

Mitf expression is sufficient to direct differentiation of medaka blastula derived stem cells to melanocytes

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Summary

Embryonic stem (ES) cell lines have provided very useful models to analyse differentiation processes. We present here the development of a differentiation system using ES-like cell lines from medaka. These cells were transfected with the melanocyte specific isoform of the microphthalmia-related transcription factor (Mitf). Mitf is a basic helix-loop-helix-leucine zipper transcription factor whose M isoform is restricted to neural crest derived melanocytes and is essential for the development of these cells in vertebrates from mammals to fish. What is not clear yet is whether Mitf is a downstream factor or a master regulator

of melanocyte commitment and differentiation. Expression of Mitf in the ES-like cells from medaka led to the induction of cells that, by morphology, physiology and gene expression pattern, were confirmed to be fully differentiated pigment cells. Mitf expression is therefore sufficient for the proper differentiation of medaka pluripotent stem cells into melanocytes.

Key words: Cell differentiation, ES cells, Medaka fish, Melanocyte, Mitf

Introduction

The mechanisms that regulate the decision of a pluripotent cell to differentiate and which lineages will be followed once differentiation is initiated are intensively studied. Embryonic stem (ES) cell lines, undifferentiated cell cultures that retain their pluripotency after long-term cultivation in vitro, have provided very useful models to study how these processes take place in vivo (Donovan and Gearhart, 2001).

However, although most results provided some indication that a variety of developmental decisions can be modelled using ES cells, attempts to direct ES cell differentiation in a homogeneous fashion are still in their infancy. In general, the methodology for inducing differentiation in mouse ES and EC cell lines involves formation of embryoid bodies, cellular aggregates that undergo a program of differentiation reminiscent of early mammalian embryogenesis (Robertson, 1987). The limitations of the embryoid bodies affect the general applicability of this approach, particularly the activation of inappropriate signalling pathways as a result of the inherent disorganisation of these structures (Rathjen and Rathjen, 2001). The development of other strategies that can promote differentiation only towards specific directions is necessary and will allow the controlled reconstruction of entire single lineages in vitro from pluripotent cells to functional, terminally differentiated progeny.

We have developed a differentiation system using ES-like cell lines (MES) from medaka (Hong and Schartl, 1996; Hong et al., 1998). These cells show similar characteristics to mouse ES cells in vitro and in vivo. Upon certain rather non-specific stimuli, such as high- or low-density culture, embryoid body

aggregation or growth factor treatment, they can differentiate in vitro into many different cell types (Hong et al., 1996), including melanocytes, neurons and myotubes. After transplantation to host embryos, MES cells participate in chimera development where they contribute as functional cells to the formation of tissues from all three germ layers (Hong et al., 1998).

Melanocytes originate as non-pigmented precursors, termed melanoblasts, from the neural crest. After migration and proliferation, they enter the epidermis and are found as mature pigmented cells in the skin as well as a range of other sites where their role as pigment-producing cells is less understood. The large amount of functional genetic data already available from this lineage has made the study of melanocyte development an attractive system for understanding how the integration of gene expression and signal transduction pathways governs the development of a specific cell lineage (reviewed by Goding, 2000). Various factors, including colony stimulating factor (SCF), endothelin 3, 12-O-tetradecanoylphorbol-13 acetate (TPA) and cholera-toxin have been shown to stimulate the differentiation of melanocyte precursors (Murphy et al., 1992; Ito et al., 1993; Ono et al., 1998). Some melanocytes were obtained after seeding undifferentiated ES cells on a bone marrow-derived stromal cell line and treating the cells with dexamethasone (Yamane et al., 1999). Mature melanocytes were obtained from a cell line of immature melanocytes treating the cells with retinoic acid (Watabe et al., 2002). An increase of expression of microphthalmia transcription factor (Mitf) was observed indicating an important role in this process. Moreover, expression of Mitf in fibroblasts conferred some characteristics of melanocytes on a

part of the recipient cells, like tyrosinase expression and dendritic morphology (Tachibana et al., 1996). Similarly, ectopic expression of *Mitf* induced transdifferentiation of neuroretina into melanocytes (Planque et al., 1999), and overexpression of *Mitf* in zebrafish embryos resulted in ectopic melanised cells (Lister et al., 1999).

