Comparative Biochemistry and Physiology, Part C xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Expression signatures of early-stage and advanced medaka melanomas

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ARTICLE INFO

Keywords:
Gene expression signature
Medaka
Melanoma
RNA-sequencing
Transcriptome
Transgenic fish model

ABSTRACT

Melanoma is one of the most aggressive tumors with a very low survival rate once metastasized. The incidence of newly detected cases increases every year suggesting the necessity of development and application of innovative treatment strategies. Human melanoma develops from melanocytes localized in the epidermis of the skin to malignant tumors because of deregulated effectors influencing several molecular pathways. Despite many advances in describing the molecular changes accompanying melanoma formation, many critical and clinically relevant molecular features of the transformed pigment cells and the underlying mechanisms are largely unknown. To contribute to a better understanding of the molecular processes of melanoma formation, we use a transgenic medaka melanoma model that is well suited for the investigation of melanoma tumor development because fish and human melanocytes are both localized in the epidermis. The purpose of our study was to gain insights into melanoma development from the first steps of tumor formation up to melanoma progression and to identify gene expression patterns that will be useful for monitoring treatment effects in drug screening approaches. Comparing transcriptomes from juvenile fish at the tumor initiating stage with nevi and advanced melanoma of adults, we identified stage specific expression signatures and pathways that are characteristic for the development of medaka melanoma, and are also found in human malignancies.

1. Introduction

Malignant melanoma is the most aggressive form of skin cancer with a rapidly rising incidence worldwide (Siegel et al., 2014). Melanoma rates have doubled in the past 30 years and an estimated 87,110 new melanoma cases will be diagnosed in 2017 in the United States (https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf). Primary melanoma tends to metastasize to many different parts of the body and at that stage current therapeutic strategies frequently fail. New treatment options were established and recently approved increasing the one-year progression free survival up to 50% (CheckMate 067 study, Larkin et al., 2017). Among these are immunotherapies (CTLA4- and PD1-inhibitor) and targeted therapies (BRAF- and MEK-inhibitors) with good response rates. However, side effects and drug resistant tumors may compromise treatment success (Chapman et al., 2011; Eggermont et al., 2016; Luke et al., 2017). In addition, there remain a large number of patients that do not benefit at all from these new treatments, e.g. patients with wild-type BRAF status. Therefore, the improvement of existing and the development of new therapies are important health care priorities. Comprehensive understanding of the genetic profile of melanoma primary tumor development and progression is a precondition for the development of new therapeutic options and strategies. Predisposing factors for malignant melanoma include exposure to ultraviolet radiation as well as pigmentation characteristics such as fair skin, a high number of nevi (Markovic et al., 2007) as well as genetic risk factors. Aberrations in different genes such as *CDKN2a*, *P53* or *BRAF* are associated with

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https://doi.org/10.1016/j.cbpc.2017.11.005
Received 15 August 2017; Received in revised form 16 November 2017; Accepted 17 November 2017

familial melanoma (Tsao et al., 2012). Genome sequencing projects of human metastatic melanomas have uncovered a large number of somatic alterations. Among these are the already known oncogenes *BRAF* (in about half of all melanomas) and *NRAS*, but also new candidates, like *NF1* and *PREX2* (Berger et al., 2012; Cancer Genome Atlas, N., 2015). The identification of such new candidate genes is very important for a better understanding of the contribution of genetic and genomic alterations to melanoma formation. Insights into melanoma tumor biology were also provided by a large number of genetic and molecular studies; however, they are only retrospective (Chin et al., 2006; Hodis et al., 2012; Sturm et al., 1994).

Cancer is a systemic disease. Therefore, the use of animal models with high anatomical and physiological similarities to humans is extremely important for the investigation of developmental and environmental factors that drive melanoma formation. Small aquarium fish, such as zebrafish or medaka are well suited for biomedical research because of several features and are well-accepted models to study melanoma development (Patton et al., 2010). Both have high fecundity, short generation time, genetic tractability and they are easy to breed in large numbers (Lieschke and Currie, 2007; Wittbrodt et al., 2002). We use pigment cell tumor-developing transgenic medaka as model for studying melanoma (Schartl et al., 2010). Melanocytes in fish, like in humans, are localized in the basal layer of the epidermis in the skin. In contrast, mouse melanocytes are located in the hair follicles and in the dermis (Gola et al., 2012; Kelsh, 2004). This anatomical conformity together with a high degree of molecular genetic conservation between medaka and human pigment cell cancers (Patton et al., 2010; Schartl et al., 2012) make medaka a highly suitable in-vivo study system.

Development of small aquarium fish models of melanoma has been proposed to produce superior experimental systems for high throughput first line drug screens (van Rooijen et al., 2017). Successful screening experiments in zebrafish have been conducted, however, these utilized established screening technologies by exposing embryos to chemicals and monitoring drug effects on normal melanocytes (Colanesi et al., 2012; White et al., 2011). So far – with a single exception (Matsuzaki et al., 2013) – efficient systems to monitor the effect of drugs that act on developing melanoma in free swimming larvae or adult animals have not been devised and drug screening protocols on larvae and adults are lacking, partly due to the difficulty of monitoring phenotypic changes of a fully developed melanoma and the long-term that such changes require to become visible. Reasoning that drug effects would be first visible at the level of gene expression, we are working toward developing such gene expression patterns that hallmark melanoma. Our ability to find gene expression readouts of melanoma development depends critically on the knowledge of transcriptional changes (i.e. expression signatures) in tumors at different stages of development.

