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MNU Induction of Neoplasia in a Platyfish Model

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SUMMARY: Interspecific hybrid crosses between members of the fish genus *Xiphophorus* have been used for over 70 years to study the genetic aspects of melanoma formation. In the well-established "Gordon-Kosswig" cross, the platyfish *X. maculatus* is outcrossed to the swordtail *X. helleri*, and the resulting backcross segregants spontaneously develop melanoma. We recently produced a distinct cross between *X. maculatus* and another platyfish species, *X. couchianus*. *X. maculatus* strain Jp 163 A is homozygous for several X-linked pigment pattern genes, including the *Spotted dorsal* (*Sd*), *Dorsal red* (*Dr*), and *Anal fin spot* (*Af*). Af is a sex-limited trait, coding exclusively for melanophores distributed on the modified anal fin or "gonopodium" in the adult male fish. Within F_1 and BC_1 hybrids (to *X. couchianus*), the Sd pigment pattern is phenotypically suppressed, whereas Dr and Af are enhanced. We exposed BC_1 hybrids to the direct-acting carcinogen *N*-methyl-*N*-nitrosourea (MNU). Treatment led to the development of schwannomas, fibrosarcomas, and retinoblastomas. In addition, numerous MNU-treated males that inherited *Af* developed a pronounced melanotic phenotype, with melanin-containing cells oftentimes totally covering the gonopodium and extending further to grow within the ventral regions of the fish. Genetic linkage analysis of the BC_1 hybrids revealed a significant ($p < 0.01$) association between *CDKN2X* genotype and the phenotypic degree of melanization. Such an association is consistent with a locus within linkage group V playing a role in the development of melanosis and delineates three genetic preconditions and a carcinogenic scheme resulting in melanosis of the ventral regions of hybrid fish. The overall study further alludes to the potential of using *Xiphophorus* fish to study carcinogenic mechanisms for tumors other than melanoma (schwannoma, fibrosarcoma, and retinoblastoma) and should enable extensive pathologic and molecular genetic studies of derived neoplastic abnormalities. (*Lab Invest 2001, 81:1191–1198*).

Animal models are an invaluable tool in understanding the complexities of neoplastic disease. Interspecific hybrid crosses between members of the fish genus *Xiphophorus* (currently comprising 22 species) have been used for over 70 years to study the genetic aspects of melanoma formation (Gordon, 1931a; Kosswig, 1927; Nairn et al, 1996b; Schartl, 1995). Typically, genetic traits, such as melanistic pigment patterns, are well regulated in the parental strains, but are inadequately regulated in hybrid fish, resulting in uncontrolled proliferation, and ultimately, in the development of neoplastic exophytic growths such as nodular melanomas. Numerous crosses between parental strains have generated hybrid fish that develop melanomas with great predictability, both for anatomical position and age of onset. Melanomas derived within such hybrid crosses are clearly genetically predetermined, as was demonstrated for several *Xiphophorus* tumor models (Nairn et al, 1996b; Schartl, 1995; Vielkind et al, 1989). A candidate tumor suppressor gene, *CDKN2X*, which maps to *Xiphophorus* linkage group V (LG V) within a genomic region implicated in melanoma tumor suppression and pigment pattern regulation, was cloned (Kazianis et al, 1998, 1999; Siciliano et al, 1976; Vielkind, 1976). This gene shows structural similarity to the human *CDKN2A* (*P16*) locus, which is presently thought to be involved in suppressing human melanoma and other tumors as well (Chin et al, 1998; Ruas and Peters, 1998).

N-methyl-*N*-nitrosourea (MNU) is an alkylating agent that methylates DNA bases at nucleophilic sites (generating N^7 and N^3 alkylpurines). The primary mutagenic lesion is believed to be O^6 methylguanine (Friedberg et al, 1995). MNU induces numerous cancers in rodents, including mammary carcinomas and thyroid tumors in rats (Ohshima and Ward, 1984; Zarbl et al, 1985), as well as thymic lymphomas in mice (Frei and Lawley, 1980; Joshi and Frei, 1970; Richie et al,

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1996). This complete carcinogen also effectively induced a wide array of tumors in *Xiphophorus* hybrids, including neuroblastomas, melanomas, and fibrosarcomas, in addition to rhabdomyosarcomas and various carcinomas, which occurred at a much reduced incidence (Schwab et al, 1978a, 1978b, 1979). The development of neuroblastomas was strongly associated with one particular hybrid cross involving *X. variatus* and *X. helleri*, and not in 64 other nonhybrid species/strains and derived hybrids (Schwab et al, 1978a, 1978b, 1979).

