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Morgan Newman, Lachlan Wilson, Esther Camp, Giuseppe Verdile, Ralph Martins, and Michael Lardelli

Abstract

Reliable animal models are required to facilitate the understanding of neurodegenerative pathways in Alzheimer's disease. Animal models can also be employed to search for disease-modifying drugs. The embryos and larvae of zebrafish are particularly advantageous for this purpose. For Alzheimer's disease, drugs that can ameliorate amyloid\(\beta\) (A\(\beta\)) toxicity have therapeutic and/or prophylactic potential. We attempted to generate a zebrafish model of A\(\beta\) toxicity that would be viable and fertile but have a highly visible pigmentation phenotype in larvae. The larvae could then be arrayed in microtiter plates to screen compound libraries for drugs acting to reduce A\(\beta\) toxicity. We used the promoter of the zebrafish \textit{mitf} (nacre) gene to drive expression of the pathological 42 amino acid species of human A\(\beta\), A\(\beta_{42}\), specifically in the highly visible melanophores (melanocytes) of transgenic zebrafish. However, the transgenic fish only showed an aberrant pigment phenotype in adults at the advanced age of 16 months. Nevertheless, our results show that alteration of zebrafish pigment pattern may be useful for analysis of toxic peptide action.

Introduction

There is considerable evidence supporting the idea that Alzheimer's disease is caused primarily by the accumulation of amyloid\(\beta\) (A\(\beta\)) peptides in the brain. The A\(\beta_{42}\) isoform is more hydrophobic than shorter forms. Consequently, it is less soluble and more prone to aggregation to form oligomers and, ultimately, amyloid plaques. Oligomerization and accumulation of A\(\beta_{42}\) is thought to result in neuronal cytotoxicity that induces neuropathological events leading to neurodegeneration. The exact mechanism by which A\(\beta\) peptide accumulation induces neurotoxicity is unclear.

Reliable animal models are required to facilitate study of the neurodegenerative pathways in Alzheimer's disease (AD). They aid in elucidation of the molecular, cellular, and pathological changes that trigger the onset of cognitive decline. Further, models are needed that can facilitate testing for compounds that affect various points of the pathogenic cascade in the hope of finding disease-modifying drugs.

Most transgenic models of A\(\beta\) action have been generated in mice. However, the utility of these mice in screening drug libraries for compounds that may combat A\(\beta\) toxicity is limited. Two invertebrate models better suited to drug screening exist. In 1995, Link generated a model of A\(\beta\) molecular pathology in \textit{Caenorhabditis elegans} by engineering these animals to express human A\(\beta_{42}\) specifically in muscle cells. These nematodes produced muscle-specific A\(\beta\) deposits that were immunoreactive with anti-A\(\beta\) antibodies. The transgenic larva displayed progressive muscle paralysis and vacuoles that were attributed to A\(\beta\) toxicity. A \textit{Drosophila} model of A\(\beta_{42}\) toxicity was developed by Crowther and colleagues in 2005. They expressed the Arctic mutant form of human A\(\beta\) in the central nervous system (CNS) and retina of transgenic flies. This resulted in intracellular accumulation of A\(\beta\) associated with progressive locomotor deficits and premature death.

The zebrafish is an advantageous model organism for studies of developmental gene function and disease processes in the nervous system. As vertebrates, they possess a brain structure similar to that found in mammals. However, their use in drug screening is facilitated by their ready availability in large numbers, their small size as embryos and newly hatched larvae, and their development outside the mother in an aqueous medium. Transgenic zebrafish can be generated through use of efficient vectors such as the Sleeping Beauty transposase system. We employed this system to generate transgenic zebrafish possessing human A\(\beta_{42}\) under the
control of the mitfa promoter that drives expression specifically in their highly visible melanophores. We aimed to produce zebrafish in which Aβ12 toxicity caused aberrant pigmentation patterns on embryos or larvae without affecting their viability or fertility. These animals could then be used to screen for drugs that suppress the transgenic phenotype. Such drugs would be candidate suppressors of Aβ12 toxicity.

Materials and Methods

Transgene construction and transgenesis

Linearized pT2-mitfa-Aβ-EGFP DNA (see Results section) was co-injected into embryos at the one-cell stage with transposase mRNA generated from the p5BRNAX plasmid. The transposase recognizes inverted repeats flanking the insert in pT2 and excises the insert for integration into the genome. Adult fish were raised from injected embryos and were then outbred individually to wild-type fish. Progeny from each mating were pooled, and genomic DNA was purified from each pool for testing by polymerase chain reaction (PCR) for the presence of the pT2-mitfa-Aβ-EGFP transgene (Fig. 1A and see PCR1 below). An improved PCR test with less nonspecific amplification (Fig. 1A and see PCR2 below) was developed to test for the transgene in tail-clips from individual aged adult fish.

