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Review

Genetics of pigment cells: lessons from the tyrosinase gene family

F. Murisier and F. Beermann

ISREC (Swiss Institute for Experimental Cancer Research),

National Center of Competence in Research (NCCR) Molecular Oncology, Epalinges, Switzerland

Summary. In mammals, the melanin pigment is produced in two cell types of distinct developmental origins. The melanocytes of the skin originate form the neural crest whereas the retinal pigment epithelium (RPE) of the eye originates from the optic cup. The genetic programs governing these two cell types are thus quite different but have evolved to allow the expression of pigment cell-specific genes such as the three members of the tyrosinase-related family. Tyrosinase, *Tyrp1* and *Dct* promoters contain a motif termed E-box which is bound by the transcription factor Mitf. These E-boxes are also found in the promoters of the corresponding fish genes, thus highlighting the pivotal role of Mitf in pigment cell-specific gene regulation. Mitf, which displays cell type-specific isoforms, transactivates the promoters of the tyrosinase gene family in both pigment cell lineages. However, specific DNA motifs have been found in these promoters, and they correspond to binding sites for RPE-specific factors such as Otx2 or for melanocyte-specific factors such as Sox10 or Pax3. The regulation of pigment cell-specific expression is also controlled by genetic elements located outside of the promoter, such as the tyrosinase distal regulatory element located at -15 kb which acts as a melanocyte-specific enhancer but also protects from spreading of condensed chromatin. Thus, by using the tyrosinase gene family as a model, it is possible to define the transcription factor networks that govern pigment production in either melanocytes or RPE.

Key words: Melanocytes, RPE, Tyrosinase, Promoter, Enhancer

Offprint request to: Dr. Friedrich Beermann, ISREC (Swiss Institute for Experimental Cancer Research), Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland. e-mail: friedrich.beermann@isrec.ch

Introduction

The coat color phenotypes have been studied for more than one century leading to the establishment of some of the first rodent inbred strains (Morse, 1978). The mouse strain DBA selected for the *dilute, brown* and *non-agouti* coat color alleles was already established in 1909 by Little (Beck et al., 2000). An albino strain was used to first demonstrate Mendelian inheritance of a genetic trait in mammals in 1903 (Castle and Allen, 1903; Beermann et al., 2004). Thus, pigment genes were "pioneers" for the exploration of mouse genetics leading to the discovery of 127 separate loci with 63 genes characterized so far (Silvers, 1979; Bennett and Lamoreux, 2003; Oetting, 2005).

Among them, the three genes of the tyrosinase-related family, namely, tyrosinase, *Tyrp1* (Tyrosinase-related protein 1) and *Dct* (Dopachrome tautomerase) were also discovered by analysis of coat color mutants (Haldane et al., 1915; Hertwig, 1942; Green, 1972). 103 spontaneous or induced phenotypic alleles have been described for tyrosinase, and they result in albinism or hypopigmentation due to absence or reduction of melanin in homozygotes (Fig. 1) (Beermann et al., 2004; Mouse Genome Database (MGD), 2005). For *Tyrp1*, 49 phenotypic alleles are known, and homozygous *Tyrp1*-mutant mice show a brown coat color phenotype on a non-agouti background instead of the wild-type black color (Fig. 1) (Mouse Genome Database (MGD), 2005). Three mutations and one targeted deletion have been described at the *Dct* gene locus, resulting in a dark grey coat color on a non-agouti black background (Fig. 1) (Guyonneau et al., 2004; Mouse Genome Database (MGD), 2005).

The pigment production machinery

In mammals, the production of the melanin pigment is achieved in two cell types of distinct developmental origins. The melanocytes of the skin, hair follicle, inner

ear, choroid, iris and ciliary body originate from the neural crest, whereas the cells of retinal pigment epithelium (RPE) originate from the optic cup of the developing forebrain (Fig. 2) (Mayer, 1973; Martinez-Morales et al., 2004). The melanin is produced and stored in specialized lysosome-related organelles called melanosomes. The three members of the tyrosinase-related family are involved in the process of melanogenesis leading to the production of either eumelanin (brown-black) or pheomelanin (yellow-red).

The first step of melanin production involves tyrosinase (monophenol monooxygenase, EC 1.14.18.1) which catalyses the transformation of tyrosine into dopaquinone. In the eumelanin pathway, the dopaquinone is then transformed into dopachrome. The further transformation of this product involves Tyrp1 and Dct, which share about 40% amino acid identity with tyrosinase, but display their own and distinct catalytic capacities (Jackson et al., 1992). Dct (Dopachrome tautomerase, EC 5.3.3.12) catalyses the transformation of dopachrome to dihydroxyindole carboxylic acid (DHICA) (Tsukamoto et al., 1992). The role of Tyrp1 (5,6-dihydroxyindole-2-carboxylic acid oxidase, EC 1.14.18.-) is more controversial since it acts as a DHICA oxydase but also stabilizes tyrosinase (Jimenez-Cervantes et al., 1994; Kobayashi et al., 1998; Garcia-Borron and Solano, 2002). When Tyrp1 and Dct functions are not impaired, the pigment produced is black. Mutation of *Tyrp1* induces the production of a brown pigment (Bennett et al., 1990). The loss of *Dct* leads to a dark grey color (Guyonneau et al., 2004). In addition, Dct might influence the balance between eumelanin and pheomelanin synthesis (Costin et al., 2005).

