Ligand-dependent tumor induction in medakafish embryos by a X*mrk* receptor tyrosine kinase transgene

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X*mrk* encodes a subclass I receptor tyrosine kinase (RTK) which has been cloned from the melanoma-inducing locus *Tu* of the poeciliid fish *Xiphophorus*. To demonstrate a high oncogenic potential *in vivo* we transferred the gene into early embryos of the closely related medakafish. Ectopic expression of the X*mrk* oncogene under the control of a strong, constitutive promoter (CMVTk) led to the induction of embryonic tumors with high incidence, after short latency periods, and with a specific pattern of affected tissues. We demonstrate ligand-dependent transformation *in vivo* using a chimeric receptor consisting of the extracellular and transmembrane domains of the human EGF receptor (HER) and the cytoplasmatic domain of Xmrk. Expression of the chimeric receptor alone does not lead to kinase activation or induction of tumors. Coexpression of the chimera with its corresponding ligand, human transforming growth factor alpha (hTGFα), however, results in the activation of the chimeric RTK. In injected fish embryos the induction of the neoplastic growth is observed with similar incidence and tissue distribution as in embryos carrying the native X*mrk* oncogene suggesting that the ligand as well as factors downstream of the RTK are required for tumor formation. In this study we show single-step induction of tumors by ectopic expression of RTKs *in vivo* substantiating the significance of autocrine stimulation in RTK induced tumors in vertebrates.

Introduction

Receptor tyrosine kinases (RTKs) have been implicated in cell transformation and human cancer. While activating mutations in RTK-derived oncogenes appear to correlate with their transforming potential, the most common cellular lesion found in human cancers seems to involve autocrine activation of overexpressed receptors (Ullrich & Schlessinger, 1990): many tumors and tumor-derived cell lines have been found to coexpress growth factors and their receptors. However, the functional significance of this correlative observation for the primary steps in tumor development remained to be elucidated. It was shown that NIH3T3 cells overexpressing the human epidermal growth factor (EGF) receptor (HER) developed a fully transformed phenotype *in vitro* that required both functional HER expression and the presence of EGF in the growth medium (DiFiore *et al.*, 1987; Velu *et al.*,

1987). Experiments *in vivo* demonstrating ligand-dependent tumor induction by an overexpressed RTK, thereby substantiating the concept of autocrine stimulation of tumor cell growth, are still lacking. To address the significance of autocrine activation of RTKs in the induction of cancer, we used the X*mrk*-RTK oncogene from the poeciliid fish *Xiphophorus maculatus*.

Melanoma formation in *Xiphophorus* is a paradigmatic case for hereditary cancer (Friend, 1993). Since 60 years this model has been used for the analysis of the factors that are instrumental for tumor formation. Early genetic work resulted in the definition of a melanoma-inducing locus, *Tu*, under the control of a regulating tumor suppressor locus, *R* (Ahuja & Anders, 1976). The X*mrk* gene, which was identified by methods of positional cloning (Wittbrodt *et al.*, 1989), is encoded by *Tu*. It is required for tumor formation, as insertional disruption of this oncogene abolishes its melanoma-inducing capacity. The structural analysis of the X*mrk* oncogene compared to its proto-oncogenic counterpart, as well as studies on expression and transcriptional control have indicated that overexpression of X*mrk* may be the prerequisite mechanism for its oncogenic activity (Adam *et al.*, 1991, 1993). This poses the question whether overexpression of X*mrk* by itself is sufficient to evoke the full neoplastic phenotype. As the ligand for the Xmrk RTK is unknown, we have previously analysed a chimeric receptor consisting of the HER extracellular and transmembrane domains and the Xmrk tyrosine kinase domain. Experiments *in vitro* and in tissue culture showed the ligand dependent activation of the Xmrk kinase (Wittbrodt *et al.*, 1992). In order to correlate such ligand dependent RTK activation *in vitro* with the process of neoplastic transformation and tumor induction *in vivo* we have attempted to perform gene transfer of the chimeric receptor in conjunction with its activating growth factor.

Small aquarium fish species such as the medaka or the zebrafish, because of their translucent embryos, offer the possibility to study the mechanisms of normal embryonic development (Marcey & Nüsslein-Volhard, 1986; Rossant & Hopkins, 1992). They are similarly suited to study phenomena that appear during this early phase of ontogenesis. Based on the assumption that overexpression of RTKs and their corresponding ligands, both of which are presumed to participate in regulating normal embryogenesis, might lead to phenotypic effects that occur in early phases of development, gene transfer studies were performed in the medakafish (*Oryzias latipes*). Cytoplasmic injection of foreign genes into early embryos led to transient

expression of the encoded proteins that was promoter-specific and stable for up to 4 weeks (Chong & Vielkind, 1989; Winkler *et al.*, 1991, 1992). This experimental system allows an efficient analysis of a large number of animals expressing the transgene during the entire embryogenesis and larval development.

In this work we have used gene transfer in a small aquarium fish species to show single-step induction of tumors of the RTK oncogene X*mrk* and growth factor-dependent tumorigenesis by a HER/Xmrk chimeric RTK coexpressed with a cognate ligand, human tumor growth factor α (hTGFα).