Mitf is a basic helix-loop-helix leucine zipper transcription factor that is expressed in multiple isoforms. Expression of *Mitf*-M is restricted to neural crest derived melanocytes and is essential for the development of these cells in mammals and in fish. Mice bearing null alleles of the gene exhibit a loss of neural crest-derived melanocytes, associated deafness and a failure of retinal pigmented epithelial cells. In zebrafish, a null mutation results in loss of dermal melanophores but does not affect the retinal cells (Moore, 1995; Yajima et al., 1999; Lister et al., 1999). It has been shown that *Mitf* regulates the expression of the *tyrosinase*, *Trp1* and *Dct* genes, the enzymes that produce the pigment melanin (for a review, see Goding, 2000). However, because *Mitf* is essential for the development of the melanocyte lineage, it must also regulate genes other than those involved in melanogenesis as these genes are not essential for melanocyte development. Hence, the unanswered question is whether *Mitf* is a crucial downstream factor or a master regulator of melanocyte commitment and differentiation. One way of addressing this question is to study the effect of *Mitf* expression on undifferentiated cells.

In this study, we have transiently transfected fish ES-like cell lines with the melanocyte-specific isoform of *Mitf*. This led to the induction of cells that, by cell shape, pigmentation, melanosome motility and gene expression pattern, were confirmed to be fully differentiated pigment cells. This indicates that *Mitf* expression is sufficient for the proper differentiation of medaka pluripotent stem cells into melanocytes.

Materials and methods

Cell culture and transfection

MES-1 and MES-2 cell lines were cultured in ESM 4 medium as previously described (Hong et al., 1998). The ES-like state of the cell lines was confirmed by transplantation to host embryos and their ability to form chimaeras, most importantly the development of donor-derived melanocytes in albino host (Hong et al., 1998). Cells were transfected with an expression vector containing the M isoform of the *Mitf* cDNA from *Xiphophorus* (Delfgaauw et al., 2003) under the control of the CMV promoter (pRc/CMV, Invitrogen). The cells were cotransfected with pCMVgfp (Hong et al., 1998), at a ratio of 10 µg DNA of the *Mitf* expression vector/1 µg DNA of pCMVgfp. After trypsinisation, the cells were resuspended in 400 µl of PBS, electroporated in an Easyject electroporator at 250 V and 600 µF, and seeded at 60% of confluence. As a control, MES cells were transfected with a vector containing the cDNA of M-*Mitf* with a point mutation that introduces a premature stop codon (Delfgaauw et al., 2003). Experiments were carried out with the MES-1 and MES-2 cell lines, with similar results. Transfection efficiencies were estimated counting the number of GFP-expressing cells 24 hours after the transfection. The values were between 10% and 15%. OLF, A2 and PSM cells were used as controls. OLF is a medaka fibroblast-like cell line (Etoh et al., 1988). A2 is an embryonic epithelial cell line (Kuhn et al., 1979) and PSM is a melanoma cell line (Wakamatsu, 1981) both derived from fish of the closely related genus *Xiphophorus*. They were cultured as previously described (Etoh et al., 1988; Baudler et al., 1997) and transfected by electroporation as the MES cell lines.

The number of melanocytes observed in a defined area of the culture dishes (2 cm²) was counted at different days after the transfection. Three replicates were counted from four different experiments. Differences were lower than 15%.

For the motility assays 0.1 mM of norepinephrine (NE hydrochloride, Sigma) was added to the medium (Uchida-Oka and Sugimoto, 2001) of transfected MES cells. Cells were later observed under the microscope to detect motile activity in the melanosomes.