Genomic structure and evolution of the tyrosinase-related family

The genomic structure of the tyrosinase-related family suggests that it has evolved from one ancestral tyrosinase gene. The duplication of this gene is probably responsible for the emergence of a tyrosinase-related gene. *Tyrp1* and *Dct* are evolutionarily more closely related to each other than to tyrosinase and thus they might have themselves duplicated from the primitive tyrosinase-related gene (Budd and Jackson, 1995; Sturm et al., 1995). The first duplication event must have taken place before the divergence of ascidians and vertebrates since two members of the tyrosinase gene family are found in *Halocynthia roretzi* (Sato et al., 1999) and in *Ciona intestinalis* (Ensembl genome browser, 2005). The second duplication event then occurred before the divergence of fishes and mammals since the three members of the family are found in Fugu (*Takifugu rubripes*) (Camacho-Hubner et al., 2000, 2002), Goldfish (*Carasius auratus*) (Peng et al., 1994; Sato et al., 1999) or Zebrafish (*Danio rerio*) (Kelsh et al., 2000; Page-McCaw et al., 2004; Ensembl genome browser, 2005).

In the mouse genome, the 5 exons of the tyrosinase

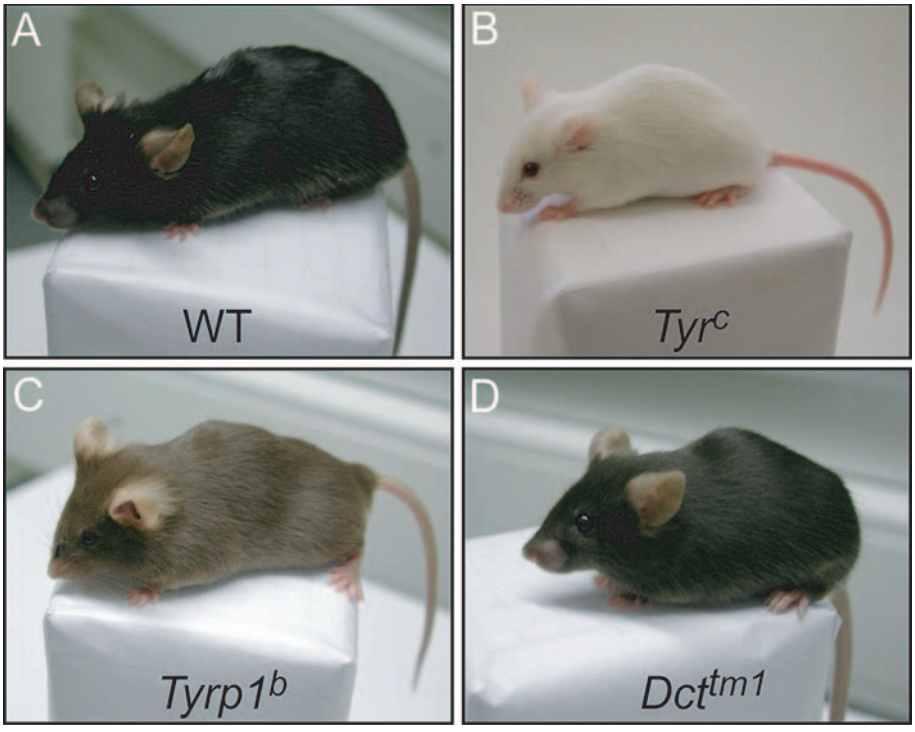

Fig. 1. Coat color phenotypes in mice (nonagouti, a/a) resulting from spontaneous or targeted mutations in the tyrosinase-related family genes. **(A)** wild-type black mouse, **(B)** *Tyr*^{c} albino mouse, **(C)** *Tyrp1*^{b} brown mouse and **(D)** *Dct*$^{tm1(cre)Bee}$ dark grey mouse.

gene are dispersed in a 66 kb region located on chromosome 7 (Fig. 3) (Kwon et al., 1987; Ensembl genome browser, 2005). In Fugu or Medakafish genomes, which are much more compact, the 5 exons are found in regions spanning less than 5 kb (Inagaki et al., 1998; Camacho-Hubner et al., 2000).

Despite its smaller size (17.5 kb), the mouse *Tyrp1* gene located on chromosome 4 has eight exons, of which the first one is non-coding (Fig. 3) (Jackson et al., 1991; Bell et al., 1995; Ensembl genome browser, 2005). The Fugu *Tyrp1* gene has a conserved structure with seven coding exons spanning 2.3 kb (Camacho-Hubner et al., 2002).

The *Dct* gene is located on chromosome 14 in the mouse and, like *Tyrp1*, has 8 exons spanning 40 kb (Fig. 3) (Jackson et al., 1992; Ensembl genome browser, 2005). This organisation is conserved in the 3 kb of the Fugu *Dct* gene (Camacho-Hubner et al., 2002).

Thus, each gene of the tyrosinase-related family has its separate genomic organisation, and the position of only one intron is conserved between all three members. Following the two successive duplication events, the three genes have probably gained new introns allowing further divergences (Jackson et al., 1991, 1992; Budd and Jackson, 1995; Sturm et al., 1995). On the other hand, each individual member of the family reveals strong conservation from fishes to mammals indicating the phylogenetic relevance of the melanin biosynthetic pathway. For instance, conserved amino acid domains are involved in the catalytic function, three-dimensional structure or sorting and localisation of the protein to the melanosome (Peng et al., 1994; Kelsh et al., 2000; Camacho-Hubner et al., 2002).

