Susceptibility to the Development of Pigment Cell Tumors in a Clone of the Amazon Molly, *Poecilia formosa*, Introduced through a Microchromosome


ABSTRACT

The Amazon molly *Poecilia formosa* is a gynogenetic fish that reproduces through the development of ameiotic diploid eggs triggered by insemination by males of related species without following karyogamy. This leads to clonal offspring. In rare cases, however, this gynogenesis is leaky, and paternal DNA in the form of small supernumerary chromosomes is included into the maternal genome. We have obtained a clone where one such microchromosome contains a pigmented locus, resulting in macromelanophore pigmentation of the carrier. Approximately 5% of these fish spontaneously develop exophytic nodular or papillomatous pigment cell tumors. The tumors display considerable differences with respect to growth characteristics and invasiveness, despite the genetic uniformity of the affected animals. Following transplantation to syngeneic hosts, a remarkable clonal variability was observed. Oncogenes that are involved in tumorigenesis in hereditary melanoma of the closely related fish *Xiphophorus* appear not to be instrumental for induction of the *P. formosa* pigment cell tumors. Moreover, a new genetic locus is defined that mediates susceptibility to pigment cell tumor development and leads to transformation of chromatoblasts.

INTRODUCTION

For melanoma, like other human tumors, not only the exposure to exogenous factors that are carcinogenic, e.g., UV light, but also the genetic constitution of the individual are now considered to determine the risk to develop cancer. Recently, genetic loci have been defined that confer increased melanoma susceptibility. In humans such "melanoma genes" have been identified on chromosomes 1, 6, and 9 (reviewed in Ref. 1). In the fish *Xiphophorus*, a situation can be generated by selective breeding where only the genetic component of melanoma formation is effective, leading to hereditary tumors in the classical platyfish/swordtail hybrid cross (Ref. 2; reviewed in Refs. 3–5). This allowed cloning of the melanoma gene and dissection of the molecular processes responsible for the transformed phenotype (reviewed in Ref. 6). However, unlike human melanomas that are marked by clonal heterogeneity, the *Xiphophorus* tumors, as a consequence of their specific etiology, are pathologically uniform and arise predictably in all individuals of a given genotype equivalent to a 100% susceptibility. That makes it difficult to analyze the interaction of exogenous factors with the pigment cell transforming genes, which initiate the process of tumor formation in this system. In this report, pigment cell tumors in a closely related fish species, *Poecilia formosa*, are described, which provide a new system to study the molecular genetics of melanoma susceptibility and may help to define a novel melanoma gene.

P. formosa is the Amazon molly, is a unisexual, all-female fish that reproduces by gynogenesis, a modified form of parthenogenesis. Sperm from males of closely related species is required to trigger the onset of embryogenesis from ameiotic, diploid eggs (reviewed in Ref. 7). Functional syngamy is thus eliminated, and all descendants from a given female contain only the maternal set of chromosomes. This leads to clones of genetically identical fish. Sometimes, however, some paternal DNA enters the clonal lineages in the form of tiny supernumerary chromosomes, thereby generating a new clone. We have obtained one clone that carries microchromosomes originating from a jet black fish strain used for breeding, the "black molly." "black mollies" have their typical pigmentation because of a specific pigment cell type, the macromelanophore. These cells are larger (up to 50-fold) than those melanophores that make up the grayish body coloration of all poeciliid fish and which are termed micromelanophores for distinction (for definitions, see Ref. 8). Micromelanophores are found in many poeciliid fishes. They constitute various prominent dark black spot patterns on the body side or the fins, the biological significance of which is still unclear. By cross-breeding and selection, a totally black strain has been obtained in the genus Poecilia, where macromelanophores constitute a closed layer in the entire integument. These fish are referred to as the "black molly." Despite the abundance of macromelanophores, no pigmented tumor lesions have been observed in black mollies, although many thousands have been bred in different laboratories. On the other hand, it is known that in the closely related genus *Xiphophorus*, malignant melanomas originate from macromelanophore spots. Here, unrestrained proliferation of the macromelanophore lineage occurs in senescent purebred fish of certain wild populations or in hybrids of specific crossings, like the well-known platyfish/swordtail system. In hybrid as well as in nonhybrid melanoma, overexpression of the Xmrk oncogene is the process that initiates tumor formation (6, 9). Xmrk encodes a receptor tyrosine kinase, closely related to the epidermal growth factor receptor. The Xmrk oncogene arose by gene duplication from the corresponding proto-oncogene and acquired a new promoter region. Deregulation of this oncogene-specific promoter is made responsible for neoplastic transformation of pigment cells.