We recently generated a cross between *X. maculatus* and another platyfish species, *X. couchianus* (Fig. 1). *X. maculatus* strain Jp 163 A is homozygous for a macromelanophore pigment pattern gene called *Spotted dorsal* (*Sd*) and a linked pterinophore locus called *Dorsal red* (*Dr*; Fig. 2A). Both loci are linked on the X chromosome and recombination between them has been rarely reported (Kallman, 1975; Kallman and Schreibman, 1971). The homologous *X. couchianus* sex chromosomes lack both *Sd* and *Dr* loci. F_1 hybrids and subsequently produced BC_1 hybrids show a pronounced phenotypic suppression of Sd, but an enhancement of Dr. The suppression of the Sd pigment pattern is a striking contrast to the severe enhancement of the Spot-sided (Sp) pigment pattern in a parallel genetic cross with the same parental species, but using *X. maculatus* strain Jp 163 B (Nairn et al, 1996b; Setlow et al, 1993, 1989). In addition, the Sd pigment pattern itself, derived from *X. maculatus* strain Jp 163 A, is typically enhanced in numerous genetic crosses, including hybrids between *X. maculatus* and *X. helleri*. Within this well-studied model, referred to as the "Gordon-Kosswig" cross, the Sd pigment pattern is enhanced to the point of extreme melanosis and development of malignancy (Morizot and Siciliano, 1983; Schartl, 1995; Vielkind, 1976).

We exposed numerous BC_1 individuals derived from the hybrid cross between the two platyfish species, *X. maculatus* and *X. couchianus*, to aqueous solutions containing the direct-acting carcinogen MNU. Melanotic hyperplasia (melanosis), retinoblastomas, fibrosarcomas, and schwannomas were produced; their incidence and histological properties are described.