Results

Transient expression of foreign DNA in fish embryos in vivo

In previous studies we showed that plasmid DNA injected cytoplasmatically into one cell of the two-cell stage embryo of medakafish persists extrachromosomally throughout embryonic and larval development (Winkler *et al.*, 1991, 1992). Due to the uneven distribution of the injected DNA during rapid cell divisions in the early fish embryo, the spatial pattern of reporter gene expression was highly mosaic. Injection of reporter genes revealed specific temporal expression patterns strictly dependent on the promoter-enhancer combinations used. This work defined the cytomegalovirus enhancer added to the Herpes simplex virus thymidine kinase promoter (CMVTk) as a strong promoter driving the expression of reporter genes at invariably high levels from late blastula onwards to posthatching stages. In order to define the spatial pattern of the CMVTk enhancer-promoter activity and to determine the degree of mosaicism of expression we injected the lacZ reporter gene under the control of CMVTk and analysed the spatial expression patterns by immunohistochemistry in embryos of early organogenesis stage (Figure 1). Whole mount immunofluorescence staining with an anti-βGal antibody revealed expression of the injected gene in small groups of cells of the embryo. Screening large numbers of embryos we could not find any restriction of CMVTk activity to specific cell types or organs of the developing embryo. Moreover we found ubiquitous activity of this heterologous promoter *in vivo*, e.g. in cells of the developing retina, in neurons of the CNS or in groups of epidermal cells (Figure 1).

Ectopic expression of Xmrk oncogene leads to neoplastic lesions during embryogenesis of medakafish

To address the question if overexpression of X*mrk* alone is sufficient for tumor induction, we constructed a minigene under the control of the cytomegalovirus enhancer/thymidine kinase promoter (CMVTk) consisting of X*mrk* cDNA sequences isolated from melanoma cells that were fused within the kinase domain to the corresponding 3′ genomic sequence (Figure 2). When compared with the control, pCMVTk Xmrk-injected embryos exhibited a considerably increased frequency of early embryonic lethality, abnormal development, and malformations (Table 1). Abnormalities generally resulted from disorganization of developing organs,
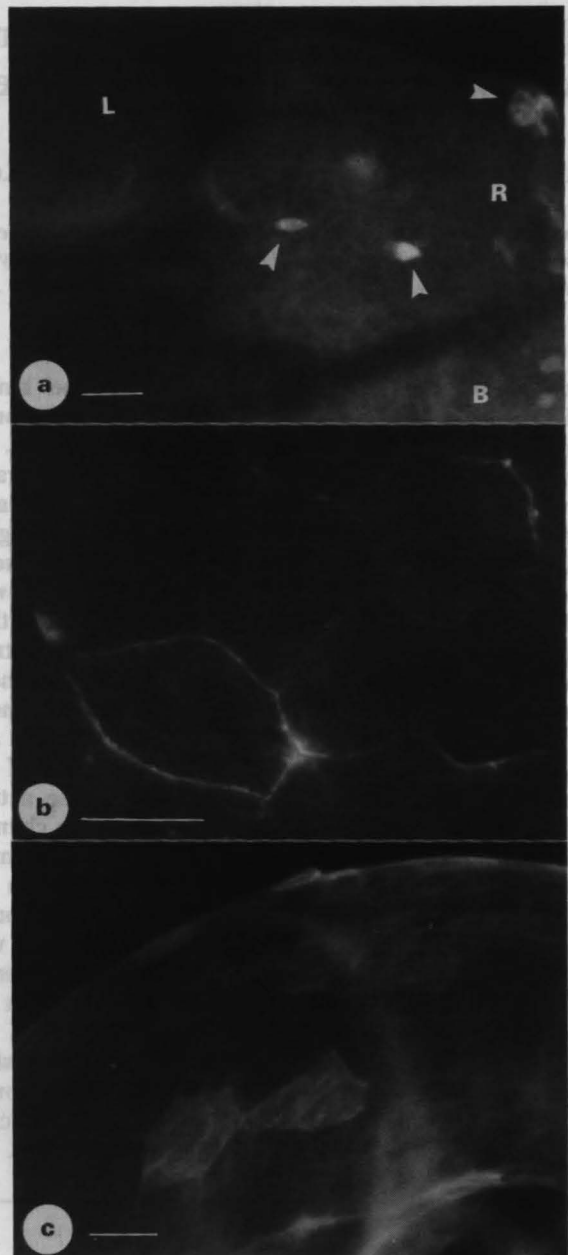

Figure 1 Transient expression of the lacZ reporter gene in 3 day old medaka embryos, that were injected cytoplasmatically at the two-cell stage with an expression vector containing the lacZ gene under the control of the cytomegalovirus (CMV) enhancer in combination with the Herpes simplex virus thymidine-kinase (Tk) promoter. Immunostaining of whole mount preparations using a monoclonal anti-lacZ antibody shows single lacZ expressing cells in the developing retina (a: marked by arrowheads), groups of expressing neuronal cells in the embryonic brain (b) and clusters of epidermal cells in the trunk region (c) of a medaka embryo at the early organogenesis (stage 28). L: lens; R: retina; B: brain. Scale bars represent 25 μm (a,b) and 50 μm (c)

primarily affecting the brain and eye. The apparent predominance of effects on the development of certain organs may possibly be interpreted on the basis of the specific differential expression characteristics of the corresponding endogeneous X*mrk* proto-oncogene during embryogenesis (Wittbrodt *et al.*,1989; Winkler & Schartl, manuscript in preparation).

