6. Summary and perspectives

Development of melanomas in Xiphophorus hybrids is due to overexpression of the receptor tyrosine kinase gene *Xmrk*. Expression of *Xmrk* is suppressed in wildtype parental Xiphophorus by an autosomal regulatory locus *R*. However, nothing is known about the *R* gene product on the molecular level. Linkage analysis revealed that *R* belongs to linkage group V and shows recombination to an isozyme locus, *ES1*, of about 30%. Emplacement of *R* into the existing gene map of several isozyme loci was not possible so far because of the non-linearity of recombination fractions in three point linkage analysis.

To test whether the tumor regulatory function of *R* is due to the cooperative action of several separate loci, Xiphophorus hybrids of higher backcrosses (2-5) were analyzed for linkage between *ES1* and *R*. These linkage analyses revealed an increasing deviation from the 30% recombination value obtained for the first backcross generation. Furthermore significant deviations from the expected 1:1 segregation of benign and malignant tumor phenotypes were observed from the third backcross generation onwards, indicating that several factors in the genome of Xiphophorus are involved in the regulation of ONC-*Xmrk* expression. To get support for this hypothesis it will be of importance in the future to analyze even higher backcross animals. Such animals finally should lose linkage between *ES1* and *R* and develop malignant melanoma at an even higher percentage. A mapping project by AP-PCR and AFLP analyses in which molecular markers for *R* were searched, yielded further indications for more than one regulatory gene. Examination of BC_1 hybrids between *X. maculatus* and *X. helleri* led to the isolation of 12 polymorphic marker sequences, which show significant linkage to *ES1*, but only two of them are distantly linked to *R* (27cM each). Furthermore *R* could not be integrated into a gene map which was established with all 12 markers. The two markers which show weak linkage to *R*, are located on different sides of *ES1*. The localization of *R* between them, however, is impossible because of the highly significant recombination values of *ES1* and the other markers with *R*. An explanation for these discrepancies could be the presence of at least two regulatory genes in this region. This could also account for the impossibility to detect more closely linked markers for *R*.

Recombination frequency between *CDKN2X*, a candidate gene for *R*, and the tumor phenotypes in classical backcross hybrids (*X. mac* Sd/*X. hell*) do not support the hypothesis that this gene might be the regulatory gene *R*. However, a role of *CDKN2X* as one of several regulatory factors seems to be more likely. Linkage analysis could also exclude *XDNMT-1* as a candidate gene for *R*. A totally different strategy like differential gene expression analysis between benign and malignant tissues of tumor bearing hybrids might offer a possibility in

the future for the identification of all the factors involved in the control of melanoma development.

Analysis of several Xiphophorus sex chromosomal mutants is expected to yield deeper insights into the regulation of ONC-*Xmrk* expression. Therefor structural bases for the different potential of various ONC-*Xmrk* alleles with respect to tumor induction and formation of macromelanophore patterns were investigated. To facilitate comparison between mutant and wildtype *Xmrk* alleles the so far uncharacterized genomic organization of the region coding for the extracellular domain of the receptor was characterized. One intron and also one exon in *Xmrk* is missing compared to the chicken EGF receptor gene. Analyses of two loss of function (lof) mutants which neither exhibit macromelanophores nor develop melanomas upon hybridization, revealed in one case loss of *Xmrk* function due to the deletion of the total oncogene locus. This provides additional evidence that *Xmrk* is indeed the gene responsible for tumor induction. A mutation that could explain the phenotype of the second lof mutant was not detectable. This could be an indication for a defect in the macromelanophore determining gene locus. Analyses of fish with altered macromelanophore patterns and/or tumor phenotype revealed that the majority shows a mutant *Xmrk* oncogene, which is the result of a recombination event between two wildtype oncogene alleles. Interestingly all intragenic crossovers are concentrating in the region of the gene coding for the extracellular domain of the receptor. This could point to a region of increased recombination activity, a so called recombination hotspot.

Using the genetic and molecular data obtained for the crossover mutants a gene order of several known important genes around ONC-*Xmrk* was established. The gene containing the genetic information for macromelanophores (*Mdl*) is located 5' of ONC-*Xmrk*, whereas the sex determining gene (*SD*) and a locus coding for different red and yellow patterns (*RY*) localize 3' of the oncogene. The gene order is *Mdl* - 5' ONC-*Xmrk* 3' - *RY* - *SD*. INV-*Xmrk* lies also 3' of ONC-*Xmrk*, but its exact localization could not be identified thus far. Analysis of the mutants "Sr crossover 30^{84}B", "Sb" and "DrLi (mut)" led to the definition of an element involved in gene regulation. This element is obviously located between the crossover points of "Sb" and "Sr crossover 30^{84}B". Differences in the malignancy of the tumors associated with the Mdl patterns Sd and Sr could be explained by such an element which should be present or functionally active in only one of the two sex chromosomal oncogene alleles. Preliminary data from DNaseI hypersensitive site mapping of native chromatin from the tumor cell line PSM point at DNA accessible for DNaseI in the region between the first intron and exon 15, which is an indication for gene regulatory regions. This is consistent with the data obtained for the crossover mutants. The aim of future experiments will be to narrow down the hypersensitive sites and to analyze an involvement of these sites in the regulation of expression of the *Xmrk* oncogene.