Here a pigment cell tumor is described in a clonal organism closely related to *Xiphophorus* where despite the fact that a similar situation is found with respect to formal genetics (namely the presence of a macromelanophore locus in a foreign genetic background), other molecular mechanisms appear to operate.

MATERIALS AND METHODS

Fish. The melanoma clone described here (designated M-clone) is derived from a strain of *P. formosa* that had acquired 2–3 microchromosomes as reported earlier (10). One of these microchromosomes carries the macromelanophore locus of the black molly (10). All fish were from the fourth to the tenth offspring generation bred from the female in which the original introgression event occurred.

All animals used in this study were propagated in population stocks of 40–100 individuals. For breeding, males from several purebred stocks of *P. formosa*.
Fig. 1. Tumors of M-clone fish with fast-growing bicolor tumor outgrowing on the left flank (a), with preponderantly xanthic papillomatous tumor on the upper jaw (b), and with cauliflower-like melanotic tumor on the upper and lower jaw (c). d. histological analysis of tumor development in the dermis of the premaxillar pharynx region. The tumor (T) is composed of highly differentiated melanized cells and nonpigmented cells. The epidermis (E) is hyperplastic. Arrows, pharyngeal teeth. e, higher magnification of the distal region of
mexicana without macromelanophore spots, derived from field-collected founder fish, were used. The fish were maintained under standard conditions as described for Xiphophorus (11) at 25–27°C with an 11-h dark/13-h light photoperiod.

**Tumor Transplantation.** Recipient fish were anesthetized in MS 222. A small incision was made through the skin into the underlying musculus carinatus dorsalis, anterior to the dorsal fin. The donor tissue (~ 1 mm³) was inserted using forceps. A second transplant was grafted to the contralateral side. To avoid extensive mobility or sudden movements that could lead to a loss of the transplant, fish were kept in the dark for the 4 following days. After this time, all grafts were firmly attached to the host. Following surgery, recipient fish were kept for 2 weeks at high salinity to exclude infection of the transplantation site. Within 4 to 6 weeks after transplantation, all grafts showed a decrease in size to approximately one-third and less. After a lag phase of another 4–6 weeks progressive growth of most transplants started.

**Chromosomal Preparations.** Chromosomes were prepared from tumorous tissue essentially as described for normal organs of P. formosa (12).

**Southern Blot Analysis.** To assay for RFLPs or additional restriction fragments that usually indicate the presence of the second, oncogenic copy of Xmrk in Xiphophorus, Southern blot analysis was performed as described (13) on DNA extracted from single fish. DNA from black molly, P. mexicana, P. latipinna, P. formosa “strain I” (14), and M-clove fish was digested with BamIII, BglIII, BsrI, EcoRI, EcoRV, HindIII, KpnI, PstI, PvuII, PstI, SacI, Sall, SmaI, SplI, XbaI, and XhoI. The filters were hybridized with the entire Xmrk cDNA (3-2E and 17-2; Ref. 15) at 42°C, 40% formamide and washed at 68°C with 0.1× SSC (1× SSC: 0.15 M NaCl 0.015 M sodium citrate).