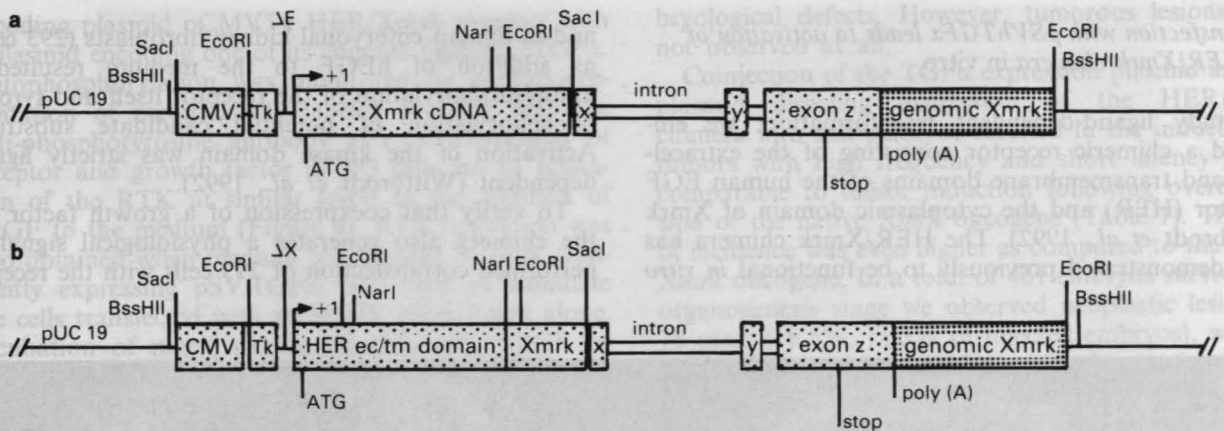In addition to embryonic lethality and abnormal organ development, we observed the formation of

Figure 2 Receptor expression constructs used for injection containing the cytomegalovirus (CMV) enhancer in combination with the Herpes simplex virus thymidine-kinase (Tk) promoter. (a) Map of pCMVTk Xmrk. The 5′ translated region of the Y-chromosomal cDNA encoding the Xmrk receptor tyrosine kinase isolated from melanoma cells was fused within the kinase domain to the corresponding genomic sequence. The original 5′ EcoRI restriction site is absent due to blunt end ligation, as indicated (ΔE). (b) Map of pCMVTk HER/Xmrk. To obtain expression of a chimeric RTK, a 2.2 kb XbaI/NarI cDNA fragment encoding the extracellular and transmembrane domain of the human EGF receptor was introduced into the vector described in a to replace the corresponding Xmrk domains (for details see Materials and methods). The original 5′ XbaI site is absent, as indicated (ΔX)

Table 1 Tumor induction in fish embryos after gene transfer of the X*mrk* oncogene and cotransfer of the HER/Xmrk chimera and hTGFα

	Total	Early embryonic lethals	Malformations and early abnormal development	Neoplastic growth
pCMVTk Xmrk	528	188 (35.6%)	108 (20.5%)	45 (8.5%)
pCMVTk HER/Xmrk	278	24 (8.6%)	27 (9.7%)	4 (1.4%)
pCMVTk HER/Xmrk and pSV hTGFα	659	198 (30.0%)	150 (22.8%)	76 (11.5%)
pSV hTGFα	199	64 (32.2%)	45 (22.6%)	0 (−)
pCMVTk CAT	122	8 (6.6%)	15 (12.3%)	0 (−)*

*In earlier experiments with more than 3000 medaka embryos injected with constructs containing the bacterial reporter genes CAT and lacZ in combination with various promoter-enhancer sequences, including SV40, CMV and RSV, not a single tumor was detected. Under the conditions used expression of the transgene occurs in 95% of the injected embryos (Winkler *et al.*, 1991, 1992). Roughly equal copy numbers of each construct were injected according to the procedure described (Winkler *et al.*, 1991)

Table 2 Developing organs affected by neoplastic growth

	Brain	Retina	Integument	Pigment cells	Epithelia*	Total
pCMVTk Xmrk	6	7	8	1	23	45
pCMVTk HER/Xmrk and pSV hTGFα	4	11	10	2	49	76
						120

*Cysts and hyperplastic growth

tumorous lesions at a high frequency (tumors in 45 (13.2%) of 340 embryos surviving at organogenesis stage; 8.5% of injected embryos). None of these phenomena have been observed in uninjected or control plasmid-injected embryos (Table 1). pCMVTk Xmrk-injected embryos developed reproducibly abnormal outgrowths at the late organogenesis stages (5–6 days of development), which originated at different sites and in different embryonal organs (Figure 3, Table 2). Approximately half of the lesions represented epithelial hyperplasias of sometimes remarkable dimensions, reaching in some cases up to about 15% of the entire embryo size. They occurred mainly in the integument, but sometimes also in the interior, where they impeded the normal development of surrounding tissues and organs.

Interestingly, tumors occurred only in those organs

that were found to express the X*mrk* proto-oncogene, like the developing brain, the embryonic retina and the integument (Winkler & Schartl, manuscript in preparation), although expression of the transgene protein product was detected immunohistochemically in tumorous as well as in no tumorous embryonic tissues. Such tissues included also those that normally do not express the X*mrk* proto-oncogene (Figure 3). This confirms the ubiquitous activity of the promoter used for X*mrk* transgene expression. To test, whether the restriction of tumor development to specific tissues is due to the localized presence of an activating growth factor and/or to the restricted function of the downstream signalling pathway in specific tissue types, we studied the ligand dependency of tumor formation by ectopically expressing a HER/Xmrk chimeric receptor together with its activating ligand, hTGFα.

Cotransfection with pSVhTGFα leads to activation of the HER/Xmrk chimera in vitro

To study ligand-dependent transformation, we employed a chimeric receptor consisting of the extracellular and transmembrane domains of the human EGF receptor (HER) and the cytoplasmic domain of Xmrk (Wittbrodt et al., 1992). The HER/Xmrk chimera has been demonstrated previously to be functional *in vitro*

and in human embryonal kidney fibroblasts (293 cells), as addition of hEGF to the medium resulted in autophosphorylation of the receptor itself and tyrosine phosphorylation of potential candidate substrates. Activation of the kinase domain was strictly ligand-dependent (Wittbrodt et al., 1992).

