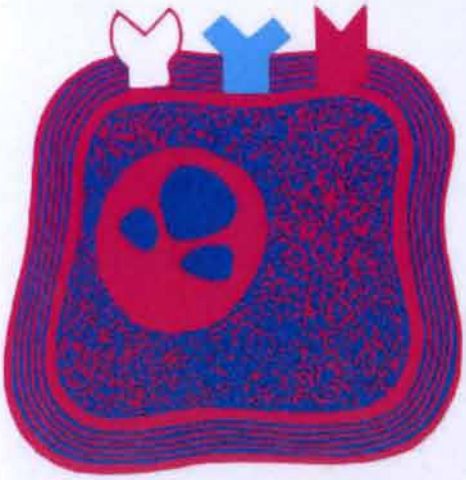
Volume 20 Number 1 Spring 2005



S. 35

Journal of Tumor Marker Oncology

the official journal of the International Academy of Tumor Marker Oncology

The International Academy of Tumor Marker Oncology Inc. Publishers

GENERAL INFORMATION

Journal of Tumor Marker Oncology will publish the discovery of new markers suitable for clinical application and basic research studies in the elucidation of the nature of markers regarding their physiological behavior and their role in malignant neoplasms.

Journal of Tumor Marker Oncology (ISSN: 0886-3849) is published six times per year by The International Academy of Tumor Marker Oncology Inc. Publisher, Schwarzspanierstr. 15, A-1090 Vienna, Austria.

Subscriptions should be addressed to the publisher and are payable in advance. Rates for personal subscriptions are US$200 per volume of 4 issues plus mailing cost. Rates for libraries and institutions are US$160 per volume of 4 issues.

Reprints can be ordered prior to printing in quantities of 50 or more. The costs per 50 reprints are US$100 plus shipping.

Information for Manuscript Submission and Instructions to Authors are given on the inside back cover of every issue.

Business Communications should be addressed to the Publisher.

Advertising inquiries should be addressed to one of the Editors.

Manuscripts should be directed to the European Editor, Dr. Georg Birkmayer, Labor Birkmayer and MEDINFO Inc., Schwarzspanierstrasse 15, A-1090, Vienna, Austria tel 43-1-4022367-0, fax 43-1-408 99 08.

Contributions to this journal are published free of charge. However, printing of coloured graphs will be charged.

All authored papers and editorial news and comments, opinions, findings, conclusions, or recommendations in the Journal of Tumor Marker Oncology are those of the author(s), and do not necessarily reflect the views of journal and its publisher, nor does their publication in the Journal of Tumor Marker Oncology infer any endorsement.

Manuscripts must be provided on diskette and as 2 print-outs.

The International Academy of Tumor Marker Oncology Inc. Publishers · Vienna, Austria

Journal of
Tumor Marker Oncology

the official journal of the International Academy of Tumor Marker Oncology

The International Academy of Tumor Marker Oncology Inc. Publishers · Vienna, Austria

JOURNAL OF TUMOR MARKER ONCOLOGY
Volume 20, Number 1, Spring 2005
The International Academy of Tumor Marker Oncology Inc. Publishers

Benignancy and Malignancy and the Significance of Terminal Differentiation in Neoplastically Transformed Cells in the Xiphophorus Melanoma Model

A. Anders

Genetics Institute, Justus-Liebig-University Giessen, HeinrichBuff-Ring 58,D-35392 Giessen, Germany, Tel.: +49-641-99-35475, Fax: +49-641-99-35469, email: Jamilah.Michel@gen.bio.uni-giessen.de

Melanoma formation in the platyfish swordtail model is encoded by numerous genes that are conducted by a platyfish derived master gene x-erbB*, which is homologous to the virus oncogene v-erbB. Benignancy and malignancy are formally determined by a key controller of cell differentiation, the Diff gene. Homozygous Diff prevents melanoma by terminal differentiation and death of transformed cells; only spots may develop. Hemizygous Diff permits development of benign melanoma by partial release of the transformed cells from terminal differentiation. Homozygous loss of Diff results in complete release of the transformed cells from terminal differentiation and cell death (apoptosis), permitting permanent melanoma growth. Diff segregates independently from the x-erbB* gene. We could bring this very important suppressor gene in line with modifications of tRNAAsn, tRNAAsp, tRNAHis, tRNATyr which contain queuosine (Q) instead of guanosine (G) in the first position of the anticodon. The more the cells are differentiated the more G is replaced by Q. The G:Q ratio specifies the risk of benignancy and malignancy of the melanoma. To modify the risk of malignancy and benignancy we treated a) Diff lacking fish that are committed to develop malignant melanoma (which are candidates of death) and b) Diff containing fish committed to develop benignant melanoma with differentiation-promoting steroid hormones such as methyltestosterone, methyltrienolone (R1881), stanozolol and trenbolone. The Diff-lacking malignant melanoma bearing fish pass over from lethal malignancy to the benign state. In contrast the Diff containing benign melanoma bearing fish pass over to malignancy. By this treatment we can either mimic or knock out the effect of the tumor suppressor gene, because the substances are more potent than Diff. The modulation of differentiation in cancer cells is the most important fact in these processes.

Introduction

The development of metazoa is based upon developmental genes that have been specified among animals from sponges to humans. A certain category of these genes, which, if amplified, overexpressed and/or released from control by impairment, loss, displacement or inactivation of multiple controller genes, may give rise to neoplasia and, therefore, collectively was termed "oncogenes". Oncogenes may code for functionally different proteins, such as the well known cellular growth factors, growth factor receptors, transcription factors, signal transducers or composed cellular growth regulators. The controller genes, collectively termed "tumor suppressor genes" or, in the reverse view, "cancer susceptibility genes", appear at least in two categories: those which suppress the oncogenes pretransformationally in the sense of "antioncogenes", and others that interfere posttransformationally by suppression of tumor growth, indicating that they actually are "oncostatic genes". Many facts of the present concept of oncogenes and tumor suppressor genes in oncology have been elaborated by means

of melanoma development in *Xiphophorus (Teleostei: Poeciliidae*) and were found to be compatible to several familial and sporadic forms of human neoplasia.

The Xiphophorus Melanoma Model

Several animal models have been used successfully in melanoma research, e.g. certain breeds of dogs and swine, which develop melanoma "spontaneously"; a South American opossum that develops melanoma at a high rate following UV exposure; guinea pigs and Syrian hamsters that develop melanoma after application of chemical carcinogens. The *Xiphophorus* model, however, covers the etiology of all kinds of melanomas and other neoplasms of neurogenic as well as of epithelial and mesenchymal origin. Another advantage of this model is that it allows the observation of the development, progression and regression of the tumor in the living animal.

