New animal models to study the role of tyrosinase in normal retinal development

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. The rescue of the albino phenotype in mammals using tyrosinase transgenes
4. Tyrosinase inducible systems and retinal development
5. Studying retinal development with tyrosinase transgenes
6. Perspective
7. Acknowledgments
8. References

1. ABSTRACT

Albino animals display a hypopigmented phenotype associated with several visual abnormalities, including rod photoreceptor cell deficits, abnormal patterns of connections between the eye and the brain and a general underdevelopment of central retina. Oculocutaneous albinism type I, a common form of albinism, is caused by mutations in the tyrosinase gene. In mice, the albino phenotype can be corrected by functional tyrosinase transgenes. Tyrosinase transgenic animals not only show normal pigmentation but the correction of all visual abnormalities associated with albinism, confirming a role of tyrosinase, a key enzyme in melanin biosynthesis, in normal retinal development. Here, we will discuss recent work carried out with new tyrosinase transgenic mouse models, to further analyse the role of tyrosinase in retinal development. We will first report a transgenic model with inducible tyrosinase expression that has been used to address the regulated activation of this gene and its associated effects on the development of the visual system. Second, we will comment on an interesting yeast artificial chromosome (YAC)–tyrosinase transgene, lacking important regulatory elements, that has highlighted the significance of local interactions between the retinal pigment epithelium (RPE) and developing neural retina.

2. INTRODUCTION

Transgenic animal models constitute a classical approach used by investigators to clarify how diseases are generated and progress. Several strategies, including conventional transgenes, genomic transgenes (artificial chromosome-type constructs such as YACs or bacterial artificial chromosomes (BACs)), and inducible transgenes systems have been developed and are widely used (1). Valuable developmental information can also be obtained from these transgenic animals, converted into excellent models to study the formation of different organs or anatomical structures, as analyzed in comparison with their corresponding wild-type counterparts.

Diseases affecting pigmentation have widely triggered the interest of investigators due to their obvious associated phenotypes, such as: alterations in the coat and eye colour pigmentation (2, 3, 4, 5). A large amount of animal models of pigmentation diseases and the molecular description of genes encoding several pigmentation functions have been reported, using mice (6, 7), fish (8, 9) and other vertebrates. The most common group of hypopigmentation diseases is albinism. Albinism is a complex syndrome characterized by the total or partial absence of pigmentation in the eye, skin and hair (10, 11)
Tyrosinase expression and retinal development

Figure 1. Albinism in mice. Functional rescue of the albino phenotype of recipient animals in transgenic tyrosinase mice. A) A newborn tyrosinase transgenic founder (on the right) can be easily detected by simple visual inspection (presence of pigment in eyes and skin), as compared with non-transgenic albino littermates (on the left). B) Adult mice corresponding to similar the genotypes shown in A. Transgenic tyrosinase mouse (left) and albino mouse (right).

First, albino individuals have an underdeveloped central retina. In albino primates, this defect corresponds to the absence of the fovea, the area of maximal visual acuity (13). In lagomorphs (rabbits and hares), the underdevelopment of the central retina in albino individuals is seen as a reduced cell density along the visual streak (14, 15). The underdevelopment of central retina is difficult to investigate in rodents, due to their poorly defined central retina (16) but it has also been documented in other mammals, such as cats (17).

Second, there is a cellular deficit in nearly all layers of the retina, most notably a 30% reduction in the rod photoreceptor numbers, as seen in albino rodents (18, 19). Interestingly, a rather similar phenotype was first observed in humans, where a thinner outer nuclear layer (ONL) of the retina (mainly corresponding to photoreceptor cell nuclei) was documented in albino subjects, as compared to normally pigmented individuals (13).

Third, chiasmatic pathways in albino individuals are abnormal. In normally pigmented mammals, many ganglion cells located in the temporal retina project their axons into the brain, at the lateral geniculate nucleus of the same side (ipsilateral or uncrossed projection) while the ganglion cells found in the nasal retina project their axons to the opposite side of the brain (contralateral or crossed projection), crossing the midline at the optic chiasm (20). This pattern of partial decussations at the chiasm forms the anatomical basis for stereoscopic vision in mammals. In albino individuals, many of the cells that would normally project ipsilaterally cross the midline inappropriately and thus project contralaterally, resulting in impaired binocular vision (Figure 2). This anomaly has been documented in a range of albino mammals including rodents (21), rabbits (22), cats (23), ferrets (24) and humans (25). The percentage of uncrossed projections is a species-specific parameter and varies from about 45 %, in primates, to 5 %, in rodents, according to their frontally or laterally placed eyes, respectively (20).