To verify that coexpression of a growth factor and the chimera also generates a physiological signal we performed cotransfection of 293 cells with the receptor
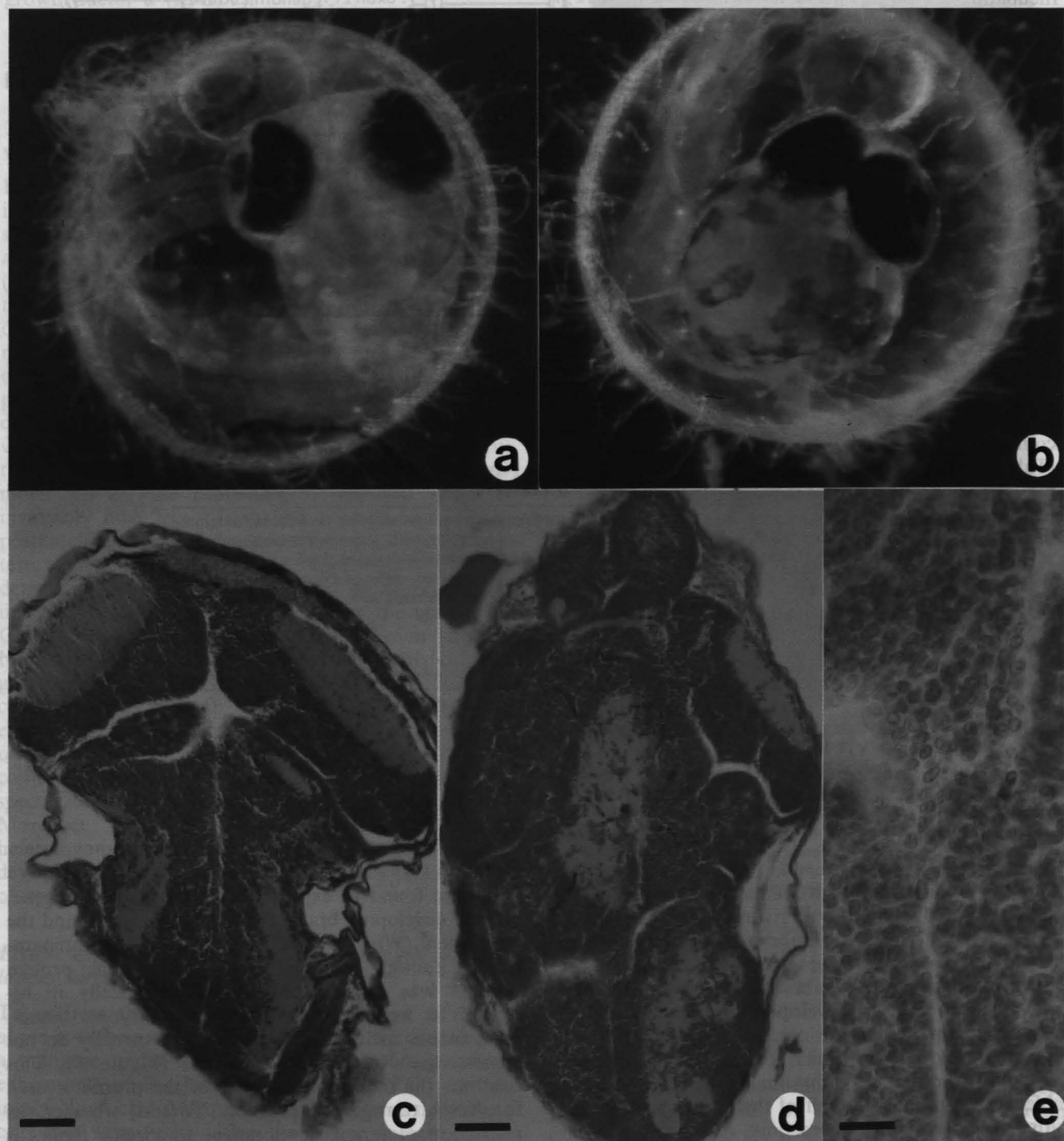
Figure 3 Noninjected control (a,b,c) and X*mrk* transgenic (b,d,e) medaka embryo (stage 35) that was injected cytoplasmatically with pCMVTk Xmrk into one cell of the two-cell stage embryo. Note expansive growth of the developing brain in the X*mrk*-injected embryo (b), leading to a latero-rostral dislocation of the eyes. Parafrontal sections (c,d) revealed uncontrolled growth in the dorsal region of the brain (see d), leading to distortion of the normal architecture of the central nervous system. Proliferation of undifferentiated cells disrupted the ventricular system (e). In frontal sections the morphology is essentially normal in the more ventral region of the brain, except for the dislocated eyes. Scale bars represent 50 μm (c,d) and 10 μm (e)

encoding plasmid pCMVTk HER/Xmrk together with a plasmid encoding one of its cognate ligands, hTGFα. Autophosphorylation was monitored by immunoprecipitation of metabolically labeled proteins with the anti-phosphotyrosine antibody αPY. Coexpression of receptor and growth factor in 293 cells led to activation of the RTK at similar levels as the addition of hEGF to the medium (Figure 4). RTK activation was also obtained when medium conditioned by cells, transiently expressing pSV TGFα, was used to stimulate the cells transfected with pCMVTk HER/Xmrk alone. Incubation of mock transfected cells with hEGF or TGFα conditioned medium did not result in any detectable phosphorylation of proteins in the size range of the receptor chimera (165 kDa). These data showed that both vectors intended to be used for gene transfer experiments into fish embryos express functional proteins and are suitable to obtain ligand dependent receptor activation. This provides the tools to activate autologous Xmrk signaling pathways in the cells of the injected embryo by stimulation with ectopically coexpressed human TGFα.