In this study, we used a pigment cell tumor developing medaka model for investigating gene expression profiles of early-stage in juveniles (3–5 weeks) and advanced melanoma in adults (6–9 months). These fish are transgenic for the *xmrk* oncogene from *Xiphophorus maculatus* under the pigment-specific promoter of the medaka *mitfa* gene. They develop aggressive melanomas, which are highly invasive into the internal organs of the body, and form large nodular tumors in the skin and at extracutanous sites (Schartl et al., 2010). The purpose of this study was to identify transcriptional profiles of juvenile fish at the initial stage of melanoma formation in comparison to nevi and advanced melanoma from adult medaka. We found a clear downregulation of critical genes from the innate and adaptive immune system in the early stage of melanoma development while an upregulation of adaptive immune response genes were detected in melanoma tumors from adults. Differentially regulated pigment cell and pigmentation specific genes were identified in both early and advanced stage melanomas while other gene expression patterns constitute specific transcriptional signatures of the respective tumor stage.

2. Material and methods

2.1. Fish maintenance

Transgenic *Oryzias latipes* (medaka) of the Carbio Tg(*mitf*:*xmrk*) strain (Schartl et al., 2010) were used as model to investigate melanoma-specific transcriptional signatures. Fish of this strain carry the cDNA from the melanoma-inducing oncogene *xmrk* of *Xiphophorus maculatus*. The expression of *xmrk* under the pigment cell specific medaka *mitfa* promotor results in formation of pigment cell tumors with up to 100% penetrance. All fish were kept under standard conditions in the aquarium facility of the Biocenter at the University of Wuerzburg in compliance with local animal welfare laws, guidelines and policies and under the authorization (55.2 – 2531.01 – 40/14) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law.

2.2. RNA-sequencing (RNA-seq)

Total RNA was isolated from whole body of 10 individual transgenic and 10 individual non-transgenic (wild-type) fish using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The age of the fish ranged from three to five weeks at a uniform total length of about 1 cm. To increase the sample size and for cross-validation we added to the dataset produced in this study the primary data from an earlier study (Schartl et al., 2015) with four wildtype and four melanoma juveniles of the same age and size, and reanalyzed them in comparison with our new data. RNA from adults (6–9 months old) was extracted from pooled fins of 11 non-transgenic medaka (strain Carbio), 2 pools (n = 5 and 10) of nevi from two independent dissections from transgenic fish (Tg(*mitf*::*xmrk*); Tg (*mitf*::*xmrk*), *p53* −/−) and three individual exophytic melanoma (Tg (*mitf*::*xmrk*)) using commercially available systems (RNeasy Kit, Qiagen, Hilden, Germany). Quality of RNA was assessed by measuring the RNA Integrity Number (RIN) using an Agilent 2100 Electrophoresis Bioanalyzer Instrument G2939A (Agilent Technologies, Böblingen, Germany). RNA samples with RIN > 8 were used for sequencing. High-throughput paired-end RNA sequencing libraries were constructed following the standard Illumina mRNA library preparation protocol (www.illumina.com; Illumina Inc., San Diego, CA, U.S.A.).

2.3. RNA-seq validation by quantitative real-time PCR (qRT-PCR)

For confirmation of RNA-seq data, selected genes were analyzed by qRT-PCR analysis. Total RNA from three to five weeks old individual transgenic and wild-type medakas (n = 4–10 in each group) was extracted from whole bodies using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA from adults (6–9 months old) was isolated from one pool (n = 9) of nevi from transgenic medakas (Tg(*mitf*::*xmrk*)) and 7–8 individual exophytic melanoma (Tg(*mitf*::*xmrk*)) using TRIzol Reagent (Thermo Fisher Scientific, Waltham, USA) according to the supplier's recommendation. After DNase treatment, total RNA (1–2 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA) and random hexamer primers, according to the manufacturer's instructions. For real-time qRT-PCR, cDNA from 25 ng of total RNA was analyzed in triplicates using SYBR Green reagent. Primer sequences are listed in Table S1. Amplification was monitored using a Mastercycler ep realplex^2 (Eppendorf, Hamburg, Germany). For quantification, expression of each gene was normalized to the housekeeping gene *ef1a1* (elongation factor 1 alpha 1) using the delta Ct method (Simpson et al., 2000). Differences in mRNA expression levels were tested for significance using Wilcoxon–Mann–Whitney *U* test or Student's *t*-test depending on the sample size and distribution. Normally distributed data with a sample size larger than 9 were analyzed by Student's *t*-test and data with a samples size smaller than 9 or

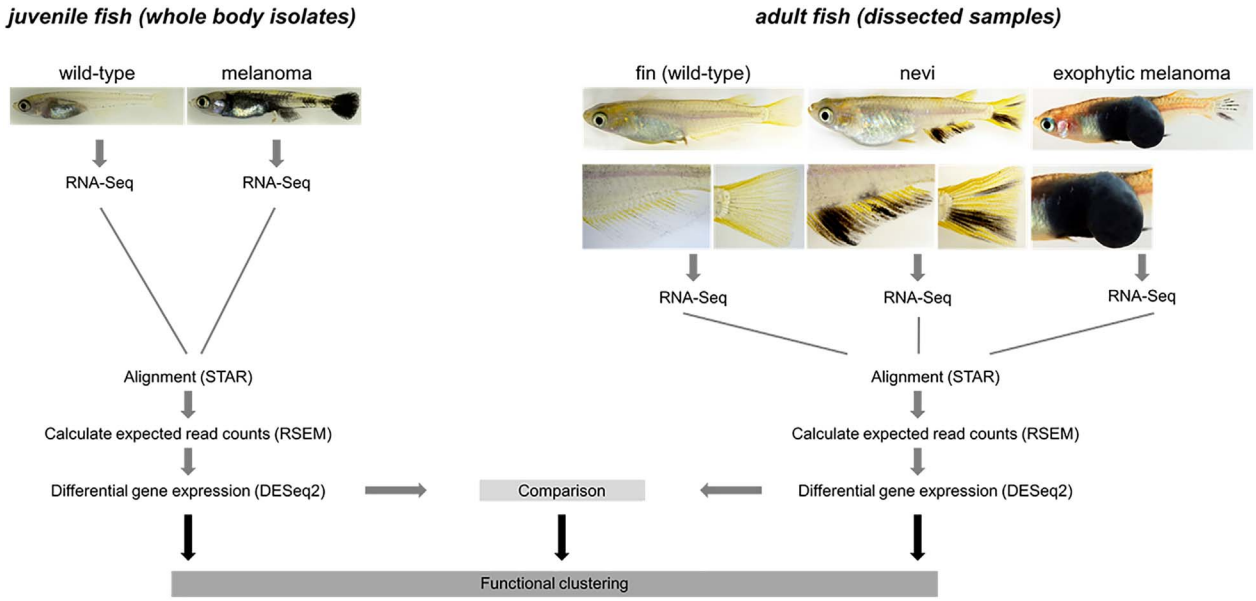

Fig. 1. Workflow of data production and analyses.

without normal distribution were analyzed using non-parametric Wilcoxon–Mann–Whitney *U* test.

