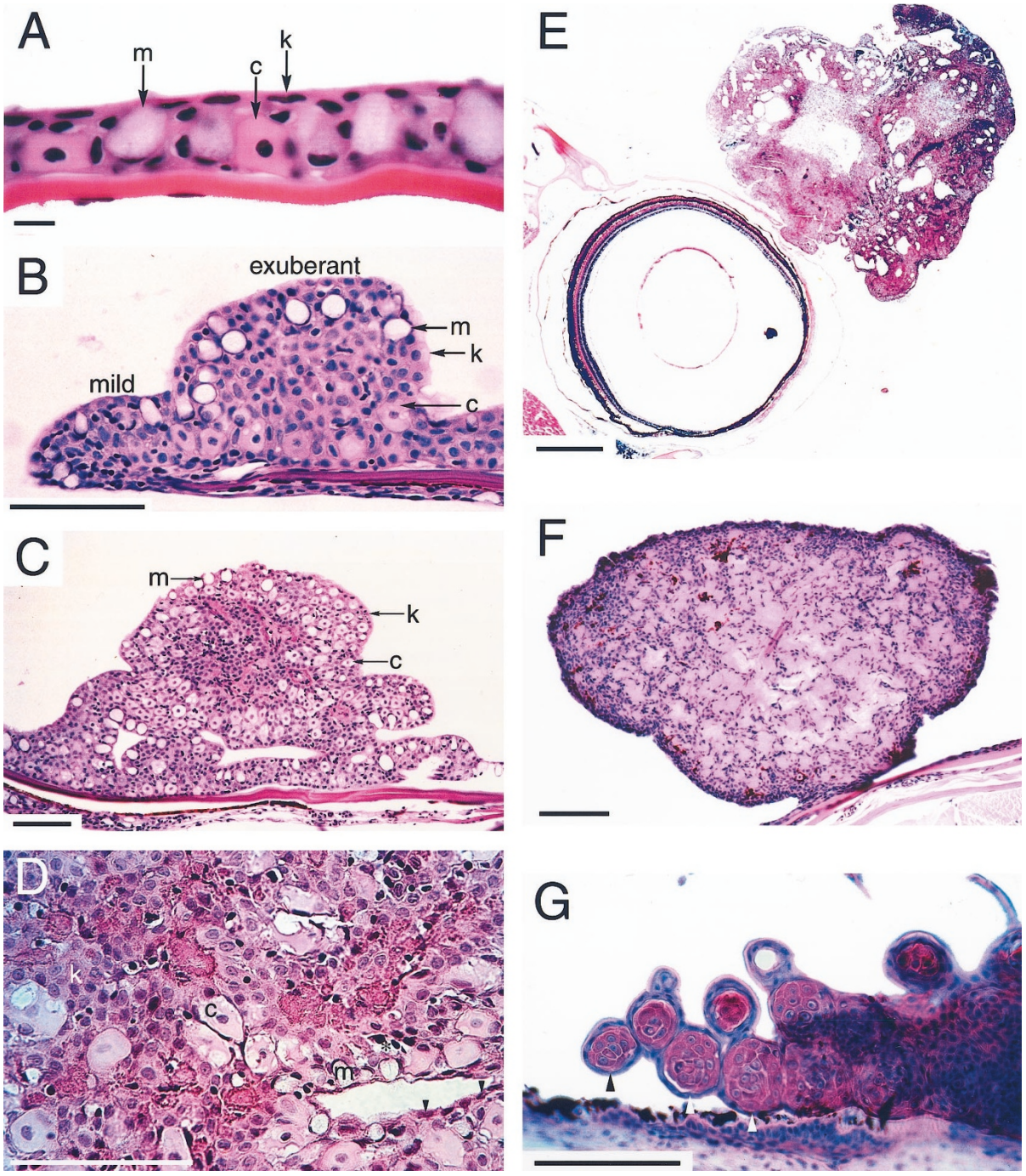


**Figure 2.**

Microscopic appearance of epidermal lesions induced by ENU. A, Normal epidermis of body scales is thin and is composed of keratinocytes (*k*), mucous cells (*m*), and club cells (*c*). Keratinocytes (*k*) are squamous epithelial cells that are red, slightly smaller than club cells, and that have intercellular desmosomes. Mucous cell nuclei are pushed to one side. Mucous cells stain light blue with hematoxylin and eosin after formaldehyde fixation (shown), and deep blue after paraformaldehyde fixation (not shown). Club cells are pink, with central nuclei and occasionally scalloped edges. Scale bar:10 mm. B, Hyperplastic epidermis was thicker than normal but was composed of the same cell types as normal epidermis. Scale bar: 100 mm. C, Sessile (broad-based) papilloma composed of keratinocytes (*k*), mucous cells (*m*), and club cells (*c*). Scale bar: 100 mm. D, Pedunculated papilloma. All except two pedunculated papillomas were composed of keratinocytes (*k*), mucous cells (*m*), and club cells (*c*). Medium-sized vascular spaces were lined by a thin endothelium (*arrowheads*). Capillaries (*) contained red blood cells which have dark nuclei and red cytoplasm. Scale bar: 100 mm. E, Pedunculated papilloma. This is a section of the largest papilloma, which measured 8 × 4 × 4 mm, and was attached to the dorsal surface of the head between the eyes of fish H by a 0.3 × 0.3 × 0.1 mm stalk. The papilloma and eye are shown; the remainder of the body extends to the left. Histologically, keratinocytes and mucous cells, but not club cells, were arranged in solid, nested, papillary, or glandular patterns. Vascular spaces were present. Scale bar: 1 mm. F, Pigmented papilloma. This papilloma variant was composed predominantly of a uniform population of keratinocytes that flatten towards the surface and that are arranged in cords around circular areas of dense collagen and basement membrane material. Numerous melanophores account for the dark brown spotted pattern seen grossly (see Fig. 1E). Scale bar: 100 mm. G, Whorls of squamous cells (squamous eddies, *arrowheads*), occurred at the distal end of spiny rays of some fins. Scale bar: 100 mm. Hematoxylin and eosin.