Xiphophorine melanomas can be classified in five groups:
Spontaneously developing by genetic combination (selective mating) (1, 2)
Mendelian inherited
Initiator induced, following treatment with carcinogens (MNU, X-ray) (3)
Promoter induced, following treatment with promoters (TPA, hormones)
Transgenerationally, developed after radiation (X-ray, UV); retroelement induced suppression of suppressors (4)
Here we will focus on the spontaneously developing melanomas.

a) Genetic Components of Melanoma Formation

The melanoma formation in *Xiphophorus* hybrids may be explained by the inheritance of two genes, an oncogene locus (*Tu*) and a suppressor gene (*Diff*) (1). Matings of a spotted platyfish (*Xiphophorus maculatus*) female with a non-spotted swordtail (*Xiphophorus helleri*) male result in benign melanoma developing F_1-hybrids (fig. 1).

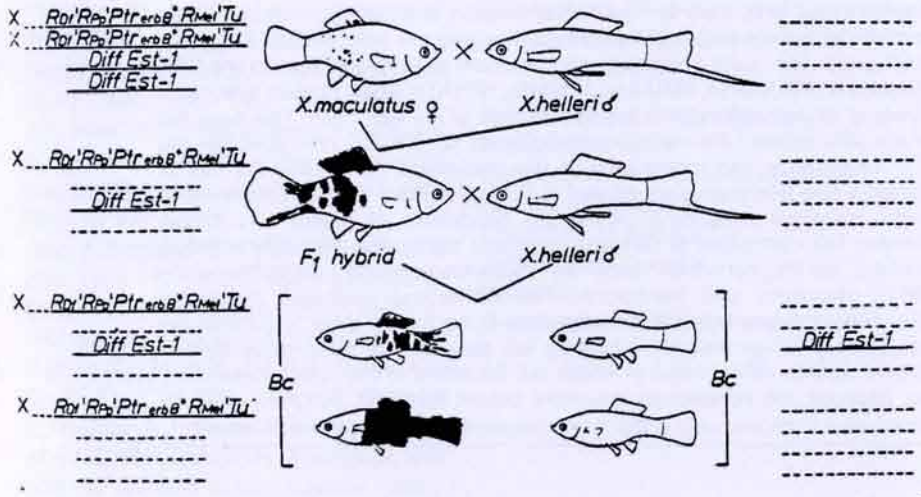


Fig.1: Schematic representation of the crossings resulting in the "spontaneous" development of spots, benign melanoma and malignant melanoma in *Xiphophorus* fish. ? ? , chromosomes of *X. maculatus*; -----, chromosomes of *X. helleri*; *Tu* tumor gene; *erb*B*, critical copy of the cellular homologue of the retroviral oncogene *verb*B; *R*Mel, impaired regulatory gene specific to pigment cells; R_{P_p} and *R*Df, impaired regulatory genes controlling *Tu* in the compartments of the posterior part of the body (P_p) and the dorsal fin (Df); *Diff*, regulatory gene controlling differentiation of neoplastically transformed cells; *Est-1*, locus for esterase-1 (a marker gene for *Diff*) of *X. maculatus*. Modified from (1).

Backcrosses of the F_1-hybrids with the swordtail result in offspring exhibiting three types of segregants: 25% develop benign melanoma like that of the F_1; 25% develop malignant melanoma consisting mainly of incompletely differentiated transformed cells, which invade other tissues (with the exception of brain, gonads and intestine), metastasize and eventually kill the fish. 50% develop neither spots nor

melanomas. The exemplified crossing experiment described in fig. 1 reveals several phenotypically identifyable platyfish derived genes which are involved in the formation, place, shape and degree of malignancy of the melanomas. Some of them are part of an aggregation of genes, which became known as "Tumor gene-complex", "*Tu*-complex" or simply "*Tu*", located at the very end of the X-chromosome of this special platyfish. When this complex is deleted neither spots nor melanomas develop.

The most prominent gene of the platyfish derived *Tu*-complex is the genetically and molecularly identified *xiphophorine x-erbB** oncogene (5), which via cell signalling conducts multiple developmental genes. This gene is a *xiphophorine* homologue of the avian erythroblastosis virus oncogene *v-erb*B, which encodes a protein-tyrosinase kinase derived from the epidermal growth factor receptor (EGF-receptor) (6). The tumor suppressor gene *Diff* is also located on a platyfish derived chromosome, marked by the locus for *Est*[1] (fig. 1), but not on the same chromosome as *x-erbB**. *Tu* and *Diff* therefore segregate independently from each other (7). *Diff* was found to be localized in linkage group V of the *xiphophorine* genome (8). So far no correlation was found to *tp53* (9). The 1:1 segregation in benignancy and malignancy is determined by the presence or absence of the *Diff* gene carrying chromosome.

b) Cellular Basis of Melanoma

The melanoma of *xiphophorine* hybrids grow out from black spots, consisting of incompletely differentiated pigment cells that can be assigned to certain inherited spots apparent in the parental wild type.

The precursors of pigment cells originate from the neural crest (fig. 2) and start migrating and differentiating at the onset of organogenesis. These cells entering their definitive place are comitted to become pigment cells and therefore may be termed chromatoblasts. They give rise to the stem melanoblasts (S-melanoblasts), which may reproduce identically throughout the life of the fish, but may also differentiate to become intermediate melanoblasts (I-melanoblasts), which irreversibly will continue differentiating to become dopa-positive advanced melanoblasts (A-melanoblasts). The A-melanoblasts differentiate to become melanocytes and, finally, melanophores. The whole system is maintained in a state of homeostatic balance between different stages of melanophore differentiation, apparently controlled by a remote regulator. Only pigment cell precursors that have reached the stage of intermediate melanoblasts, I-melanoblasts, can undergo neoplastic transformation. On the other hand, A-melanoblasts, melanocytes and melanophores, which can easily be recognized by their shape, phenoloxidase activity, and content of melanin, were not found to undergo neoplastic transformation, indicating that they are too far advanced in differentiation as to become transformed. The only competent cells for the transformation activity of the *Tu*-complex are, therefore, only the I-melanoblasts (11).

The I-melanoblasts, after being transformed to TrI-melanoblasts (all transformed cells are designated as Tr-cells) differentiate to easily recognizeable large, dopa- positive TrA-melanoblasts. These cells differentiate to the heavily pigmented Tr-melanocytes, which differentiate to the final stage of giant melanophores. They stop dividing like normal melanophores and undergo a process of aging followed by removal through macrophages. The most significant difference between Tr-melanophores and normal melanophores is that the transformed cells are not subjected to distance dependent regulation. Their lobules and dentrites interlace with each other, thus forming compact three dimensional accumulations of neoplastically transformed cells (1).

Breeding of x*iphophorine* albinos developing completely amelanotic melanomas show that the well understood biochemistry and molecular biology of melanin synthesis is independent from melanoma formation (fig. 3).