Preparation of RNA and RT-PCR

Transfected cells were harvested at different time points. Seeding of

Table 1. Primers and PCR conditions

Gene	Accession number	Primer sequence (5'→3')	T _a (°C)	Number of cycles	Product size
Tyrosinase	D29686	Tyra, AAGGAGTGTCTGCCAGTGTGG; Tyrc, TGTGCCTGTGGTGATGACGTA	58	35	369 bp
Trp1	AF072305	Trp1b, GATCGTGGATGATGATGTGG; Trp1c, GCCCGAAGACGTCCGAGTA	58	35	500 bp
Dct (TRP2)	AV177491	Trp2d, GTGCGTCTGCTTCAACCTTAAGC; Trp2b, CCCGACTTGGTGAGGCTG	60	35	248 bp
Ednrb	AB057354	Ednrb1, GACTGCAGTTTCTACACGC; Ednrb2, CAG CTT TGA TGC TGC AGC TC	51	40	440 bp
Fms	*	Fms1, TGACAGTGGGATTTCCAGCAC; Fms2, TAGAATTTAGAGTCCACAGGC	56	35	490 bp
Kit	BJ012204	Kit5, GGACGGGGTACATGCCCATG; Kit6, ATGCAGGTGGCTCTGCGTTT	60	35	421 bp
Aim1	AF332510	Baim1, TCCTCCACAGTATGGTTATG; BAIM2, AACAGGTAGGCCTTGATTGG	54	35	396 bp
Tbx2	AJ298301	Tbx2a, AAGGTGCGAATAAACGGCC; Tbx2b, GTTCTGTAGCGTACCG	56	35	389 bp
P53	AF212997	P531, TGCAGTCCCGATTCAAAAATATTTT; P532, CCACGTGCTCCGTCTTCTTGTAG	64	35	500 bp
Myf5	BJ012067/ B J494813	Olamyfup, CTTCCTCAAGGTGGAGATCCT; Olamyfdown, GCGTCAGTGGGTATAACAGC	62	35	578bp
ErbB3	AF072310	ErbB31, TTGGGGACCAAACGAGCATC; ErbB32, CAAAATGAGTGGGACATTGAG	61	35	302 bp
Sox10	AJ410688	Sox10-1, AAGCAGCAGCAGCATCACAG; Sox10-2, CTGCTTCTGTGGTCTGATGT	53	35	441 bp
NCAM	GOLWno3 546_p09.g1	NCAM-1, GTTCGTTTCAAAAATACAAAGC; NCAM-2, CGTTTACAATGACCTTGATG	55	35	139 bp
Enolase	GOLWno4 142_i13.b1	Eno-1, GTTAGCCATCGTCTGGTG; Eno-2, CCCAACTCTCTTCAATTC	55	35	150 bp
AP2	BJ001734	Ap2-1, GGAAATAGGGGAATGGCAGG; Ap2-2, GACGCTGTGTTCTTGGATA	51	40	519 bp
Snail2	AV670822	Snail2-1, TCTACCTGCCACCTCTGAA; Snail2-2, TGAGCGGATGTGCATCTTCA	51	35	319 bp
OlaMitf	GOLWno4 461_k14.b1	Memim-1, TCTGTCAACTACTTTGGTAG; Memim-2, TGTTGATTTGAAGTCTGTAT	53	35	231 bp
XmaMitf	AF475090	MiEx4for, GTCATCGACGACATCATCAGC; MiEx7rev, CCAGCTCTTGTATGCGGTCG	64	35	384 bp
Actin	S74868	Mact-1, TTCAACAGCCCTGCCATGTA; Mact-2, GCAGCTCATAGCTCTTCTCCAGGGAG	60	30	349 bp

*Primers were derived from the zebrafish sequence AF240639.