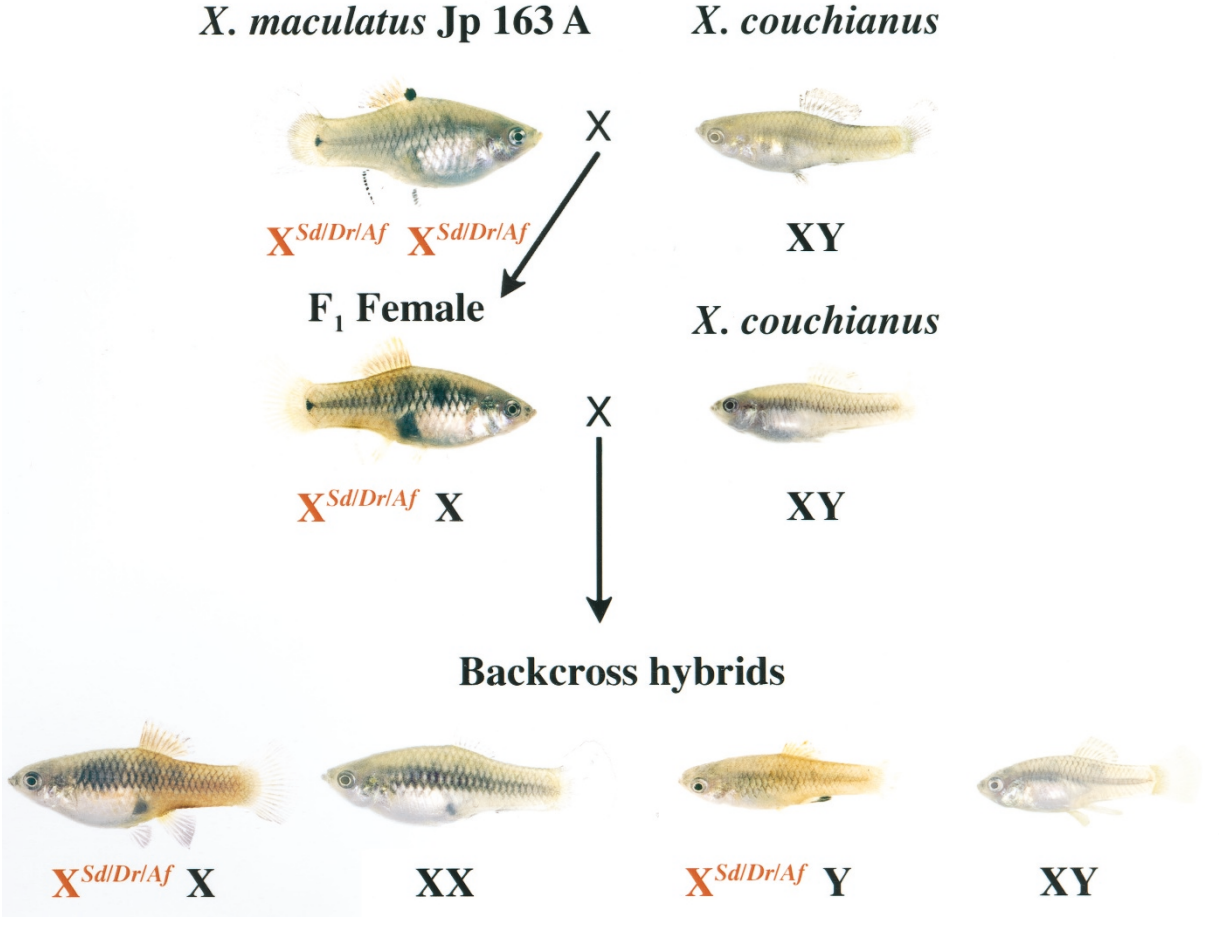

Figure 1.

Generalized depiction of the (*Xiphophorus maculatus* Jp 163 A × *X. couchianus*) × *X. couchianus* cross. A *X. maculatus* individual, homozygous for the *Sd* macromelanophore pattern locus and the pterinophore locus *Dr*, is mated to a *X. couchianus* lacking both loci. The resulting F_1 hybrids show a phenotypic suppression of Sd but an enhancement of Dr. This pattern of respective suppression and enhancement is preserved in the BC_1 hybrid progeny. Within male offspring, the sex-limited pigment pattern locus *Af* is inherited and expressed by half of the male progeny. This pigment pattern is carried by the X chromosome derived from *X. maculatus* and is also not present in *X. couchianus*. For simplicity, only the genotypes for *X. maculatus* are depicted.