Ligand dependent induction of embryonic tumors by ectopic coexpression of HER/Xmrk and hTGFα in vivo

Injection of pCMVTk HER/Xmrk alone into two-cell stage medaka embryos resulted in a dramatic decrease of phenotypic effects during early embryo development and in a significant reduction in tumor incidence when compared with embryos injected with pCMVTk Xmrk (Table 1). The chimeric receptor, however, was abundantly and ubiquitously expressed, as shown by immunohistochemistry (Figure 5). We observed tumorous lesions only in four out of 278 injected embryos all of which were characterized as epithelial hyperplasia of the integument ('cysts').

Expression of the corresponding ligand hTGFα alone resulted in a high incidence of early em-

IP: αPY

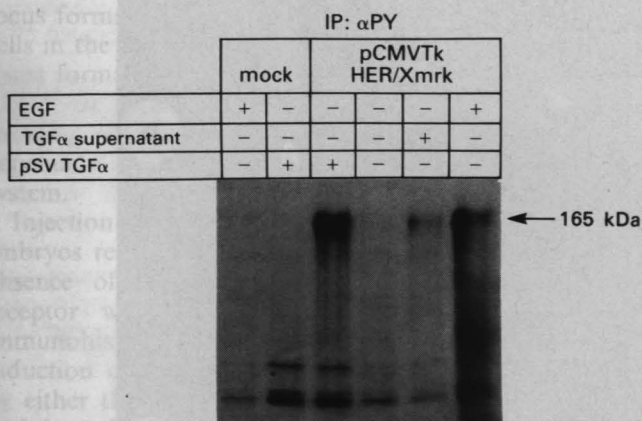|	mock		pCMVTk HER/Xmrk			
EGF	+	−	−	−	−	+
TGFα supernatant	−	−	−	−	+	−
pSV TGFα	−	+	+	−	−	−

← 165 kDa

Figure 4 Ligand dependent receptor autophosphorylation *in vitro*. Equal numbers of 293 cells untransfected ('mock'), transfected with pCMVTk HER/Xmrk alone or cotransfected with pSV TGFα were metabolically labeled with [^{35}S]methionine and were incubated in the presence (+) or absence (−) of 50 ng ml^{-1} EGF or with the supernatant of 293 cells transiently transfected with pSV TGFα. The cell lysates were used for immunoprecipitation with a monoclonal antibody directed against phosphotyrosine ('αPY'). The molecular weight of the signal representing the phosphorylated form of the receptor chimera is indicated in kDa

bryological defects. However, tumorous lesions were not observed at all.

Coinjection of the TGFα expression plasmid and the plasmid directing expression of the HER/Xmrk chimera into fish embryos resulted in the induction of tumors with high frequency and short latency period comparable to tumor induction following overexpression of the native Xmrk oncogene (Table 1). The rate of incidence was even higher as compared to the native Xmrk oncogene. In a total of 461 embryos surviving at organogenesis stage we observed neoplastic lesions in 76 cases (16.5%; 11.5% of injected embryos), whereas the incidence was 13.2% with the native Xmrk oncogene. Despite the ectopic expression of the activating ligand, the tumors obtained in these experiments were of the same histiotype as observed in Xmrk injected embryos (Table 2). As with Xmrk more than half of the lesions observed in the coinjected embryos affected epithelia by cystic and hyperplastic growth.

Discussion

We have used a novel experimental system to study the function of oncogenes in vertebrates by a transgenic model. Small aquarium fish, like the Japanese medaka (*O. latipes*), provide a promising and powerful system to analyse the regulation and functional significance of potentially oncogenic genes for the process of tumor formation. They are easy to breed in the laboratory, have a short generation time and produce large amounts of eggs perfectly suited for gene transfer by microinjection. The high transparency of the externally developing embryos allows an easy observation *in situ* at the single cell level of the complete embryonic development of large numbers of embryos. Cytoplasmic injection of plasmid DNA into one cell of the two-cell stage medaka embryo results in transient expression of the injected gene during embryogenesis with a spatial expression pattern, that is highly mosaic. This provides an important advantage of transient transgenic systems compared to stable transgenic animals when oncogenes are analysed for their function in spontaneous tumorigenesis (Weinberg, 1989). In stable transgenic animals virtually all cells of a given tissue express the activated oncogene instead of small isolated groups of activated oncogene-bearing cells that interact with non-transformed neighbour cells. The mosaic pattern of expression in the transient medaka expression system, however, mimics the actual situation of tumorigenesis *in situ*, where an initiated cell or cell clone is in contact with a normal, non-transformed environment.