Nucleic acid purification/preparation

Genomic DNA preparation from zebrafish tail-clips was performed essentially as described by Reinhart and Bogerd, with adult fish anesthetized in 168 µg/mL tricine solution before tail-clipping. Proteases were removed by phenol/chloroform extraction and then ethanol precipitation. RNA was extracted from adult zebrafish skin using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). RNA samples were precipitated with lithium chloride (Ambion, Austin, TX) and re-dissolved in water before treatment with RNase to remove any traces of contaminating DNA. These were then reverse transcribed into cDNA as previously described in Newman et al.

Polymerase chain reactions

PCR1: 30 cycles of 94°C 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C using primer pair 1: 5’GCAATGTGTTGGGCCGA3’ and 5’TACGGCTATGCAACCCG3’.

PCR2: 30 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 72°C using primer pair 2: 5’GGGTATCTAGAAGACAAAGT3’ and 5’TTCGCTACGCTATGACAC3’.

Nested PCR for detection of expression of mitfa. The initial PCR used primers 5’GGTTCATGCGAATGGACTT3’ and 5’GGGGAAGAAGCAGATCCG3’ with 30 cycles of 20 s at 98°C, 20 s at 63°C, and 30 s at 72°C (25 µL total volume). Two microfactors of the initial PCR was then placed into a second, nested PCR using the primers 5’CAGATCCGATCTTCCAGCA3’ and 5’ATAACGAGCCAGGATGTC3’ with 30 cycles of 20 s at 98°C, 20 s at 65°C, and 30 s at 72°C (25 µL total volume).

Whole-mount in situ hybridization

Embryos were fixed in 4% formaldehyde in the embryo medium before manual de-chorionation. Whole-mount in situ transcript hybridization (WISH) was then performed essentially as described by Jowett using a digoxigenin-labeled antisense EGFP RNA probe.

Results

To construct a transgene for expression of human Aβ12 peptide specifically in zebrafish melanophores, we fused an optimized secretory signal peptide sequence (“HMM” see Barash et al.) to DNA coding for human Aβ12 followed by a stop codon. This coding sequence was then inserted into a derivative of the Sleeping Beauty vector pT2R6 containing a ~1 kb fragment of the zebrafish mitfa promoter (GenBank accession AF211990, and see Dorsky et al.). The insertion of the HMM-Aβ12 fusion into the pT2 derivative was performed such that it separated the vector’s EGFP coding sequences from the mitfa promoter. The resultant transgene was named pT2-mitfa-Aβ-EGFP (Fig. 1A). Transcripts from the mitfa promoter of pT2-mitfa-Aβ-EGFP should include the vector’s EGFP coding sequences, but these would not be translated.

To generate transgenic fish, linearized pT2-mitfa-Aβ-EGFP DNA was co-injected into embryos with transposase mRNA at the one-cell stage. At 24 hpf a subset of the injected embryos was fixed for WISH against EGFP coding sequence. This should detect transcripts from the transgene that include the nontranslated EGFP coding sequence. As expected, this revealed cells expressing the transgene in positions consistent with migrating melanophores (e.g. see Fig. 1B). Extended staining was required to reveal these cells (32 h at 4°C and 8 h at room temperature), implying that the cloned mitfa promoter is not highly active and/or that the transcripts are relatively unstable.

Ten adult fish were raised from injected embryos and were then outbred individually to wild-type fish. Progeny from each mating were pooled before extraction of genomic DNA to test for the presence of the transgene (see PCR1 in Materials and Methods section). This revealed that two of the original injected adult fish were transmitting the transgene to their progeny. Out-crossed progeny from these fish were then grown to adulthood. Transgenic individuals were detected from among the progeny using a PCR (PCR1) on genomic DNA purified from the fish after tail-clipping. Ultimately, two independent lines of transgenic fish, line 1 and line 2, were established.

We could not discern any abnormalities in embryos and larvae from lines 1 or 2, when visible pigmentation developed after 24 h of development. Therefore, these transgenic lines were not useful for screening of compound libraries to find drugs that ameliorate Aβ toxicity. However, by 16 months of age some adult fish in both lines began to show loss of stripes of melanin pigmentation but retention of normal reflective pigment (in iridophores) and yellow pigmentation (in xanthophores). In many cases, small foci of melanin pigmentation could be observed, especially in the fins of the stripeless fish (Fig. 1D lower panel). We do not know whether this represents cells with abnormal melanosome distribution or cells dying in a manner consistent with a progressive, age-dependent phenomenon (or both).

