ADGRE5 ACTS AS A MELANOMA TUMOR SUPPRESSOR IN NATURALLY HYBRIDIZING *XIPHOPHORUS*.

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Abstract

The *Xiphophorus* genus has long been studied for its development of melanoma. *xmrk* has been identified as a potent oncogene. The recent discovery of *adgre5* as a candidate tumor regulator gene in naturally hybridizing *Xiphophorus birchmanni* and *X. malinche* has shed new light on the genetic basis of melanoma. This study aimed to functionally test the role of the *adgre5* alleles from each hybridizing species by analyzing their effect independently on cell growth and migration *in vitro* and melanoma development *in vivo*. *In vitro* experiments showed that cells with the *X. birchmanni* allele grew and migrated slower than those with the *X. malinche* allele. *In vivo* experiments using transgenic medaka confirmed that melanoma development was only inhibited in the presence of the *X. birchmanni* allele of adgre5. These findings provide new insights into the genetic basis of melanoma development in Xiphophorus and highlight the importance of *adgre5* as a potential therapeutic target for the treatment of melanoma.

Introduction

The genus *Xiphophorus* is the oldest animal system for the study of melanoma. Almost a century ago, it was discovered that certain hybrids of the platyfish (*X. maculatus*) and swordtails (*X. hellerii*) develop a highly malignant melanoma (C 1928, G. 1928). From the classical crossing experiments, theoretically, there are two genetic explanations to how hybrid melanoma is generated: (i) certain individuals of the platyfish carry an oncogene controlled by a tumor suppressor. In this case, the oncogene effect on the platyfish is only a local dysplasia of melanocytes visible as a black pigment spot of macromelanophores. The oncogene and the tumor suppressor are located in different linkage groups, thus, when a platyfish hybridizes with a swordtail that lacks both genes, some individuals will express the oncogene without the control of the tumor suppressor and therefore develop malignant melanoma. (ii) instead of the platyfish having an oncogene repressed by a tumor suppressor, the platyfish has a pigmentation gene that interacts with a tumor enhancer from the swordtail. In that case, the pigmentation gene makes a minor contribution to pigment cell proliferation, only to become malignant once it interacts with the tumor enhancer (M 2008). Besides *X. maculatus* and *X. hellerii* there are several artificial hybrids withing the genus (reviewed in (Walter 2001) which have been used to study melanoma.

Biochemical data and transgenic experiments have identified *xmrk* as a potent tumor gene, whose expression is necessary and sufficient for tumor development in *Xiphophorus* (Schartl M 1999, Schartl M 2010). While the melanoma driving oncogene (*xmrk*) has been

identified and isolated almost thirty years ago (Wittbrodt J 1989, Wittbrodt J 1992), a candidate tumor regulator gene was only recently identified using the naturally hybridizing *X. birchmanni* and *X. malinche* system: *X. birchmanni* has *xmrk* and is polymorphic for a black spot in their caudal fin, comparable to the hyperplastic melanocyte spot of the platyfish. *X. malinche* lacks *xmrk* as well as this phenotype, and hybrids between them can develop melanoma. Powell et al. 2020 performed an admixture mapping study of these hybrids and revealed that melanoma occurs when *xmrk* interacts with *adgre5*, on chromosome 5 (Powell D 2020). (That region on chromosome 5 is also known as *cd97*. However, *adgre5* is the HUGO Gene Nomenclature Committee approved name, and will be used throughout the manuscript). *adgre5* is a member of the G- protein couple transmembrane receptors, with varied roles in cell adhesion and signaling (Yona S 2008). *adgre5* plays a vital role in the adhesion and migration of healthy immune cells, however, it is also a mediator of invasion in a variety of human cancers (reviewed in (Safaee M 2013)). Accordingly, *adgre5* is upregulated in RNA-seq data of invasive melanoma in *X. birchmanni-X. malinche* hybrids, and five aminoacid changes, including one that occurs in a conserved epidermal growth factor–like calcium binding site, exist between *X. birchmanni* and *X. malinche* (Powell D 2020), signifying its association with melanoma development.

