RESEARCH ARTICLE

Endothelin signalling in iridophore development and stripe pattern formation of zebrafish


ABSTRACT

Colour patterns of adult fish are composed of several different types of pigment cells distributing in the skin during juvenile development. The zebrafish, Danio rerio, displays a striking pattern of dark stripes of melanophores interspersed with light stripes of xanthophores. A third cell type, silvery iridophores, contributes to both stripes and plays a crucial role in adult pigment pattern formation. Several mutants deficient in iridophore development display similar adult phenotypes with reduced numbers of melanophores and defects in stripe formation. This indicates a supporting role of iridophores for melanophore development and maintenance. One of these mutants, rose (rse), encodes the Endothelin receptor b1a. Here we describe a new mutant in zebrafish, karneol (kar), which has a phenotype similar to weak alleles of rse with a reduction in iridophore numbers and defects of adult pigment patterning. We show that, unlike rse, kar is not required in iridophores. The gene defective in the kar mutant codes for an endothelin-converting enzyme, Ece2, which activates endothelin ligands by proteolytic cleavage. By morpholino-mediated knockdown, we identify Endothelin 3b (Edn3b) as the ligand for endothelin receptor signalling in larval iridophores. Thus, Endothelin signalling is involved in iridophore development, proliferation and stripe morphogenesis in larvae as well as adult zebrafish. In mammals the pathway is required for melanocyte development; therefore, our results indicate a previously unrecognized close evolutionary relationship between iridophores in zebrafish and melanocytes in mammals.

KEY WORDS: karneol, Iridophores, Endothelin-converting enzyme, Endothelin signalling, Pigment pattern formation

INTRODUCTION

Adult zebrafish display a characteristic body and fin pigmentation pattern of alternating dark stripes and light interstripes. Three pigment cell types are known to build this pattern: black melanophores, yellow xanthophores and silvery iridophores. Melanophores covered by loose “blue” iridophores dominate the dark stripes, whereas dense iridophores covered by xanthophores generate the light stripes (Frohnhöfer et al., 2013; Hirata et al., 2003; Hirata et al., 2005). These pigment cells are derived from the neural crest, a transient embryonic structure in vertebrates which also contributes to many other tissues, e.g. bone, cartilage and the enteric nervous system (Le Douarin and Dupin, 2003). In zebrafish the larval pigmentation pattern is composed of iridophores and melanophores arranged into a dorsal, a ventral and a yolk-sac stripe, whereas melanophores align along the horizontal myoseptum into lateral stripes; xanthophores cover the entire larval body (Eisen and Weston, 1993; Kelsh, 2004; Raible and Eisen, 1994). The adult striped pattern develops during metamorphosis mainly by newly differentiating pigment cells in the dermis (Johnson et al., 1995; Kirschbaum, 1975; Parichy, 2003; Parichy et al., 2009). Iridophores reach the dermis at the horizontal myoseptum, proliferate and spread to create a series of dense ridges of interstripes that serve melanophores to accumulate into stripes (Frohnhöfer et al., 2013; Singh et al., 2014). Xanthophores closely follow iridophores in stripe development. Melanophore progenitors proliferate while migrating along spinal nerves into the skin where they melanise to form the dark stripes. Both iridophores and melanophores are derived from segmentally arranged postembryonic stem cells located close to dorsal root ganglia while the origin of metamorphic xanthophores is not known (Budi et al., 2011; Dooley et al., 2013; Hultman and Johnson, 2010; Singh et al., 2014).