2.4. Bioinformatics and statistical analyses/Pathway and transcription regulator enrichment

For the samples from the juvenile whole-body RNA extractions sequencing reads were filtered using an in-house data processing pipeline (Garcia et al., 2012). Briefly, sequencing adaptors were firstly removed from sequencing reads. Processed sequencing reads were subsequently trimmed and filtered based on quality scores by using a filtration algorithm that removed low-scoring sections of each read and preserved the longest remaining fragment. For the adult samples two independent sequencing runs were done: (1) one pool of nevi (N1) with melanoma M1, and (2) a second nevus pool (N2) with melanoma M2 and M3 and pooled fins (F). Adapter sequences from reads were trimmed using leeHom (Renaud et al., 2014). These reads were then mapped with the RNA-seq aligner STAR (Dobin et al., 2013) to the Ensembl medaka genome version 88. Gene abundance was calculated by RSEM (Li and Dewey, 2011) and resulting expected read counts were used for detection of differentially expressed genes using the bioconductor package DESeq2 (Love et al., 2014). Genes from juvenile wild-type and Tg (*mitf*::*xmrk*) medaka with log2fold change (log2FC) < -1 or $> +1$ and an adjusted p-value ≤ 0.05 were considered to be differentially expressed. For detection of differentially expressed genes in samples from adult fish both sequencing runs were treated separately and a log2fold change of < -1 or $> +1$ was used. Only genes showing consistent regulation in both sequencing runs were considered further. To exclude false positives due to fin (F) specific genes within the nevus tissues in regulated genes in adult melanoma samples (M) compared to nevi (N), the log2FC fin/nevus was required to be not 0 according to the following formula:

$$((M > N) \text{ AND } (\log 2FC(F \text{ vs. } N) < -1) \text{ OR } ((M > N) \text{ AND } \log 2FC(F \text{ vs. } N) > 1))$$

for genes upregulated in melanoma and

$$((M < N) \text{ AND } (\log 2FC(F \text{ vs. } N) < -1) \text{ OR } ((M < N) \text{ AND } \log 2FC(F \text{ vs. } N) > 1))$$

for genes downregulated in melanoma.

Heatmaps with dendrograms were generated using the R/Bioconductor package 'made4'. Medaka genes were matched to human orthologs using the Ensembl tool BioMart (http://www.ensembl.org/biomart/martview/662764dc0a1ef355f59d8c648d5196be). WebGestalt (http://www.webgestalt.org/webgestalt_2013/) and DAVID (https://david.ncifcrf.gov/) functional enrichment analysis web tools were used for subsequent functional classification (default settings) KEGG pathways that are overexpressed in melanoma samples were extracted and analyzed with the Search&Color Pathway tool (http://www.genome.jp/kegg/mapper.html).

2.5. Histopathology

Cryostat sections of medaka melanomas were stained with hematoxylin and eosin.

3. Results and discussion

The purpose of this study was to get insights into melanoma specific gene expression patterns from the initiation of tumor formation up to advanced melanoma. We first analyzed whole body RNA-seq data from juvenile medaka to establish expression profiles of early tumor development by comparing wild-type and transgenic fish. Second, we compared nevi and advanced melanoma to find expression patterns that distinguish the premalignant and advanced malignant state. Finally, we compared early melanoma expression profiles with advanced melanoma (Fig. 1).

3.1. Establishment of gene expression signatures of early melanoma development

First, the whole-body gene expression profiles from juvenile wild-type medaka were compared to fish with early melanoma formation. Differentially expressed genes were identified and used to define gene expression signatures representative of early-stage melanoma. Whole body RNA-seq was employed because of the impossibility to dissect out the invasive early melanoma in the very young animals. We have shown previously that whole body RNA-extracts are suitable to detect initiating melanoma specific gene expression patterns and moreover to detect the host response to the early cancer stages (Schartl et al., 2015).