The association between melanoma and ancestry at *xmrk* and *adgre5* is not random. To increase the risk of melanoma, there must be at least one copy of *X. birchmanni xmrk* and *adgre5* must be homozygous for the *X. malinche* allele. In fact, heterozygous individuals for *adgre5* had smaller spots than *X. malinche* homozygous individuals, which results in lower probability of becoming invasive. This result can be interpreted in two ways: (i) the *X. malinche* allele of *adgre5* is a tumor enhancer that interacts with *xmrk* and triggers a change from benign to malignant pigmentation, or (ii) malignant melanoma only arises in the absence of the tumor

suppressor *X. birchmanni* allele of *adgre5*. These two hypotheses, although they would produce the same pattern of ancestry associations, imply very different subjacent mechanisms explaining those patterns. Whereas the first hypothesis suggests the action of a tumor enhancer, the second one advocates for the presence of a tumor suppressor. These differences are crucial in the repercussions they may have for future research and therefore this study focuses on functionally testing the role of different *adgre5* alleles by analyzing their effect independently on cell growth and migration and *in vivo* tumor development.

Methods

Cell culture and generations of stable adgre5 expression lines

The murine melanocyte cell line (Melan-a) offers an excellent equivalent non-tumorigenic line for studies of the cellular and molecular basis of melanoma malignancy (Bennett D 1987), particularly for understanding the mechanisms triggering the change from benign pigmentation to malignant melanoma. Melan-a cells were cultured in DMEM with pyruvate (Gibco #), supplemented with 10% FCS, 1% penicillin/streptomycin and maintained at 37°C, 5% CO_2 with 100% humidity.

To generate doxycycline-inducible expression cell lines, the vector pSB-ET-iE (M. Gessler, Dept. of Developmental Biochemistry, University of Wurzburg) was used, which allows integration of genes by *sleeping beauty*-mediated transposition. Dox-inducible promoters enable

precise regulation of the expression of the targeted gene, facilitating elegant experimental designs. Here, the responsive T6 promoter drives expression of *adgre5* and EGFP, with an IRES site between them (Figure S1a). After transposition, these cells were selected with 1 μg/ml puromycin for 2 weeks.

X. malinche and *X. birchmanni* alleles of *adgre5* were amplified using primers with XbaI and ClaI restriction enzyme sites. PCR amplification was performed using cDNA from tissue samples of organisms of *X. birchmanni* and *X. malinche*. The High Fidelity Q5 (New England Bio Labs #M0491S) was used for PCR amplification. The respective restriction enzymes were used to clone the PCR product into the vector, and wild-type melan-a cells were transfected using Fugene transfection reaction (according to the manufacturer's protocol). As a result, pSB-ET-iE_*X. malinche_adgre5*, and pSB-ET-iE_*X. birchmanni_adgre5* stable cell lines were generated. No significant differences were observed in the expression levels of *adgre5* between melan-a cell lines containing the different alleles (Figure S2, ANOVA p value > 0.05 for differences between *X. birchmanni* and *X. malinche* adgre5 alleles).

RNA extraction, cDNA synthesis and qPCR

RNA extraction, cDNA synthesis, and qPCR were performed to measure the gene expression levels of *adgre5* in the different melan-a cell lines. Total RNA was extracted from freshly harvested cells or cell pellets that were stored at -80°C, using TRIzol reagent according to the manufacturer's protocol. DNase I digestion was carried out for 1 hour at 37°C to eliminate any contaminating DNA. RNA concentration was determined using a NanoDrop spectrophotometer. RevertAid First Strand cDNA Synthesis Kit and random hexamer primers were used to reversely transcribe 100-4000 ng of RNA according to the manufacturer's instructions. Fluorescence-based

RT-qPCR was performed using SYBR Green reagent and analyzed with a Mastercycler ep Realplex. Gene expression levels were normalized to a housekeeping gene (*Hprt* fwd: ACTGGCAACACTAACAGGACT *Hprt* rev: TGTTGTTGGATATGCCCTTG, *adgre5* fwd: CATCCGGCCCCTTTACTTGT *adgre5* rev: GGGCCAAAGAAGCTCCAGAT) using the delta-delta Ct method, and the success of transfection was confirmed by checking for GFP expression using an inverted fluorescence microscope with a 63x objective (Figure S1c). Three independent replicates of cells were used for each treatment and each cell line.