The populations of each transgenic line of fish were the outbred progeny of single individuals heterozygous for the transgene, so one would expect approximately half the fish in these populations to possess the transgene (unless multiple
transgene inserts were present in a line). At 16 months the line 1 population had 7 of 30 fish and the line 2 population had 3 of 22 fish that lacked normal dark stripes. At 19 months line 1 had 10 of 30 fish and line 2 had 6 of 22 fish that lacked normal dark stripes. The final observations were made at 24 months, where 11 of 30 fish (33%) of the line 1 population and 6 of 22 fish (27%) of the line 2 population lacked normal dark stripes. In contrast, the progeny of a nontransgenic sibling of the founders of transgenic lines 1 and 2 all showed normal pigmentation at 24 months of age (21 fish observed).

The late onset of the apparent pigment loss phenotype of the mitfs-Aβ-EGFP transgene implies that the transgene is active in melanophores (or their precursors) in adult skin. (However, it is possible that early transgene expression might predispose aged melanophores to premature loss, e.g., by sensitizing these cells to accumulated stress.) Since it has not yet been demonstrated formally that the endogenous mitfs promoter is active in the skin of adult wild-type zebrafish, we performed a nested reverse transcription (RT)-PCR test to detect mitfs transcripts. No amplified product was evident after agarose gel electrophoresis of the reaction products. However, when 2 μL of the initial PCR was then placed into a second, nested PCR a signal indicating the presence of mitfs cDNA was evident (Fig. 1C). Hence, mitfs is transcribed in adult zebrafish skin.

A similar nested RT-PCR was used to detect expression of the mitfs-Aβ-EGFP transgene in the skin of affected transgenic fish. Total cellular RNA was extracted from the excised dorsal skin of individual transgenic fish from line 1. However, despite performing RT-PCR with three separate sets of initial and nested PCR primer pairs, we were unable to detect the presence of transgene transcripts (data not shown). This may be due to a combination of factors (see Discussion section).

To support that the age-dependent stripe loss was due to the presence of the transgene, we tested the transgenic status of eight stripeless fish and eight fish with stripes in the line 1 population as well as three fish from a wild-type population. An improved PCR test with less nonspecific amplification, PCR2, was performed on DNA samples prepared from the clipped tails of these fish. All stripeless fish and six of eight striped fish were shown to be transgenic (Fig. 1E, F). As expected, the PCR test was negative for the wild-type fish (data not shown). Thus, presumed expression of Aβ12 in the melanophores of zebrafish results in noticeable loss of melanin pigmentation by 16 months. If this represents melanophore

FIG. 1. (A) The pT2-mitfs-Aβ-EGFP transgene. The transposon construct in the Sleeping Beauty vector. The binding positions of the primer sets used for polymerase chain reaction (PCR) tests are indicated above the transgene. Arrows at the end of the transgene are the inserted repeats recognized by the transposase mRNA. (B) Whole-mount in situ transcript hybridization for transcripts containing EGFP coding sequences in a 24 hpf embryo. A lateral view (slightly oblique, sagittal optical section) at the midregion of the yolk extension (ye). Dorsal is to the top. Arrowheads indicate putative immature melanophores lacking pigmentation. The upper indicated cell was observed to lie near the level of the horizontal myoseptum in the region between the notochord (nc) and the myotome (myo), consistent with the melanocyte migration pattern zebrafish (Jesuthasan, 1996). The indicated cells appeared somewhat dendritic. (C) Nested reverse transcription (RT)-PCR to detect mitfs in the skin of wild-type adult zebrafish. Lane 1 is the PCR product from wild-type skin; lane 2 is the negative (no RNA/cDNA) control. M indicates the DNA marker ("2-Log DNA Ladder"). (D) Adult zebrafish at 16 months. Upper panel: a representative normal adult zebrafish. Lower panel: a transgenic fish that had specific loss of melanophores. (1) and (2) are enlarged images of corresponding areas of the caudal fin (see boxes). (E, F) PCR on genomic DNA from zebrafish tail clips to detect the presence of the transgene. Eight striped fish (E, lanes 1–8) and eight stripeless fish (F, lanes 1–8) from an outcross of line 1 were tail clipped, and genomic DNA was tested for the presence of the transgene. "-" indicates detection of transgene and "-" indicates no detection. (F) Lane 9 is blank, lane 10 is a negative control (no DNA) reaction, and lane 11 is from a reaction containing pT2-mitfs-Aβ-EGFP plasmid DNA as a template as a positive control. M indicates the DNA marker, which is "2-Log DNA Ladder" supplied by New England BioLabs (Ipswich, MA). Color images available online at www.liebertonline.com/zeb.
loss, it may occur by their premature death and/or failure to maintain a precursor cell population (if these also express mitfα). Unfortunately, by the time the pigmentation phenotype was observed at 16 months, these fish were no longer fertile, thus precluding propagation of the transgenic lines for further observation.