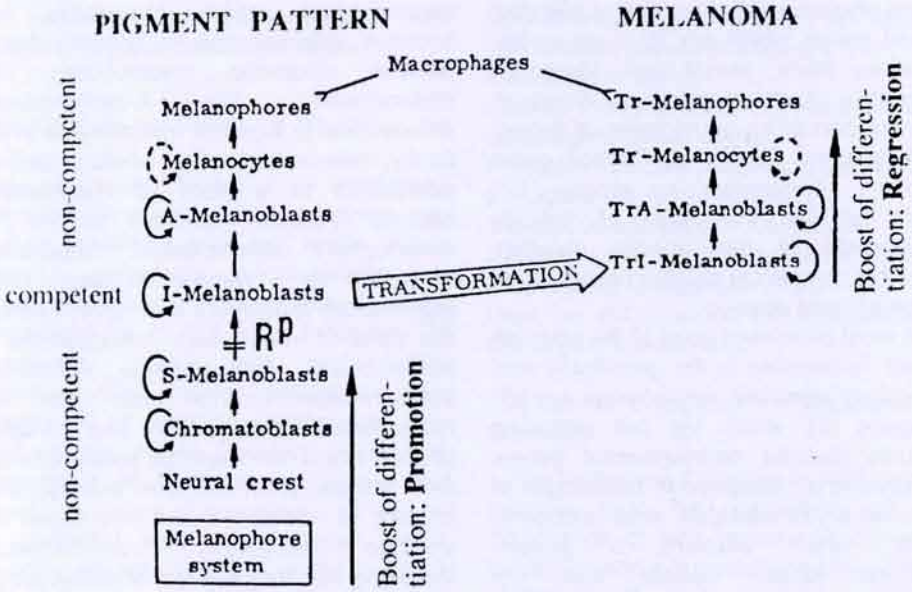


Fig. 2: Schematic presentation of differentiation of normal and neoplastically transformed pigment cells. S-, I- and A-melanoblasts are stem cells, intermediate and advanced melanoblasts, respectively. The Tr cells represent the transformed cells. Only I-melanoblasts are competent for neoplastic transformation. RP, regulatory gene that blocks pigment cell differentiation. The boost of differentiation in precursos cells overcomes the stop of differentiation leading to tumor formation. Boost of differentiation in Tr-melanoblasts and Tr-melanocytes is caused by tumor regression. Macrophages attack melanophores and Tr-melanophores. Modified from (10).

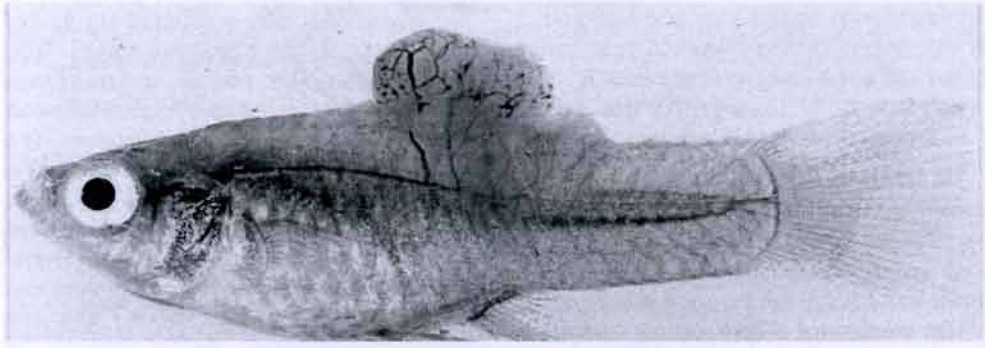


Fig. 3: Highly backcross albino carrying an amelanotic melanoma that completely lacks melanine. Note the vascularisation which normally is hidden under the melanine.

c) Relationship between Neoplastic Transformation, Proliferation and Differentiation

Neoplastic transformation of pigment cells has been proposed to be a normal process occurring continuously in the skin of the wild spotted platyfish. The transformed cells (Tr-cells), however are restrained from further proliferation by terminal differentiation exerted by two copies of the *Diff* gene, which represents the normal homozygous condition (*Diff*/*Diff*, Fig. 1). These terminal differentiated cells are later removed by macrophages. If *Diff* is present in single dosage in the hybrids (*Diff*/ -), the Tr-cells differentiate slowly, and, therefore are permitted to proliferate slowly, thus giving rise to benign melanoma. If, however, both copies of *Diff* are lacking in the hybrids (-/-), the majority of the Tr-Cells remain incompletely differentiated, proliferate permanently, and give rise to malignant melanoma of multicellular origin. Terminal differentiation and the removal of melanoma cells, therefore, is antagonistic to the permanent supply of melanoma cells by proliferation.

d) *Diff*-dependent characters of benignancy and maligancy

The clear cut 1:1 segregation in BC hybrids developing either benign or malignant melanomas (fig. 4) provided the opportunity to distinguish between benignancy and malignancy in more detail by means of morphological, histological, cytological, fine structural, biochemical and molecular studies. The majority of the cells in the benign melanomas are well differentiated, whereas those of the malignant melanomas are poorly differentiated and stop differentiating (tab. 1) (13). Based on the stage of differentiation the tumors in the *Diff*-lacking group grow rapidly and invasively. Vascularisation was observed, as well as high enzyme activity, condensed chromatin and high thymidine incorporation, features that we could not find in the *Diff* carrying group. Nevertheless, these attributes are not dependent on the *Diff* gene, but are rather epiphenomena, which are due to the stage of differentiation in the tissues. But they could be used as tumor markers.

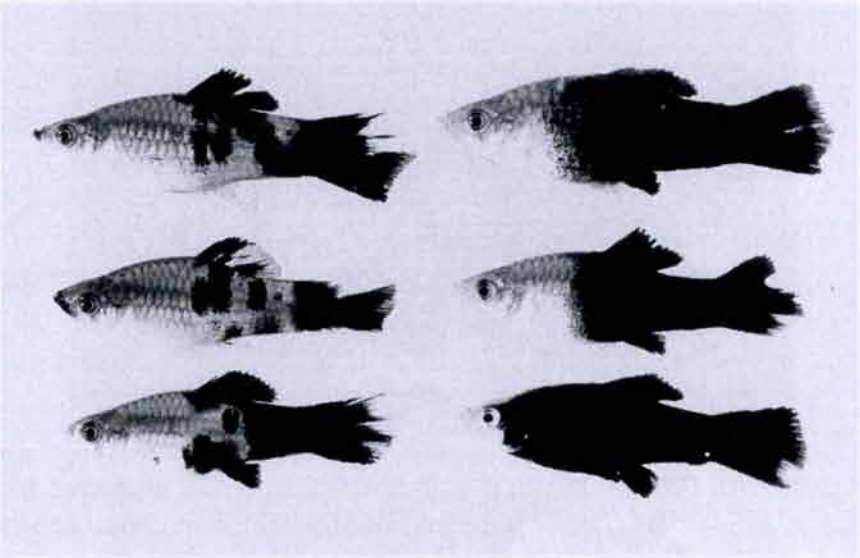


Fig. 4: Melanoma bearing backcross segregants according to the schematic drawings of fig. 1. Left: benign melanoma-bearing fish containing the *Diff* gene; right: malignant melanoma-bearing littermates that lack the *Diff* gene.