Finally, some cell proliferation abnormalities are found in developing albino retina. In the neural retina of albino animals there is a cell cycle alteration during development, reflected by a transient increase of proliferation and a subsequent increase of apoptosis, that results in a deficit of cells in nearly all layers of the adult albino retina (26, 27, 28, 29).

The most common form of albinism, oculocutaneous albinism type-I (OCA-I), is due to mutations in the tyrosinase gene, encoding the key enzyme in melanin biosynthesis (30, 31, 32). Around 90 different mutations that cause albinism have been mapped in the human tyrosinase gene (33, 34 and http://www.cbc.umn.edu/tad), and, in mice, the majority of the 102 known mutant alleles of the mouse tyrosinase gene correlate with some degree of tyrosinase functional impairment and, hence, albinism (7). The most common mutation producing albinism in mice is C103S, present in most of the different laboratory albino mouse strains (35, 36, 37) (i.e. CD1, Swiss, SLJ, NMRI, FVB, BALB/C). Indeed, the mouse tyrosinase gene and the albino mutation, that correlate with lack of functional tyrosinase protein, represents one example of a mutation whose phenotype remains unaltered regardless of the genetic background (independent of the action of modifier genes) in which the albino mutation is present (38). Apart from the null or low enzymatic activity of these mutant proteins, it has been demonstrated that non-functional tyrosinase forms are likely to be retained in the endoplasmic reticulum and thus do not reach their target subcellular organ, the melanosome (39, 40).

Numerous phenotypic studies, carried out using classical approaches demonstrated the direct relationship
Tyrosinase expression and retinal development

Figure 2. Schematic representation of visual pathways in a pigmented and an albino animal with frontalized eyes (i.e. a primate). Left drawing, a pigmented individual showing the presence of crossed and uncrossed retinal ganglion cells axonal projections going from the eye to the lateral geniculate nucleus (LGN), in the brain, through the chiasm. Right drawing, an analogous representation of an albino individual, showing a severe decrease in the uncrossed visual pathway and a corresponding increase in the crossed axonal fibres.

Figure 3. The mouse tyrosinase locus and associated tyrosinase transgenes. The mouse wild-type tyrosinase allele (Tyr⁺), with 5 exons (shown as numbered black rectangles), a promoter (shown with an arrow) and its locus control region (LCR), including the described A and B target sequences for nuclear proteins corresponding to the enhancer core unit of the LCR (84, 85, 88). Below, two YAC-tyrosinase constructs are represented corresponding to the entire tyrosinase expression domain (YRT2 construct, 49) and a derivative transgene, lacking the LCR (YRT4 construct, 85). The YAC-vector ends are represented with triangles. The two transgenic constructs used in the tetracycline-inducible tyrosinase transgenic mice (61) are displayed above the wild-type endogenous tyrosinase allele, with indications of the relevant regulatory and coding DNA sequences derived from the mouse tyrosinase locus.

between the amount of pigment in the RPE and the size of the uncrossed retinal axon projection, regardless of the locus affected (41, 42, 43). The expression of the mouse tyrosinase gene is tightly regulated during development. It is expressed from 16.5 days post-coitum (dpc) in melanocytes from hair bulbs and skin, originating from migrating neural crest cells, and from 10.5 dpc in the RPE cells, that develop in the eye from the original optic cup (44, 45). The aim of this review is to discuss different animal models recently generated that have been applied to study the role of the tyrosinase gene in mammalian retinal development, that is, how the expression of the tyrosinase gene (and hence, the production of melanin in the RPE) is required for normal mammalian retinal development.