In this study, we demonstrate that the Xmrk gene which has previously been shown to be required for melanoma formation in the poeciliid fish Xiphophorus is also sufficient for induction of the neoplastic phenotype of certain cells. Using gene transfer into early fish embryos we could show that ectopic expression of this RTK under the control of a strong, ubiquitously active promoter resulted in the induction of tumorous growth already during embryonic development. The short latency period of 4−6 days and the high frequency of tumor incidence, which ranged from 10−20% in independent experiments (Table 1 and data
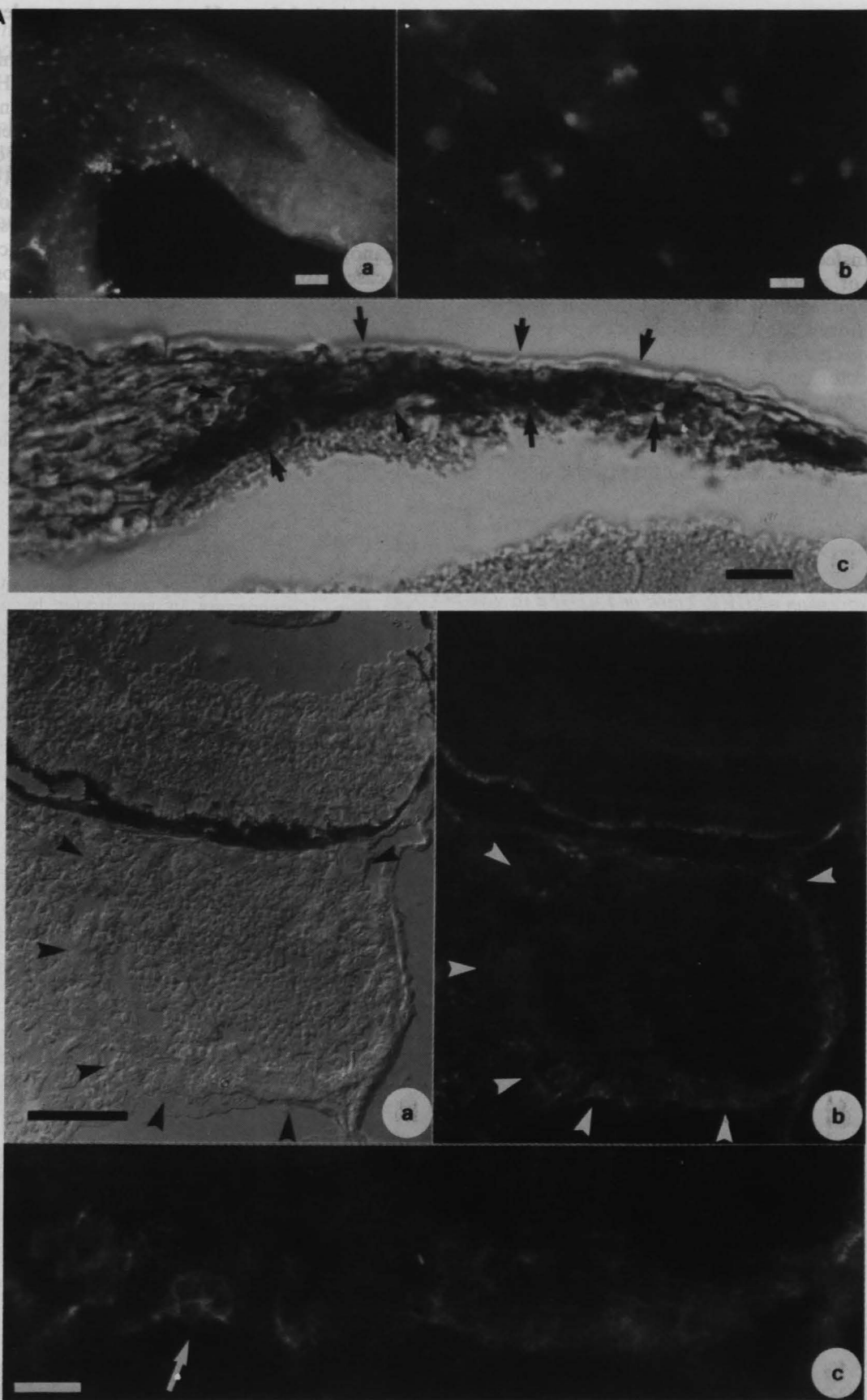
Figure 5 (A) Immunohistochemical detection of HER/Xmrk protein expression in non-tumorous medaka embryos that were injected with pCMVTk HER/Xmrk at the two-cell stage. (a) Whole mount preparation of the trunk region of an injected embryo, stage 28. The non-homogeneous pattern of expression is due to the mosaic distribution of the injected DNA as a general feature of the transient *in vivo* expression system (Winkler *et al.*, 1991, 1992). (b) Higher magnification view of a showing single mesenchymal cells expressing the chimeric receptor. (c) Paraffin section of the peduncular region of a tumor free stage 33 embryo. Arrows indicate the cell surface expression of the transgene protein in clusters of mesenchymal cell. Scale bars represent 50 μm (a) and 10 μm (b,c). (B) Tumor-bearing medaka embryo (stage 34) coinjected with pCMVTk HER/Xmrk and pSV TGFα at the two-cell stage showing nodular tumorous growth caudal of the right eye. (a) Nomarski image of a cryostat frontal section depicting an

not shown), suggests a dominant role for the X*mrk* gene in the oncogenic events leading to tumor formation. The results with many other oncogenes tested in transgenic animals are in line with a multistep process of cancer development, involving several mutational events that are necessary for induction and maintenance of the neoplastic phenotype (reviewed in Vogelstein & Kinzler, 1993). In contrast, our results provide evidence that an activated RTK oncogene alone might lead to cancer with only few, if any additional events required. Our findings are consistent with the effect of an activated *neu* oncogene in transgenic mice, which at high frequency led to the development of mammary tumors (Muller *et al.*, 1988). This confirms the possibility of a dominant role for tyrosine kinase oncogenes in tumorigenesis. Overexpression of the nonactivated, proto-oncogenic form of *neu* also resulted in tumors in transgenic mice, however with frequencies lower and latency periods longer than with the activated form (Guy *et al.*, 1992), indicating that overexpression of a RTK alone might not be sufficient for the inductive event. At present, it remains unclear, whether in our studies the transforming capacity of the overexpressed X*mrk* was due to activating mutations in the oncoprotein or to the simultaneous expression of the as yet unidentified X*mrk* ligand in embryonic tissues. The X*mrk* oncogene shows a low level of constitutive autophosphorylation activity after transfection into heterologous expression systems (Wittbrodt *et al.*, 1992). Whether this is the effect of an activating mutation in the extracellular or transmembrane domains, and what the significance for tumor induction might be, can only be answered after the X*mrk* proto-oncogene is available for comparative expression studies.