Diff / -	- / -
benign melanoma	malignant melanoma
well differentiated slow growing non-invasive no vascularisation Tr-melanophores prevail many macrophages low thymidine incorporation low enzyme activity less complex glycosphingolipids	poorly differentiated fast growing invasive vascularisation Tr-melanoblasts and -melanocytes prevail few macrophages high thymidine incorporation high enzyme activity more complex glycosphingolipids
tumorous and non-tumorous tissues dipersed chromatin diffusable *Diff* product	tumorous and non-tumorous tissues condensed chromatin no *Diff* product
first position of the anticodon of $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, $tRNA^{Tyr}$ contains predominantly:	
Queuosine	Guanosine

Table 1: The effect of *Diff* in tumorous and non-tumorous tissue. Modified from 13.

The most convincing data supporting the *Diff* dependent control of pigment cell differentiation come from transplantation experiments, in which tissue from malignant melanoma was transplanted to recipients either lacking or carrying the *Diff* gene. If the recipient is lacking the *Diff* gene, the melanoma tissue grows. If, however, the *Diff* gene is present in the recipient, the cells of the malignant melanomas become terminally differenttiated; they regain their distance dependent regulation, and later on they are removed by macrophages (fig. 5) (11, 12, 13). Thus the effect of *Diff* on the differentiation of neoplastically transformed cells can be traced to a diffusable substance. The nature of this substance is unknown at present (tab. 1, *Diff* in tumorous and non-tumorous tissue). Li *et al.* could show that the products of tumor suppressor genes inhibit cell proliferation by activation of cell differentiation in the tumor cells (14).

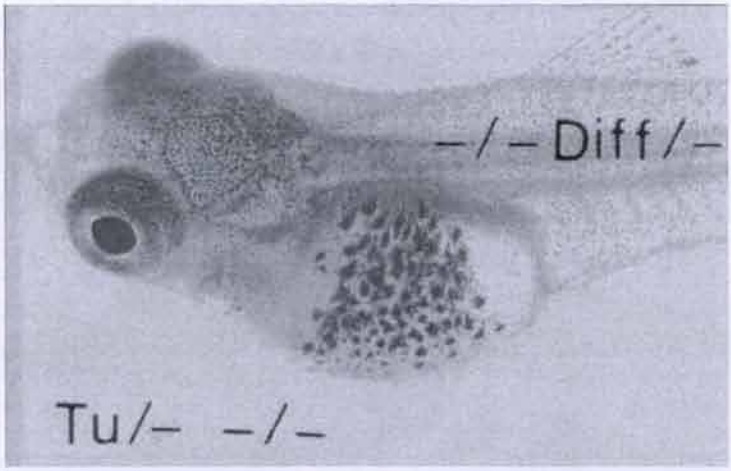


Fig. 5: Chimera composed of tissues containing the *Tu*-complex, but lacking *Diff*, and tissues containing *Diff*, but lacking the *Tu*-complex. Note *Diff* dependent terminal differentiation and distance regulation of the *Diff*-lacking transformed cells. From Schartl, 1979.

e) Modified tRNAs in *Diff*-Dependent Differentiation

There is considerable indication for the involvement of tRNAs containing modified nucleosides in the process of cell differentiation in eubacteria, slime molds and in neoplastic tissues of vertebrates (15). The modified nucleosides are $tRNA^{Asn}$, $tRNA^{Asp}$, $tRNA^{His}$, $tRNA^{Tyr}$, that contain queuosine (Q) instead of guanosine (G) in the first position of the anticodon (position 34). The more the cells are differentiated, the more replacement of G by Q is observed in position 34.

The method to estimate the G:Q ratio in a given population of the tRNA family consisted of following the replacement of guanine in position 34 by a labeled guanine excerted by a guanine-transglycosylase (insertase) of *E. coli*.

The results obtained in *Xiphophorus* by measurement of [^{3}H]-guanine incorporation into the tRNA family, differing in the ratio of G:Q in position 34, are summarized in Figure 6. The graphs show the kinetics of the exchange of G34 of the tRNA family by [^{3}H]-guanine, which is the reaction used to evaluate the amount of (Q^-)-tRNA. The fish genotypes and phenotypes are identical to those shown in fig. 1.

In accordance with the findings of many investigators working with other differenttiation systems, [^{3}H]-guanine incorporation is high in tRNAs from malignant melanoma that consist predominantly of poorly differentiated cells (13). In contrast, the incorporation is lower if the tRNAs are derived from benign melanoma that consist predominantly of well differentiated cells. Therefore, tRNAs of malignant melanomsa have a higher amount of G in place of Q than those of the benign melanoma (fig. 6).

To decide whether the distinct difference in G:Q ratios between benign and malignant melanoma is *Diff*-dependent or represents an epiphenomenon of benignancy and malignancy, the skin of non-tumorous littermates that segregate into animals carrying *Diff* and lacking *Diff*, like the tumorous fish, in a 1:1 ratio (see fig. 1), was used for analysis. The *Diff*-lacking segregants (specified by the lack of

esterase-1) always had higher amounts of Q-lacking tRNA than the *Diff*-carrying animals. The skin of the parent animals used for the initial crosses showed the same differences: *X. helleri*, which lacks the *Diff* gene, has a high [^{3}H]-Gua incorporation (*i.e.*, is G-rich), whereas *X. maculatus*, which contains the *Diff* gene, has a lower [^{3}H]-Gua incorporation. From these results, we suggest that the difference of G:Q ratios between benign and malignant melanoma is no epiphenomenon of benignancy and malignancy but is closely related to the primary effect of the *Diff* gene that in tumorous fish converts the malignant to the benign state.

