Phenotypic Conversion of Malignant Melanoma to Benign Melanoma and Vice Versa in *Xiphophorus**

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INTRODUCTION

Melanoma formation in *Xiphophorus* is mediated by a "tumor gene" (*Tu*).[1] *Tu* becomes active, if distinct *Tu*-specific regulating genes (*R*) are eliminated, but only one distinct stage of pigment cell differentiation is competent for neoplastic transformation. The onset of melanoma formation, the location of the melanoma on the body of the fish, and the degree of malignancy of the melanoma are determined by the genotype of the individual. Cytologically, the degree of malignancy of the melanoma depends on the ratio of incompletely differentiated melanoma stem cells that are capable of dividing (i.e., transformed (T)-melanoblasts, T-melanocytes) to terminally differentiated melanoma cells that are incapable of dividing (i.e., T-melanophores). The more incompletely differentiated cells are present in the melanoma, the higher is the degree of malignancy. Melanomas consisting predominantly of terminally differentiated cells are benign.[2] Genetic analysis revealed that the presence of a "differentiation gene" (R_{Diff}) results in the benign phenotype of the melanoma, while the absence of R_{Diff} leads to the malignant phenotype of the melanoma.[3]

The objective of our experiments was to find out whether an influence on melanoma cell differentiation may lead to a change in the degree of malignancy of the melanoma. For this purpose we chose two different approaches: 1) We investigated whether differentiation of melanoma cells can be triggered by nontumorous cells. Therefore we transplanted malignant melanoma into *Tu*-free nontumorous embryos. 2) We investigated whether an exogenous agent, like a hormone, which is added to the aquarium water, may trigger melanoma cell differentiation.

MATERIALS AND METHODS

Fish

All stocks of *Xiphophorus* were raised in our laboratory under standard conditions (25° C, 12 hours artificial light/24 hours).

The following genotypes were used:

a) *Xiphophorus helleri*, stock derived from wild fish from Rio Lancetilla (Mexico). These animals are considered *Tu*-free. They are nontumorous.

* This paper contains part of the dissertations of A. Schartl and M. Schartl.

b) Breeds representing the same genetic background as (a), however, homozygous for the albino gene (*a*/*a*) and hemizygous for the *Tu*-containing gene complex that codes for the *Lineatus* pattern (Tu^{Li}). The Tu^{Li} gene is derived from *X. variatus* by introgression. These animals spontaneously develop amelanotic, benign melanoma on the body side at the age of 2–3 months due to the presence of Tu^{Li} and the lack of most *R*-genes.

c) Tu^{Li} breeds like (b); however, nonalbino but homozygous for the golden gene (*g*/*g*). These animals spontaneously develop melanotic, benign melanoma on the body side at the age of 2–3 months due to the presence of Tu^{Li} and the lack of most *R*-genes. Because of the homozygocity of *g* the differentiation of melanoma stem cells is delayed and only a limited number of these cells are available.

d) Breeds like (a), however, is hemizygous for the *Tu*-containing gene complex that codes for the *Spotted Lepper* (named after K. Lepper who has selected the mutant) pattern (Tu^{Sp^L}). The Tu^{Sp^L} gene is derived from *X. maculatus* by introgression. These animals spontaneously develop melanotic malignant melanoma on the whole body, the fins, and occasionally at the eyes at the age of 2–4 weeks due to the presence of Tu^{Sp^L} and a total lack of *R*-genes.

Transplantation procedure

Females of the viviparous teleost *Xiphophorus* were sacrificed at day 2 or 3 of pregnancy. Only embryos that were in the stage of early histogenesis (stages 9–12 according to Tavolga)[4] were used. The embryos were removed from the ovary and washed 4 times for 5 min in sterile phosphate-buffered saline (PBS; 5.82 g NaCl, 0.15 g KCl, 1.05 g Na_2HPO_4, 0.15 g KH_2PO_4) per 1 l agua dest.) containing antibiotics (500 IU penicillin/ml, 500μg streptomycin/ml and 7.5μl amphotericin B/ml).