To approach the question of ligand dependent tumor induction *in vivo* we analysed the tumorigenic capacity of a receptor chimera consisting of the HER extracellular and transmembrane domain and the Xmrk kinase. Our previous studies had shown that this receptor, combining mammalian with fish domains, is functional *in vitro* (Wittbrodt *et al.*, 1992). Moreover, this chimera showed a high transforming activity in the focus formation assay, when transfected into NIH3T3 cells in the presence of the inducing ligand, hEGF. No focus formation was observed without the addition of hEGF or after transfection of the wildtype Xmrk, showing that transformation by this RTK is ligand-dependent in the context of a heterologous *in vitro* system.

Injection of the chimeric receptor into early medaka embryos resulted in a very low tumor incidence in the absence of its corresponding ligand. However, the receptor was abundantly expressed, as shown by immunohistochemistry (Figure 5). The low frequency induction of tumorous lesions may have been caused by either the presence of a fish growth factor binding with low affinity to the HER extracellular domain, thus activating the Xmrk cytoplasmic domain, or the ligand-independent activation of the RTK signaling

potential due to high level overexpression. Embryos that were injected with pSVhTGFα alone exhibited a high incidence of early embryological defects. This suggested the possibility of human TGFα activation of an endogeneous RTK that is crucial in early developmental stages. No tumors, however, were detected in later embryonic stages, larvae or young fish. In the mouse, ectopic expression of TGFα after gene transfer into the germ line led to predisposition for the development of tumors of various histological types (Jhappan *et al.*, 1990; Matsui *et al.*, 1990; Sandgren *et al.*, 1990; Takagi *et al.*, 1992). Tumor development, however, only occurred in adult animals, which suggested the necessity of a second event in addition to growth factor overexpression.

Coexpression of the HER/Xmrk receptor and hTGFα resulted in the induction of tumorous growth with a high incidence and short latency period comparable to the results obtained by plasmid-mediated X*mrk* oncogene overexpression (Table 1). The tumors observed in both sets of experiments showed the same tissue distribution (Table 2). In the *neu*-oncogene carrying transgenic mice the tissue specificity of malignant tumor development, namely restriction to the mammary gland, was due to the promoter specificity directing *neu* expression, although an influence of the tissue context was noted (Muller *et al.*, 1988). However, in the experiments reported here, an ubiquitously active promoter was used, that led to RTK expression in a much wider spectrum of cell types and tissues than those finally involved in tumorous lesions. This suggests that activation of the cell-specific signaling pathways through X*mrk* or the HER/X*mrk* by either the endogenous X*mrk* ligand or ectopically expressed TGFα, respectively, caused the pathogenic effects. The identification and characterization of the X*mrk* ligand, as well as the signal transduction machinery downstream of this RTK will help to understand the activating mechanisms of a RTK, that results in the induction of tumor growth in a single step.

Our results clearly demonstrate the potential of RTKs in the *in vivo* situation, under conditions of abnormal overexpression and autocrine activation, to induce malignant transformation of normal cells in an intact organism. This provides direct evidence for a mechanism that has been proposed to be involved in the development of cancer in humans.

Materials and methods

Embryos

Medakafish (*Oryzias latipes*; Teleostei: Cyprinodontidae) were purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA). Adult fish were maintained under standard conditions (Kirchen & West, 1976) with an artificial photoperiod (10 h of darkness, 14 h of light) to induce reproductive activities. Clusters of fertilized eggs were collected 1–2 h after the onset of light and kept in

encapsulated tumor (marked by arrowheads) adjacent to the eye bulb. (b) Immunohistochemical detection of HER/Xmrk expression (marked by arrowheads) in the marginal zone of the lesion. (c) Higher magnification view of the marginal zone. Arrow indicates cell surface expression of HER/Xmrk. Scale bars represent 50 μm (a) and 10 μm (c). Sections were treated with a monoclonal antibody (MoAb 108.1) directed against the receptor domain of the human EGF receptor. Sections were either incubated with a secondary antibody coupled to FITC (A a,b; B b,c) or horseradish peroxidase (A c)

Yamamoto's saline (0.75% NaCl, 0.02% KCl, 0.02% CaCl$_2$ pH 7.3; Yamamoto, 1961) prior to injection. Injected medaka embryos were reared in a medium containing 0.1% NaCl, 0.003% KCl, 0.004% CaCl$_2 \times 2\,H_2O$, 0.016% MgSO$_4 \times 7\,H_2O$, and 0.0001% methylene blue and transferred to aquarium water immediately after hatching. Embryos were staged according to Kirchen and West (1976).

Expression plasmids

To analyse transient expression of reporter genes in vivo a plasmid was injected into fish embryos containing the β-galactosidase (lacZ) gene under the control of the cyto-megalovirus (CMV) enhancer in combination with the Herpes simplex virus thymidine-kinase (Tk) promoter. The lacZ gene together with a SV40 polyadenylation sequence was removed as XhoI/KpnI fragment from the vector pUC-lac20 (Michiels et al., 1989) and cloned into a pUC 118 derivative containing the CMVTk promoter/enhancer (Friedenreich & Schartl, 1990).