the cells after the electroporation was considered time zero. Total RNA was isolated with the Trizol reagent (GibcoBRL) from tissues or cell cultures and 2 μ g were used for reverse-transcription using random primers. PCR was run for various genes using the primers and the conditions described in Table 1. One tenth of the single strand cDNA products was used for each PCR amplification. Primers were derived from medaka sequences available from public data bases (Table 1). *XmaMitf* primers were derived from the Xiphophorus sequence and were specific for the transgene, no amplification was obtained with medaka skin cDNA (data not shown). *OlaMitf* primers are specific for the M-isoform of medaka Mitf. They were designed from genomic sequence reads of the medaka whole genome shotgun sequence project available at <http://shigen.lab.nig.ac.jp/medaka/genome/indexen.html> Medaka actin was used for calibrating RNA amounts. Several independent transfections were carried out with no variation in the expression results. Every PCR was repeated at least four times.

Results

MES-1 and MES-2 cells (Fig. 1A,B) were co-transfected with plasmid expression vectors for the Xiphophorus M isoform of Mitf and for GFP. A mutant Mitf did not induce any phenotypic change in MES cells compared with non-transfected controls. However, after transfection of the M-*Mitf* isoform, the cells reproducibly started to adopt a melanocytic morphology (larger size and dendritic shape) and to produce melanin. First, from day three to five, the differentiating cells showed only some dendrites and were scarcely pigmented (Fig. 1C,D), later they presented more dendrites, were strongly pigmented and larger in size (Fig. 1E).

Only GFP expressing transfected cells underwent the transition to pigment cell morphology (Fig. 1F). Thus, it can be excluded that a global nonspecific differentiation process was taking place. The non-transfected cells retained their typical ES-like morphology: small in size and round or polygonal in shape. Therefore it is apparent that Mitf is the inducer responsible of the differentiation process observed.

The number of melanocytes in the transfected cultures increased over time (Fig. 1G) until it reached a plateau at day ten (Fig. 2). At this time, the expression of the transgene decreased, probably owing to loss of the transfected DNA. This indicates that, as expected, the differentiation process is cell autonomous and that the differentiated cells stop dividing. The percentage of cells that differentiated represented between 30% and 50% of the transfected cells, as determined 24 hours after transfection. The transfected non-differentiated cells retained their ES cell-like morphology and continued growing and dividing as the non-transfected cells. Therefore, the ES cells tended to overgrow the differentiated cells at the end of the 3-week observation period.

Transfection of *Mitf* into melanoma cells (PSM), or embryonic epithelial cells (A2) did not lead to any morphological change (Fig. 2). When *Mitf* was introduced into a fibroblast cell line (OLF) a minimal effect was seen. At day 10, a few pigmented cells appeared (Fig. 1H), representing $\sim 0.1\%$ of the transfected cells.

In lower vertebrates, melanocytes differentiate further to a stage that is most prominently characterised by the ability of