Small pieces (1 mm^3) of tissue of extremely malignant melanoma and of normal skin were washed in PBS + antibiotics 4 times for 30 min. Subsequently the tissue was treated with trypsin/EDTA (0.5% trypsin, 0.2% EDTA, 0.7% NaCl dissolved in sterile agua dest.) for 2 hours. Transplantation was performed with the aid of a Leitz micromanipulator. The graft was placed in an area of the ventrolateral region of the embryo, which is bordered by the duct of Cuvier, the vitello-caudal vein, the postcardial vein, and the yolk sac portal sinus. After transplantation the embryos were cultivated *in vitro* in an artificial medium (5.8 g NaCl, 0.15 g KCl, 1.05 g $Na_2HPO_4 \cdot 2H_2O$, 0.15 g KH_2PO_4, 0.08 g $CaCl_2$, 0.08 g $MgCl_2 \cdot 6H_2O$, 0.25 g glucose, 0.25 g casein dissolved in sterile aquarium water containing 0.2% chick embryo extract (GIBCO, Grand Island, USA), 100 IU penicillin/ml, 100 μg streptomycin/ml, and 2.5 μl amphotericin-B/ml) and raised to fertile adults according to the method described by Haas-Andela.[5]

Hormone treatment

Neonate and adult fish were treated by adding a solution of 17-methyl-testosterone (EGA-Chemie, Steinheim, FRG) in ethanol (1 mg/ml) to the aquarium water. Fish were treated during a period of 8–12 weeks with different dosages ranging from 2 μl to 20 μl per liter aquarium water and per day. Controls were kept under the same conditions and treated with the appropriate dosage of pure ethanol.

Determination of malignancy

The degree of malignancy of each melanoma was determined either histologically or

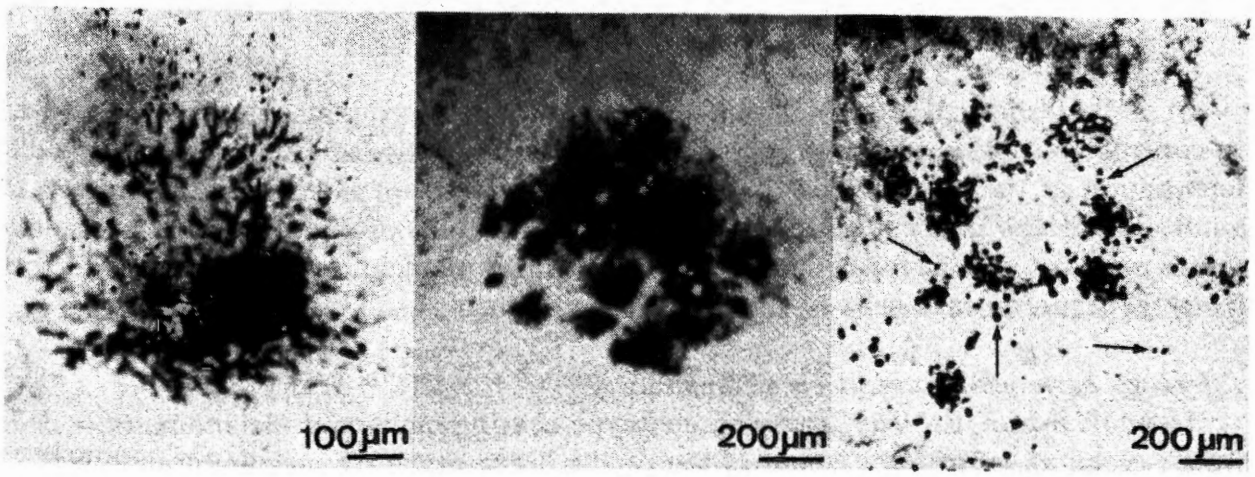
phenotypically according to the cirteria given by Anders, Diehl, Schwab, and Anders.[2)]

RESULTS

Fate of melanoma following transplantation

Extremely malignant melanoma from 2-month-old fish of the Tu^{Sp^L}-carrying genotype was used as donor tissue. This tumor usually leads to the death of the donor fish within 2–3 weeks. In control experiments nontumorous skin from fish of the same genotype was used for transplantation. All recipients were embryos of the *X. helleri* wild fish stock (*Tu*-free).

Two to three days after transplantation of malignant melanoma into nontumorous embryos ($n = 558$) T-melanoblasts and T-melanocytes migrated from the graft (Fig. 1a), populated the host, and in several cases ($n = 222$) led to formation of small, localized metastasis-like tumors (secondary melanoma), which were mostly located on the operculum and near the dorsal fin anlage. Five to six days after transplantation we observed an increased differentiation of melanoma stem cells to terminally differentiated T-melanophores in all grafts and secondary melanomas (Fig. 1b). This process resulted in a retardation of the growth of both the tumor grafts and the secondary melanomas. The terminally differentiated T-melanophores were subsequently removed by macrophages (Fig. 1c), which led to a partial regression of the grafts and the secondary melanomas. About 14 days after transplantation all grafted melanomas could be classified as benign; some of them showed total regression within the following 2–3 weeks, while others remained throughout the whole life of the recipient as extreme benign melanomas.



a *b* *c*

FIG. 1. Fate of neoplastically transformed pigment cells grafted into nontumorous embryos of *X. helleri*.

a: Graft, 3 days after transplantation. Melanoma cells migrate and populate the skin of the host.

b: Graft, 10 days after transplantation, consisting mainly of terminally differentiated T-melanophores.

c: Macrophages (→) in the area of the host's skin where the graft was located. Groups of macrophages show the shape of the removed T-melanophores.