3. THE RESCUE OF THE ALBINO PHENOTYPE IN MAMMALS USING TYROSINASE TRANSGENES

The first reported rescue of the mouse albino phenotype, and the confirmation of the association of the tyrosinase gene (Tyr) with the classical albino locus (C), came from pioneer experiments with transgenic mice performed in the late 1980s. A number of different transgenic mouse lines were obtained on albino mouse genetic backgrounds, using standard (plasmid-type) tyrosinase constructs driven by limited DNA fragments of 5' upstream regulatory sequences of the mouse tyrosinase locus. These transgenic mice showed the rescue of the hypopigmented phenotype, though with a high variability in the degree of pigmentation between independent lines, and, further, they did not usually reach faithful melanin levels as found in wild-type pigmented animals (46, 47, 48). The subsequent use of larger genomic fragments, encompassing most if not all the mouse tyrosinase expression domain, for the generation of transgenic animals (mice and rabbits) resulted in stronger pigmentation phenotypes. Indeed, the use of a 250 kb YAC, named YRT2, covering the whole mouse tyrosinase locus resulted in transgenic animals that were indistinguishable from wild-type pigmented individuals (49, 50, reviewed in 51).
The phenotypic analyses in tyrosinase transgenic animals clearly demonstrate that tyrosinase, directly or indirectly, has a fundamental role in retinal development. Although the actual mechanism underlying this process remains unknown, this role can be mediated by the end-product (melanin) or by intermediate metabolites of the melanin biosynthetic pathway in which tyrosinase is involved (27). Interestingly, mutations in other genes encoding proteins involved in the formation of melanosomes, the organelles in which melanin is produced, or in other proteins necessary for melanin synthesis lead to the visual abnormalities associated with tyrosinase-albinism (20, 55, 56, 57). An increase of tyrosinase protein using conventional tyrosinase transgenes in a pigmented wild-type mouse results in a concomitant increase in tyrosinase enzymatic activity, but it does not lead to an increase in the appearance of phenotypic traits associated with normal tyrosinase function (58). For instance, there were no additional ipsilaterally-projecting axonal fibres generated, suggesting that tyrosinase is required to maintain the fibre ratio, but not to specify the number of retinal ganglion cells that will project ipsilaterally (58). This observation suggests that an upper threshold of tyrosinase activity also exists, since higher values of enzymatic activity do not result in an increase of phenotypic traits commonly associated with normal tyrosinase function. These results, along with reports in which differences in rod numbers or in central retina development are not observed, irrespective of pigmentation levels (59, 60), potentially suggest that a given threshold of tyrosinase activity has to be reached in order to avoid visual (retinal) abnormalities commonly associated with albinism.

4. TYROSINASE INDUCIBLE SYSTEMS AND RETINAL DEVELOPMENT

Without a functional tyrosinase enzyme no melanin can be synthesized, resulting in an albino phenotype. To address the question of how much tyrosinase activity is necessary to avoid the generation of visual abnormalities, two independent experimental approaches using tyrosinase inducible transgenes have been generated (61, 62). In the most recent study, tyrosinase expression was induced in a mouse albino genetic background using the TET-ON system (61), a binary transgenic approach in which the expression of the tyrosinase gene is ultimately controlled by the exposure to a drug, doxycycline (63) (Figure 3 and Figure 5). Doxycycline interacts with a reverse tetracycline-controlled transcriptional transactivator protein (rtTA), provided by the HSTyrTET transgenic construct, and promotes the binding of rtTA to its target DNA sequence (TRE, tetracycline responsive element), included in the second transgenic construct (TRETyBS), which has been engineered to drive the expression of a tyrosinase cDNA under the control of the inducible TRE element, eventually resulting in an increase of transcription of the tyrosinase gene being controlled.

In these double transgenic tyrosinase-inducible mice, minor basal uninduced tyrosinase expression was detected, pointing to the existence of limited leakiness of this inducible system, as reported in similar experiments.
Figure 5. Tyrosinase inducible systems in transgenic mice. A) Generation of transgenic mice with inducible tyrosinase expression using the tetracycline system. Two independent transgenic mouse lines were produced. First, Tyr-rtTA, expressing the reverse tetracycline-controlled transactivational transactivator protein (rtTA, TET-ON) under the control of mouse tyrosinase regulatory sequences. Second, TRE-Tyr, expressing a mouse tyrosinase cDNA under the control of TRE, an inducible promoter. The most adequate lines were selected and crossed. The resulting double transgenic mouse displayed an enhanced eye pigmentation upon induction with doxycycline (61). None of these mice showed any detectable traces of pigment in skin. B) Generation of transgenic mice with inducible tyrosinase expression using the lac system. Two independent transgenic mouse lines were produced. First, β-actin-lacI, expressing the lac repressor protein ubiquitously. Second, Tyr-lacO, expressing a mouse tyrosinase cDNA under the control of a mouse tyrosinase promoter in which several lac operator sequences have been inserted. The resulting double transgenic mouse recovered skin and eye pigmentation upon induction with IPTG (62).