Cell growth assay

In triplicate, cells were counted and seeded at equal density (1-2 x 10^3 cells/well) in 96-well plates. Cells were treated with either dox 0 or dox 500 (500 mg/ml) by adding the respective dox concentrations to cell media. On days 2, 3, and 4 after treatment, 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a ratio of 1:5 (MTT:medium). The medium was aspirated after 2 hours of incubation at 37°C, and 150 µl of DMSO was added to each well. The plate was then incubated on a shaking device at room temperature for 15 minutes. A microplate reader was used to measure formazan accumulation by reading the optical density at 590 nm with a reference filter of 620 nm. Quintuples for each treatment, species, and duration of the experiments were performed. Cell growth was calculated by subtracting the optical density at 590 nm observed in dox 0 cells from the optical density at 590 nm observed in dox 500 induced cells. An ANOVA and a Tukey post hoc test were used to determine the statistical differences between *adgre5 X. birchmanni* and *X. malinche* alleles in cell growth.

Transwell migration assay

To assess cell migration, cells seeded at $2x10^3$ per well previously starved in 1% dialyzed FCS for 24 hours, were seeded in the upper layer of uncoated transwell inlays with 8 µm pore diameter in 24-well plates. The cells were treated with either dox 0 or dox 500 (500mg/ml) for 24 hours. To stimulate migration, medium containing 10% FCS and either dox 0 or dox 500 (500mg/ml) was added to the lower layer of the transwell, and the cells were allowed to migrate for 16 hours. Each assay was performed in triplicate per cell line and per dox treatment. Non-migrated cells were removed by cotton swabs, and the migrated cells were fixed with methanol, stained with 0.2% crystal violet dye in 2% ethanol for 15 minutes, and washed with PBS. The membrane was cut out of the transwell inlay, embedded with Mowiol (polyvinyl alcohol) on microscope slides, and images were captured. The migrated cells were counted under a microscope, and migration was calculated as the difference in the number of cells that migrated in dox 500-induced cells minus the number of cells that migrated in dox 0 cells.

Isolation of the *Xiphophorus adgre5* and construction of expression vectors with *tyrp fugu* promoter

Although the *mitf* promoter can drive the expression of a powerful oncogene (i.e. *xmrk*), it may not be suitable for a tumor modifier candidate gene. Additionally, recent evidence from Dr. Frederik Helmprobst (personal communication, paper in review) suggests that the *mitf* promoter is relatively weak. Therefore, *adgre5* was tested driven by a stronger pigment cell specific promoter: *Fugu* rubripes Tyrosinase-related Protein 1 gene (*tyrp*) (Zou J 2006). *Tyrp* is an enzyme required for melanin synthesis and is specifically expressed in melanocytes or melanophores(F 1996).

To isolate the *adgre5* gene from *Xiphophorus*, a high-fidelity PCR (using Q5 Taq enzyme) was performed from cDNA extracted from either *X. birchmanni* or *X. malinche* using overhang primers containing the XbaI restriction enzyme cutting site. Each cloning step was performed according to manufacturer's protocols and plasmids were extracted using Quiagen Mini Prep Kit. Plasmids were sequenced to verify that no mutations occurred during cloning.

mCerulean was extracted with its promoter and a bGH poly(A) site using KpnI restriction enzyme from the brainbow vector (kindly provided by Brigitta Wilde). The pIsceI-typr-pa 3 plasmid the containing the *tyrp fugu* promoter (kindly provided by Dr. Mateus Adolfi) was linearized with KpnI restriction enzyme and ligated them together. The resulting vector was subsequently digested with XbaI and ligated to the previously digested with XbaI *adgre5* PCR fragments. Thus, specific plasmids were created containing either the *X. malinche* or *X. birchmanni* alleles of *adgre5* under the expression of the *tyrp fugu* promoter (Figure S3)

Generation of stable transgenic medaka line

Animal models are imperative to evaluate their importance in the context of the whole organism. Moreover, the advantage of establishing transgenic organisms is that we can study the isolated effects of the induced gene without other unknown possible genetic interactions. To generate stable transgenic lines, the injection meganuclease protocol was used since it was demonstrated to be more effective than injecting just the plasmid, as it reduces mosaic expression, increases frequency of positive founder fish and increases germline transmission rates (Thermes et al., 2002). One-cell stage of the *tg*(*mitf*:*xmrk*) medaka embryos (*Oryzias latipes* strain: Carbio) were injected into the cytoplasm with approximately 15–20 pg of total DNA plasmid in a volume of 500 pl injection solution containing I-SceI meganuclease. Adult F0 fish

were mated with each other and the offspring were tested for the presence of the transgene by screening of blue eyes under UV light (mCerulean effect).