The differential expression (DE) analysis of whole body RNA-

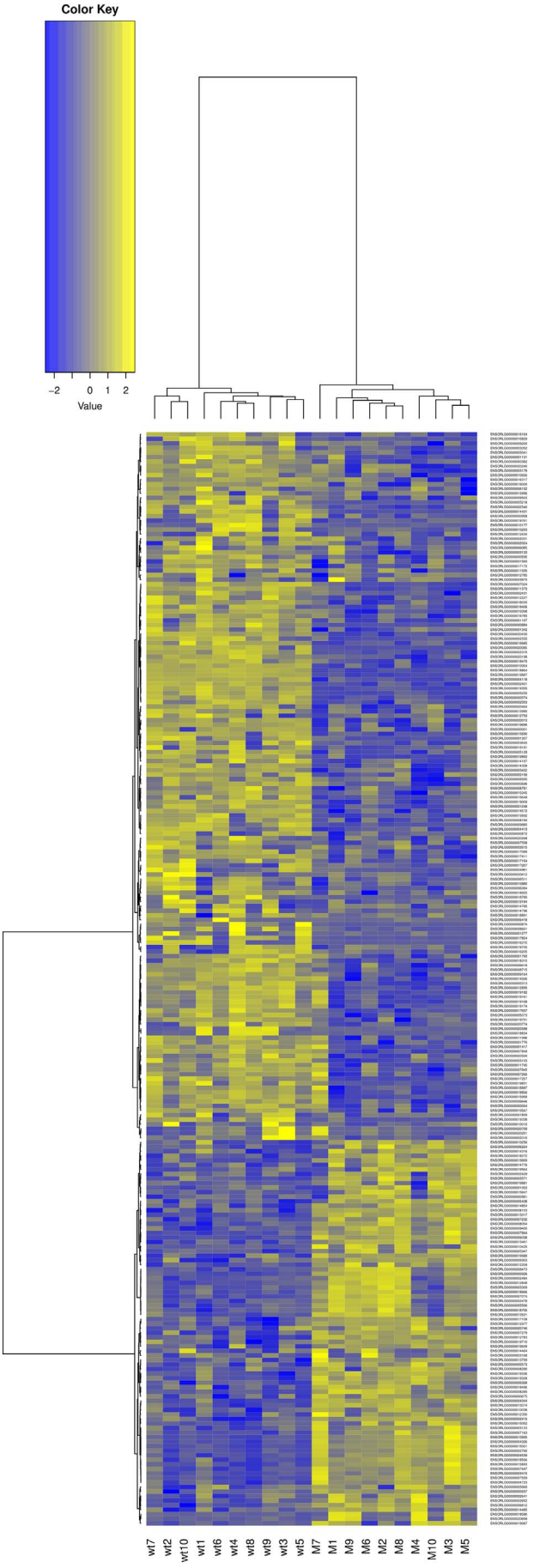

Fig. 2. Hierarchical clustering of juvenile wild-type and melanoma initiating medakas. Heatmap of 241 transcripts differentially expressed between juvenile wild-type and tumor (mitf::xmrk) fish (log2FC < −1 and > +1; adjusted p-value < 0.05). Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). Heatmap color displays the z-score ranging from blue (z-score of −2 and above) to yellow (z-score of +2 and below). The relative expression for each gene is represented by color intensity, with blue indicating low expression and yellow indicating high expression levels. (wt: wild-type, M: melanoma). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracts revealed 156 downregulated and 85 upregulated genes in juvenile transgenic fish (n = 14) compared to wild-type fish (n = 14) (Table S2). This confirms our earlier results (Schartl et al., 2015) that it is possible to obtain regulated gene profiles from whole body RNA isolates of healthy and tumorous fish. Subsequent hierarchical clustering of differentially expressed genes confirmed two distinct groups (Fig. 2). Enriched GO terms and KEGG pathways were extracted. Ten overrepresented GO terms were identified for the overexpressed genes in the melanoma cohort, most of them associated with processes of melanin synthesis and pigmentation (Table S3). Among the upregulated genes are *tyrp1* (tyrosinase-related protein 1), *pmel* (premelanosome protein), *tyr* (tyrosinase), *slc24a5* (solute carrier family 24 member 5) and *mitf* (microphthalmia-associated transcription factor). The tumorigenic phenotype is a result of *xmrk* expression that induces development of pigment cell tumors leading to hyperpigmentation already some days after hatching. Within 1–3 weeks, the hyperpigmented skin areas progress to malignant tumors, evident as invasion of pigment cells into the underlying musculature and further into internal organs (Schartl et al., 2015). However, at this time point typical cancer-associated GO terms or enriched KEGG pathways are not evident in line with a very early stage of tumor development. On the other hand, expression of the melanoma driver oncogene *xmrk* was clearly detectable at high levels in all tumor fish samples. An upregulation of *kif5b* (kinesin family member 5B) was observed as well. A correlation between *KIF5B* (and *KIF4A*) overexpression and skin cancer has been reported earlier as well as its benefit as a potential prognostic marker in the context of tumor development and progression (Liu et al., 2013; Yu and Feng, 2010). Remarkably, we found a number of immune system associated GO terms enriched for downregulated genes in the juvenile transgenic fish (Table S4) confirming our earlier studies and supporting the hypothesis that early melanoma development is accompanied by a suppression of the immune system and/or immune-associated processes (Schartl et al., 2015). Some of the downregulated genes like *tlr3* (toll-like receptor 3), *c3* (complement component 3) or *c4b* (complement component 4B) belong to the innate immune system that functions as first line of defense against pathogens and tumors (Marcus et al., 2014), while others, e.g. *irf1* (interferon regulatory factor 1), *tnfrsf14* (TNF receptor superfamily member 14), *hla-dma* (major histocompatibility complex, class II, DM alpha), *il1r1* (interleukin 1 receptor, type I) are part of the B and T cell mediated adaptive immune response. Differential expressions in these gene sets are strong evidence for a suppressed innate and adaptive immune system during early stages of melanoma development. A selection of the above described differentially expressed genes was validated and confirmed by qRT-PCR (Table S5A).

3.2. Gene expression signatures of advanced malignant melanoma in adults

Next, the gene expression patterns of microdissected nevi and advanced stage melanoma from adult medaka were analyzed. Differentially expressed genes were defined and used to establish characteristic expression signatures. A total of 1829 genes were consistently upregulated and 899 genes downregulated in melanomas (Table S6). Of the 2728 differentially expressed genes 1578 matched to human orthologs and were used for further enrichment analysis. The differentially expressed genes were hierarchically clustered. The