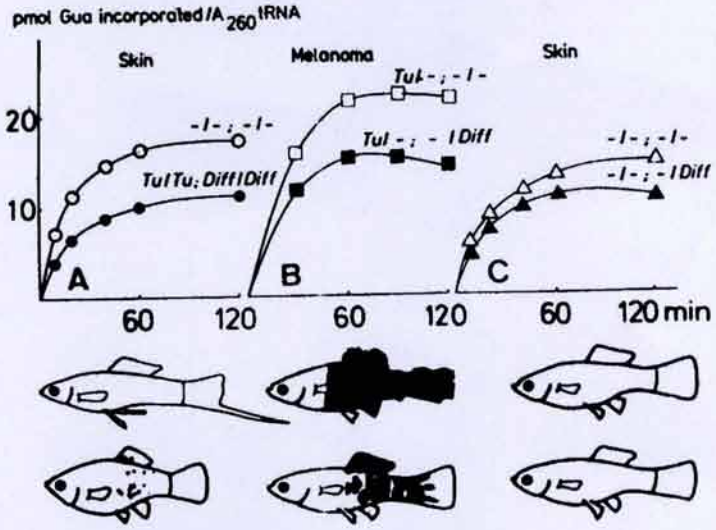


Fig. 6: Incorporation of [^{3}H]-guanine in position 34 of tRNA for Asp, Asn, His and Tyr of *Xiphophorus* catalyzed by tRNA-guanine-transglycosylase (insertase) of *E. coli*. The graphs show the kinetics of the exchange of G34 of tRNA by [^{3}H]-guanine, a reaction used to evaluate the amount of (Q^-)-tRNA. Accordingly shown are the fish below the curves. These fish correspond to those shown in fig. 4. High incorporation of [^{3}H]-guanine in *Diff*-lacking animals corresponds to to a low content of Q, whereas low incorporation of [^{3}H]-guanine in *Diff*-containing animals corresponds to a high extend of Q. A. Skin of purebred *Xiphophorus*: ?, *X. helleri*; ●, *X. maculatus;* B. Melanoma of BC segregants: □, malignant; ■, benign; C. Skin of nonmelanomatous BC segregants: ?, lacking *Diff*; σ, containing *Diff*. Note that comparable *Diff*-containing animals always have a lower G content and a higher Q content than *Diff*-lacking ones. Data from (13).

f) Modifying Benignacy and Malignancy

Most of our knowledge about experimental influence on benignancy and malignancy comes from studies on fish genotypes that, on the one hand, are genetically ready for "spontaneous" melanoma formation, but, on the other hand, cannot move on to melanoma formation because their pigment cell differentiation is retarded or blocked at the stem cell stage. As a consequence, neoplastic transformation cannot go on because the cells that are competent for neoplastic transformation are lacking (fig. 7). We tested a large variety of agents, some of which were supposed to be carcinogens and we found that they actually were promoters of cell differentiation that move the retarded pigment cell precursors to the competent stage, thus indirectly giving rise to melanoma development (fig. 8). Several tester strains for the detection of tumor promoters have been bred. The analysis of the oncogene machinery (measurements of $pp60^{x\text{-}src}$ kinase activity and inositol lipid turnover in the brain and other tissues) has shown that it is running even faster in the tumor free susceptible animals than in malignant melanoma bearing fish (16). It appears that the molecular and biochemical machinery leading to neoplasia operates in the tumor free fish without forming melanomas. The machinery, however, becomes immediately

effective as the competent cells are made available by promotion of cell differentiation achieved by tumor promoters. Table 2 lists a selection of substances which have been tested: positive promoters are for the most part hormones, *i.e.* androgens and estrogens. From 897 treated animals 783 developed melanomas. This means that there is a nearly 100% effect in the tested animals. Furthermore a large variety of agents was tested: e.g. TPA, a well known promoter, and questionable substances, such as betel nut extract (fig. 8), which were positive in the test (17). It is evident that the tumor suppressor gene *Diff,* which is present in the susceptible fish lines, does not prevent promotion. This opens the possibility to modify benignancy and malignancy experimentally by treatment, because treatment is obviously stronger than the tumor suppressor *Diff.*

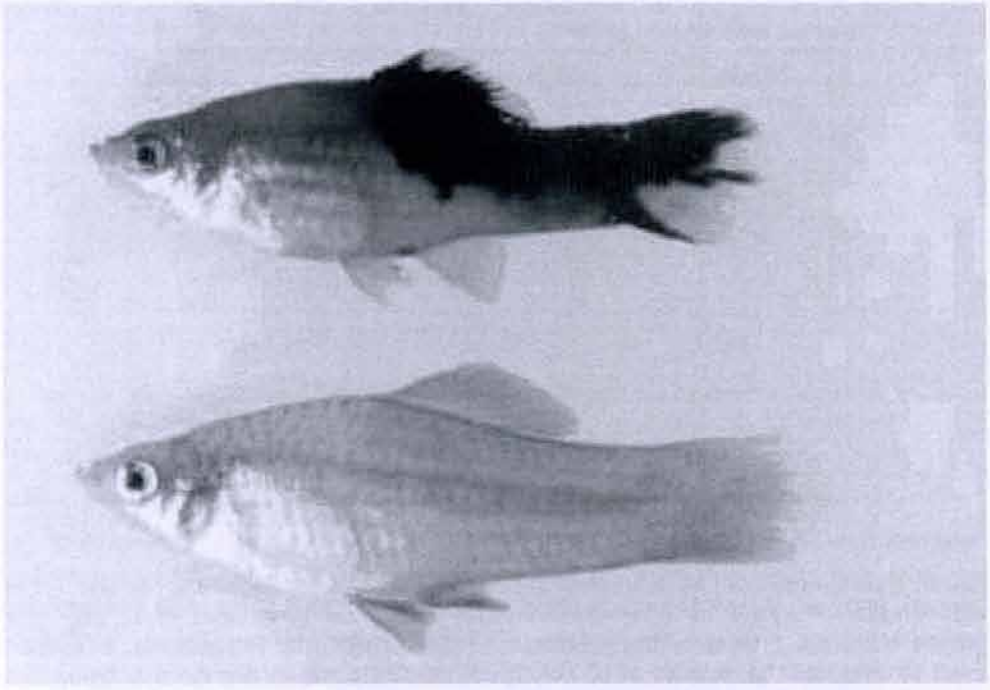


Fig. 7: Expression of the *Tu*-complex. Above: control; below: strain with retardation in pigment cell differentiation (tester strain for promoting agents).

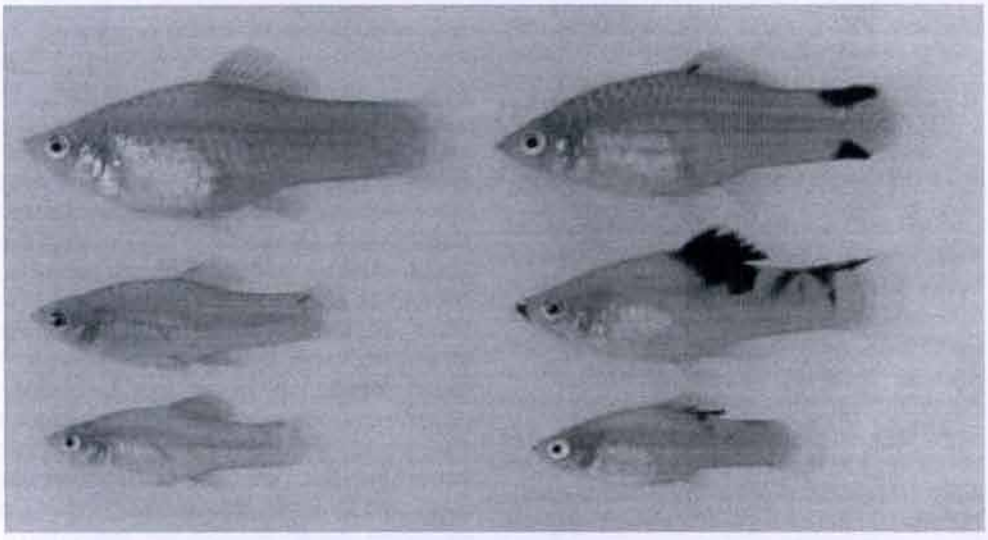


Fig. 8: left: untreated control containing the *Tu*-locus and the gene for retardation in pigment-cell differentiation; right: start of melanoma development following treatment with betel nut extract.