Conservation in the promoters of the tyrosinase-related family

Evolutionary conserved DNA motifs are also found in promoters or more distal regions of the tyrosinase family genes. Moreover, the mechanisms controlling pigment cell-specific gene expression appear to be conserved from fishes to mammals since promoters of heterologous origin are active in either mouse or fish. The mouse tyrosinase promoter is able to drive expression to pigment cells in medakafish (Hyodo-Taguchi et al., 1997), and the fugu tyrosinase promoter is able to drive expression to melanocytes and RPE in transgenic mice (Camacho-Hubner et al., 2000). Quail and turtle tyrosinase promoters have also been successfully used to drive specific expression to mouse melanocytes (Sato et al., 2001). *Tyrp1* and *Dct* promoters from fugu have been shown to be active in a mouse melanoma cell line (Camacho-Hubner et al., 2002). This indicates that some of the pigment cell-specific transcription factors and their related binding sites are well conserved in vertebrates.

Sequence analysis of fish tyrosinase promoters reveals the presence of E-box motifs (CANNTG), which are known binding sites for bHLH-LZ transcription factors. In particular, a CATGTG motif is present in the 5' upstream sequence of the zebrafish tyrosinase, medakafish tyrosinase, fugu tyrosinase, fugu *Tyrp1* and fugu *Dct* genes (Inagaki et al., 1998; Camacho-Hubner et al., 2000, 2002; Camp et al., 2003). This sequence has been shown to be bound by the Microphthalmia (Mitf) transcription factor which is a key player in the establishment of the pigment cell lineage (Aksan and Goding, 1998; Hou et al., 2000). Another E-box containing motif termed M-box (AGTCATGTGCT), located at -104 to -93 bp from the mouse tyrosinase start site, was shown to be conserved in more closely related species such as the turtle, quail, chicken, mouse and human (Jackson et al., 1991; Lowings et al., 1992; Bentley et al., 1994; Ganss et al., 1994c; Sato et al., 2001). This 11-mer motif is also present in the human and mouse *Tyrp1* promoters (Jackson et al., 1991;

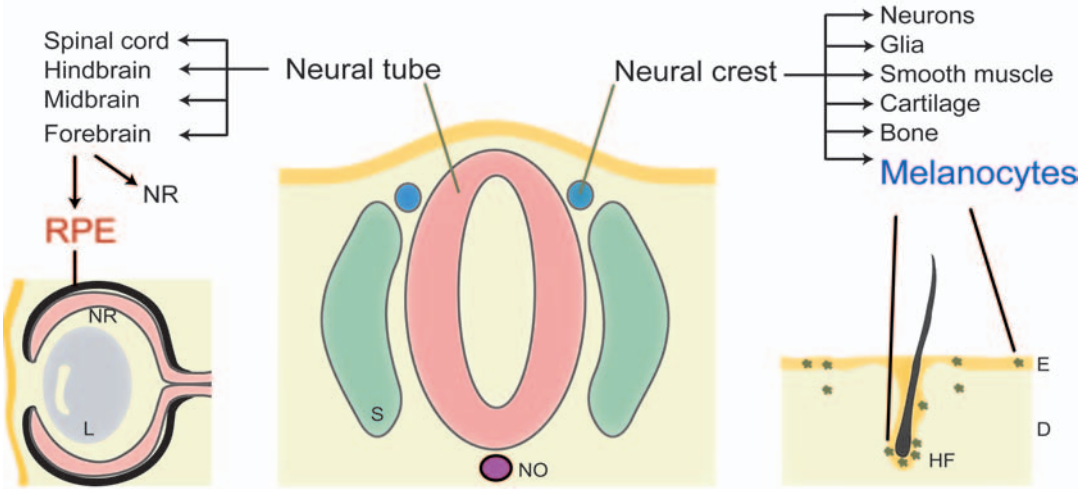

Fig. 2. Distinct developmental origins of pigment cells. The cells of the retinal pigment epithelium (RPE) originate from the optic cup of the developing forebrain, whereas the melanocytes of the skin, hair follicle, inner ear, choroid, iris and ciliary body originate from the neural crest. NR: neural retina; L: lens; S: somite; NO: notochord; E: epidermis; D: dermis; HF: hair follicle.

Shibahara et al., 1991; Yasumoto et al., 1997). A very close homologue of the M-box is also found in mouse and human Dct promoters (Yokoyama et al., 1994; Budd and Jackson, 1995). This strong conservation between species and between the three members of the family underlines the pivotal role of E-boxes in pigment cell gene regulation. This process clearly involves the bHLH-LZ transcription factor Mitf (Bentley et al., 1994; Ganss et al., 1994c).

Another element termed MSE (melanocyte specific element, GTGTGA), which is probably involved in restriction of expression to melanocytes, is found in fugu and mouse *Tyrp1* promoters (Yavuzer and Goding, 1994; Camacho-Hubner et al., 2002). In fugu *Dct*, a distal element containing an HMG consensus binding site has been shown to correspond to the human Dopachrome tautomerase distal enhancer 1 (DDE1) (Amae et al., 2000; Camacho-Hubner et al., 2002). In the mouse, this element has been shown to be bound by Sox10, which is a crucial transcription factor for the development of vertebrate neural crest cells (Mollaaghababa and Pavan, 2003; Jiao et al., 2004; Ludwig et al., 2004; Wegner, 2005).