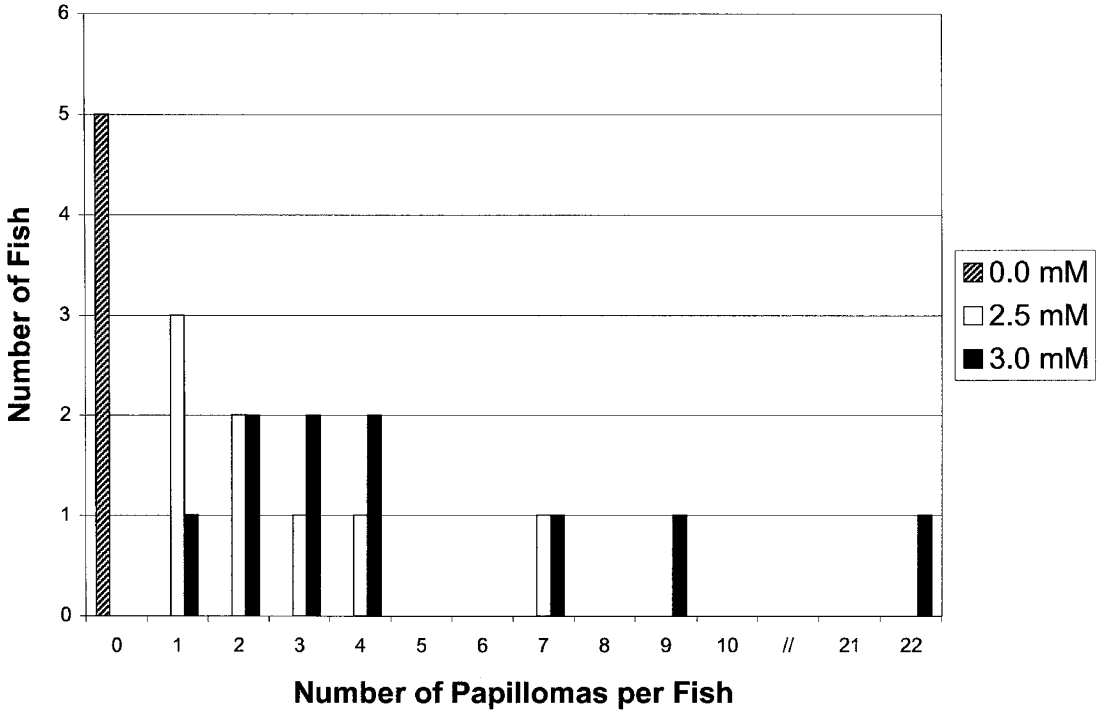


**Figure 3.**
Number of papillomas per fish. A greater number of papillomas tended to be associated with fish dosed with ENU at 3.0 mM compared with those dosed at 2.5 mM. None of five control (0.0 mM) fish developed tumors.

at the edges (Fig. 2D), and have central nuclei. In addition to these three cell types, two papillomas, on Fish S and V, also contained few scattered melanophores (melanocytes) (not shown). The histologic range of induced epidermal lesions included epidermal hyperplasia (Figs. 1B and 2B), flat papillomas (0.2 to 1.2 mm) (Figs. 1, A, C, and D; and 2, B and C), and pedunculated papillomas (1.2 to 8 mm in greatest dimension) (Figs. 1E, and 2, D to F). None of the papillomas were invasive.

Two papillomas were histologically different. One of these, the largest papilloma, was an 8 × 4 × 4 mm pale, pink, fleshy, pedunculated mass on the dorsal head between the eyes of Fish H (Fig. 2E). Microscopically, it was composed primarily of keratinocytes admixed with a small number of mucous cells. Club cells were absent (not shown). The second papilloma variant occurred near the dorsal fin in Fish N (Fig. 1E). Grossly, this papilloma was 1.5 × 1.2 × 1 mm, and pedunculated. Microscopically, the keratinocytes in this lesion were basaloid, and arranged in trabecular and cylindromatous patterns. In contrast to the other lesions, club cells were few, mucous cells were absent, and numerous scattered melanophores (melanocytes) accounted for the grossly pigmented pattern (Figs. 1E and 2F). The absence of either club cells or mucous cells in these variants makes the keratinocyte the only consistent cell type in these papillomas.

Progression from non-dysplastic papillomas to dysplastic papillomas to malignant skin tumors has been studied in the mouse (Berenblum, 1954; Berenblum and Shubik, 1949; Boutwell, 1964; DiGiovanni, 1992; Friedewald and Rous, 1944; Yuspa, 1998). In SENCAR mouse skin, ENU functions as a tumor initiator at lower doses, but as a complete carcinogen at higher doses (O'Connell et al, 1987). It is unknown whether ENU-induced papillomas in zebrafish would progress to carcinomas by the use of higher or more frequent doses of ENU, tumor promoters, or DNA alkyl adduct repair enzyme inhibitors, such as the $O^6$-alkylguanine transferase inhibitor, $O^6$-benzylguanine (Lijinsky et al, 1994; Tong et al, 1997).

ENU-induced zebrafish skin papillomas described here differ histologically from carcinogen-induced mouse skin papillomas. First, mouse papillomas consist of keratinocytes (Bogovski, 1994; Peckham and Heider, 1999); whereas, the zebrafish papillomas often also contain mucous and club cells (Fig. 2, B to D). Second, mouse papillomas exhibit hyperkeratosis (Bogovski, 1994; Peckham and Heider, 1999), which is absent in the zebrafish papillomas. Third, the stroma of mouse papillomas is fibrovascular and exhibits papillomatosis (Bogovski, 1994; Peckham and Heider, 1999); whereas, the stroma of the zebrafish papillomas consists of a more delicate vasculature (Fig. 2D).