Recently, a crucial role of iridophores in stripe formation in the skin of the body was demonstrated. Mutants deficient in iridophores show a strong reduction of melanophore numbers and defects in stripe formation (Frohnhöfer et al., 2013; Krauss et al., 2013; Patterson and Parichy, 2013). Four genes were so far found to be required for iridophore development, shady (shd) encoding Leukocyte tyrosine kinase (Ltk), rose (rse) encoding Endothelin-receptor b1a (Ednrb1a), transparent (tra) encoding the mitochondrial protein Mpv17 and bonaparte (bnp) encoding the transcription factor basonuculin-2 (bnc-2) (Krauss et al., 2013; Lang et al., 2009; Lopes et al., 2008; Parichy et al., 2000). In strong alleles of shd or rse only the first stripes, 1D and 1V, develop, albeit broken into spots; in weak alleles, some residual interstripe formation is observed, and the striped organization of the melanophores is better preserved. Chimeric animals obtained by blastula transplantations revealed that in the case of shd, rse and tra the genes are autonomously required in iridophores, while mutant melanophores and xanthophores are not affected and can form normal stripes when confronted with wild-type iridophores (Frohnhöfer et al., 2013; Krauss et al., 2013). This indicates that in all three cases the loss of iridophores causes the observed complex phenotypes, and that differentiated iridophores sustain melanophore development and stripe formation. In contrast to this, bnp seems to be required in a non-chromatophore cell type (Lang et al., 2009; Patterson and Parichy, 2013).

Both shd and rse encode plasma membrane receptor proteins. Ltk belongs to the class of receptor tyrosine kinases; it is expressed...
broadly in pre-migratory neural crest cells with gradual restriction to developing iridophores during later embryonic stages. Mutations in shd lead to a lack of iridophores throughout all developmental stages, which led to the assumption that Ltk function is required for the specification of iridophores (Lopes et al., 2008). Ednrb1a belongs to a family of G-protein coupled receptors. Mutations in rse, although the gene is expressed in pigment cells during early zebrafish development, do not show defects in embryonic iridophores (Parichy et al., 2000), possibly due to redundancy, as zebratil contain a second paralog, ednrb1b. For both receptors, the activating ligands have not been identified yet in zebratil. In mammals, where Endothelin signalling promotes the development of melanocytes, mutations in Ednrb or its ligand Endothelin 3 (Edn3) lead to a reduction of melanocytes, as well as aganlization caused by a strong reduction in sensory gut neurons (Baynash et al., 1994; Gariepy et al., 1996; Hosoda et al., 1994; Kunieda et al., 1996; Metallinos et al., 1998; Santschi et al., 1998). Similar reductions in the number of melanocytes were observed in mice carrying a knock-out allele of the endothelin-converting enzyme 1 (Ece1). This enzyme cleaves the inactive Endothelin precursor proteins to produce the biologically active 21-aa peptide ligands (Yanagisawa et al., 1998). In humans, mutations in EDN3 and EDNRB cause Waardenburg-Shah syndrome (Waardenburg syndrome type IV) hallmarked by defects in pigment cell specification (Kishimoto, 2002). Genetic linkage was determined as described previously (Geisler et al., 1996; Hofstra et al., 1996).

Here we investigate the function of the endothelin pathway in pigment pattern formation in zebratil. We describe karneol (kar), a new iridophore-deficient mutant that shows an adult-specific phenotype similar to weak rse karneol mutants. Chimeric animals reveal that in contrast to rse, shd and tra, kar is not required in iridophores or in any other chromatophore type. We identified a mutation in the endothelin-converting enzyme 2 (ece2) gene resulting in a premature stop and the loss of the C-terminal peptidase domain containing the catalytic centre of the enzyme. As neither rse nor kar mutants develop pigmentation defects in larvae, we investigated the role of endothelins in larval pigmentation. By expression analysis and morpholinio knockdown we identify Edn3b as potential ligand of rse acting in iridophore development in zebratil. Thus Edn3 signalling, which in mammals is involved in melanocyte development, affects specifically iridophores in zebratil.

**MATERIALS AND METHODS**

**Fish husbandry**

Zebrafish were maintained as described earlier (Brand et al., 2002); we used the following genotypes: karneol (kar), nacre^WT^, pfeffer^mu236b^, Tuebingen, albino^bd^, Tg(b-actin:GFP) and WIK. Embryos and larvae were staged as described previously (Kimmel et al., 1995). Staging of juveniles was done according to Parichy et al. (Parichy et al., 2009). The experiments with zebratil conform to the regulatory standards relevant for Baden-Württemberg, Germany.

**Transplantations**

Chimeric animals were generated as described previously (Kane and Kishimoto, 2002).