In some cases ($n = 13$) the melanoma cells were growing in a nonoverlapping fashion (Fig. 2). They formed a pattern which is typical for nontransformed pigment cells and which has never been observed in autologous neoplastically transformed pigment cells of *Xiphophorus*.

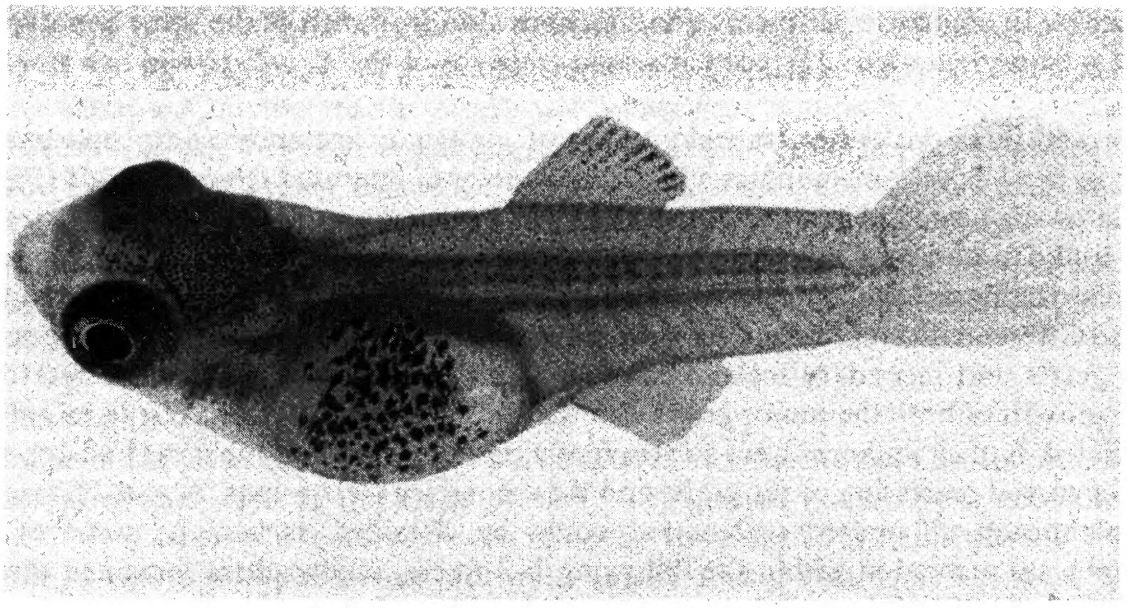


FIG. 2. A 3-week-old neonate fish of *X. helleri* with transplanted T-melanophores. The neoplastically transformed pigment cells form a pattern which is typical for nontransformed pigment cells.

In grafts of normal skin consisting only of nontransformed pigment cells which served as controls ($n = 86$), we also observed the migration of cells from the graft, but the differentiation of the transplanted donor pigment cells was not increased as compared to autologous donor pigment cells. Thus we concluded that the promotion of cell differentiation is an effect specific for neoplastically transformed pigment cells, mediated by factors from the nontumorous cells of the recipient embryo.

Effects of hormone treatment on melanoma

In adult fish of the Tu^{Sp^L}-carrying genotype bearing malignant melanoma ($n = 317$) we observed an increased amount of terminally differentiated T-melanophores after 3–4 weeks of testosterone treatment. These cells were immediately removed by macrophages. The differentiation of the melanoma stem cells that are capable of dividing into terminally differentiated melanoma cells that are incapable of dividing was promoted by testosterone. This resulted in a phenotypic conversion of malignant melanoma to the benign state (Fig. 3). Macrophage activity led to melanoma regression, which was most prominent in the fins, the abdominal part of the body, and the caudal part of the trunk. No regression was observed in the peduncle of the tail. After 3 months of treatment all malignant melanomas were reduced to extreme small, benign melanomas.