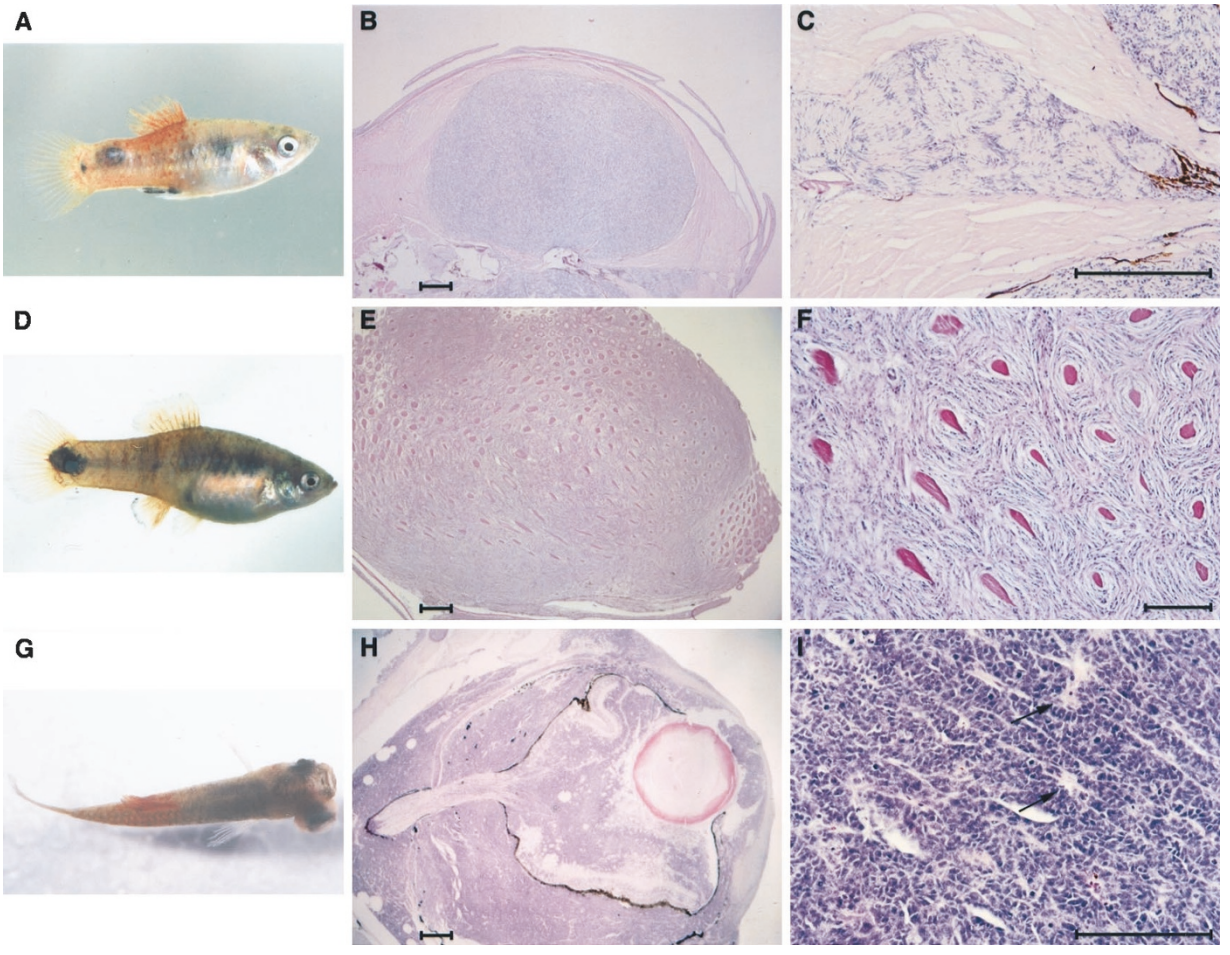

Figure 2.

Development of schwannomas, fibrosarcomas, and retinoblastomas in *N*-methyl-*N*-nitrosourea (MNU)-treated fish. A, BC_1 hybrid fish showing development of a schwannoma on the caudal peduncle. B, A well-circumscribed schwannoma derived from the fish in A. C, Area of typical palisades. D, Example of a fish developing fibrosarcoma on the base of the caudal fin. E, Low-magnification image of a fibrosarcoma infiltrating muscular structure. F, Muscle bundles compressed by whorled masses of densely packed spindle cells. G, Photograph of a fish taken from above showing protrusion of the right eye. H, The retinoblastoma in the fish depicted in G, with tumor cells invading the inner and outer surface of the retina, the sclera, and the optic nerve. I, Higher magnification photomicrograph showing tumor cell histological characteristics. The *arrows* delineate the area that shows a rosette-like arrangement. Scale bars are 200 μm.

Results and Discussion

General Description of the Animal Model

BC_1 hybrid fish were produced between *X. maculatus* and *X. couchianus*, using either F_1 hybrid females or males. Figure 1 depicts one crossing scheme used to generate such hybrid fish, while following the inheritance of the sex chromosomes which harbor the *Sd* macromelanophore locus, the *Dr* pterinophore locus (Kallman, 1975), and a pigment pattern locus referred to as *Anal fin spot* (*Af*, also referred to as *Anal fin black* [*Ab*]) (Gordon, 1931b; Schartl et al, 1995). The *Sd* and *Dr* loci are tightly linked on the X chromosome of *X. maculatus*, and recombination is rare (Kallman, 1975). Within the F_1 and BC_1 hybrids to *X. couchianus*, the Dr pigment pattern is sharply overexpressed, so that the fish are distinctly orange in color. Pterinophore cells that are usually restricted to the dorsal fin in the parental *X. maculatus* strain Jp 163 A are widespread over the body, including the trunk. In contrast, the Sd pigment pattern is phenotypically suppressed, because only 13.6% (21/154) Dr-bearing backcross hybrids also phenotypically express Sd.

A total of 276 BC_1 fish were raised with 171 BC_1 individuals used as untreated controls. Treatment of 105 BC_1 fish with the direct-acting carcinogen MNU yielded several types of neoplasms, including schwannomas, fibrosarcomas, and retinoblastomas (Table 1). None of the control fish developed any discernible neoplasms. In addition, MNU treatment resulted in numerous individuals with melanosis. The histopathological features of these abnormalities are described below.