For expression of the Xmrk oncogene a cDNA containing the 5′ portion of the translated region of the Y-chromosomal encoded Xmrk isolated from melanoma cells was fused within the kinase domain (SacI site at 3016, see Wittbrodt et al., 1989) to the corresponding genomic sequence (SacI site at 809, see Adam et al., 1991) to improve the expression in vivo. This Xmrk minigene was cloned as a 7.6 kb EcoRI fragment in pBluescript II (Stratagene) under the control of the CMVTk promoter/enhancer. For expression of the chimeric RTK, a HER 2.2 kb XbaI/NarI cDNA fragment originating from CMVneoHER (Wittbrodt et al., 1992) was introduced in pCMVTk Xmrk to replace the 5′ extracellular and trans-membrane encoded domains of Xmrk.

Microinjection

Medaka embryos were injected cytoplasmatically into one cell of the two-cell stage embryo as previously described (Winkler et al., 1991). Approximately 500 pl (equivalent to 25 pg) of plasmid DNA were microinjected cytoplasmatically into one cell of the two-cell stage embryo. DNA was dissolved to a final concentration of 50 μg ml^{-1} in Yamamoto Ringer saline containing 0.1% phenol red. Plasmid-DNA was injected in either supercoiled or linearized conformation, however no significant differences in expression patterns or incidence of tumor formations have been observed (data not shown, and Winkler et al. (1992) for similar result with reporter gene constructs). For linearization the Xmrk minigene has been removed from prokaryotic vector sequences by digestion with BssHII (see Figure 2).

Histology

For histological examinations embryos were fixed for 24 h in Bouin's fixative (saturated picric acid in H$_2$O:formaldehyde (37%):glacial acidic acid 15:5:1), embedded in paraffin and thin sectioned. 10 μm sections were stained with acid alizarine blue/anilinblue-orange G and mounted in Entellan (Merck).

For immunohistochemistry embryos were fixed for 12 h at 4°C in 4% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4 (PB). During the washing procedure in PB (three times, 5 min each) chorions and yolk sacs were removed. The fixed embryos were incubated in 20% sucrose at 4°C over-night prior to embedding in OCT (Tissue Tek, Miles Inc.). 10 μm frozen sections were cut on a cryostat and stored at −20°C until staining. For peroxidase staining the air dried slides were incubated for 20 min in methanol:3% H$_2$O$_2$ (4:1) to block endogeneous peroxidase activity. To prevent un-specific antibody binding all slides were treated with 1%

normal horse serum in PB for 30 min at room temperature. The mouse monoclonal antibody MoAb 108.1, directed against the extracellular domain of the human EGF receptor (Lax et al., 1989) was added at a dilution of 1:1000 and incubated for 60 min. After washing with PB/1% horse serum (three times, 15 min each) sections were incubated with a biotinylated secondary horse antimouse IgG diluted 1:200 in PB/horse serum for 30 min. After washing slides were incubated in a 1:200 solution of avidin coupled to FITC or horseradish peroxidase in PB/horse serum for 30 min. In the latter case after washing with PB sections were stained for 5 min with diaminobenzidine (0.6 mg ml^{-1} in PB) sup-plemented with 0.03% H$_2$O$_2$ and counterstained with a 1% light green solution in water for 10 min.

For whole mount immunostainings, embryos at early organogenesis (stage 28) were fixed in paraformaldehyde as above. After washing in PB chorions and yolk sacs were removed. Embryos were incubated in a blocking solution containing 1% normal horse serum, 1% BSA, 1% DMSO and 1% Triton X-100 in PB for 30 min at 4°C with gentle shaking. The mouse monoclonal antibodies anti-β-galactosidase (Boehringer Mannheim) or 108.1 (Lax et al., 1989) were added in a dilution of 1:1000 and incubated for at least 12 h at 4°C with gentle shaking. After washing with blocking solution (four times, 30 min each), embryos were treated with a biotinylated secondary horse anti-mouse antibody (Vector laboratories)(1:200 in blocking solution) for another 12 h at 4°C with shaking. After washing as above embryos were finally treated with avidin-FITC (1:200 dilu-tion in blocking solution) for 3–6 h, washed and mounted in a medium containing 10% (w/v) polyvinylalcohol, 25% (w/v) glycerol and 2.5% 1,4-diazo-bicyclo-(2.2.2)-octane (DABCO) in 0.1 M Tris-Cl pH 8.5, to reduce fading.

Metabolic labeling and immunoprecipitation

293 human embryonic kidney fibroblasts were transfected with expression vectors as previously described (Wittbrodt et al., 1992). Cells were grown in DMEM containing 10% fetal calf serum. 24 h after transfection cells were incubated with 1 ml of methionine-free medium containing 1% dialyzed fetal calf serum, 2 mM L-glutamine, antibiotics and 50 μCi ml^{-1} [^{35}S]methionine per 3 cm dish. The incubation was continued for 16 h.

For detection of tyrosine phosphorylation, transfected and metabolically labeled cells were stimulated for 10 min in the presence of 100 ng ml^{-1} hEGF, TGFα and mock conditioned medium respectively. Proteins were immunoprecipitated after lysis with monoclonal antibody αPY as described (Wittbrodt et al., 1992). Briefly, after lysis of cells supernatants were incubated on ice for 5 min and then centrifugated at 14 000 g for 5 min. The supernatants were diluted 1:3 with HNTG (20 mM HEPES pH 7.2, 150 mM NaCl, 10% glycerol and 0.1% Triton X-100) supplemented with 100 mM NaF and 100 μm sodium orthovanadate and incubated with 4 μl αPY antiserum and 20 μl of protein A-Sepharose (1:1 slurry in HNTG buffer) for 2 h at 4°C. The immunoprecipitates were washed three times in 0.5 ml HNTG buffer. Samples were boiled for 5 min, centrifuged and analysed by fluorography after separation on 7.5% SDS-polyacrylamide gels.

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