**Genetic mapping**

Genetic linkage was determined as described previously (Geisler et al., 2007). For validation of the candidate gene, ece2, genomic DNA or total RNA was prepared from fin clips using TRIzol Reagent (Invitrogen) according to manufacturer’s protocol. Reverse transcription was performed using total RNA and the Omniscript RT kit (Quagen). The following primers were used:

T1315: 5'-AGAGAGGAGACACAGTCCG-3'  
T1316: 5'-AGAGGAGACACAGTCCG-3'

T1317: 5'-AGAGAGGAGACACAGTCCG-3'  
T1318: 5'-ATGCCACACTCTATCTATCC-3'

T1319: 5'-CATCACAAGAACCGGACAC-3'

T1320: 5'-CTCTTTTCTGACAGATTC-3'

T1321: 5'-CGACAGTGGAGCTTACAC-3'

T1322: 5'-TCAAATCTCAGTGGTTC-3'

T1357: 5'-GATCAATGAAATCCGCAG-3'

T1358: 5'-GATCAATGAAATCCGCAG-3'

**Phylogenetic analysis**

The alignment of amino acid sequences was generated using ClustalW. Sequences with the following accession numbers were used: Ece1 human: NP_001388.1; Ece1 mouse: NP_955011.1; Ece1 chick: NP_990048.1; Ece1 zebrafish: NP_00071260.1; Ece1 human: NP_004812.7; Ece1 mouse: NP_067281.2; Ece1 chick: XP_422744.3; Ece1 zebrafish: ENSDARG00000060549; Ece2 human: NP_055083.3; Ece2 mouse: NP_808810.1; Ece2 chick: XP_003641814.1; Ece2 zebrafish: KJ622365.

**RNA in situ hybridization**

Templates for RNA probes were amplified by RT-PCR using an antisense DNA oligo containing a T7 promoter sequence on its 5’ end. The following oligos were used:

edn3b: 5’-CATCACAAGAACCGGACAC-3’ and 5’-TGGATCTCTAA-TGCACAACCTATAGGGGTAACGCCTTC-3’

edn3a: 5’-CGCTCCGAAAGCGCTGTTG-3’ and 5’-TGGATCTCTAA-TGCACAACCTATAGGGGTAACGCCTTC-3’

pnp4a: 5’-GCACGACCTGGTTTCTCAC-3’ and 5’-TGGATCTCTAA-TGCACAACCTATAGGGGTAACGCCTTC-3’

**Image acquisition**

Adult fish were briefly anaesthetized with 0.004% MS-3222 (Sigma) or fixed over night at 4 °C in 4% PFA/1% glutaraldehyde and imaged with Canon D5MarkII/Macro 100. The angle of illumination was adjusted individually to allow optimal visualization of iridophore pigmentation. Images of larvae were taken on a Discovery stereo microscope (Zeiss). Photographs were processed in Adobe Photoshop.

**Morpholinio injections**

The following antisense morpholinos for edn3b were obtained from Gene Tools:

edn3b AUG-MO: 5’-GTGACATCGAGGATCTAGTTAGCCAT-3’

edn3b splice-MO: 5’-TCACTGACAAAGAAGACTTACAC-3’

edn3b splice mismatch-MO: 5’-TCACTGACAAAGAAGACTTACAC-3’

Morpholinos were injected into Tuebingen and albino^bd^ one-cell stage embryos. Embryos were either fixed at 48 hpf and subjected to RNA in situ hybridization or analyzed at 5 dpf for pigment cell defects. Pools of 10 injected or control embryos were used for RT-PCR to test the efficiency of the splice morpholino using the following primer pairs:

edn3b forward: 5’-GATGAGGATGTCCAGAAC-3’

edn3b reverse: 5’-GTGTTGACATCGAGGATGATG-3’

eefla1 forward: 5’-GAGGAAATCCACGAAAGAGATC-3’

eefla1 reverse: 5’-AGGTCACAACCACATACCAGG-3’

**RESULTS AND DISCUSSION**

**Adult kar mutants are deficient in iridophores and melanophores**

In an ENU mutagenesis experiment for adult pigmentation phenotypes we identified a mutant with a strong reduction of iridophores and melanophores (Fig. 1A,B), which we named...
karneol (kar) after the semi-precious stone. The kar mutant phenotype is similar to that of weak rse alleles, e.g. rse\(^{AN17X}\) (Fig. 1C), whereas strong rse alleles, e.g. rse\(^{LF802}\), lead to an even further reduction of iridophores and melanophores (Fig. 1D). Both, kar and weak rse mutants develop two dark stripes, 1D and 1V, containing fewer melanophores than wild type and only remnants of 2D and 2V. Due to these similarities we performed complementation analysis between rse and kar; double heterozygotes show no mutant phenotype and we conclude that we identified a new locus required for iridophore development in adult zebrafish.