Development of Schwannomas, Fibrosarcomas, and Retinoblastomas in MNU-Treated Fish

Schwannomas are benign tumors composed entirely of Schwann cells, which are derived from the neurilemma of a nerve fiber. Schwannomas developed at an incidence of 2.8% (3/105) and were typically found on the caudal peduncle region of the fish (between the

Table 1. Incidence of Lesions in BC_1 Fish Treated with MNU[a]

Lesion	MNU (n = 105)	MNU (%)
Schwannoma	3	2.8
Fibrosarcoma	7	6.6
Retinoblastoma	4	3.8
Unrestricted melanosis[b]	11	10.4

MNU, *N*-methyl-*N*-nitrosourea.

[a] Five-week-old fish exposed to 1.0 mM, 2 hours, on Days 0, 2, 4, and 6. No neoplasms occurred in 171 untreated control fish.

[b] Unrestricted melanosis is defined as aggregations of melanin containing cells that are found outside the confines of the gonopodium. Typically, such cells grow within the integument of the ventral and mid-ventral flanks of the fish (see text and Fig. 3).

dorsal and caudal fins). All were unilateral, well-circumscribed, whitish lesions with globular configurations (Fig. 2A). Microscopically, the tumors were encapsulated by dense bundles of muscle (Fig. 2B). Some areas exhibited compact fascicular tissue (Antony A pattern) and typical palisades resulting from stacked arrays of nuclei alternating with nuclear fibrillar zones composed of cell processes (Fig. 2, B and C). Mitotic figures were absent.

Fibrosarcomas are malignant neoplasms derived from mesenchymal cells. Fibrosarcomas were typically found on the caudal peduncle area (Fig. 2D), although several growths also occurred elsewhere, such as on the operculum and the dorsal trunk above the midlateral line. They developed at an incidence of 6.6% (7/105), and several individuals displayed multiple exophytic tumors dispersed throughout the body surface. Microscopic examination showed packed spindle-shaped fibroblasts infiltrating surrounding structures (Fig. 2E). Muscle bundles were compressed by whorled masses of densely packed spindle cells (Fig. 2F).

Retinoblastomas are an intraocular malignant neoplasm derived from embryonic neuronal cells. Retinoblastomas were harvested when protrusion of the eye was evident (Fig. 2G) and observed in 3.8% (4/105) of the MNU-treated BC_1 hybrids. The retinoblastomas were all unilateral. Microscopic examination revealed tumor cells resembling neuroblasts that were invading the inner and outer surface of the retina, the sclera, and the optic nerve. In one case, the tumorous cells had spread along the optic nerve to the brain (Fig. 2H). In another individual, tumor cells were found in the middle part of the head invading the muscle associated with the opposite eye. The tumors were intensely cellular and densely packed, and, in some instances, round neoplastic cells with hyperchromatic nuclei, scant cytoplasm, and abundant mitoses were randomly distributed (Fig. 2I). Moderately differentiated rosette-like structures were distinguishable in two of the neoplasms (Fig. 2I, *arrows*). Generally, all the retinoblastomas were well vascularized.

Development of Melanosis after MNU Treatment

Analysis of phenotypic segregation data within BC_1 hybrids (generated using both F_1 males and females) revealed that the *Af* pigment pattern locus resides on the X chromosome derived from *X. maculatus*. This gene is linked to *Dr* with an estimated recombination rate of approximately 3.4% (2 recombinants within 58 informative fish). Within the *X. maculatus* strain Jp 163 A, *Af* codes for melanophores distributed within the modified anal fin or "gonopodium" exclusively within adult male fish; thus, it is a sex-limited trait. The melanic cells are usually restricted toward the tip of the gonopodium, and most are concentrated on the most ventral fin rays (Fig. 3A). Unique to the F_1 and BC_1 hybrids described herein, Af is phenotypically enhanced, and in many cases melanic cells are distributed along the entire length of the gonopodium. *X. couchianus*, the other species used, does not harbor the Af pigment pattern.