In control fish ($n = 98$) 17 animals showed a partial, spontaneous regression of the

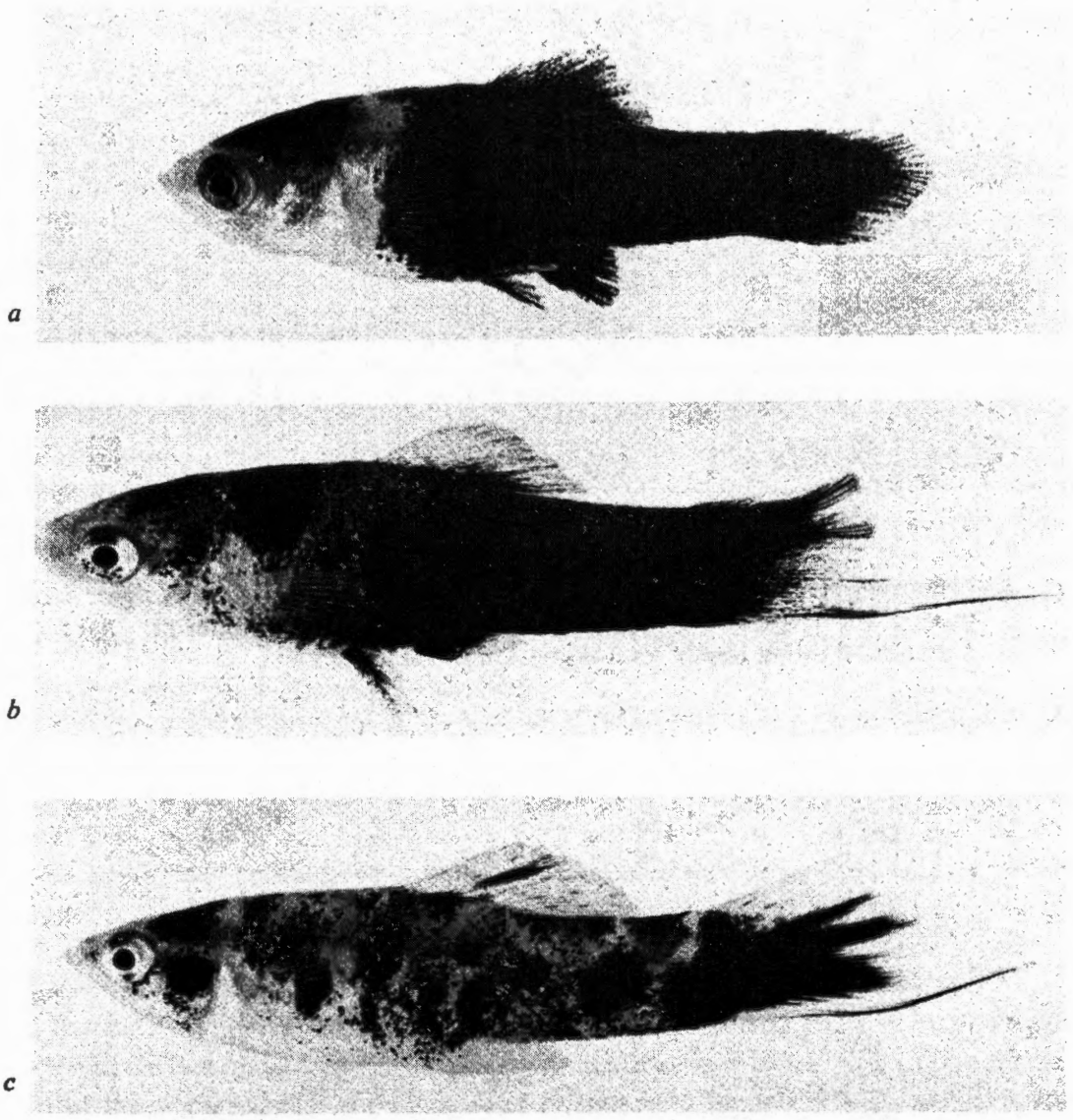


FIG. 3. Regression of malignant melanoma following treatment with 17-methyltestosterone:
a: a fish of the Tu^{SPL}-carrying genotype, untreated;
b: the same animal after 3 weeks of treatment (2 μl per liter aquarium water per day); and
c: the same animal after 8 weeks of treatment.

melanoma, which, however, was never as prominent as in treated fish. This phenomenon happens infrequently in this genotype.[1)]

Treatment of adult fish of the Tu^{Lt}-carrying golden *X. helleri* (*g*/*g*) ($n = 47$) bearing benign melanoma led to nearly total regression of the tumor (Fig. 4). We observed the same mechanism as described above, namely, promotion of cell differentiation, as the underlying cause for the effect of testosterone treatment. It might be possible that in this genotype only small amounts of melanoma stem cells are available (see Materials section) and nearly all stem cells have been pushed to complete differentiation. This would result in a lack of proliferating tumor cells, leading to the pronounced melanoma regression that we observed.