All animal studies have been approved by the authors' Institutional Review Board (Animal Welfare Officer of the University of Wurzburg). Adult fish were maintained under standard conditions (Kirchen and West, 1976) with an artificial photoperiod (10 hours of darkness, 14 hours of light) to induce reproductive activity. Clusters of fertilized eggs were collected 0.5–1 hour after the onset of light and kept in a rearing medium containing 0.1% NaCl, 0.003% KCl, 0.004% CaCl2 × 2H2O, 0.016% MgSO4 × 7H2O, and 0.0001% methylene blue.

F1 Fish (8 *tg*(*mitf*:*xmrk)+tyrp:Xmal*, and 7 *tg*(*mitf*:*xmrk)+tyrp:Xbir* were anesthetized in MS-222 and photographed with a Nikon D300 digital camera with a Tamron SP 90mm F/2.8 1:1 Macro lenses. Individuals were quantified for hyperpigmented melanin area from the images using ImageJ (Schneider et al., 2012). Data fit normality principles and therefore it was analyzed with an ANOVA and a Tukey post hoc test.

Results

To assess the potential role of *adgre5* as a tumor modifier gene and to discriminate between the action of a tumor enhancer or suppressor mechanism, cell growth and migration assays were performed. Tumor enhancer genes are expected to cause cells to grow and migrate more, whereas tumor suppressor genes are expected to cause the opposite effect. Melan- a cells transfected with the *X. birchmanni* allele of *adgre5* showed less growth than melan-a cells transfected with the *X. malinche* allele of *adgre5* after 3 and 4 days of cell growth (Figure 1a, ANOVA p value < 0.0001, Tukey post hoc pvalues, X.bir-X.mal day 2 = 0.49, X.bir-X. mal day

3 = 0.0006, X.bir-X.mal day 4 = 0.0019). Furthermore, Melan-a cells transfected with the *X. birchmanni* allele of *adgre5* migrated less than melan-a cells transfected with the *X. malinche* allele of *adgre5* (Figure 1 b, t test p value = 0.00582)