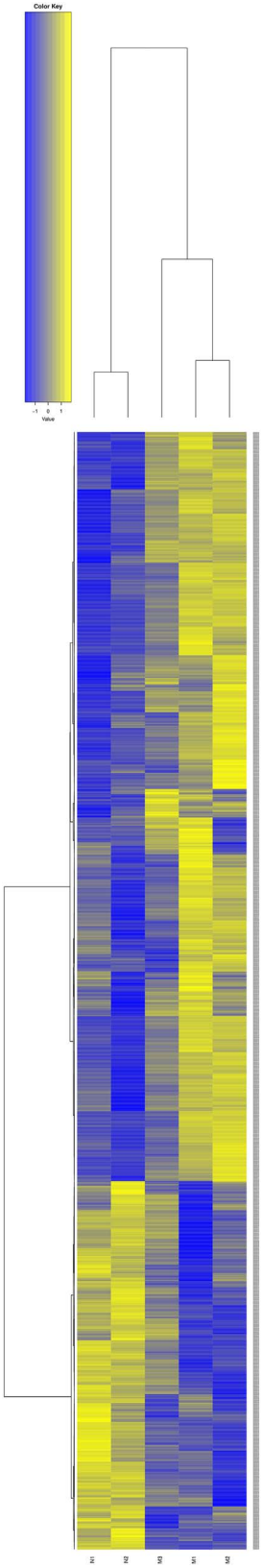

Fig. 3. Hierarchical cluster analysis applied to nevi and melanoma data set.
Heatmap of 2728 differentially expressed genes between fin, nevi and exophytic melanoma from adult mitf::xmrk medakas (log2FC < -1 and $> +1$; adjusted p-value < 0.05). Columns represent pools of several samples from fin, nevi and three individual exophytic melanoma tumors, while rows represent differentially expressed genes (y-axis). Heatmap color displays the z-score ranging from blue (z-score of -2 and above) to yellow (z-score of $+2$ and below). The relative expression for each gene is represented by color intensity, with blue indicating low expression and yellow indicating high expression levels. (F: fin, N: nevi, M: melanoma). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

heatmap (Fig. 3) shows a clear separation of nevus samples from melanoma samples. 40 (Table S7) and 18 (Table S8) enriched GO terms were found in the overexpressed and downregulated genes, respectively ($R > 2$; adjusted p-value ≤ 0.01). Expectedly, signaling pathways downstream of Xmrk (epidermal growth factor signaling pathways G0:0038127, GO:0007173, ID 04010, ID 04012) were upregulated. Overexpressed genes from GO terms related to melanin pigmentation (0048770, 0042470, 0046148, 0042438, 0006582, 0042440) as well as from the enriched KEGG pathways 'melanogenesis' and 'tyrosine metabolism' (Table S9) include genes like *tyr*, *dct* (dopachrome delta-isomerase, tyrosine-related protein 2), *tyrp1* or *rab38* (member RAS oncogene family). These upregulated genes represent the hypermelanized phenotype of the medaka melanoma. They indicate that terminal differentiation to fully melanized cells still occurs in highly proliferative and aggressive tumors. Further enriched KEGG pathways in melanoma samples include three different pathways in cancer. The enriched pathways 'cell cycle' and 'nucleotide metabolism' are indicative of the proliferative and transcriptionally highly active state of the tumors (Table S9). Several pathways and GO terms enriched for melanoma upregulated genes indicate the high metabolic activity of the tumors (e.g. 'glycolysis', 'gluconeogenesis', 'carbohydrate biosynthetic process') (Table S7). Several pathways related to the adaptive immune response are upregulated in the melanoma samples (Table S9). This points to the presence of host immune cells in the dissected tumors and an ongoing immune reaction (Fig. S1). The pro-tumorigenic expression signature includes upregulation of *mitfa* (microphtalmia-associated transcription factor a) and *dusp4* (dual specificity protein phosphatase 4). Interestingly, *piwil1* was upregulated from 2 to 40-fold in melanoma. This is in agreement with an earlier study that found upregulation of the piRNA processing machinery in medaka melanoma and a high number of abundantly expressed melanoma-specific piRNAs (Kneitz et al., 2016). piRNAs with tumor-specific functions have been reported in enigmatic cases; however, their roles in mediating aspects of the neoplastic phenotype remain largely unclear (Hashim et al., 2014; Kwon et al., 2014; Suzuki et al., 2012). In this context the medaka model may provide a useful tool to increase our understanding of the relevance of piRNAs for melanoma development.

In the advanced melanoma apoptosis is downregulated (Table S10), which is a common feature of many cancers (Brown and Attardi, 2005; Hanahan and Weinberg, 2000). A number of downregulated genes from enriched GO terms in melanoma samples (Table S8) are associated with intermediate filament and cytoskeleton organization. These include two members of the keratin (*krt*) gene family, which are involved in structural stability and cytoskeletal organization of epithelial cells. Members of the *KRT* gene family are often implicated in epithelial-mesenchymal transition (EMT). EMT is a process of phenotypic changes of cells and plays an important role in carcinoma development. EMT induces reorganization of cytoskeletal components, loss of cell polarity, cell-cell adhesion and keratin expression (Klymkowsky and Savagner, 2009). The diminished *krt* expression in the melanoma samples suggests a link between increased cell growth, migration, invasiveness and EMT processes. Consistently, the KEGG pathway 'regulation of actin cytoskeleton' has an increased number of regulated genes, e.g. *hrasls* (*HRAS-like suppressor*), *nhe1* (Solute Carrier Family 9 Member A1), *irsp53* (BAI1-associated protein 2), *arp2/3* (actin-related protein 2/3 complex subunit 4), and *gsnb* (gelsolin b) (Fig. 4). The enriched KEGG pathway 'ECM-receptor interaction' (Fig. 5) in melanoma comprises upregulation of the *laminin* gene family and several *integrins*, and downregulation of *col11a1a* (collagen, type XI, alpha 1a), *col17a1a* (collagen,