Despite the leakiness observed, an increase in the eye pigmentation (but not skin pigment) was found in the group of induced animals that were exposed to doxycycline since the first week of embryonic development, eventually resulting in the correction of the abnormal ipsilateral projections, mostly absent in albino individuals, in most transgenic mice that were analysed (61). Remarkably, this phenotypic correction is achieved without reaching the melanin levels that are characteristic of wild-type pigmented individuals, thus supporting the hypothesis that a given tyrosinase enzymatic activity threshold needs to be present, to rescue chiasmatic abnormalities. However, it was not possible to verify the corresponding correction in rod photoreceptor deficits, most likely due to the high variability observed in the analyses or to the requirement for much higher tyrosinase activity (higher pigmentation) changes than those obtained in order to observe some rod number correction (59, 61).

Similar results have been obtained with an independent inducible approach, reported earlier, in which the tyrosinase gene expression was regulated by the lactose analogue isopropyl beta-d-thiogalactopyranoside (IPTG) (62, 67) (Figure 5). In this alternative tyrosinase-inducible animal model, the authors turned back to one of the first inducible systems that were initially proposed for regulating gene expression in mammalian cells (68), based on the use of the bacterial lac repressor protein and its ability to target lac operator sequences and block transcription in the absence of IPTG, a reagent that binds to the lac repressor and triggers its release from the target DNA lac operator sequences, thereby allowing transcription. The original system was shown to be largely non-functional or reproducible in transgenic mice, due to a number of reasons, including the silencing of lac repressor-containing transgenes due to strong hypermethylation effects that were normally observed (69). Subsequently, it was reported that lac repressor coding regions in transgenic constructs could be activated if the entire lac repressor DNA sequence was re-encoded using mammalian codons, instead of the prokaryotic original codons, prone to become hypermethylated in mice (70). In this regard, using fully functional lac repressor transgenes, in combination with tyrosinase transgenes, driven by a tyrosinase promoter in which several lac operator sequences were inserted, the authors could show inducible tyrosinase expression, and hence pigmentation, both in the skin (though with obvious variegation) and in the eye, in double transgenic mice exposed to IPTG (62). In the experiments reported with IPTG-inducible tyrosinase transgenic mice the authors showed some reversible regulation, since most of the pigment in the skin (but not apparently in the eye) was lost in the adulthood, after removal of IPTG soon after birth. Similar to what it was shown with tetracycline-inducible tyrosinase transgenic mice, the IPTG-inducible tyrosinase...
Tyrosinase expression and retinal development

transgenic mice supported also the correction of the uncrossed (ipsilateral) chiasmatic pathway that is mostly absent in albino mice (71) and that early induction of tyrosinase expression was required to allow proper retinal development, including the generation of both crossed and uncrossed retinal ganglion cell axonal projections into the brain (61, 71).

In summary, the IPTG-inducible tyrosinase transgenic mice were more efficient in promoting tyrosinase expression (and hence, pigmentation) in skin melanocytes, but showed a similar behaviour to the tetracycline-inducible tyrosinase transgenic mice in retinal pigment epithelium cells. This last model did only show variation in pigmentation in the eye, but not in skin, possibly related to the low transgene expression and inherent toxicity of the transactivator rtTA protein (61, 72). Both models were comparably useful to address how tyrosinase expression is required to sustain normal retinal development in mammals (61, 71).

Although the universal use of IPTG-inducible transgenic models has been discussed as a valid strategy for the efficient regulation of gene expression (73), there are only few other examples available (i.e. 74). The main disadvantage of the IPTG strategy, in comparison with the tetracycline approach, is that it requires first the insertion of several lac operator sequences within the promoter to be regulated, in order to work efficiently. Moreover, there is no general rule regarding where these sequences need to be inserted within promoter regions to reproducibly interrupt the normal transcription process, after binding of the corresponding lac repressor protein. However, this potential problem was solved in the case of the tyrosinase inducible model (62). In contrast, the vast majority of tetracycline-inducible transgenic models rely on the use of the same minimal promoter, TRE, carrying several target binding sites for the tetracycline-controlled transcriptional activator (tTA) and the rtTA transactivator proteins, and have already been shown to work reproducibly in a large number of experiments (75, 76, 77), although they are also not exempt of limitations and requirements for optimization (78, 79, 80).