The tyrosinase promoter

Different lengths of tyrosinase upstream sequence driving a tyrosinase minigene have been used in transgenic mice, allowing the identification of regions involved in gene regulation (Beermann et al., 1990, 1991; Yokoyama et al., 1990). It was demonstrated that a 270 bp upstream region is sufficient for specific but weak expression in melanocytes and RPE (Kluppel et al., 1991). This "minimal promoter" contains an M-box (position -104 to -93) which is a binding site for Mitf. Two other elements have been defined as positive (position -245 to -230) and negative regulatory elements (position -193 to -125) but their roles have not been defined so far (Ponnazhagan and Kwon, 1992; Ganss et al., 1994b, c). The analysis of the human tyrosinase promoter has highlighted the presence of another E-box (position -12 to -7), which is perfectly conserved in the mouse promoter. This initiator E-box also recruits MITF transcription factor (Bentley et al., 1994; Yasumoto et al., 1994, 1997; Aksan and Goding, 1998). A third conserved E-box element has been found in the human tyrosinase upstream sequence (position -1861 to -1842) termed tyrosinase distal element (TDE) which also interacts with MITF (Yasumoto et al., 1994, 1997; Aksan and Goding, 1998). The initiator E-box has been shown to be essential for the activation of the tyrosinase promoter by MITF. The M-box, and more importantly the TDE, act to further increase the level of tyrosinase expression (Fig. 4) (Bentley et al., 1994; Yasumoto et al., 1994, 1997).

The CATGTG E-box motif is recognised by MITF but also by other members of the bHLH-LZ family. It has been reported that a 5' flanking T residue is required for the binding of MITF to the E-box, thus increasing the binding specificity. Moreover, this conserved flanking T residue surrounds the initiator E-box, the M-box and the TDE (Aksan and Goding, 1998). The ubiquitously expressed bHLH-LZ transcription factor USF1 (Corre et al., 2004) is also able to bind efficiently the tyrosinase E-boxes (Bentley et al., 1994; Yasumoto et al., 1994; Bertolotto et al., 1996), and mediates UV-induced tyrosinase expression (Galibert et al., 2001). Thus, MITF might be responsible for constitutive tyrosinase expression whereas USF1 might be involved in the UV response.

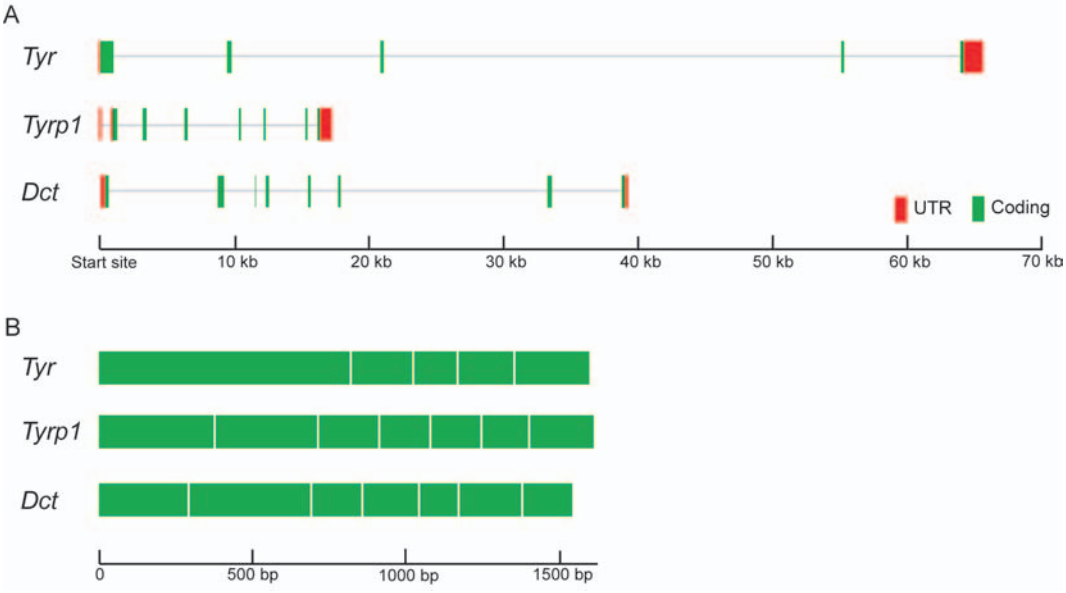

Fig. 3. The mouse tyrosinase-related family. **A.** Genomic organisation with translated and untranslated regions of exons indicated. **B.** Schematic representation of coding sequences with indication of exon-intron structure.

The transcription factor TFE3, which belongs to the bHLH-LZ family, has a strong homology with MITF. This factor is expressed in a number of different tissues including melanocytes. It has been shown that overexpression of TFE3 stimulates tyrosinase expression through binding to the M-box and initiator E-box. However, the binding of endogenous TFE3 to the M-box was not detected. Thus, the functional relevance of the interaction of TFE3 with the tyrosinase promoter remains to be elucidated (Verastegui et al., 2000).

The transcription factor SP1 has been shown to bind and transactivate the human tyrosinase promoter at position -45. Moreover, it was demonstrated that Sp1 binds the mouse tyrosinase promoter at the same relative position to the M-box. Sp1 is probably not involved in the control of cell-specific expression but might act as a sensor of differentiation, which is characterized by an increase of tyrosinase expression (Bentley et al., 1994; Rieber and Strasberg Rieber, 1999).