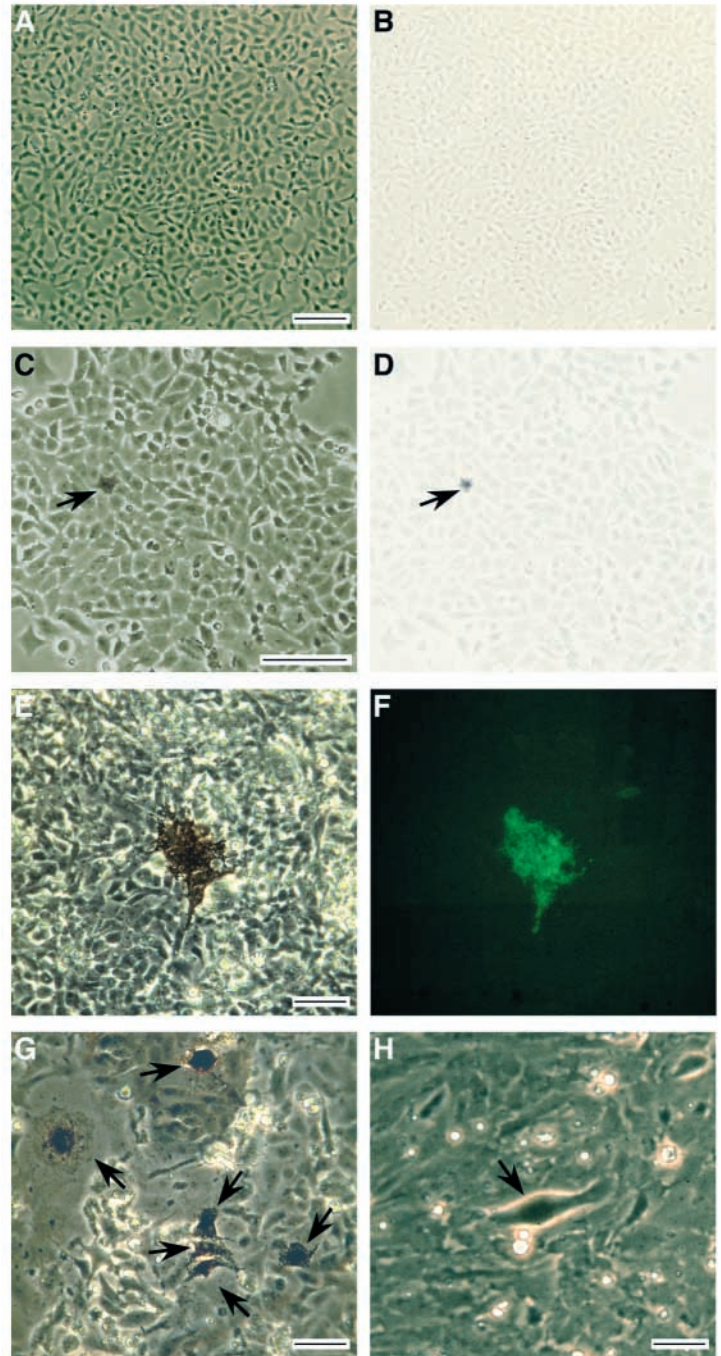


Fig. 1. Transfection of Mitf into ES-like cell and fibroblast cell lines of medaka. (A) Non transfected MES-1 cells. (B) Bright field view of A; (C) MES-1 cells 4 days after transfection with *XmaMitf* and *GFP* vectors (arrow shows a faintly pigmented cell). (D) Bright field view of C. (E) Terminally differentiated melanocyte seven days after the transfection of MES-2 cells. (F) Dark field showing GFP expression. (G) Melanocytes in MES-1 transfected cells 12 days after the transfection (arrows indicate melanocytes). (H) Pigmentation in OLF cells transfected with *XmaMitf* (arrow indicates pigmented cell). Scale bars: 100 μ m.

the melanosomes to be translocated from the periphery of the cells to the center, thus giving the cells a lighter or darker appearance. This process is mediated by a special architecture

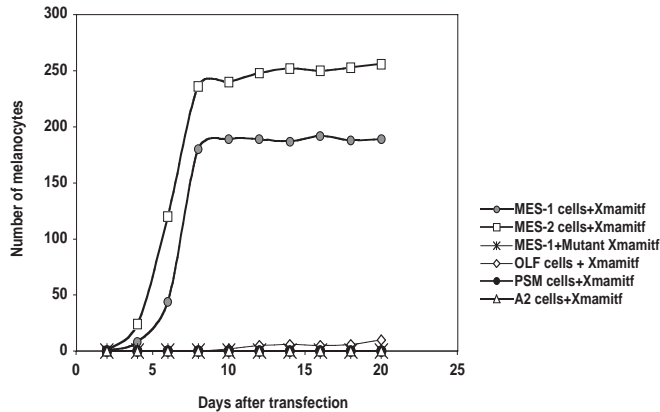


Fig. 2. Number of melanocytes observed in a defined area of the culture dishes after transfection of the different cell lines with *XmaMitf*. Absolute numbers are given because non transfected cells continue proliferating as usual, thus a percentage value of melanocytes does not really reflect the increase in the number of differentiated cells observed over time.