**Expression Analysis.** RNA was extracted from tumors and normal organs of individual fish using the TRIZOL reagent (Life Technologies, Inc., Grand Island, NY) according to the suppliers’ recommendations. Reverse transcription was done with 2 μg of total RNA using Superscript II reverse transcriptase (Life Technologies, Inc.). cDNA from 10 ng (actin) to 500 ng of total RNA was used for competitive PCR using a cloned, shorter (40–50 bp) PCR product from Xiphophorus as competitor [according to Förster (16) and Grassi et al. (17)]. The reliability of the expression data was determined by comparison to a dilution series of the internal control DNA and to the actin mRNA content of each tissue sample. For reverse transcription, the following gene-specific primers were used: Xmrk Ins1, 5′-GAA GTT GGT CTC C-3′; e-src SrcRT, 5′-CGT ACT ACT CTG TC-3′; c-yes YesRT, 5′-CTA AAT CCC AGG AC-3′; and actin Act3, 5′-TGA TCT GCT CTC C-3′.

**RESULTS**

P. formosa that carry two to three microchromosomes originating from the normal chromosome complement of the black molly strain are heavily spotted or even totally jet black due to the presence of macromelanophores. In the P. formosa clone described here (referred to as the M-clone), macromelanophores occur in the dermis at very high densities, often in multiple layers. They are also frequent in the peritoneum, the meninges, and the connective tissue of the blood vessels. Many macromelanophages are present in headneurons, eventually forming large macromelanophore centers. Some macromelanophores, either from the dermis or the peritoneum, invade along the septae of the body muscles. All of these features of heavy macromelanophore pigmentation are also found in the black molly strain, from which the microchromosomes of the M-clone are derived. M-clone fish, however, in contrast to the black molly, develop prominent exophytic pigmented tumors (Fig. 1, a–c). Most tumors obtained thus far were dark black; however, some had sharply bordered, intensively yellow areas. The tumors were found in ~5% of adult fish (51 tumors sampled from ~1000 fish bred thus far). Contrary to the heritable tumors of Xiphophorus hybrids, the age of onset of visible tumor formation varied considerably within M-clone fish. Earliest appearance was seen in 3-month-old fish, whereas in other cases, the fish were senescent (over 1 year of age). Some tumors showed extremely slow growth over up to 4 months, others reached a considerable volume within 2–3 weeks. Although fish of the M-clone are pigmented all over the body and the fins, the site of tumor development was nonrandom. Significantly, more tumors appeared on the left operculum [12 of 51 tumors recorded for this compartment, compared to 1 of 51 on the right operculum (binomial test, n = 13, P = 0.04, two-tailed)]. The second most frequent sites were the snout (upper jaw, n = 10; lower jaw, n = 4) and the dorsocranium (10 of 51). All other tumors were located on the anterior trunk. No melanoma occurred in the peduncle, which is a very frequent site for melanoma in Xiphophorus. Very frequently, fish developed multiple tumors (up to three).

Transplantation (n = 12) to recipients of the original P. formosa clone from which the female that acquired the microchromosomes was derived, or to unpigmented sibling fish from the M-clone that had lost the pigmentation locus carrying the microchromosome (Ref. 10; data not shown), revealed a marked clonality of the tumors. Grafts that originated from the same donor tumor and were put to contralateral sites on the same host showed greatly different in their growth. In the most extreme case, one of them showed no growth at all, while the other become a prominent secondary tumor on the host within 4 weeks after the lag period (Fig. 2). Two grafts derived from a dark black tumor turned totally yellow during the progressive growth period. No tumor appeared elsewhere except the site of transplantation, indicating that the tumors did not progress to the metastasis stage.

By histopathological appearance, all tumors analyzed (n = 20) were identified as pigment cell neoplasia. They originated in the dermis and consisted mainly of three cell types, although these differed in relative abundance. Cells with a heavy melanin pigmentation represent differentiated melanophores. These usually built up a multiple cell layer in the stratum spongiosum below a hyperplastic epi-
Fig. 2. Transplanted tumors, 4 months after grafting. a, progressively growing tumor; b, highly differentiated, melanized tumor cells (arrow) from the graft on the contralateral side of the host. c, low magnification of a cross section through the malignant tumor. M. muscles; T. tumor. d, similar histological appearance of the outgrowing graft as the donor tumor. Note distribution of melanized cells in nests and in the peripheral stratum spongiosum (arrows) and the hyperplastic epidermis (E). e, invasion of the graft via the septae into the muscles, in between scale pockets, and along the periost of the baseosts (b) of the dorsal fin rays. S. scale; c–e. H&E stains; bar, 50 μm.