We found a 100% incidence of skin tumors after three 1-hour treatments within a 1 week period. A 100% incidence of skin tumors was also reported in the SENCAR mouse after a minimum of 27 weekly doses of ENU to shaved skin (O'Connell et al, 1987). It is noteworthy that the three ENU doses here induced no carcinomas in zebrafish. However, a minimum of 15 weekly topical treatments with ENU were required to induce skin carcinomas in mice (O'Connell et al, 1987). It is also interesting that ENU induced such a high incidence of skin tumors in zebrafish without

detectable tumors in other organs of high cell turnover, such as gut or testis. The difference in tumor induction between skin and internal organs may be due to either a lower tissue concentration secondary to systemic absorption, or less efficient repair of DNA alkylation damage in skin (Tong et al, 1997).

Angiogenesis was evident in papillomas larger than 1 mm (Fig. 2D). Angiogenesis has been described in benign papillomas induced with 7,12-dimethylbenz[*a*]anthracene (tumor initiator) and 12-*O*-tetradecanoylphorbol-13-acetate (tumor promoter) in mice (Bolontrade et al, 1998).

Raised papillomas of the skin have not, to the best of our knowledge, been reported previously in the zebrafish. The most similar reported zebrafish epidermal tumor is an inverted squamous papilloma that involved the dermis in a zebrafish exposed as an embryo to 3000 parts per million N-nitrosodiethyl–amine (Tsai, 1996). Zebrafish tumors other than papillomas have been induced by N-nitrosodiethylamine, aflatoxin B1, methylazoxymethanol acetate, N-methyl-N′-nitro-N-nitrosoguanidine, or 7,12-dimethylbenz[*a*]anthracene through solution exposure or feed, but never at a 100% frequency (Hendricks, 1996; Stanton, 1965).

Virally-induced epidermal papillomas have been reported in fish since the 16th century when "epithelioma papillosum" (also known as "carp pox") was a scourge of European carp farmers (Hofer, 1904). Such papillomas coalesce as they spread over the surface of carp as white plaques, and develop focal exophytic masses (Wildgoose, 1992). These papillomas are caused by *Herpesvirus cyprini* (also cyprinid herpesvirus 1, CHV) (Sano et al, 1991), regress in warm summers or when held above 20° C (Sano et al, 1993b), and persist below 15° C (Sano et al, 1993a). Viruses have also been associated with papillary epidermal plaques in other fish species (Wolf, 1988), such as walleye, in which a retroviral etiology is suspected (LaPierre et al, 1998).

A chemical etiology is suspected for papillomas in a number of fish species that live in polluted environments. These papillomas are typically exophytic rather than plaque-like (Baumann et al, 1996; Grizzle et al, 1988; Russell and Kotin, 1957). Evidence of chemical etiology of epidermal papillomas has been found in brown bullhead (*Ameiurus nebulosus*) (Black et al, 1985; Black, 1983a, 1983b; Brown et al, 1973; Folmar et al, 1995; Lucke and Schlumberger, 1941; Obert, 1997; Poulet and Spitsbergen, 1996; Poulet et al, 1993, 1994), channel catfish (*Ictalurus punctatus*) (Chen et al, 1996), and medaka (*Oryzias latipes*) (Hyodo-Taguchi and Matsudaira, 1987). Thus, papillomas in some fish have an epizootiologic and experimental basis for a chemical etiology, but have all occurred at frequencies much lower than that seen here.

Other tumors found in our ENU-treated zebrafish included a 1.5 × 1 × 0.9 mm malignant peripheral nerve sheath tumor in the distal tail of Fish M. A satellite nodule (0.25 × 0.25 × 0.2 mm) occurred 0.5 mm proximal to the main tumor. The histologic diagnosis is supported by features including wavy to plump spindle cells formed whorls, loose myxoid (Antoni B), and more cellular (Antoni A) areas, nuclear palisades, rare Verocay bodies, focal large dark atypical spindle-shaped nuclei, infiltrative border, and mitoses (two per ten 400x fields; not shown) (Enzinger and Weiss, 1995; Hruban et al, 1990). Peripheral nerve sheath tumors have also been induced by ENU in the rat (Kindler-Rohrborn et al, 1999; Koestner et al, 1971; Nikitin et al, 1991; Perantoni et al, 1987). Two cavernous hemangiomas were also found in the mutagenized zebrafish. One was a 1 × 1 × 1 mm subcutaneous lesion in the head of Fish M, and the other was a 5 × 3 × 3 mm lesion which completely replaced the soft tissues of the proximal tail of Fish V (not shown). These mesenchymal tumors were too few to firmly establish a relationship to the ENU treatment.