of the cytoskeleton and is regulated by external physiological signals, e.g. norepinephrin (NE), light, etc. With this response, the pigment cells mediate the colour change of fish and amphibia. Hence, for a functional assay the transfected MES cells were treated with NE in order to check the ability of the melanocytes to aggregate the pigment granules. The effect was clearly observed already within 30 minutes after adding the drug, (Fig. 3A,B). Two hours later, in many cells the pigment organelles were completely aggregated and retracted to the perinuclear compartment (Fig. 3C). The same result was obtained by strong illumination of the melanocytes (data not shown). This indicates that the differentiated cells were fully functional pigment cells able to respond to physiological signals coming from their environment.

For a molecular characterisation of the differentiation process, the expression of various genes was tested by RT-PCR. At first, known target genes of *Mitf* were analysed including the tyrosinase gene family: tyrosinase, tyrosinase related protein 1 (*Tyrp1*) and dopachrome tautomerase (*Dct* or *Tyrp2*), which are the enzymes involved in melanin synthesis. A clear induction of the three genes was observed (Fig. 4A). No induction was detected in the cells transfected with the mutant *Mitf* (Fig. 4B). Concerning the timecourse of the induction, an increase in the expression level of the three genes was detected over time, with *Dct* being the earliest marker. Expression of this gene was already detected 2 hours after transfection. *Tyrp1* was the last of the three genes to be expressed, being barely detectable at 8 hours and clearly visible at 24 hours. In contrast to *Tyrp1* or *Dct*, Tyrosinase expression decreased at 10 days. This is also observed for the transfected *Mitf* and could be due to the loss of the transgene.

To analyse whether there is any effect of the transgene on regulation of the endogenous gene, the expression of the medaka *Mitf* gene was determined at all time points. There was a low level of *OlaMitf* expression in the non-transfected cells, which did not change after introduction of the *Mitf* transgene at any time (Fig. 4A).

Four genes were tested that are known markers of melanocyte differentiation. Kit, a type III receptor tyrosine

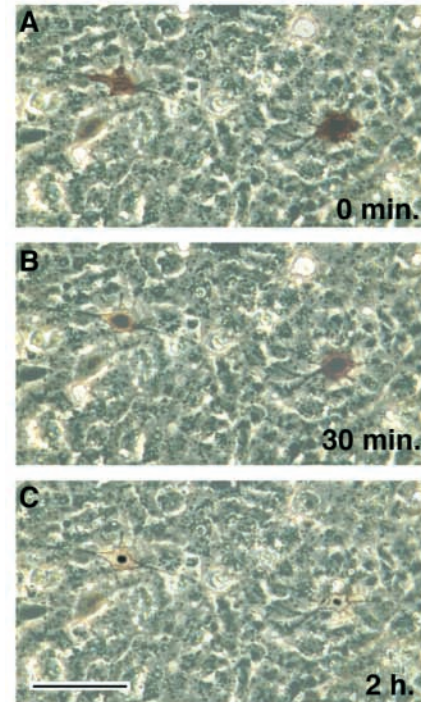


Fig. 3. Melanocytes differentiated from *XmaMitf* transfected MES-1 cells and treated with norepinephrine (NE). (A) Before adding NE. (B) Thirty minutes after, the melanosomes start to aggregate. (C) Two hours later, the melanosomes are completely aggregated around the nucleus. Scale bar: 100 μ m.