dermis. Star-shaped melanophores were also found equally scattered throughout the tumor mass. A certain fraction of the unpigmented cells stained blue in Schmorl’s ferri-ferricyanide reaction for melanin precursors (Fig. 1), indicating that they are undifferentiated melanoblasts and melanocytes. The third type, Schmorl-negative cells, contained many vacuoles and appeared to represent the cell type responsible for the yellow pigmentation, the xanthophores. Immunohistochemical staining with Ki-S1 (α-topoisomerase) showed varying amounts of proliferating cells scattered all over the tumor, indicating different stages of tumor progression (data not shown). Connective tissue fibers were interspersed, eventually forming compact layers. In general, the tumors were well vascularized. In tumors of high
differentiation stage, melanomacrophages were frequent. These sometimes were released through the epidermis (Fig. 1). Many tumors were well differentiated, with the main tumor mass showing exophytic growth. Tumors on the upper jaw typically had papillomatous arrangements and had only a low tendency to invade the subjacent tissues. However, frequent local invasion of tumor cells into the epidermis and underlying connective tissue as well as the cranial and trunk musculature was observed for the tumors located at the opercula and the body side. In total, 12 of the 20 tumors analyzed grew invasively and were classified as malignant. All tumorous fish showed a heavy extradermal melanin pigmentation in the peritoneum and the meninges exceeding the pigmentation of non-tumor-bearing fish. In the trunk, occasionally proliferating, heavily melanized tissue originating from the melanophores of the peritoneum invaded along the septae between the fibers of the skeletal muscles (Fig. 1, g and h).

Following transplantation, those tumors that grew progressively had the same histological appearance as tumors in situ (Fig. 2). However, the proportion of the three main cell types could change considerably, leading to overabundance of melanophores or xanthophores. All transplant-derived tumors analyzed were invasive and well vascularized. Melanomacrophage centers, which are barely detectable in wild-type pigmented P. formosa, were abundant in the headnephros of the recipients.

Ultrastructural analyses of eight tumors confirmed that one of the cell types constituting the tumors was melanophores (Fig. 3). Less differentiated pigment cells recognized by the presence of premelanosomes were scarce. Highly differentiated melanophores showed the typical irregular shaped lobulated nuclei exhibiting nuclear pockets (19). Another abundant cell type was characterized by spherical or oval cytoplasmic organelles. They incorporate pleomorphic internal structures, in particular granular, filamentous, membranous, and amorphous materials in various degrees. Most are apparently limited with a single membrane, but sometimes even two membranes were discernable. The ultrastructure of these organelles matches perfectly the pigment granules described for xanthophores in P. latipinna (20) and for leucophores of the guppy, P. reticulata (21). The xanthopterine content of these vesicles would then account for the yellow color in some areas of the tumors. Onion skin-like arrangement of membranes as described for pterinosomes was only rarely seen. Some cells had pleomorphic nuclei. Occasionally, macrophages with ingested pigment organelles, mitotic and apoptotic cells, were found. Remarkably, destruction of the basal membrane occurred, leading to breakdown of the dermal/epidermal border, thus allowing invasion of tumor cells (Fig. 3b).

To verify that tumor formation is indeed related to the presence of the macromelanophore locus-containing microchromosome, metaphase spreads were obtained from a fast-growing tumor (Fig. 4). From 14 metaphases analyzed, four had one microchromosome, eight had two microchromosomes, and two had three microchromosomes. In normal organs (gills, kidney, and spleen) from non-tumorous, heavily spotted M-clone fish as well as in the healthy organs of the non-tumor-bearing fish, the proportion was similar (e.g., 9:12:1). Fish that had lost the pigmentation locus-containing microchromosome and wild-type P. formosa never developed neoplasia.