5. STUDYING RETINAL DEVELOPMENT WITH TYROSINASE TRANSGENES

The tissue-specific expression of the mouse tyrosinase gene is controlled by proximal promoter regulatory elements (81, 82), that include binding sites for microphthalmia, the key nuclear factor responsible for tyrosinase transcription (66) and, in retina development, a factor that determines the RPE fate of original optical vesicle cells (83). However, additional regulatory elements operating at a higher (chromatin) level are required for the appropriate expression of the mouse tyrosinase expression domain, most importantly a locus control region (LCR) (84, 85), located at –15 kb, that has been shown to contain, at least, composite enhancer elements (84, 86, 87) associated with boundary elements (88) and scaffold/matrix-attachment regions (89). The deletion of this LCR in YAC-tyrosinase transgenes, a YAC construct called YRT4 (Figure 3), results in transgenic mice displaying hypopigmentation, mostly seen in hair and skin, that appears lightly pigmented, in patches, but not apparently in eyes (85) (Figure 6). The variegated expression of the tyrosinase transgenes in skin, iris and choroid (29), correlated with a mosaic distribution and delayed pigmentation in the retina, suggesting that the absence of the LCR did not alter the tissue-specific expression pattern of the mouse tyrosinase construct but the probability by which the tyrosinase construct could be efficiently transcribed, a feature previously observed in other mammalian loci and transgenes in the absence of LCR or LCR-like regulatory elements (90, 91). Interestingly, upon detailed investigation of mice carrying YAC-tyrosinase transgenes lacking the LCR, a peculiar variegation pattern was found in the RPE, in which central areas of the retina appeared essentially devoid of melanin, while pigmentation progressively increased to almost normal levels in more peripheral retinal regions (29, 92) (Figure 7). YRT4 transgenic mice have been used to ask whether the tyrosinase gene expression, and hence pigmentation, in RPE cells had only a regional impact on the adjacent developing neural retina or, in contrast, the effects were blurred and evenly distributed across the retina (92). In these mice, the number of rod photoreceptors in peripheral retinal areas, where the RPE was heavily pigmented, were similar to that seen in wild-type pigmented animals, while in central retinal areas, mostly devoid of melanin, the number of rods was similar to that found in albinos. Also, the ipsilateral projection that arises from the temporal pigmented periphery in YRT4 animals was normal. These results were consistent with the hypothesis that RPE-neural retina interactions are local and that presumably relevant molecules or metabolites are exchanged between RPE and adjacent developing neural retina (92).

6. PERSPECTIVE

The application of diverse strategies using animal models allows the study and characterization of interesting
Tyrosinase expression and retinal development

Figure 7. Analysis of the retina of the YRT4 transgenic mouse. (A) Histological retinal section of an adult YRT4 transgenic mouse counterstained with Cresyl Violet (29). Note the discontinuous pigmented and non-pigmented retinal pigment epithelium (RPE) cells (arrow). A few pigmented melanocytes can also be observed in the surrounding choroid (CHO). (B) Whole-mount retina of an adult YRT4 transgenic mouse (29, 92) showing the gradient of pigmented RPE cells from central regions, next to the optical nerve (ON), increasing towards more peripheral regions. The black tissue visible in the centre of the retina, next to the ON, corresponds to the rests of surrounding pigmented choroid.

It is also unknown the factor involved in retinal development whose presence correlates with tyrosinase activity. Currently, a provocative model points towards 3,4-dihydroxy-L-phenylalanine (L-DOPA), an intermediate metabolite in the melanin biosynthetic pathway, whose accumulation is reduced in albino individuals (27), and can control cell cycle (96). The addition of L-DOPA in vitro to developing albino mouse eyes can correct proliferation and cell-death defects to figures similar to those found in wild-type pigmented individuals (27). Moreover, there is an increasing number of indirect evidences, related to other visual abnormalities supporting this idea (97, 98, 99, 100). Nevertheless, this hypothesis remains to be addressed in vivo. In particular, experiments need to be performed in albino recipient animals to study whether albino-associated visual abnormalities could be rescued upon a regulated increase of retinal L-DOPA levels, without the involvement of pigmentation. Finally, the study of alterations in gene expression profiles in albino retinal development can lead to discover the function of genes involved in specific roles in normal retinal development, by comparison with the gene expression profiles in wild-type pigmented animals.

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Tyrosinase expression and retinal development

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Tyrosinase expression and retinal development


Tyrosinase expression and retinal development


Abbreviations: BAC, bacterial artificial chromosome; dpc, days post-coitum; IPTG, isopropyl beta-d-thiogalactopyranoside; LCR, locus control region; L-DOPA, 3,4-dihydroxy-L-phenylalanine; OCA-I, oculocutaneous albinism type I; ONL, outer nuclear layer; RPE, retinal pigment epithelium; rtTA, reverse tetracycline-controlled transcriptional activator; TRE, tetracycline responsive element; tTA, tetracycline-controlled transcriptional activator; UTR, untranslated region; YAC, yeast artificial chromosome

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