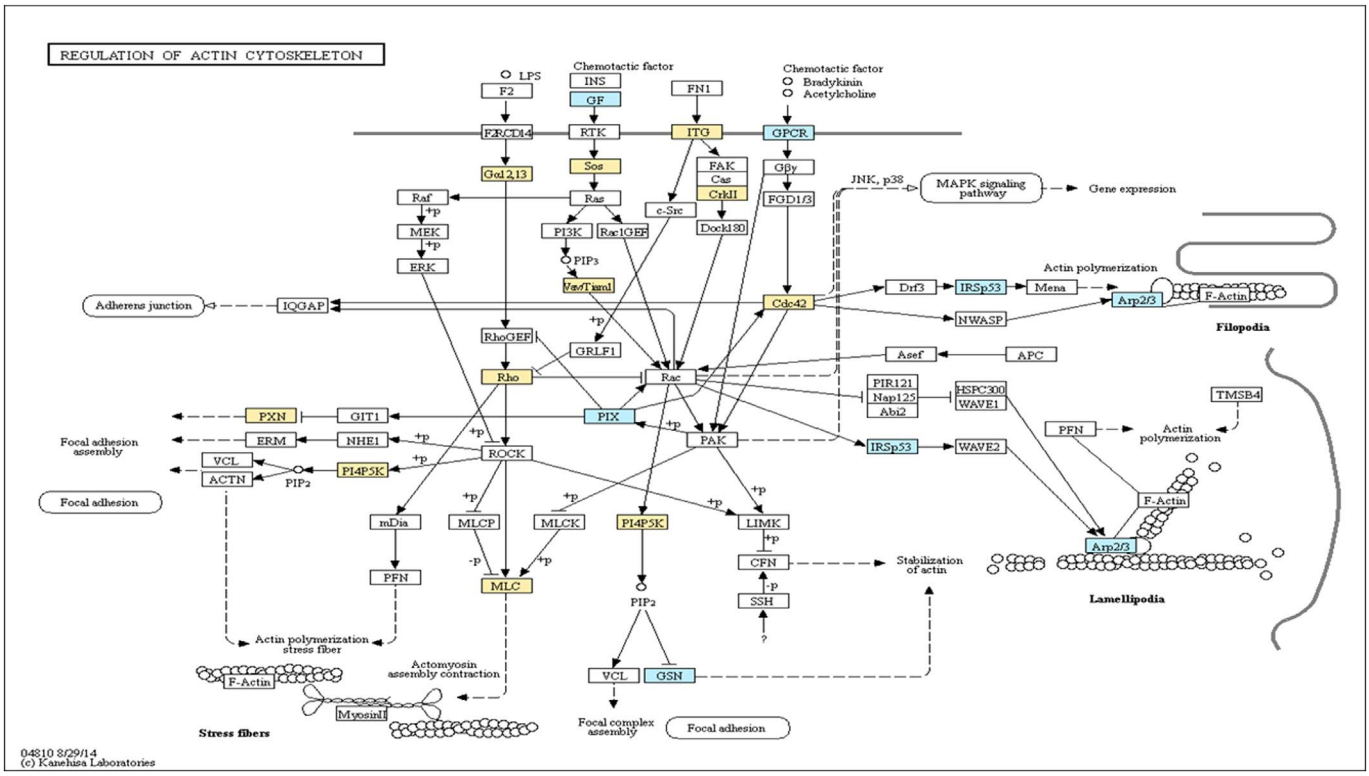

Fig. 4. Enriched KEGG pathway of Regulation of actin cytoskeleton in adult melanoma.
Melanoma samples from adult fish show an increased number of regulated genes. Upregulation: yellow colored; downregulation: blue colored. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