To analyze for expression of the oncogenes/proto-oncogenes that are most frequently involved in melanoma formation in the closely related genus, Xiphophorus quantitation of transcript abundance was done by competitive RT-PCR.3 The oncogenic version of Xmrk is heavily overexpressed in spontaneous hybrid and nonhybrid melanomas of Xiphophorus and is the causative melanoma-inducing gene. By Southern blot analyses, DNAs from heavily spotted M-clone fish were compared with wild-type pigmented P. formosa and other fish of the genus Poecilia. Using 17 different restriction enzymes for digestion and the entire cDNA of the Xmrk gene as hybridization probe, no RFLP or additional restriction segments, which usually indicate the presence of the second, oncogenic copy of Xmrk in Xiphophorus (22)4 was detected, either in M-clone fish or in the paternal black molly strain. The hybridization banding pattern of M-clone fish and P. formosa was identical in each case (data not shown). Expression of the

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3 The abbreviation used is: RT-PCR, reverse transcription-PCR.
4 S. Weis and M. Schartl, The macromelanophore locus and the melanoma oncogene Xmrk are separate genetic entities in the genome of Xiphophorus, manuscript in preparation.
**DISCUSSION**

The tumors described here all occurred in a certain clone of the gynogenetic fish *P. formosa*, that had acquired a microchromosome from a bisexual related fish, the black molly. Such introduction of some paternal DNA into the unisexual amazon molly has been observed now quite frequently (in ~1% of all matings). Only if the macromelanophore pigmented black molly was involved, pigmentation was observed in resulting new clones, having acquired a microchromosome.

Loss of this microchromosome, conversely, leads to a loss of the macromelanophore pigmentation, thus demonstrating the presence of the macromelanophore locus on this microchromosome. The tumors of the M-clone fish all consisted of cells of the melanin-containing pigment cell (the melanophore) and the pteridine-containing pigment cell (the xanthophore) lineage. Because both are derived from a common precursor, the chromatoblast (24, 25), this one most likely is the neoplastically transformed, proliferating cell, the derivatives of which still have the potential to differentiate to some extent. The tumor would thus be diagnosed as a chromatoblastoma. A tumor of similar mixed pigment cell composition has been described in the nibe croaker, *Nibea mitsukurii*, except that besides the melanophore lineage, the iridophore lineage constituted the tumor cells (26), and incidentally, in some snakes (27). Also, in the well-studied melanoma of *Xiphophorus*, sometimes areas composed of cells from the xanthophore/pterinophore lineage are found. This phenomenon is rare in the SD line but very frequent in the DrLi mut and ArSr lines. Cells without any phenotypic trait of pigment cells isolated from the dermal tissue of xanthic goldfish or from goldfish erythrophoroma can be induced to differentiate either toward the melanin- or carotene/pterine-containing pigment cell lineage (28–30). Whereas in these in vitro studies the common origin of both cell types was unequivocal, it cannot be excluded that transdifferentiation, well known in amphibian pigment cells (31), from the melanophore to the xanthophore lineage may occur in the solid tumors. This would be an alternative explanation for the abundance of two different pigment cell lineages in the tumors.

UV-induced thyroid carcinomas have been reported in the Amazon molly, although no genetic factors were apparent in these studies (32). Together with the pigment cell tumor system, this could define the Amazon molly as a new model fish for experimental cancer research; in particular, the genetic uniformity of a clonal organism has a lot of potential for investigations on interactions of the tumor with the host and the ability to identify genetic factors involved in carcinogenesis.