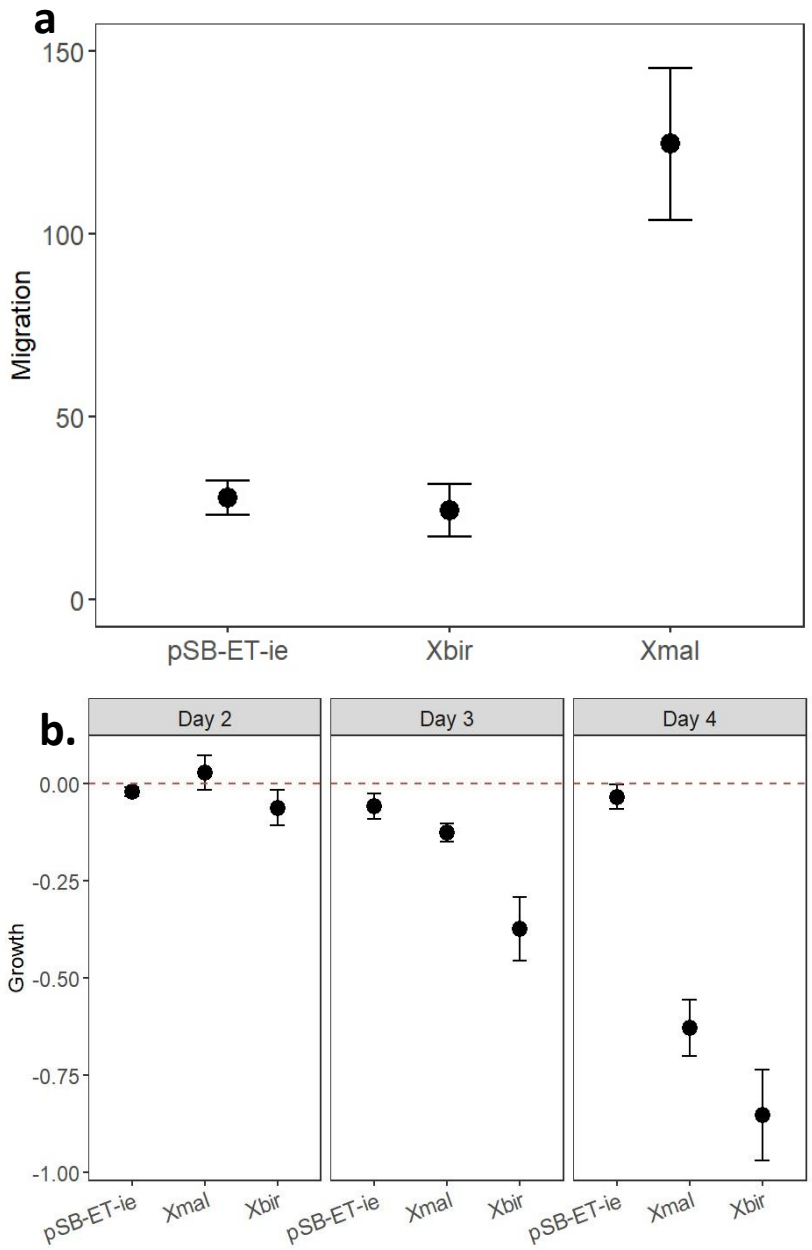

Figure 1. a) Cell growth (calculated as the difference in optical density at 590 nm observed in dox 500 induced cells minus the optical density at 590 nm observed in dox 0 cells.) for each cell

line and for each duration of the growth experiment. The dashed red line indicates no difference in growth between dox treated and untreated cells. b) Migration assay (calculated as the difference between number of cells that migrated in dox 500 induced cells minus the number of cells that migrated in dox 0 cells). Xmal = stable cell line transfected with the *X. malinche* allele of *adgre5*. Xbir = stable cell line transfected with the *X. birchmanni* allele of *adgre5*. Asterisk means the difference is statistically significant. The plot shows the mean, and whiskers indicate two standard errors of the mean.

Stable transgenic medaka lines were developed to study the interaction between the *adgre5* and *xmrk* in vivo. If *adgre5* acts as a tumor suppressor it is expected that *X. birchmanni adgre5* transgenic fish develop less melanoma. On the contrary, if *adgre5* acts as a tumor enhancer *X. malinche adgre5* transgenic fish develop should develop more melanoma. Transgenic lines were established using the genetically mixed and not inbred Carbio medaka strain which develop pigment cell tumors with a 100% penetrance (Schartl et al., 2010). Xmrk expression medaka, which also contained the *X. birchmanni* allele of *adgre5*, *tg(mift:xmrk)+tyrp:X.bir_adgre5*, showed significant reductions of pigmentation compared to those which had the *X. malinche adgre5* allele, *tg(mift:xmrk)+tyrp:X.mal_adgre5*, or the non-injected controls *tg(mitf:xmrk)* (Figure 2, Figure 3. ANOVA p value < 0.0001, Tukey post hoc p values, *tg(mift:xmrk)+tyrp:X.bir_adgre5- tg(mitf:xmrk)* < 0.00001, *tg(mift:xmrk)+tyrp:X.bir_adgre5- tg(mift:xmrk)+tyrp:X.mal_adgre5* < 0.00001). *tg(mift:xmrk)+tyrp:X.mal_adgre5* and *tg(mitf:xmrk)* medaka lines did not show significant differences between them (Tukey post hoc p values, *tg(mift:xmrk)+tyrp:X.mal_adgre5-tg(mitf:xmrk)* = 0.85).