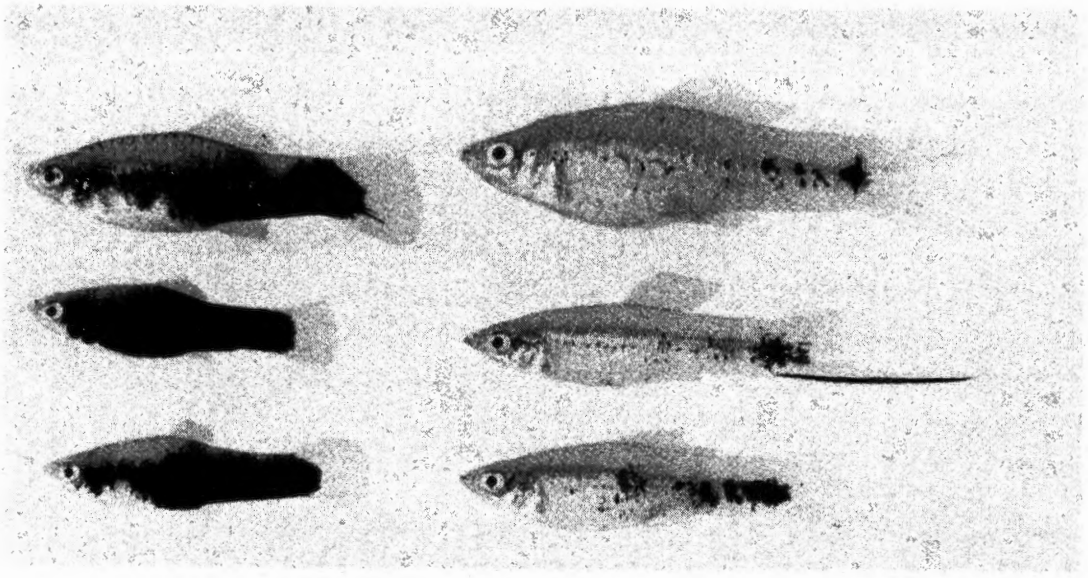


FIG. 4. Fish of the Tu^{Li}-carrying golden *X. helleri* (*g*/*g*). Left: untreated fish; right: fish treated with 17-methyl-testosterone (4 μg per liter aquarium water per day) for 3 months showing nearly total regression of the melanoma.

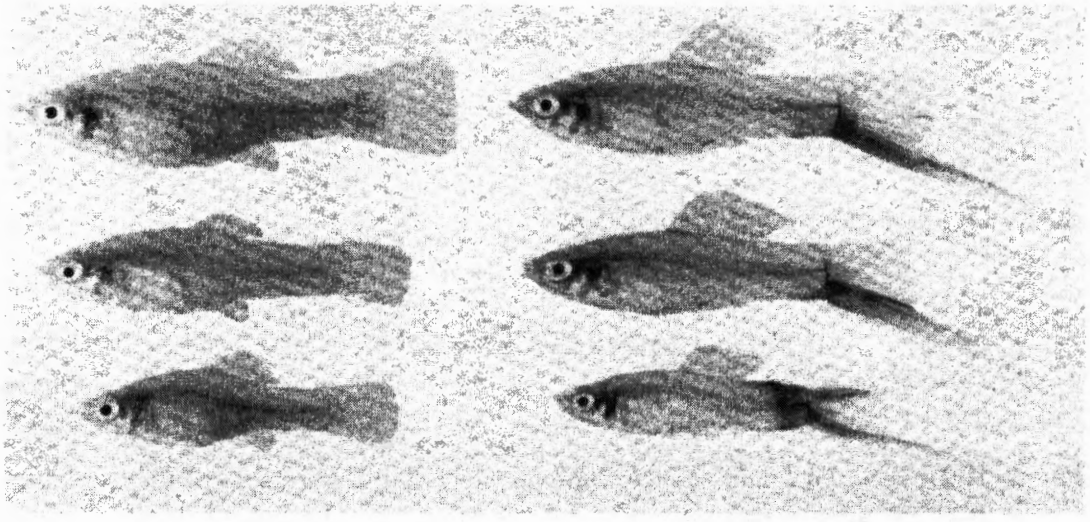


FIG. 5. Fish of the Tu^{Li}-carrying albino *X. helleri* (*a*/*a*). Left: untreated fish; right: fish treated with 17-methyl-testosterone (2 μg per liter aquarium water per day) for 3 months, showing malignant amelanotic melanoma in the peduncle of the tail.