The well-characterized melanomas of *Xiphophorus* have some peculiarities that are due to their exclusively hereditary origin. In fish of the same genotype, the tumor rate is 100%, all melanomas originate from the same site, and all are classified as a similar malignancy. The tumors of the M-clone show a marked individual difference with respect to malignancy, growth characteristics, and to the body compartment where they arise, although the fish are clonal and thus genetically identical by descent. In principle, all macromelanophores of all M-clone fish are alike and should be equally prone to tumor formation. This indicates that additional somatic alterations occur that initiate tumor formation. The relatively high frequency (especially if compared to the zero tumor rate of the black molly) and the preference for certain compartments are connected to the presence

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**Table 1** Expression of Xmrk, c-yes, and c-src in melanoma and nontumorous organs of M-clone *P. formosa*

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<sup>a</sup> - <5 fg/100 ng of total RNA; +, 5–50 fg/100 ng; ++, 50–100 fg/100 ng; ++++, 200–300 fg/100 ng.

<sup>b</sup> The highest abundance of the mRNA is given; however, the values differed considerably between the four individuals analyzed for all three transcripts. We think that the advanced stage of the tumorous disease or other pathogenic effects are the reasons for the pronounced interindividual differences that were not noticed in the other organs.

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<sup>6</sup> A. Schartl and M. Schartl, unpublished observation.
of the microchromosome in the *P. formosa* genome. Thus, the microchromosome contributes an increased susceptibility to develop cancer to its carrier. We propose that the microchromosome indeed contains a pigment cell tumor susceptibility locus of thus far unknown biological nature.

From the formal genetics point of view in the M-clone of *P. formosa*, a situation is found that is comparable to the genetic make-up of platyfish/swordtail backcross hybrids, where the swordtail was used as the recurrent parent. In both cases, the macromelanophore locus from one species or strain is introduced into the genetic background of a different and apparently considerably divergent genotype (being another species or population). In the M-clone fish, the microchromosome derived from the black molly becomes part of the foreign genome of *P. formosa* and is activated through the interaction with another genetic background. Support for this concept comes from the finding that hybridization of the black molly with another species, the guppy, *P. reticulata*, or the sailfin molly, *P. velifera*, also rendered melanoma-bearing hybrids (33, 34). By histological appearance, both tumors were similar to the M-clone tumors, although no contribution of another pigment cell lineage in addition to melanophores was noted.

In *Xiphophorus* during the crossing procedure leading to melanoma-bearing hybrid offspring, chromosomes of the platyfish that are proposed to contain regulatory loci suppressing the transforming capacity of the *Xmrk* oncogene are substituted by the homologous chromosomes of the swordtail, which do not harbour loci that are effective on *Xmrk* activity (2, 6). Thus, overexpression of *Xmrk* can occur in the macromelanophore lineage of such genotypes. In the chromatoblastoma of *P. formosa*, the *Xmrk* oncogene, the overexpression of which leads to melanoma in *Xiphophorus*, is apparently not of a similarly crucial function. A duplicated version is not found (as in all *Xiphophorus* genotypes that have the predisposition to develop melanoma following the appropriate crossings), nor is overexpression of the proto-oncogene found, which after acquiring activating somatic mutations could also be effective in tumor induction.7 Also cytoplasmic tyrosine kinases of the src family, which are frequently affected in *Xiphophorus* melanoma (23), showed no elevated levels of expression in M-clone chromatophoromas. Another thus far unidentified oncogene must be instrumental in neoplastic transformation of the macromelanophores of M-clone fish. This is intriguing in the light of

the histopathological similarities, the extremely close relationship of *P. formosa* and *Xiphophorus*, and the analogy of the formal genetic situation. It is tempting to generalize the speculation that different molecular pathways may lead to phenotypically and, by all other means indistinguishable, tumors.

What is the biological nature and the relationship of the susceptibility gene and the oncogene? Because the microchromosome appears to be fully retained as a structural unit in the tumor cells, loss of a gene, as would be typical for tumor suppressor genes seems, unlikely. The tumor susceptibility contributed by the microchromosome is highly cell type specific because no other cancer has thus far been found. To answer the question of whether the susceptibility locus is identical to the transforming gene or whether it only removes a control from a melanoma gene, like the situation for *Xmrk* of *Xiphophorus*, what the nature of the genes involved is and knowledge about the identity of these genes are required. Cloning of the microchromosome following microdissection from metaphase spreads offers an approach for this in the future.

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