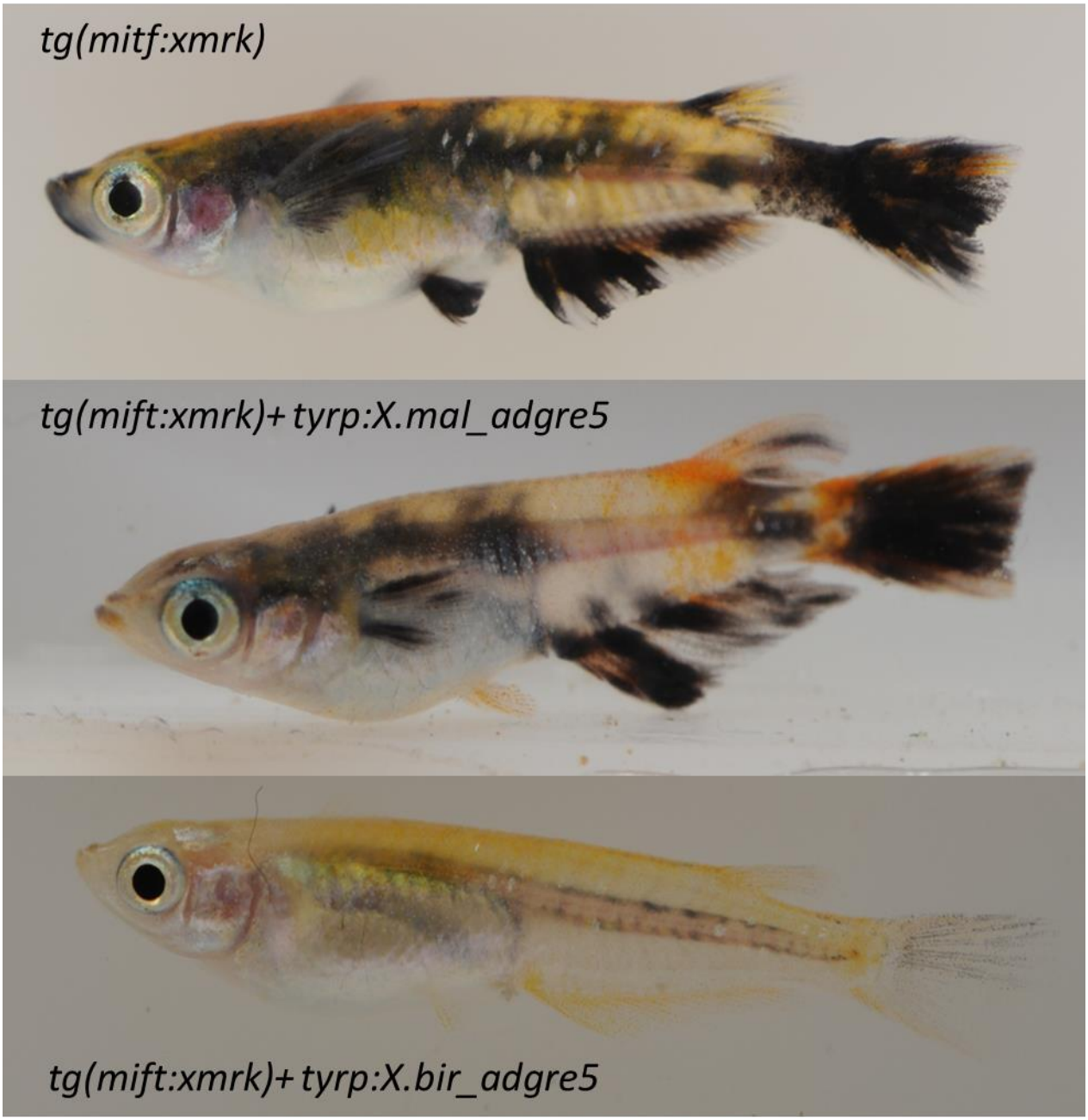

Figure 2. Close up photographs of the different transgenic lines. All individuals photographed of *tg(mift:xmrk)+tyrp:X.mal_adgre5*, *tg(mift:xmrk)+tyrp:X.bir_adgre5* are F1s, *tg(mitf:xmrk)* are more than 30 generations old.