In MNU-treated fish, all pigmented lesions were derived from the Af pigment pattern, with melanin-containing cells oftentimes totally covering the gonopodium and proceeding to penetrate into the ventral regions of the fish. Figure 3 depicts two exemplary BC_1 fish that had been treated with MNU. In the first (Fig. 3B), the enhancement of Af was not pronounced, because melanization is restricted to the gonopodium. Histology showed pigmented melanocytes and melanophores located near and around the bony rays of the gonopodium (Fig. 3D). However, the second fish shown (Fig. 3C) had developed radially spreading melanosis derived from Af, and the cells had proliferated to encompass the neighboring pelvic fins and extended into the body. The pronounced melanosis was no longer restricted to the gonopodial region. However, the melanocytes and melanophores were restricted to the integument, exhibiting a distinct lack of invasiveness (Fig. 3E). Uniform, dense spindle-pattern melanin pigment was distributed along and restricted to the dermis and basal layer of the gonopodial skin. No invasion of body tissues was detected in any of the restricted Af individuals. Ten additional such BC_1 fish revealed similar pathological characteristics. The overall lack of invasiveness derived from Af is in stark contrast to the vast majority of melanotic neoplasms derived from *Xiphophorus* hybrid models, where invasion of underlying tissues and exophytic growths are commonly observed (Reed and Gordon, 1931; Schartl et al, 1995; Sobel et al, 1975).

Genotypic Association of LG V and Melanosis

Eleven of 28 (39%) MNU-treated males that inherited Af developed a pronounced (ie, unrestricted) melanotic phenotypic enhancement similar to that in the individual shown in Figure 3C. All of them were genotyped as homozygotes for *CDKN2X* using the PCR (Mullis et al, 1986). In addition, we genotyped 9 of the individuals that had developed restricted pigmentation within the gonopodium, and 7 of them were heterozygous. Together, these results are sig-

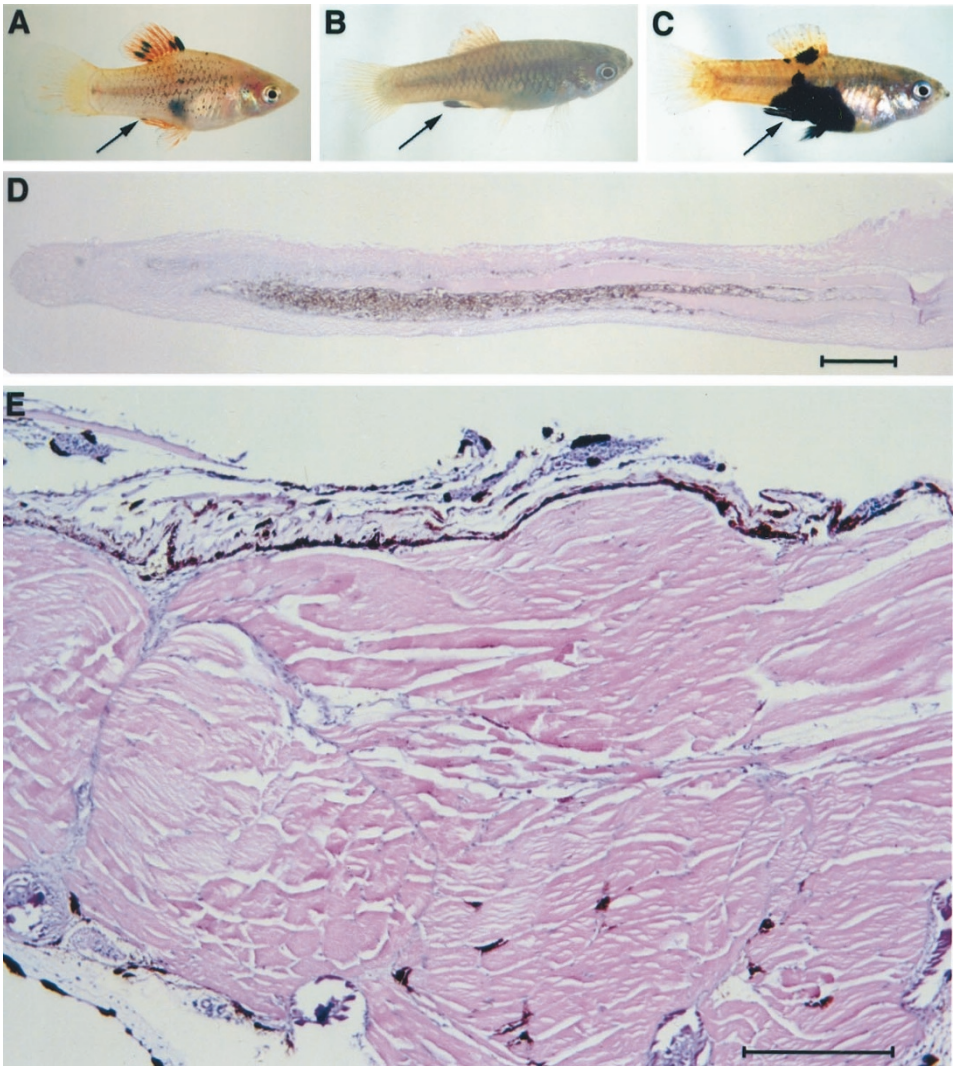

Figure 3.