Neonate fish of the Tu^{Li}-carrying albino *X. helleri* (*a*/*a*) ($n = 96$), which never spontaneously show any melanoma at this age but usually develop benign melanoma at the age of 3–4 months, developed colonies of neoplastically transformed pigment cells after 4 weeks of testosterone treatment. After 3 months about 75% of the treated animals bore amelanotic, malignant melanoma in the peduncle (Fig. 5). In control fish ($n = 104$) only sexually active adult males aged about 18 months infrequently developed spontaneous amelanotic malignant melanoma in the tail. This may be due to a high endogeneous testosterone level in these fish.

Besides the alteration of the degree of malignancy of melanoma, all animals after 1–2 weeks of testosterone treatment showed phenotypic alterations: males exhibited an in-

crease in the expression of male secondary sex characters (i.e., gonopodium, "sword"-like caudal fin process). Females, as well as immature fish of both sexes, developed male secondary sex characters. All treated fish exhibited exceedingly aggressive behavior. This agrees with the results of Dzwillo and Zander[6] that 17-methyl-testosterone added to the aquarium water is incorporated by the fish and has cytobiological effects.

DISCUSSION

The experiments show that an acceleration of cell differentiation leads to a change in the degree of malignancy of melanoma. In transplantation experiments, diffusible factors from nontumorous cells specifically promote the differentiation of neoplastically transformed pigment cells. A comparable result was described by Sachs[7] in the myloid leukemia system in mice. A biochemically identified "macrophage and granulocyte inducer" (MGI), which is produced in nontumorous cells, specifically promotes the differentiation of leukemia stem cells to terminally differentiated macrophages and granulocytes. This action of MGI has the same effect on leukemia as that which we observed for the action of the embryonic factors on melanoma development in *Xiphophorus*: phenotypic conversion of malignancy to the benign state.

In the hamster melanoma system it has been reported that a diffusible factor from nontumorous cells induces density-dependent inhibition of the growth of melanoma cells.[8] Those melanoma cells show the same growth characteristics as nontransformed pigment cells *in vitro*. In *Xiphophorus* we found a similar phenomenon *in vivo*: transplanted melanoma cells showed in some cases density-dependent inhibition of growth, forming a pattern in the skin of the fish which is typical for nontransformed cells. It would appear that regulation of growth of the melanoma cells in these cases is also mediated by diffusible factors from nontumorous cells.

In the view of the results presented here and earlier results on genetic control of cell differentiation,[3] it may be possible that the diffusible factors in *Xiphophorus* are homologous to the gene product of R_{Diff}. The presence of the diffusible factors in our experiments evoked the same effects as the presence of R_{Diff} in the genome of a melanoma-bearing fish; in both cases the melanoma is of a benign phenotype.

Like the diffusible agents from nontumorous embryonic cells, testosterone influences the degree of malignancy of melanoma by promotion of cell differentiation. Treatment with testosterone, however, leads to opposite effects depending on the stage of differentiation of the melanoma cells. In the melanoma of adults, where most cells are in an advanced stage of differentiation and already neoplastically transformed, the cells become stimulated to complete differentiation, and thus render the malignant melanoma benign. On the contrary, in neonate melanoma, where most cells are in an early stage of differentiation and not yet competent for neoplastic transformation, the cells become competent via differentiation. This process results in a higher degree of tumor malignancy.

The dual effects of treatment with sex steroids[9,10] or other agents like retinoids[11,12] on tumor development have also been reported for mammals and humans, but have not been explained so far. Further experiments will show if tumor treatment in *Xiphophorus* via promotion of cell differentiation is a valuable therapy. In any case the developmental stage of the tumor must be a prime consideration.

Acknowledgments

We thank Dr. M. Schwab, Giessen, for critical suggestions and Mrs. E. Silomon-Pflug for typing the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 103 "Zellenergetik und Zelldifferenzierung," Marburg, by Stiftung Volkswagenwerk, and by Justus-Liebig-Universität Giessen.

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