Agents	Dose µg/l	Promotion		
		Survivors	Melanoma prov.	Melanoma %
Androgens				
Testosterone	2	96	72	75
Methyltestosterone	2	139	139	100
Trenbolone	2	268	220	82
Stanozolol	2	149	139	93
Estrogens				
Ethinylestradiol	2	110	95	86
Diethylstilbestrol	2	20	19	95
Antiestrogens				
Tamoxifen	6	55	50	91
Retinoic acid	6	60	49	82
TOTAL		897	783	90
Cortisone	6	163	0	0
$H_2S_2O_8$	0.25 ml/l	180	0	0

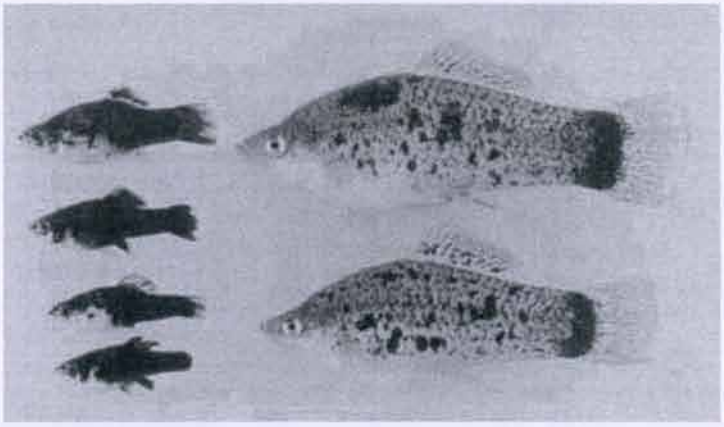
Tab. 2: Induction of melanoma following treatment with promoting agents. Modified from (18, 19).

Malignancy to Benignancy

To provoke cancer remission, more than 5000 malignant melanoma bearing fish were treated, which without such treatment would have died within a few months. Following treatment with agents known or suspected to promote differentiation, such as the androgens methyltestosterone, trenbolone, testosterone, stanozolol and R 1881, as well as the estrogens estrone, and progesterone, 50-60% of the death candidates survived (tab. 3) (18, 19). High survival rates were also observed after treatment with cortisone and $H_2S_2O_8$ (2258 treated, 1478 survivors = 65%). This result is remarkable, because these agents have no promoting activity (tab. 2). This means, that treatment of cancer with these therapeutic agents is not likely to induce another cancer in the animal.

The recovered animals showed three months after treatment a remission of malignant melanomas even down to spots (fig. 9a, b). Terminal differentiation was induced in the cancer cells, which stopped dividing. The treatment mimics perfectly the effect of the *Diff* gene. Even candidates of rapid death could be cured and raised up to maturity. Their offspring, however, develop malignant melanomas according to their genetic constitution indicating that the recovery takes place without genetic changes (fig. 10).

effe
ava
diff
pro
sub
pos
hor
Fro
mel
nea
Fur



a) estrogen, left before treatment, right after treatment.

b) androgen; left: before treatment; right: after treatment.

Fig. 9: Malignancy to benignancy.Treatment *of Xiphophorus* with hormones leads to regression.

Fig.
pigm

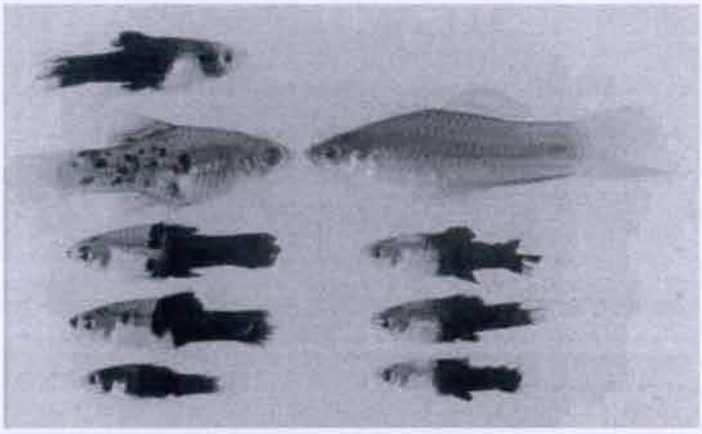


Fig. 10: Upper fish before treatment, lower fish after treatment. Offspring according to genetic constitution (malignant melanoma).

Fig
pigme
betel

Benignacy to Malignancy

The experiments with the promoter sensitive animals opened the possibility to experimentally influence the action of the *Diff* gene. In order to test this, extreme benign melanoma bearing animals, which exhibit only small black spots, were treated with the same substances that we used for tumor regression and promotion. One should assume that one gets an overexpression of the suppressor effect because in the treated malignant melanoma bearing fish the agents worked in this direction (fig. 9). But to our surprise we got the opposite effect: a wakening up of dormant tumors. After treatment, the spots started developing towards the malignant state of melanoma (fig. 11). We have tested more than 2000 fish bearing extreme benign melanoma with the same agents that we used for tumor regression (tab. 4). With R 1881, trenbolone, stanozolol and methyltestosterone we induced a very strong progression from benign to malignant melanoma. More than 90% of the treated animals showed an activation of dormant tumors. The weaker androgens testosterone and nortestosterone were less active; only 50% of the treated animals showed a progression. Treatment with estrogens (estrone and DES), as well as cortisone and $H_2S_2O_8$ did not induce the slightest progression.

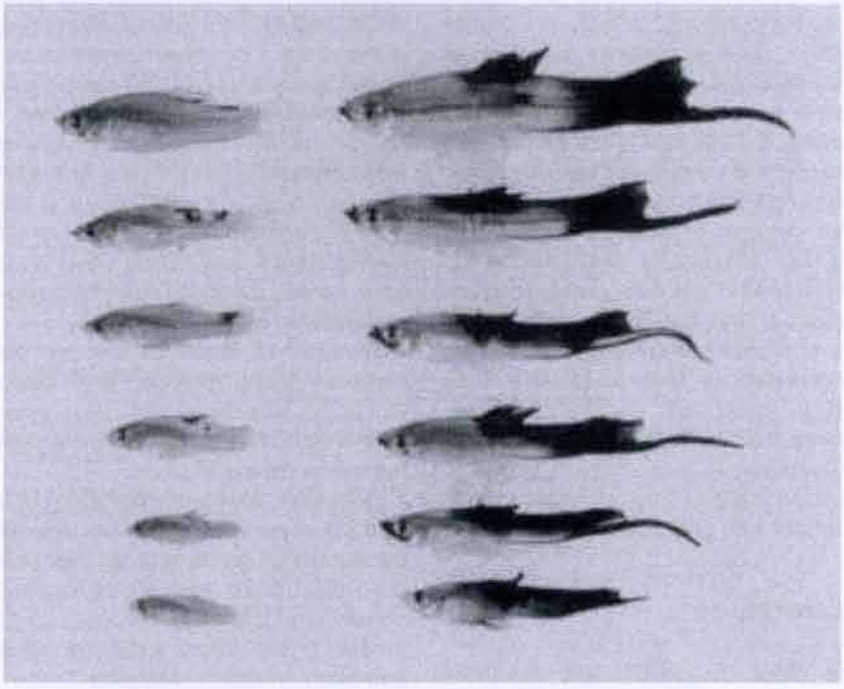


Fig. 11: Benignancy to malignancy. Progression after treatment. Left: before treatment; right: three month after treatment with stanozolol.