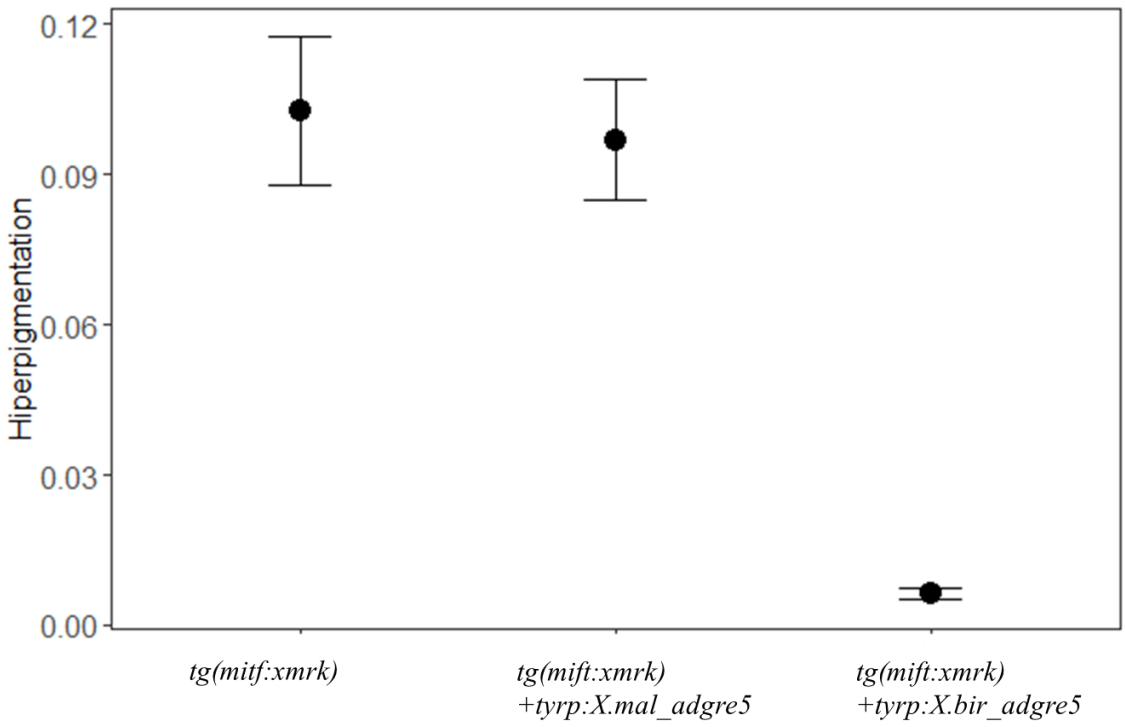

Figure 3. Hyperpigmentation area (corrected by standard length) for each transgenic line generated. Asterisk means the difference is statistically significant. The plot shows the mean, and whiskers indicate two standard errors of the mean.

Discussion

This study focuses on functionally testing the role of the *X. birchmanni* and *X. malinche* alleles by analyzing their effect independently on cell growth/migration in vitro and melanoma development in vivo. Melan-a cells are a well-established line of normal mouse melanocytes (Rossato F 2014) and provide an excellent non-tumorigenic line for studying the cellular and molecular basis of melanoma malignancy(Bennett D 1987), particularly for studies aimed at understanding the mechanisms triggering the change from benign pigmentation lesions to malignant melanoma. Two main characteristics of cancer are unlimited cell growth and potent metastatic properties (W 1956). Therefore, it is expected that tumor enhancer genes cause cells to grow and migrate more whereas tumor suppressor genes cause the opposite effect. Even though melanoma modifying genes are easiest to investigate in cell culture models, animal models are imperative to evaluate their importance in the context of the whole organism. Transgenic organisms isolate effects of the induced gene from other unknown possible genetic interactions. The lack of differences the area covered by melanoma cells between the *tg(mift:xmrk)+tyrp:X.mal_adgre5* and *tg(mitf:xmrk)*, as well as the striking reduction of pigmentation of *tg(mift:xmrk)+tyrp:X.bir_adgre5*, indicate that *adgre5* acts as a tumor suppressor. Moreover, pigment cells transfected with the *X. birchmanni* allele of *adgre5* caused cells to grow slower and migrate less than those transfected with the *X. malinche* allele of *adgre5*.