Otx2, a paired-type homeobox transcription factor, is able to bind and transactivate the mouse tyrosinase promoter through consensus sequences located at -1943 and at -413. Moreover, when assayed together, Otx2 and Mitf act in a synergistic fashion (Martinez-Morales et al., 2003). Since Otx2 expression is restricted to the retinal pigment epithelium during adulthood, this interaction seems not to be relevant for the melanocyte lineage (Fig. 4).

Taken together, these data underline the complex integrative process that takes place on the tyrosinase promoter allowing for a tightly regulated control of gene expression. This process becomes even more complex knowing that the central player, Mitf, is placed downstream of the c-Kit/MAP kinase pathway, the Wnt pathway, the stress response pathway or the MC1R/cAMP pathway (Goding, 2000; Widlund and Fisher, 2003; Steingrimsson et al., 2004; Vance and Goding, 2004).

The *Tyrp1* promoter

The first *Tyrp1* exon in human and mice is an untranslated sequence, which together with the first intron, seems to be required for efficient gene expression but does not confer pigment cell specificity (Jackson et al., 1991; Shibata et al., 1992; Sturm et al., 1995). The *Tyrp1* promoter (up to -1.3 kb) combined with the first intron was shown to confer expression in a melanocyte cell line (B16) but not in 3T3 cells. Moreover, further deletions in the promoter revealed that most of the cis-acting sequences are located between the positions -322 and -44 (Lowings et al., 1992). The in vivo situation appears to be quite different since the *Tyrp1* promoter (up to 4.5 kb) drives detectable transgene expression only to RPE but not to melanocytes (Raymond and Jackson, 1995; Schmidt et al., 1999; Mori et al., 2002). This probably reflects the absence of important melanocyte-specific regulatory sequences located either upstream or downstream of the promoter.

As for tyrosinase, the *Tyrp1* promoter contains a M-box (AGTCATGTGCT) located between positions -44 and -34 in the mouse gene (Jackson et al., 1991; Lowings et al., 1992). Microphthalmia is able to bind to this M-box leading to a strong transactivation of the promoter (Yasumoto et al., 1995, 1997; Aksan and Goding, 1998; Bertolotto et al., 1998). There is some evidence that other members of the bHLH-LZ family such as Usf1, TFE3 or TFEB bind also to this M-box (Yavuzer and Goding, 1994; Aksan and Goding, 1998; Verastegui et al., 2000). Another E-box-like element (CAAGTG), located at position -233 in the mouse, is also transactivated by Mitf but to a lower extent (Bertolotto et al., 1998).

Extensive series of mutations and deletions have been performed on the *Tyrp1* promoter leading to the identification of another genetic element located between position -322 and -299. This region was shown to contain an octamer motif which is recognized by the ubiquitous OCT1 transcription factor and potentially by other related POU domain containing members (Lowings et al., 1992). Two negative regulatory elements have been mapped to the initiator site and to position -240 (respectively MSEi and MSEu). A melanocyte-specific factor termed MSF has been shown to interact with two GTGTGA motifs located on these elements but was not required for repression. It has been proposed that MSF acts as an antirepressor involved in the control of *Tyrp1* expression in melanocytes (Yavuzer and Goding, 1994). Further experiments have shown that the repressor that binds to MSEi and MSEu is the T-box transcription factor Tbx2 which is expressed in different tissue including melanocytes (Carreira et al., 1998). Thereafter, MSF has been shown to be the Pax3 transcription factor. Moreover, Pax3 overexpression induced an up-regulation of *Tyrp1* promoter activity (Galibert et al., 1999). Beside its role on *Tyrp1* expression, Pax3 has been shown to be essential for melanocyte development (Hornyak et al., 2001).

As in tyrosinase, the transcription factor Otx2 is able to bind and transactivate the human *TYRP1* promoter. This interaction takes place through two bicoid/Otx consensus elements located at position -595 and -142 (Martinez-Morales et al., 2003). The interaction of Otx2 with the *TYRP1* promoter indicates that the control of pigment cell-specific gene expression is achieved through different modalities in melanocytes and RPE (Fig. 4).

The *Dct* promoter

A stretch of 447 bp of human *DCT* promoter has been shown to be sufficient for high and melanocyte-specific expression in cell culture. Moreover, the level of expression was similar to that obtained with much larger constructs (up to 6.7 kb) (Yokoyama et al., 1994). In transgenic mice, the use of either 3.7 or 1.7 kb of mouse promoter sequence was sufficient to specifically drive expression to endogenous Dct-expressing sites (Steel et

al., 1992; Pavan and Tilghman, 1994; Mackenzie et al., 1997; Zhao and Overbeek, 1999; Hornyak et al., 2001; Guyonneau et al., 2002). Similar to tyrosinase and *Tyrp1*, *Dct* is expressed in RPE and melanocytes. In addition, *Dct* is also expressed in the telencephalon, but the relevance of this finding has not been resolved (Steel et al., 1992; Pavan and Tilghman, 1994).