Discussion

Three types of pigment cells differentiate from the neural crest during zebrafish embryo development: the starkly visible black melanophores, the less obvious yellow xanthophores, and the silvery reflective iridophores. Zebrafish nacre mutants lack all melanophores and have marginally reduced xanthophores and increased iridophore numbers. The zebrafish mitfα/nacre gene is a duplicate of an ancestral ortholog of the human MITF gene. MITF is a key regulator of the melanocyte/melanophore lineage and is involved in the differentiation, growth, and survival of pigment cells. Importantly, Mitf is expressed in adult mouse hair follicles and MITF is expressed in adult human skin since melanocytes are distributed throughout the epidermis. This study provides RT-PCR evidence for expression of endogenous mitfα in adult zebrafish skin. We presume that this occurs primarily or exclusively in melanophores since, in humans, the TYROSINASE (gene required for melanin pigment formation in melanocytes) is activated by binding of the MITF transcription factor. This idea is supported by the apparent late manifestation of the toxic effect of Aβ12 expression in zebrafish; that is, aberrant pigmentation was not observed until 16 months of age (although the possibility exists that expression of Aβ12 from the mitfα promoter in melanophores or melanophore precursors may predispose melanophores or their precursors for cell death at a later time point).

Transcripts from the mitfα-Aβ12-EGFP transgene could be detected in injected embryos at 24 hpf (Fig. 1B). The positions of the cells expressing the transgene were consistent with what we know of melanophore differentiation and migration, supporting that the transgene is active in these cells, at least at this early time point. Our failure to detect transcripts of the transgene by RT-PCR in the skin of affected adults may be due to one or a combination of factors. For example, the transgene may be transcribed at relatively low levels compared to the endogenous mitfα gene due to possible lack of enhancer elements in the cloned mitfα promoter. Indeed, detection of the transgene by WISH in the as yet pigmentless melanophores of the 24 hpf embryo required an extended staining reaction.) Also, the insertion sites of the transgene in lines 1 and 2 may have been suboptimal for promoter activity, and/or the transgene transcript may be relatively unstable. These factors, combined with the small proportion of all adult skin cells that melanophores represent and the ongoing toxic effects of the transgene on the melanophores in these aged fish, may have reduced the concentration of transgene mRNA to levels below which our RT-PCR could not amplify it. Nevertheless, we think that it is highly improbable that the observed pigment loss phenotypes are artefactual since only fish possessing the transgene were affected and the presence of the transgene specifically affected melanophores rather than the other two pigment cell types (xanthophores and iridophores) in which mitfα is not expressed. (Note, mitfα expression has been observed in some cells that may be xanthophore precursors, but xanthophores do not require mitfα activity to differentiate.) Unfortunately, the age and infertility of the transgenic fish prevented us from propagating them for future analysis and limited the number of individuals we had available to study. We note that in the only previously published work to have used the mitfα promoter in germline transgenics, Patton et al. only reported expression of their oncogenic transgene within tumor tissue and not in embryos (see Fig. 3C of Patton et al.), and detection of the action of the transgene in adult fish was by observation of changes in pigmentation pattern alone.

Melanocyte stem cells (MSC) exist in humans and mice, as evident through the mammalian hair follicle cycle. Age-associated graying is a result of loss of MSCs from the hair follicle. The existence of MSCs in zebrafish adults has been proposed since melanophores can be observed throughout the regenerating caudal fin 4 days after its amputation. Thus, the delayed phenotype observed in the transgenic fish expressing human Aβ12 could be due to the presence of MSCs during development and in adults producing new melanophores as the transgenic ones die. It is possible that, as the fish age, the ability of MSCs to generate melanophores and new MSCs declines, such that the toxicity of human Aβ12 eventually reduces MSC and melanophore numbers. Therefore, the age-related loss of melanophores due to human Aβ12 toxicity may be similar to age-associated graying in humans, which is due to loss of MSCs.

The mitfα promoter has previously been used to analyze the function of the human BRAF gene activity in melanoma formation using zebrafish, but we believe that our study is the first example of using this promoter to examine the function of a protein not commonly associated with melanophore biology. Future work will examine the effects of increased Aβ toxicity and whether this can overcome melanophore regeneration to produce an early phenotype more amenable to screening of libraries of chemical compounds.

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Disclosure Statement

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