Development of the Af pigment pattern and melanosis induced by MNU. A, A *X. maculatus* strain Jp 163 A male fish, showing the Af pigment pattern at the distal gonopodial region. The Sd pigment pattern is manifested by melanic spotting on the dorsal fin. B, BC_1 hybrid fish with Af melanization restricted to the gonopodium. C, An MNU-treated fish with a radially spreading melanosis that is not restricted to the gonopodial region. D, Longitudinal section through the gonopodium showing melanization around the bony rays of the gonopodium. E, Cross section of the anterior caudal peduncle showing pronounced hyperpigmentation within the integument with a lack of invasiveness. Scale bars are 200 μm.

nificant ($\chi^2 = 12.8$, $p < 0.01$) because *CDKN2X* genotypes correlate with the phenotypic development of restricted or unrestricted melanization derived from the Af pigment pattern (18 parentals, 2 recombinants, logarithm of odds [LOD] = 3.19). Such a result is striking and consistent with a locus within LG V playing a role in the development of melanosis and being associated with the Af pigment pattern. There have been several previous reports (involving different genetic crosses and pigment patterns) of an association of *Xiphophorus* LG V genotypic data and melanoma formation and for spontaneous and UV-induced melanotic hyperplasia, although involvement with the Af pigment pattern has not been previously noted (Ahuja et al, 1980; Fornzler et al, 1991; Kazianis et al, 1998; Morizot and Siciliano, 1983; Siciliano and Wright, 1976).

Summary

MNU treatment of 105 hybrid fish derived from crossing two platyfish species resulted in the induction of several neoplasms at incidences between 2.8% and 6.6% (Table 1). These neoplasms did not develop in 171 control fish. In addition, 10.4% of treated backcross hybrids exhibited melanosis. We can clearly identify three genetic preconditions that must be met before melanosis develops in BC_1 hybrids. Firstly, the individuals must inherit an X chromosome derived from the southern platyfish, *X. maculatus*, carrying a pigment pattern locus referred to as *Af*. Secondly, afflicted fish are always males, as the Af pigment pattern is sex-limited to them, and only such fish develop the restricted (to the gonopodium) or unrestricted melanosis. Thirdly, melanosis is significantly associated with the lack of inheritance of an autoso-

mal locus derived from *X. maculatus*. *Xiphophorus* LG V harbors a locus referred to as *Diff* which has been implicated in determining the state of differentiation of melanistic pigment cells (Ahuja et al, 1980; Siciliano et al, 1976; Vielkind, 1976). In addition, strong associations have been shown between LG V genotypes and spontaneous or UV-induced melanoma formation in several distinct *Xiphophorus* genetic crosses, all involving the platyfish *X. maculatus* (Kazianis et al, 1998). The tumor suppressor gene candidate *CDKN2X* maps to this autosomal region (Nairn et al, 1996a) and has shown strong genetic associations with melanotic phenotype and even stronger associations with melanoma formation (Kazianis et al, 1998; Nairn et al, 1996a). Thus, *CDKN2X* is considered a candidate for the classically defined *Diff* tumor suppressor gene (Kazianis et al, 1999). We examined BC_1 hybrid male fish that had been exposed to MNU and had developed restricted or unrestricted melanistic pigmentation for *CDKN2X* alleles. The results significantly implicate this genomic region, because all BC_1 fish developing unrestricted melanosis were homozygotes for *X. couchianus CDKN2X* alleles and lack *X. maculatus* genomic copies. Conversely, individuals not developing the unrestricted hyperpigmentation (even after MNU treatment) were heterozygous for *CDKN2X*. We are currently continuing studies of *CDKN2X* and examining the genomic region encompassing this locus to address potential mechanisms leading to melanoma formation. Recent studies have indicated that *CDKN2X* allelic copies derived from differing species may show variable expression in cells of the melanocytic pathway, with the *X. maculatus* allele showing the highest expression in such cells (Kazianis et al, 1999, 2000). Such a simple mechanism may account for the development of melanoma in select crosses and is possibly responsible for the melanosis observed in the cross between *X. maculatus* and *X. couchianus*. We are actively investigating this mechanism and others to fully assess the role of *CDKN2X* in the cross between *X. maculatus* and *X. couchianus*, described herein.