Analysis of the human *DCT* promoter sequence reveals the presence of an M-box element (GGTCATGTGCT) located at position -138 to -128 (Yokoyama et al., 1994). The M-box has been shown to be essential for efficient *DCT* expression as shown by site-specific deletion. Another E-box was found at position -346 to -340 (Bertolotto et al., 1998). However, the binding and transactivation of the *DCT* promoter by MITF is still under debate since different studies performed with either the human or mouse Mitf on the human promoter came to contradictory and opposite conclusions (Yasumoto et al., 1997; Bertolotto et al., 1998; Schwahn et al., 2005). This discrepancy might reflect species-specific regulation or the need of specific cofactors for binding and transactivation of the *Dct* promoter by Mitf. In this way, it has been shown that LEF1 cooperates in a synergistic manner with MITF to transactivate the human *DCT* promoter. Moreover, the LEF1 binding site is found adjacent to the M-box (Yasumoto et al., 2002). Besides the M-box and the LEF1 binding site, a third cis-acting region is required for the transctivation by MITF and LEF1. This region was located at position -249 to -233 and was shown to contain a motif similar to a cAMP-responsive element (CRE-like) (Bertolotto et al., 1998; Yasumoto et al., 2002). Interestingly, ß-catenin, another partner of the Wnt signalling pathway, was able to enhance the synergistic interaction of LEF1 and MITF on the *DCT* promoter (Yasumoto et al., 2002). Thus, MITF is able to act as a nuclear mediator for LEF1/Wnt signalling in melanocytes (Saito et al., 2003).

In vitro and *in vivo* experiments have shown that Sox10 acts as a transcriptional activator of the human and mouse *Dct* promoter (Britsch et al., 2001; Potterf et al., 2001; Lang et al., 2005). The mouse *Dct* promoter contains several Sox10 consensus binding sites. Three of them, located at positions -141, -292 and -323, have been shown to be targeted by SOX10. On one of these sites, adjacent to the M-box, the interaction of Mitf and SOX10 was analysed, revealing a strong synergistic transactivation of the *Dct* promoter by both transcription factors (Ludwig et al., 2004). A series of deletion studies have shown that a region, located at positions -447 to -415 in the human *DCT* gene, was required for pigment cell-specific expression (Yokoyama et al., 1994). This element was termed *Dct* distal enhancer (DDE) and was shown to contain a potential binding site for HMG transcription factors such as SOX10 or the TCF/LEF1 family (Amae et al., 2000). A region located at position -459 to -426 in the mouse *Dct* promoter corresponding to the human DDE was shown to contain two Sox consensus binding sites at positions -438 and -426. The mutation of either one of these sites decreased the transactivation of *Dct* by SOX10 by 2-fold (Jiao et al., 2004).

The transactivation of the *Dct* promoter by either Sox10, Mitf or the combination of both has been shown to be repressed by the Pax3 transcription factor. This occurs by interactions within the region that contains the M-box, the Lef1 and Sox10 binding sites (Fig. 4). Mitf and Pax3 act as competitors for binding to the Dct promoter. Pax3 in collaboration with Lef1 recruits the Groucho co-repressor Grg4 inducing the repression of the *Dct* promoter. Moreover, ß-catenin abolished Pax3 repressing activity by displacing Grg4 and Pax3 from the promoter. It has been proposed that Pax3 initiates the melanocyte lineage-specific programme by activating expression of specific factors such as Mitf but also prevents terminal differentiation by repressing Mitf from activating downstream genes such as *Dct* (Lang et al., 2005).

Estrogen receptor-α has been shown to interact with the *DCT* promoter through an ERE motif overlapping with the M-box. Moreover, estrogen receptor-α in cooperation with p300 synergistically interacts with MITF to induce a strong *DCT* transactivation (Schwahn et al., 2005). In the RPE, the transcription factor OTX2 has been shown to bind the human *DCT* promoter at position -104 to -99. Moreover, overexpression of OTX2 in RPE cell lines induced a transactivation of the *DCT* promoter (Takeda et al., 2003).

Control of gene expression by distal regulatory elements

In addition to the promoter, specialized sequences such as insulators, boundary elements, locus control regions (LCRs), repressors or enhancers are often required to ensure tight control of gene expression in time and space. Evidence for the presence of such distal regulatory sequences in the pigmentation genes came from transgenic rescue experiments. When the mouse tyrosinase promoter was used to drive a tyrosinase minigene, 270 bp were sufficient to drive tyrosinase gene expression to pigment cells and thus induced pigmentation in albino mice. However, even with larger fragments (2.1 kb or 5.5 kb), the pigmentation obtained remained often weak and showed integration site dependence (Beermann et al., 1990, 1992; Yokoyama et al., 1990; Kluppel et al., 1991; Ganss et al., 1994b). On the other hand, a yeast artificial chromosome (YAC) containing 155 kb of 5' upstream sequence was shown to completely rescue the albino phenotype in transgenic mice (Schedl et al., 1993). Since these mice where indistinguishable from black wild-type mice, it was concluded that the YAC contained all regulatory sequences necessary for tyrosinase expression.

Further substantial evidence for the presence of important upstream sequences in the tyrosinase gene came from analysis of the *chinchilla-mottled* mutation ($Tyr^{c\text{-}ch}$). This mutant was obtained from the progeny of

a neutron-irradiated male and displayed transversal grey stripes on the back. Molecular analysis revealed a rearrangement in the sequence located upstream of the tyrosinase start site starting from -5 kb up to at least -30 kb. The amount of tyrosinase mRNA analysed by Northern blot was shown to be significantly reduced in the grey areas of the mutant mice (Porter et al., 1991). Active genetic elements are often associated with DNase I hypersensitive sites indicating accessibility for protein binding. Such analysis, performed on the tyrosinase upstream sequence, revealed the presence of a melanocyte-specific hypersensitive site (HS) at position -15 kb (Porter et al., 1991; Ganss et al., 1994a; Porter and Meyer, 1994).