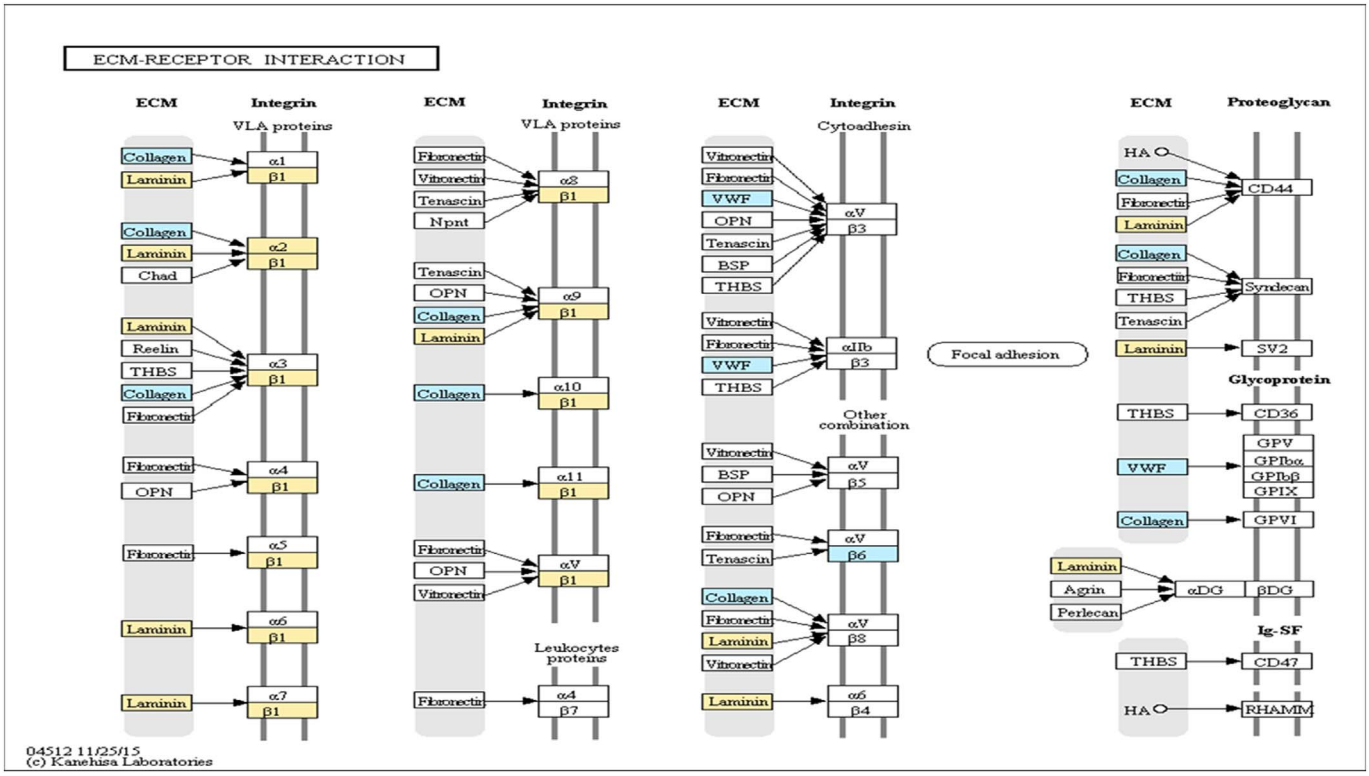

Fig. 5. Enriched KEGG pathway of ECM-receptor interaction in adult melanoma.
Members of the *laminin* gene family and several *integrins* (*β1*, *α2*) are upregulated (yellow colored) and members of the *collagen* gene family and *vwf* are downregulated (blue colored) in melanoma samples compared to nevi samples from adults. The *integrins itgb6*, *itga8*, *itga6-like* are also downregulated (not shown in the figure). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

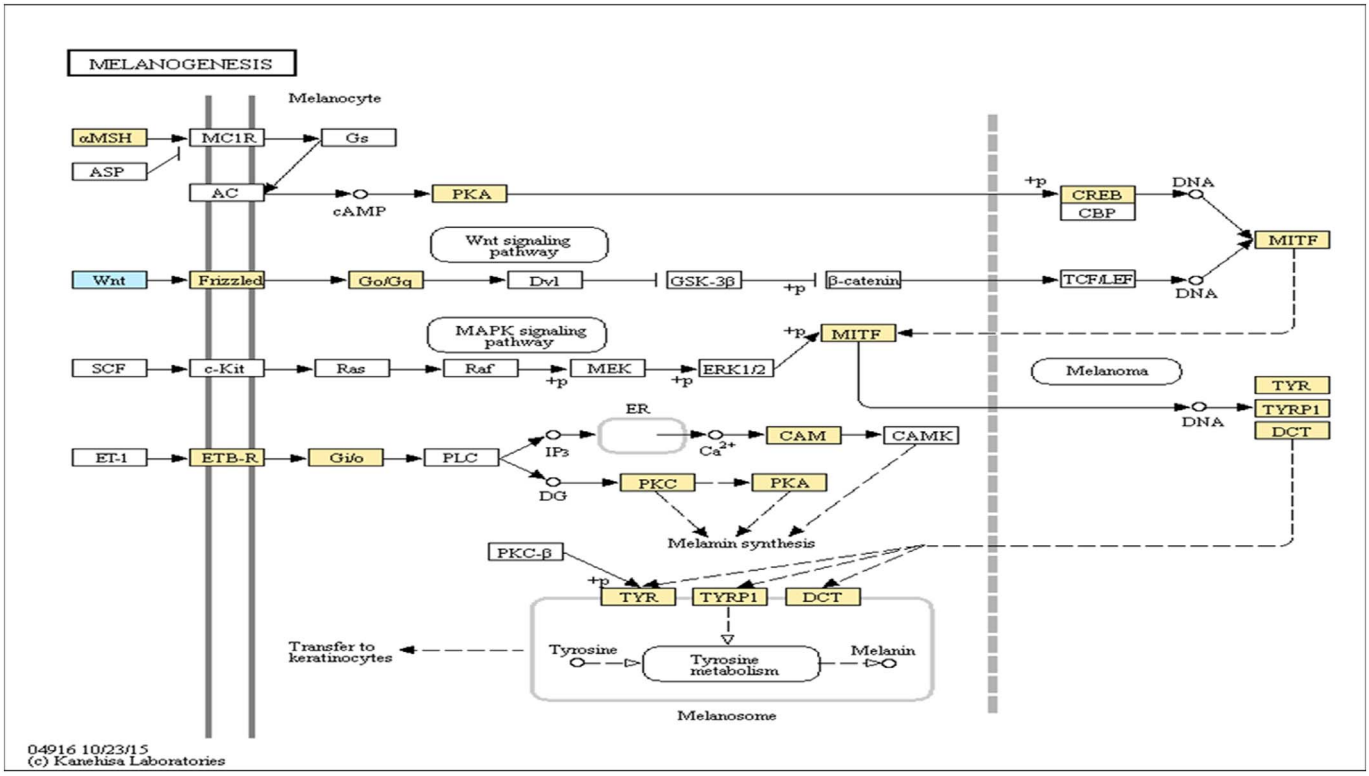

Fig. 6. Melanogenesis pathway.
Several pigmentation-associated genes like *mitf*, *tyr*, *tyrp1* and *dct* are upregulated (yellow colored) in early and advanced melanoma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

type XVII, alpha 1a), *col5a3a* (collagen, type V, alpha 3a), *col5a3b* (collagen, type V, alpha 3b) and *itgb6* (integrin, beta 6), *itga8* (integrin, alpha 8) and *itga6l* (integrin, alpha 6, like) and *vwf* (von Willebrand factor). Members of the *laminin* gene family are part of the extracellular matrix (ECM) affecting cellular processes (e.g. cell proliferation, differentiation, adhesion and migration) and are known to be involved in tumorigenic mechanisms (Givant-Horwitz et al., 2005; Ioachim et al., 2002; Oikawa et al., 2011). They are highly expressed in melanoma cells during invasion and metastasis (Oikawa et al., 2011). A diminished plasticity of ECM was observed in several tumors accompanied by VEGF and TGFβ-induced vessel growth and development of inflammation. ECM receptors are necessary for the interaction between cell adhesion and ECM that can influence cell proliferation, migration or apoptosis (Lu et al., 2012; Ozbek et al., 2010; Schmidt and Friedl, 2010). The increased *laminin* expression levels in our melanoma samples are in line with the aggressive phenotype of the advanced tumor stage. Laminins are recognized by integrin-receptors like *itgb1*, which is also upregulated. Besides laminin, collagens are also important components of the ECM. They confer tensile strength, elasticity of the skin and are involved in cell adhesion and migration (Rozario and DeSimone, 2010). The results of the validation of selected differentially expressed genes by qRT-PCR were in accordance with the RNA-seq analysis (Table S5B).