In addition to the above-mentioned genetic preconditions, unrestricted melanosis is strongly associated with MNU treatment, because untreated individuals ($n = 171$) did not develop this phenotype. Hypothetically, MNU is acting on at least one other genetic locus that is expected to have antiproliferative properties. Such a gene would be a mutational target in MNU-treated animals in order for melanosis to become established, but the consequences would only be manifested in fish of a specific genetic heritage.

The discovery of schwannomas, fibrosarcomas, and retinoblastomas in the BC_1 hybrids, albeit at a lower incidence, opens the additional possibility of studying the underlying genetic background necessary for induced development of these neoplasms as well. In addition, we may be able to identify the genomic targets after MNU treatment. We are currently exploring this, using hybrids between *X. maculatus* and *X. couchianus*, and in several other crosses. In general, the use of small aquarium teleosts, such as the genus *Xiphophorus*, zebrafish, and Japanese medaka, should prove extremely fruitful in the study of inducible neoplasia.

Materials and Methods

Experimental Animals and MNU Treatment

Animal care was performed in accordance with institutional guidelines. Parental stocks and hybrids used in this project were derived from the *Xiphophorus* Genetic Stock Center (Southwest Texas State University, San Marcos, Texas). Female *X. maculatus* (strain Jp 163 A, Rio Jamapa, Mexico) individuals, homozygous for the X-chromosomal Sd and Dr pigment patterns, were mated to *X. couchianus* males (Huasteca Canyon, Mexico). Both male and female F_1 hybrids were subsequently crossed to *X. couchianus* to produce BC_1 hybrids. Artificial insemination protocols were not necessary to produce the F_1 and BC_1 fish.

MNU was stored in ISO-PAC vials (Sigma, St. Louis, Missouri) and frozen until use. All treatments were carried out in glass beakers under fume hoods to minimize contamination. Five-week old fish were caught in disposable nets and transferred to 1.5 L clean (filtered) aquarium water within 2.0 L beakers. Freshly prepared 100 mM MNU solution (pH 4.5, citrate buffer) was appropriately diluted to 1.0 mM (final concentration) and added to the beakers containing the fish. After a 2-hour exposure, the fish were rinsed in MNU-free aquarium water and returned to their home aquaria (5- to 30-gallon tanks). This exposure was repeated every other day, with up to four treatments. The survival rate at 2 months post-exposure was 87.7%.

Euthanasia of Fish and Processing for Histology

BC_1 hybrid fish usually were killed at 14 months of age unless accelerated development of a tumor required earlier euthanasia. They were scored for pigmentation phenotypes, sex, standard length, and the development of abnormal external growths. Euthanasia was performed with the commonly used anesthetic MS-222 (tricane methane sulfate) diluted to an aqueous solution of 0.06%. All lesions, along with surrounding normal tissues were excised and then fixed in 10% buffered formalin. After paraffin embedding, 4- to 5-μm–thick sections were cut and stained with hematoxylin and eosin.

DNA Isolation and CDKN2X *Genotyping*

High MW DNA was extracted from snap-frozen samples of the gills, or from preserved (w/95% ethyl alcohol) tailfin samples using a Puregene kit (Gentra Systems, Minneapolis, Minnesota). A PCR-based assay was used to determine *CDKN2X* genotypes of backcross progeny. Primers P16F8 (5'-TAAACCAGAACAACTAAGTGG-3') and P16R16 (5'-CTGTATTGCTCTTCGTCCA-3') were specifically designed to amplify the 5' flanking and exon 1 regions of

the *CDKN2X* gene. The 5' flanking region contains a variable copy number GT-repeat that is highly polymorphic between species, enabling quick and reliable allele-specific genotyping. The PCR conditions conformed to those previously published (Kazianis et al, 1998). Amplification from *X. maculatus* (Jp 163 A) resulted in a 1220-bp product, whereas a 1079-bp band was derived from *X. couchianus*. PCR products were generally run on 1.8% agarose gels, stained with ethidium bromide, and visualized with an IF-500 Gel Documentation System (Alpha Innotech, San Leandro, California).

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