Albino rescue experiments performed with a YAC in which the HS has been deleted gave rise to mice with weak and variable pigmentation. Moreover, mosaic patterns of expression were observed in both melanocytes and RPE indicating that the transgene is not protected from positional effects. The HS-bearing element is thus crucial for proper tyrosinase expression and was defined as a locus control region (LCR) (Montoliu et al., 1996; Gimenez et al., 2001). The combination of the tyrosinase promoter and 3.6 kb of upstream regulatory sequence to drive a tyrosinase minigene resulted in grey to black hair pigmentation. The level of transgene expression was proportional to copy number indicating that the 3.6 kb fragment confers position independence (Ganss et al., 1994a; Porter and Meyer, 1994). In the eye, the enhancer was shown to be active in pigment cells of neural crest origin as present in the choroid and the iris. Porter and Meyer (1994) have observed a lower enhancer activity in the RPE compared to the choroid whereas no differences were reported by Ganss et al. (1994). However, it was noticed that the 3.6 kb fragment was conferring integration-site independent expression in both RPE and melanocytes (Ganss et al., 1994a; Porter and Meyer, 1994). Transgenic experiments using the lacZ reporter gene have shown that the tyrosinase promoter alone drives detectable but weak expression to RPE whereas the 3.6 kb distal regulatory element was necessary to confer strong expression in melanocytes. In addition, the presence of the 3.6 kb element not only failed to act as an enhancer in the RPE,

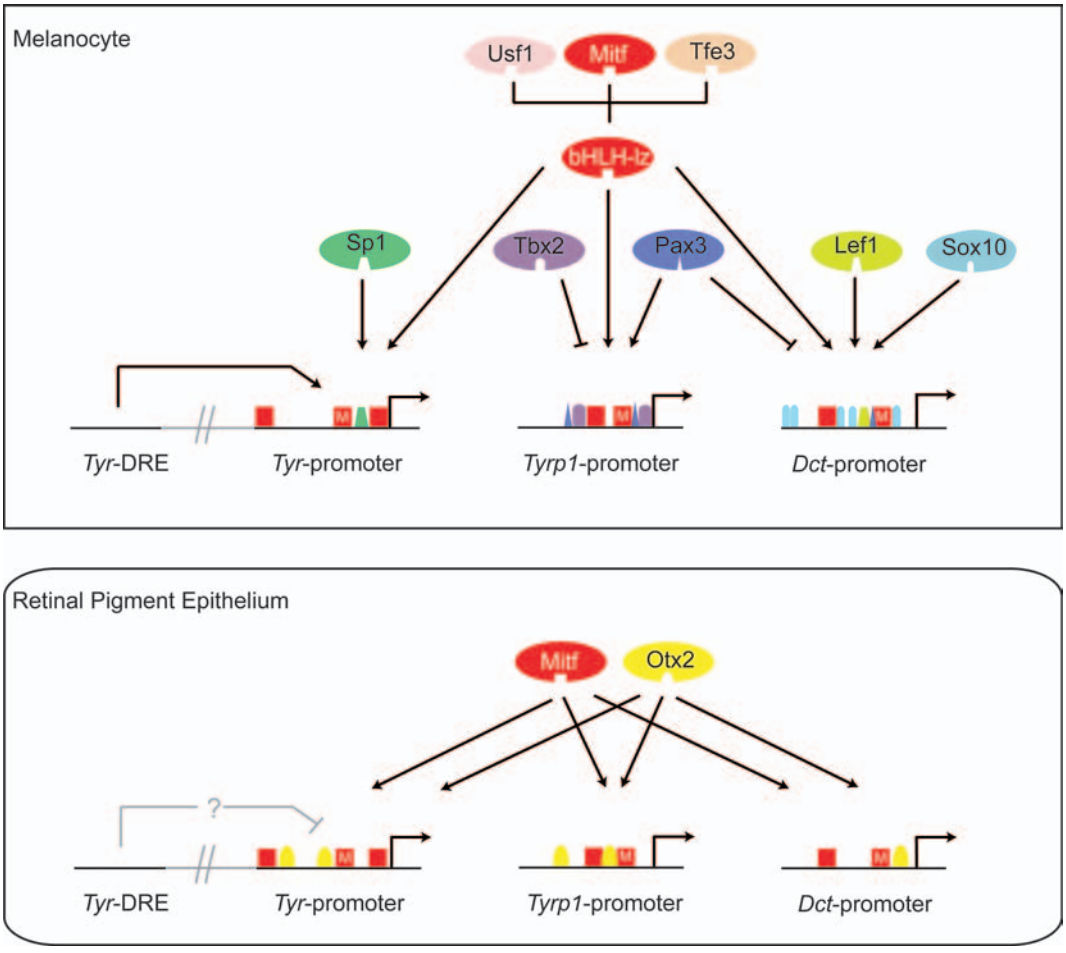

Fig. 4. Differential regulation of the tyrosinase-related family genes in the two pigment cell lineages. Ubiquitously expressed transcription factors such as Sp1, pigment cell-specific factors such as Mitf or cell-type specific factors such as Otx2 or Sox10 are active players of the transcription factor networks that control pigment production in either RPE or melanocytes. Multiple binding sites for these factors are thus found in the promoters of the tyrosinase family genes thus allowing for a tight control of gene expression. *Tyr*: tyrosinase; *Tyrp1*: tyrosinase-related protein 1; *Dct*: dopachrome tautomerase; DRE: distal regulatory element.

but rather repressed expression (Camacho-Hubner and Beermann, 2001).