Metamorphosis of the body pigmentation pattern in kar mutants starts similar to wild type with the appearance and proliferation of iridophores in X0. At stage PB (Fig. 2A,E) there is a slight reduction in their number, but less pronounced than in strong rse mutants (Fig. 2I). This reduction becomes more apparent later in metamorphosis (Fig. 2A–H). In addition, from stage SP onwards the number of blue iridophores that spread dorsally and ventrally during juvenile stages is reduced in kar mutants (Fig. 2C,G). Unlike in strong rse mutants iridophores in kar mutants form thin ridges in the middle of the interstripe regions X0 and X1V (Fig. 2H,L). These ridges typically persist into adulthood in X0, while those in X1V dissolve during later stages. Similar to strong rse mutants, kar mutants show a reduction in the number of melanophores from stage SP onwards (Fig. 2C,G,K). In conclusion, both kar and rse are required for iridophore proliferation and aggregation during stripe formation, which argues for a role of kar in the Edn signalling pathway.

kar encodes Endothelin-converting enzyme 2

To identify the molecular lesion causing the kar mutant phenotype, we mapped the mutation using microsatellite markers. Three markers on chromosome 15 showed strong linkage with the mutant phenotype. One marker, G40280, was fully linked with no recombinant amongst the 46 mapping fish; the other two markers, z4396 and G39890, were less closely linked with 10 and 15 recombinants, respectively (Fig. 3A). This places the mutation on the beginning of chromosome 15 in an interval of less than 8.5 Mbp. The fully linked marker G40280 lies in an intron of the gene encoding Endothelin-converting enzyme 2 (Ece 2), which is a
strong candidate for kar. We sequenced cDNA of this transcript (GenBank accession: KJ622365), which corresponds to the Ensembl gene RNASEQG00000017608 without the long 3’ UTR. At bp 1456 of the CDS (genomic position Zv9:15:4049885) we identified a C to T substitution, which leads to a premature stop codon resulting in a truncation of the predicted protein at amino acid 487 with the complete loss of the C-terminal thermolysin-like peptidase domain, which contains the zinc-coordinating residues and the catalytic centre of the enzyme (Fig. 3B–D). A phylogenetic analysis of Endothelin-converting enzymes from several vertebrates (human, mouse, chick and zebrafish) confirmed that the gene encodes indeed the zebrafish homologue of Ece2 (Fig. 3E). To further demonstrate that the identified base substitution is indeed the mutation causing the kar phenotype, and not simply a polymorphism in the zebrafish genome without further consequence, we sequenced the site in 49 wild-type fish from different backgrounds. All of them showed a C at the relevant position. In addition we also sequenced the progeny of heterozygous kar mutant incrosses. All fish with the kar phenotype were homozygous for the identified substitution (n=96). Therefore we consider kar to be a loss-of-function allele of ece2.

kar is not required in iridophores nor other chromatophores

The rse and shd gene products are required within iridophores, but not in melanophores nor xanthophores (Frohnhofer et al., 2013). The similarities of kar with weak rse alleles prompted us to test if kar is also required in iridophores, or in any other chromatophore type. To this end we transplanted cells between embryos of different genotypes during the blastula stage. Transplantations of kar cells into rse hosts resulted in chimaeras with large patches of wild-type pattern and normal sized stripes (Fig. 4C). This indicates that kar mutants provide normal iridophores to rse mutant fish, and that kar is not required in iridophores.