Agents	Treated animals (n)	malignant to benign		
		n	%	effect
Androgens				
Methyltestosterone	221	139	63	+
Trenbolone	457	273	60	+
Testosterone	272	141	52	+
Stanozolol	416	195	47	+
R 1881*	76	36	47	+
Estrogens				
Estrone	203	112	55	+
Progesterone	177	97	55	+
DES	269	19	7	-
	total : 2.091			
others				
Prednisone	189	54	29	?
Retinoic acid	408	206	50	+
Cortison	225	152	67	+
$H_2S_2O_8$	2.023	1326	65	+
Dexamethasone	248	45	18	?
	total : 3.093			
	? 5.184			

* Dose :10^{-12} in the tank

Table 4: Tumorregression. Induced in malignant melanoma bearing fish (lacking *Diff*). *Diff* phenocopied.

It turns out that the powerful androgens have a very strong effect on tumor progression. The suppressor, instead of being supported (in contrast to the results obtained in tumor regression), is switched off, allowing the differentiation of pigment cell precursors to competent cells for tumor formation. This is the same process that is observed in tumor promotion, in which the pigment cell precursors are retarded in differentiation and are then pushed toward differentiation by treatment. While this process of promotion is not age restricted, the progression is age restricted, since only fish in young age can transfer their melanomas from a benign to a malignant state. Obviously, in older fish the effect of the suppressor gene *Diff* is stronger than is the treatment with androgens.

Discussion

In the fifties, Butenandt and Kaufman suggested that steroid hormones might be endowed with the capacity to induce neoplasia by a mechanism that today is called tumor promotion. Siciliano and Perlmutter (20, 21) suggested that crossing conditioned "spontaneous" melanomas in *Xiphophorus* might depend upon the inductive effects of steroid hormones in the fish. We nevertheless could not confirm any influence of endogenous hormones on spontaneously developing melanomas. In several hundred of tested fish we never observed a difference in the expression of melanoma, neither in males or in females, though we could observe an influence of extra gonadal steroid hormones applied to very young fish that are far away from sexual maturity. During their sensitive period, endogenous hormones are not available, which may explain that we do not find an influence of endogenous hormones on melanoma development. The influence on secondary sex determination, however, is not age restricted, because even fertile females react to androgen treatment by developing a sword and a gonopodium, secondary sex characters for males. The fertility, however, is not disturbed by this process.

As demonstrated in tab. 3 we induced a regression of melanomas with androgens and estrogens (fig. 9a, b). The effect of these steroids is due to an induction of terminal differentiation in the permanently dividing melanoma cells. The stop in differentiation is cancelled, thus leading to tumor regression. This is mimicry of the effect of the *Diff* gene. On the other hand, following treatment of *Diff*-carrying animals with extragonadally applied very strong androgens, we observed a progression of melanomas (fig. 10), being obviously due to a switch off of the *Diff* gene. It turns out that extragonadally applied steroid hormones have a very strong effect on the *Diff* gene that controls the expression of melanomas.

Because of these factors we have to consider many players that directly or indirectly influence the key events of differentiation in the development and the regression of melanomas.

In cancer research we are confronted with the paradox that therapeutic and preventive drugs sometimes induce tumors (22). We could test these contradictory effects in the *Xiphophorus* melanoma model. If the target cells for melanoma formation are arrested in their differentiation before they reach the competent stage for neoplastic transformation - which is the case in promoter sensitive fish lines - tumor formation is prevented. After applying agents with promoting effects, we induce a boost of differentiation in precursor cells that transfers them into a competent stage. Then transformation and tumor development may start (fig. 2).

On the other hand - and this demonstrates the double-edged sword of these substances - when we apply the same agents on malignant melanomas, we induce a boost of differentiation in the incompletely differentiated melanoma cells (fig. 2), leading to terminal differentiation. These cells stop dividing, they can enter aging and finally are removed by macrophages. The result is a tumor regression, the opposite effect from the previous experiments, induced by the same substance. The target cells in both experiments are different: in case of promotion, the arrested precursors of pigment cells are pushed to go on in differentiation, and, because these cells become available for transformation, this leads to melanoma formation. In case of melanoma regression, the permanently dividing melanoblasts and melanocytes, which have stopped differentiating, are the targets (fig. 12). This stop is cancelled, and the cells enter the pathway of differentiation leading to tumor regression. Because differentiation is a one-way street

that always leads from poorly to more or less terminally differentiated cells, we can influence this process only in this direction.

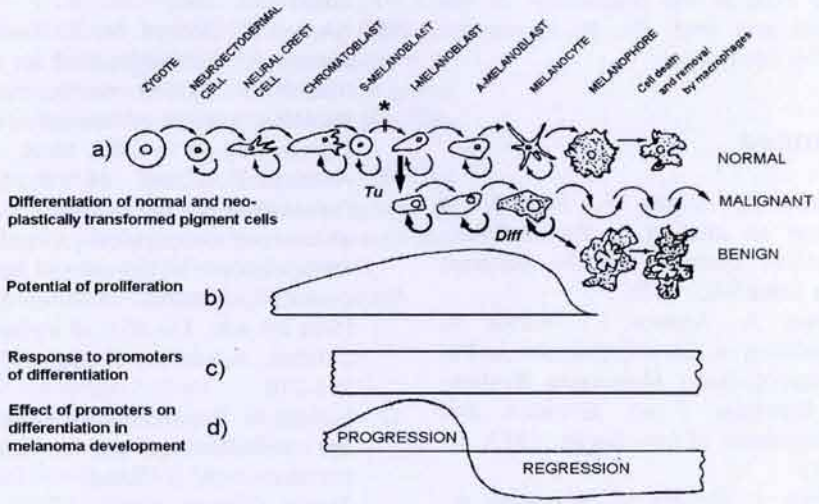


Fig. 12: Chronology of different steps in cell differentiation in normal and neoplastically transformed pigment cells (a) and the sensitive phase for agents inducing either progression and promotion or regression of melanoma (d).The target cells for promotion and progression are poorly differentiated cells, which can not go on in differentiation to the competent stage for neoplastic transformation. The target cells for regression, however, are the permanently dividing melanoblasts and melanocytes, which are pushed forward by treatment to the non dividing melanophores which end in tumor regression. This gives an explanation for the two-edged sword of many of the tested agents.
* stop of differentiation in promotor sensitive fish lines

The last point requiring discussion here is the relation between the immune system and the differentiation of the neoplastically transformed cells. Transplantation experiments in which the tissue of malignant melanoma was transplanted on the skin of tumor free animals (23) have shown, that the tumor tissue will start growing, thus giving rise to large tumors. After about two months the tumor is suddenly attacked by macrophages that are the immune competent cells for melanophores and disappears completely within 24 hours. Obviously, in this experiment the transplanted cells reach the stage of terminal differentiation simultaneously and immediately are attacked. Whereas poorly differentiated melanoma cells escape the immune system and are able to divide indefinitely, terminally differentiated cells, neoplastically transformed or not, provide a target for macrophages. The immune system obviously is not determined to destroy poorly differentiated cells and this concerns cancer cells and stem cells of the different tissues, present in an organism.