Using a reporter gene in cell culture, the 3.6 kb fragment was shown to act as a transcriptional enhancer in melanoma cells (Porter et al., 1999; Giraldo et al., 2003). Moreover, a central 200 bp sequence was shown to contain critical regulatory sequences for enhancer activity. DNAse I footprinting, gel retardation experiments and sequence analysis performed on the 0.2 kb core-enhancer have revealed the presence of potential binding sites for transcription factors such as AP1 or Creb but also for an undefined melanocyte-specific factor (Ganss et al., 1994a). The use of a 1 kb fragment encompassing the 0.2 core-enhancer in albino rescue experiments was shown to increase the level of expression but showed integration site dependence (Porter and Meyer, 1994). This indicates that the 200 bp fragment acts as an enhancer but that other upstream sequences located in the 3.6 kb fragment are necessary for integration site independence. The 5' region of the 3.6 kb fragment was shown to contain a scaffold/matrix attachment region (S/MAR) which might help to protect from position effects (Porter et al., 1991). In albino rescue experiments, a YAC carrying specific mutations in the core-enhancer showed a phenotype less pronounced than when the entire LCR was deleted indicating the existence of several functional units (Giraldo and Montoliu, 2002). It has been proposed that the 3.6 kb fragment contains at least four different active regions: one core sequence which acts as an enhancer specifically in cells of neural crest origin, one S/MAR region, located upstream of the core, which protect from position effects and is active in RPE and melanocytes, one RPE specific enhancer which maybe overlap with the S/MAR and one RPE repressor located in the 3' region of the 3.6 kb fragment (Porter et al., 1999). Moreover, a combination of experiments using a reporter gene in mouse, Drosophila and cell culture have shown that the 5' end of the tyrosinase LCR has boundary activities that protect from spreading of condensed chromatin (Giraldo et al., 2003).

The tyrosinase distal regulatory element was shown to be conserved in the human genome (position -8 kb to -10 kb) and to act as an enhancer in melanocytes cell lines (Fryer et al., 2003; Regales et al., 2003). So far, it is not clear whether the tyrosinase distal regulatory element is specific to mammals or whether it is also present in lower vertebrates.

Control of gene expression in melanocytes and RPE

Most of the transcription factors that control gene expression of the tyrosinase-related family are either expressed in neural crest-derived melanocytes or in optic cup-derived retinal pigment epithelium. For instance Sox10, Pax3 or Tbx2 are not expressed in the RPE whereas Otx2 is absent from melanocytes (Goulding et al., 1991; Bovolenta et al., 1997; Bondurand et al., 1998; Sowden et al., 2001). The Mitf transcription factor is found in both RPE and melanocytes but displays cell type-specific isoforms. The Mitf-M isoform is exclusively expressed in melanocytes and specific depletion of this isoform leads to a black-eyed white phenotype (Yajima et al., 1999). Mitf-A, which is the predominant isoform expressed in the RPE, differs from Mitf-M in its amino-terminus (Amae et al., 1998; Hershey and Fisher, 2005). Differences were also observed between RPE and melanocytes regarding the activity of the tyrosinase distal regulatory element (Porter et al., 1999; Camacho-Hubner and Beermann, 2001). The transcription factor networks controlling the pigment production are thus very different when comparing melanocytes and RPE (Fig. 4). It has been proposed that the genetic network involved in pigment production has evolved initially in the context of primitive eye function and then was adapted by the neural crest to form melanocytes (Arnheiter, 1998; Martinez-Morales et al., 2004).

Conclusion

The members of the tyrosinase-related family represent a good model for accessing numerous aspects of pigment cell biology as well as more general processes. During evolution, the emergence of *Tyrp1* and *Dct* by duplication of a primitive Tyrosinase gene or the establishment of the two distinct pigment cell-types are key steps in the establishment of the mammalian pigment system. Comparison of melanocytes and RPE transcription factor networks regulating pigment production is thus instrumental in understanding the evolution of pigment cells. More generally, the recruitment of a very specialized biological processes such as pigment production by two cell-types of different origins represents an interesting mechanism of evolution.

Pigment production is achieved in fully differentiated cells. However, *Dct* is already expressed in melanocyte precursors whereas tyrosinase and *Tyrp1* are expressed later (Nishimura et al., 2002). The analysis of the network controlling tyrosinase, *Tyrp1* and *Dct* expression thus gives precious information on the development of pigment cells from stem cells to terminally differentiated pigment cells. Sox10, Pax3, Mitf or partners of the Wnt signalling pathway have been shown to be involved in such a process (Lang et al., 2005).

The regulatory region controlling gene expression is not only composed of a promoter placed immediately upstream of the first exon. More distal elements that act as enhancers or protectors from chromatin compaction are also critical for proper and specific control of expression. The tyrosinase distal regulatory element, which is crucial for expression in melanocytes, clearly highlights this fact. The tyrosinase gene might have gained this distal regulatory element during evolution thus allowing for "recruitment" of active genes involved in pigmentation to the melanocyte lineage.

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