Using nac:pfe mutant hosts (Fig. 4B) the resulting chimaeras, after transplantations of kar mutant cells, also developed normally patterned regions (Fig. 4D). Here the only host-derived pigment cells are iridophores, due to mutations in nacre/mitfa, required for melanophore development, and in pfeffer/fms, which acts in xanthophores. The rescue of the pigmentation pattern in this situation indicates that kar mutant melanophores and xanthophores are not affected and behave normally when confronted with wild-type host cells, as is the case for rse (Frohnhofer et al., 2013). However, transplantations of cells from nac:pfe into kar mutant hosts led only to the development of very small patches of dense iridophores and some local improvement of the kar residual pigment pattern (Fig. 4E,E’), showing that iridophores can only poorly develop in the kar mutant environment. Transplantations of GFP-marked cells from nac:pfe mutants into kar hosts showed donor-derived GFP-positive cells in the vicinity of these small patches of dense iridophores without all iridophores being labelled.

Taken together, these results demonstrate that there is no cell autonomous requirement for kar activity in any of the chromatophores. Instead kar promotes iridophore development in a non-cell autonomous manner, which is in agreement with the molecular nature of Ece2 as the identified kar gene product. Enzymes of this class proteolytically cleave inactive Pro-endothelins to produce active Edns. These secreted peptide ligands then bind to receptors, EdnrA, located in the plasma membranes of target cells and activate down-stream signalling. Our data suggest a paracrine mode of endothelin signalling during pigment patterning. Iridophores express the receptor Ednrb1a (Lang et al., 2009), the rse gene product, which in kar mutant embryos...
cannot be fully activated due to the lack of processed ligand. The ligand and the processing enzyme, Ece2, are likely to be produced by non-pigment cells in the vicinity of the iridophores. However, we cannot rule out that in the wild-type situation iridophores also express ece2 and contribute to the signalling in an autocrine manner.

There is considerable redundancy in the endothelin pathway in vertebrates. In zebrafish six genes encode Edn ligands, five Ednrs and three Eces (Braasch et al., 2009). The differences in the severity of the phenotypes in rse and kar mutants could be explained by this redundancy. One of the other two enzymes could also process ligand in the vicinity of iridophores leading to some residual signalling activity via the rse receptor in kar mutants. Redundancy and/or sub-functionalization could also explain the lack of defects during larval development in rse and kar mutants, where other receptors and enzymes might function.

Edn3b is required for larval iridophore development

In mammals Ednrb and Edn3 are involved in the development of melanocytes (Baynash et al., 1994). We investigated the expression of the two zebrafish edn3 paralogs during larval stages. The expression of edn3a at 24 hpf and 48 hpf is not detectable above background, whereas edn3b is strongly expressed in the epidermis (Fig. 5). To examine a potential role of edn3b in pigment cell development, we performed knockdown experiments by injection of morpholinos into one-cell stage embryos. Injection of morpholinos targeting either the translation start site or a splice site of edn3b pre-mRNA, led to a significant reduction of iridophores at 48 hpf, as measured by RNA in situ hybridization using pnp4a as an early and specific marker for iridophores (Lang et al., 2009) (Fig. 6A). We further analysed this phenotype by counting iridophores based on their reflective properties in the dorsal trunk of 5 dpf larvae (Fig. 6B). Both morpholinos resulted in similar reductions of iridophores at 5 dpf. The control 5 bp mismatch splice morpholino did not show such an effect. We did not detect defects in melanophore development in the larval pattern (data not shown). RT-PCR analysis of edn3b confirmed the activity of the splice morpholino (Fig. 6C). These results suggest that Edn3b is specifically required for iridophore but not for melanophore development during early larval stages. In contrast to this, edn3 signalling in mammals is specifically
required for melanocyte migration and maintenance in dermis and epidermis and the establishment of the enteric nervous system. In zebrafish there is evidence that a fraction of the adult melanophores share a common precursor with iridophores (Singh et al., 2014). It is conceivable that this population is homologous to melanocytes in amniotes. However, the majority of melanophores in zebrafish are independent of iridophores and we suspect that other paralogs of genes coding for endothelin signalling components are involved in their development.

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Competing interests

The authors have no competing interests to declare.

Author contributions

J.K., H.G.F. and C.N.V.-V. conceived and designed the experiments. J.K., H.G.F., U.I. and C.N.V.-V. wrote the manuscript.

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