In summary, we report that ENU induced epidermal papillomas in all adult male zebrafish treated in a widely-used mutagenesis protocol. The high tumor incidence, combined with the strength of zebrafish as a genetic model system, establish the potential of the zebrafish as a model for the study of skin neoplasia.

## Materials and Methods

### *Mutagenesis*

Germ line mutations were induced in adult male zebrafish with N-ethyl-N-nitrosourea (ethylnitrosourea, ENU; Sigma, St. Louis, Missouri) as described (Mullins et al, 1994). Wildtype zebrafish males were obtained from North American wholesalers, and a subset was chosen for mutagenesis based on a high fertility rate in pairwise matings. A total of 30 zebrafish males were treated at 7 to 9 months of age with either 0.0 mM ENU (6 control males, designated A to E), 2.5 mM ENU (12 males, designated F to M), or 3.0 mM ENU (12 males, designated N to W). Treatment consisted of 1-hour immersions at room temperature (22° C to 23° C) every 72 hours for a total of three treatments. The ENU solution was freshly prepared within 1 hour of each treatment by a combination of stirring and crushing of powder aggregates. ENU solutions were inactivated by bringing them to 10 mM NaOH; containers were decontaminated in 250 mM NaOH 10% sodium thiosulfate overnight; and surfaces with any potential for aerosol contamination were covered and sprayed with 10 mM NaOH after the mutagenesis. The control group was treated with buffer only (0.03% Instant Ocean, Aquarium Systems, Mentor, Ohio; in 10 mM sodium phosphate, pH 6.7). All 30 fish survived this mutagenesis regimen well, although the 3.0 mM group exhibited signs of physiologic stress, such as huddling and poor feeding, compared with the other two groups. One of the control males and two of the 2.5 mM males died before our final inspection for tumors.

### *Fish Care*

The fish were raised in a recirculating system (water turnover approximately 4 times/hour/tank) containing dechlorinated tap water mixed approximately 50:50 with reverse osmosis filtered water to a hardness of 68 to 85

parts per million. Approximately 10% of the water was replaced daily. Treated and untreated fish had identical diets: once per day of 2-day-old brine shrimp (Argent, Redmond, Washington; Gold grade), and once per day of a coarsely ground mixture of equal parts by volume of Wardley Staple flake food (Wardley, Secaucus, New Jersey), 450 grade floating Trout Pellets (Rangen, Buhl, Idaho), Trout and Salmon Starter (Rangen), freeze-dried krill (Argent), and freeze-dried tubifex worms (Wardley). Fish were euthenized with tricaine methane sulphonate, as described (Westerfield, 1995).

### *Gross and Microscopic Pathology*

After several months of breeding, these fish were kept in isolation for 10 to 12 months and scored for tumors, at 17 to 21 months of age. After euthanasia, whole fish were fixed in 10% neutral buffered formalin for at least 1 day. Fish H, J, M, S, U, and V were decalcified for 24 hours in 22.5% formic acid 300 mM sodium citrate, and the remaining fish were decalcified for 1 hour in Cal-EX (1.35 N HCl, 0.003 M Sodium EDTA, Fisher Scientific, Pittsburgh, Pennsylvania). Decalcified fish were returned to formalin in a tissue processing cassette, dehydrated, and impregnated with paraffin, and stained with hematoxylin and eosin according to standard protocols (Luna, 1968).

### *Photography*

Gross photography was done using a Nikon F2 camera (Tokyo, Japan) with a Nikkor 55 mm f3.5 micro lens and a Honeywell (Morristown, New Jersey) slave Strobonar 200CA flash. Low-power photomicroscopy was done on a Leica M10 with a MPS60 camera, and higher power photos made on a Zeiss Axioscope with an MC 100 spot camera, and a variety of objectives. Slides were scanned using a Polaroid SprintScan 35 Plus scanner (Cambridge, Massachusetts) and adjustments made using Adobe Photoshop (San Jose, California).

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