3.3. Comparison of the gene expression profiles of early and advanced melanoma

First, similarly regulated genes in juvenile melanoma fish (compared to wildtype fish) and in melanoma samples from adult fish (compared to nevi) were extracted. Eighteen genes were upregulated and six genes were downregulated (Table S11). Most prominently, an upregulation of pigmentation-associated genes, including the melanoma antigen *mlana*, the photopigment melanopsin (*opn4xa*), several pigmentation genes and *mitf* marks the common expression signature of early and advanced melanoma. This result is in line with the hyperpigmented phenotype of the samples from both groups (Fig. 6), which employs the melanin-producing pathway. MITF is a key regulator of melanocytes and its high expression levels in melanoma are linked to a proliferating tumor phenotype (2017). The gene *g6pd* (glucose-6-phosphate dehydrogenase) is commonly downregulated. It has been reported that expression of G6PD is increased in many different tumor types including breast cancer (Polat et al., 2002), leukemia (Batetta et al., 1999), colon cancer (Van Driel et al., 1999) or liver and prostate cancer (Rao et al., 1997; Tsouko et al., 2014), but its role in melanoma is unclear. On the other hand, tumor cells are often characterized by a state of redox imbalance and G6PD is involved in the maintenance of the redox balance. Proteomic and micro-array analyses of the *xmrk*-driven melanoma from the related *Xiphophorus* fish model also pointed to a deregulated antioxidative capacity (Lokaj et al., 2009; Teutschbein et al., 2010). The downregulated expression of *g6pd* in malignant tumor samples could mean a reduced antioxidative capacity of tumor cells and therefore high levels of oxidative damage as described in G6PD deficient mice (Jeng et al., 2013). Such reduced antioxidative capacity is generally seen as being pro-tumorigenic (Pantavos et al., 2015; Rathan Shetty et al., 2015). Experimental studies in well-defined in-vitro systems are required to test this hypothesis.

Another gene, which is consistently downregulated in medaka melanoma is *cathepsin E* (*ctse*). It has been shown that repression of CTSE is associated with increased risk of mammary carcinogenesis and linked to poor prognosis in breast cancer (Kawakubo et al., 2014). In *ctse*-deficient mice B16 melanoma cells show more profound growth, viability and metastasis compared to wild-type and CTSE over-expressing transgenic mice (Kawakubo et al., 2007).

Next, differences in gene expressions between juvenile and adult melanoma were analyzed. Seventeen genes were downregulated and 11 genes upregulated (Table S12). Initial stage melanoma from juvenile

fish showed an upregulation of *tgm5* (*transglutaminase 5*). The transglutaminase enzymes crosslink proteins by formation of isopeptide bonds and act as a stabilizing component for the adhesion of melanoma cells with extracellular matrix proteins under conditions of wall shear stress induced by blood laminar flow (Menter et al., 1991). Further changes in the ECM, which might be related to the highly invasive phenotype of the initial stage melanoma are indicated by frequent GO terms. Overexpression of *tgms* in juvenile fish during early-phase melanoma is in accordance with the gene expression pattern of another transglutaminase enzyme, TGM2, in different cancer types (Iacobuzio-Donahue et al., 2003; Mehta et al., 2004; Miyoshi et al., 2010) including melanoma (van Groningen et al., 1995). Moreover, high TGM2 expression is regarded as negative prognostic marker in different cancer types and is often described in the context of advanced tumor stages, metastasis formation as well as drug resistance (Fok et al., 2006; Herman et al., 2006; Mehta et al., 2004).

4. Conclusions

In agreement with an earlier study (Schartl et al., 2015) we find a strong downregulation of critical genes from the innate and adaptive immune system in the early stage of melanoma development. This appears to be a “host” response of the juvenile fish that is elicited by the melanoma in its initial stage and likely facilitates establishment of the tumor. This motivates further studies with experimental modulation of the immune system during the early phase of tumorigenesis in medaka.

In the advanced melanoma of adult fish besides the expected upregulated oncogenic signaling pathways we found as characteristics of this stage pronounced differential expression of ECM components, which may reflect the remodeling of the ECM during epithelial-mesenchymal transition in the processes of tumor invasion. We also noted an upregulation of adaptive immune response genes indicative of an ongoing “immune surveillance”. Small aquarium fish melanoma models can tolerate the malignant tumors for quite a while and are not heavily affected by the cancerous disease with respect to their general health and the metastatic phenotype often is not as pronounced as in mice.

We found as major common features of the early and advanced stage melanoma differential regulation of pigment cell and pigmentation specific genes, while the expected changes in expression of proliferation and oncogenic signaling genes remained undetectable by the currently possible approach. Expression of *tgm5* in the juvenile samples, as detected by the whole-body sequencing strategy appears to provide an early prognostic biomarker. With respect to immune genes, it is evident that the early phase is more characterized by tumor-induced suppression of the innate immune response, while during late tumor development an active adaptive immune response is apparent.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2017.11.005.

Acknowledgments

This work was supported by NIH grant 1R24OD018555 to JHP, MS, RW, and WW, R24-OD-011120 to RW, funding from the Max Planck Society to MD, BN and JK and by the Melanoma Research Network of the Deutsche Krebshilfe e.V. grant no. 109716 (German Cancer Aid) to MS and MK.

Declaration of interest

The authors declare that they have no competing interests.

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