Regarding cancer treatment under these aspects, it has to be concluded that terminal differentiation is the first step in tumor regression, rendering the cells prone to attacks by the immune system. Differentiating agents (retinoic acids and others) therefore are very effective in tumor therapy.

It follows that differentiation is the most important process in the development and therapy of cancer. The key goal of ongoing cancer research is the development of sensitive methods for detecting genetic and molecular changes in malignant tissues, in the hope that these methods will permit earlier diagnosis of the disease by tumor markers.

Acknowledgements

I thank the president of the Justus-Liebig-University Giessen and Prof. Dr.

Renkawitz of the Genetics Institute for their support. My thanks also go to Dr. J. Michel and Dr. M. Henze for valuable discussions and their help in the preparation of the manuscript and Prof. Dr. R. Glaser for reading the manuscript.

References

1. Anders A, Anders F. Etiology of cancer as studied in the platyfish-swordtail system. Biochim Biophys Acta 516:61-95, 1978.
2. Anders A, Anders F., Klinke K. Regulation of Gene Expression in the Gordon-Kosswig Melanoma System, In: Schröder J ed, Genetics and Mutagenesis of fish, Berlin, 1973, pp 33-63.
3. Anders, F, Schartl M, Barnekow A, Schmidt CR, Lüke W, Jaenel-Dess G, Anders A. The genes that carcinogenes act upon. Hamatol Blutransfus 29: 228-251, 1985.
4. Roushdy J, Michel J, Petry H, Anders A, Anders F. Paragenetic suppressors of suppressorgenes - a new class of oncodeterminants. J Cancer Res Clin Oncol 125: 123-133, 1999.
5. Zechel C, Schleenbecker U, Anders A, Anders F. v-*erb*B related sequences in Xiphophorus that map to melanoma determining Mendelian loci and overexpress in a melanoma cell line. Oncogene 3: 605-617, 1988.
6. Wittbrodt, J, Adam D, Malitschek B, Mäueler W, Raulf F, Telling A, Robertson SM, Schartl M. Novel putative receptor tyrosine kinase encoded by the melanoma-inducing *Tu* locus in Xiphophorus. Nature 34:415-421, 1989.
7. Ahuja MR, Schwab M, Anders F. Linkage between a regulatory locus for melanoma cell differentiation and an esterase locus in Xiphophorus. J Hered 71:403-407, 1980.
8. Kazianis S, Gutbrod H, Nairn RS, Mcintire B, Della Colleta L, Ronald B, Richard W, Borowsky L, Woodhead AD, Setlow RB, Schartl M: Localisation of a CDKN2 gene in linkage group V of Xiphophorus Fishes defines it as a candidate for the Diff tumor suppressor. Genes, Chromosomes & Cancer 22:210-220, 1998.
9. Kazianis S, Gan L, Della Coletta L, Santi S, Morizot DC, Nairn RS. Cloning and comparative sequence analysis of TP53 in Xiphophorus fish hybrid melanoma models. Gene 212:31-38, 1998.
10. Anders F, Schartl M, Barnekow A, Anders A. Xiphophorus as an in vivo model for studies on normal and defective control of oncogenes, Adv Cancer Res 42:191-275, 1984.
11. Anders F, Diehl H, Scholl E. Differentiation of normal melanophores and neoplastically transformed melanophores in the skin of fishes of genus Xiphophorus. In: Spearman R, Riley PA eds, The Skin of Vertebrates, London, Academic Press, 1980, pp 211-218.
12. Schartl M. Experimentelle Modulation der Zelldifferenzierung neoplastisch transformierter Zellen. Doctoral Thesis, Giessen, 1979.
13. Anders A, Dess G, Nishimura S, Kersten HA. Molecular approach to the study of malignancy and benignancy in melanoma of Xiphophorus, In: Bagnara J, Klaus SN, Paul E, Schartl M eds, Pigment Cell 1985, Tokyo, University of Tokyo Press, 1985, pp 315-324.
14. Li Z-R, Hombach R, Mundipalli A, Bloch A. Tumor suppressor proteins as regulators of cell differentiation. Cancer Res 58:4282-4287, 1998.
15. Kersten H. Modified nucleosides and Cancer, In: Nass G ed, Recent Results in Cancer Research, Berlin, Heidelberg, New York, Springer, 1983, pp. 255-263.
16. Anders A, Gröger H, Anders F, Zechel Ch, Smith A, Schlatterer B, Discrimination of initiating and promoting carcinogens in fish. Ann Rech Vet 22:273-294, 1991.
17. Stich HF, Anders F. The involvement of reactive oxygen species in oral cancers of bethel quid/tobacco chewers. Mutation Res 214:47-61, 1989.
18. Anders A, Gröger H, Fleming Ch, Schneider H, Reuschler A, Anders F. Development and regression of neoplasia in the Platyfish-Swordtail-Model. Proceedings XVI Internatl Cancer Congr New Delhi 1994, Vol 1, Bologna, Monduzzi Editore, pp 133-13, 1995.
19. Anders A, Fleming Ch, Roushdy J, Henze M. Benignancy and malignancy. Anticancer Res 15:1649, 1995.

20. Siciliano MJ, Perlmutter A. Maternal effect on development of melanoma in hybrid fish of the genus Xiphophorus. J Cancer Inst 49 (2): 415-421, 1972.
21. Siciliano MJ, Perlmutter A, Clark E. Effect of sex on the development of melanoma in hybrid fish of the genus Xiphophorus. Cancer Res 31 (6): 725-729, 1971.
22. Anders F. Contributions of the Gordon-Kosswig melanoma system to the present concept of neoplasia. Pigment Cell Res 3: 7-29, 1991.
23. Anders A, unpublished data, 2000

Correspondence
Dr. Annerose Anders
Genetics Institute
Justus-Liebig-University Giessen
Heinrich-Buff-Ring 58
D-35392 Giessen
Germany
Tel.: +49-641-99-35475
Fax: +49-641-99-35469