ADGRE5 is a prototypic member of the Adhesion class of G protein-coupled receptors (Adhesion GPCRs), which plays vital roles in numerous developmental processes as well as in

tumorigenesis. Although it has been demonstrated that *adgre5*, under apoptotic conditions, can increase tumor cell viability by inhibition of caspase activation and modulation of anti- and pro-apoptotic members of the BCL-2 superfamily(Hsiao C 2015), it has also been demonstrated that G protein-coupled receptors inhibit melanoma tumor growth and metastasis (Xu L 2006). In fact, a recent review (T 2020) proposes Adhesion G protein–coupled receptors as candidate metabotropic mechanosensors and novel drug targets for numerous cancer types because they are surface molecules that act as mechanosensors. Information about *adgre5* function could also be expected from loss of function studies. Recently, it was demonstrated that *xmrk* can be suppressed when co-expressed with a known potent tumor suppressor in medaka transgenic lines: *cdkn2ab (Regneri J 2019)*. Future studies to further characterize the role of *adgre5* as a tumor suppressor are much needed. For example, a tissue-specific knock-out of medaka adgre5 could cause even more malignant melanoma phenotypes, in analogy to the knock out of *cdkn2ab* .

The study by Powell et al. in 2020 was the first to propose a candidate hybrid incompatibility identified to the single-gene-level in naturally occurring hybrids. Almost simultaneously, another important example of hybrid incompatibility single-gene-level identification came from the closely related artificial hybrids between *X. maculatus* and *X. helleri (Powell D 2020)*. Lu et al., 2020 successfully identified *rab3d* as a tumor suppressor candidate gene. Future studies should focus on experimentally testing the role of *rabd3 (Lu Y 2020)*. Transgenic studies with the medaka *tg*(*mitf*:*xmrk*) line as well as cell culture experiments, should be particularly informative.

The results of both studies are interesting because they propose different genes, 7 Mb apart in the same chromosome, interacting with *xmrk* as responsible for the hybrid

incompatibility in a closely related pairs of hybrids. This suggests that a melanoma incompatibility involving *xmrk* originated independently in two distinct lineages. Schartl 2008 proposed that R (as it was called the then unknown tumor regulator gene) "could have preexisted before *xmrk* arose and would have suppressed the melanoma from the moment when the oncogene arose". The most parsimonious hypothesis is that *xmrk* originated in a common ancestor to all *Xiphophorus* and that it has been repeatedly lost in several branches of the tree. In contrast, it appears that all species have *R*, but different alleles, because different hybrid crosses have different levels of *xmrk* suppression (J 1962, F 1967). Indeed, the recent findings suggest that R "different alleles" are actually different genes.

There has been renewed interest in the search for Bateson-Dobzhansky-Muller hybrid incompatibilities since we realized that hybridization is more common than previously thought (N 2002, Schumer M 2014). However, few studies have been able to precisely identify the interacting genes responsible for the incompatibility(D 2010). Of those that have, most identify candidate genes by using hybrids between crosses of model species that no longer hybridize naturally (Lee H 2008, Bikard D 2009, A 2018, Yu X 2018). The list of the studies that have been able to pinpoint single gene effects grows significantly when considering which ones are actually able to experimentally test them and effectively assign a causal relationship between the gene interaction and the hybrid incompatibility(Trachtulec Z 2008, Mihola O 2009).

As far as we know, this is the only study that has experimentally validated a candidate gene involved in a hybrid incompatibility in species that currently hybridize naturally. Further studies of this kind could shed important light on how incompatibilities affect speciation, specifically whether mapped incompatibilities trigger divergence between species or arise after gene flow has ceased. The results of this study demonstrate that *adgre5* acts as a tumor modifier

and highlight the importance of following mapping studies with functional cell culture and in vivo studies. Future research focused on characterizing the specific mechanism by which adgre5 suppresses xmrk holds great promise for biomedical research. In 2021, there were 106,110 new cases of melanoma and 7,180 deaths in the U.S. due to melanoma, which accounts for the vast majority of skin cancer deaths, despite only accounting for approximately 1% of all skin cancer cases (American Cancer Society, 2021). Insights into how a tumor suppressor gene can counteract an oncogene will be of tremendous value in the development of melanoma treatments.

Acknowledgments

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