kinase (RTK) expressed in melanocytes and melanoblasts, is essential for the development of neural crest-derived melanocytes. In mammals, it has also an important role in haematopoiesis and germ cell development (Parichy et al., 1999; Hou et al., 2000). *Fms*, another type III RTK, is a closely related RTK to *Kit*. In mouse, it is essential for the development of macrophage and osteoclast lineages; in zebrafish it is required for pigment pattern development (Parichy et al., 2000; Parichy and Turner, 2003). *Aim1* is a putative integral melanosomal membrane protein that probably functions in transporting certain substances required for melanin biosynthesis (Fukamachi et al., 2001). The endothelin receptor b (*Ednrb*) is important for the proliferation and differentiation of neural crest derived cells, including melanocytes (Opdecamp et al., 1998). For *Kit*, *Fms* and *Aim1*, a clear induction of expression in the transfected cells was observed (Fig. 4A). *Fms* is the earliest appearing marker and *Aim1* is the last (Fig. 4A). The pattern of *Ednrb1* was different. This gene is expressed at low levels already in the non-transfected cells. Two hours after transfection there was a clear induction of this gene. By 4 hours, the expression was downregulated again.

As an early differentiation marker *Tbx2* was tested. This gene is a member of the T-box transcription factor family that is expressed in melanoblasts and melanocytes and is a known target of *Mitf* in mouse (Carreira et al., 1998; Carreira et al., 2000). It was clearly induced (Fig. 4A) as early as 4 hours after *Mitf* transfection.

As a general marker of cell differentiation, P53 expression was tested. P53 is a phosphoprotein that plays a general role

in controlling cell proliferation, growth arrest and apoptosis. It acts as a tumour suppressor gene and has also been implicated in regulating cellular differentiation (Sabapathy et al., 1997). Induced expression of P53 was observed 2 hours after the transfection (Fig. 4A).

In order to exclude the possibility that an unspecific upregulation of gene expression is taking place in the *Mitf* expressing cells, the expression of *Myf5*, a bHLH transcription factor that plays a key role in the skeletal muscle lineage, was tested. No induction of this gene was observed (Fig. 4A).

Melanocytes progress in their development in vivo through a precursor stage, the neural crest cell, which can also differentiate to peripheral neurons and glia – the Schwann cell cells. Occasionally, two or three neuron-like cells per culture dish were observed after *Mitf* expression. Hence, the expression of two pan-neuronal markers, NCAM and neuron-specific enolase was assayed. Both genes were not detected in any of the *Mitf* or mutant *Mitf* transfected cell lines (Fig. 4A,B).

The intimate relationship between melanocytes and glial lineages suggested by the ability of Schwann cells to dedifferentiate to generate melanocyte progenitors (Dupin et al., 2003) suggested the possibility that some Schwann cell differentiation might occur in our cultures. Therefore a typical Schwann cell marker, *ErbB3*, which is the receptor for the growth factor neuregulin (Garratt et al., 2000) was tested. This gene is not expressed in the ES-like cells and was not induced in *Mitf*-transfected MES during differentiation (Fig. 4A). *Sox10* is a transcription factor required for the development of neurons, glia and pigment cells. There was a transient expression at 24 hours for this gene, which did not occur in the control transfections (Fig. 4A,B). *Sox10* is expressed in differentiated Schwann cells (Jessen and Mirsky, 2002); however, it is rapidly downregulated in differentiating melanophores (Dutton et al., 2001). Obviously, if there is some differentiation of Schwann cells, it is a minor fraction and below the detection limit.

The transient expression of *Sox10*, a typical neural crest gene, could be also an indication that the *Mitf* transfected ES cells express some neural crest cell markers. To address this, the expression of two other neural crest markers was analysed. *AP2* is a transcription factor that regulates cell growth, differentiation and programmed cell death (Hilger-Eversheim et al., 2000). *Snail2* is a transcription factor involved in the formation of premigratory neural crest cells (Locascio et al., 2002). In the case of *AP2* a clear transient induction of the expression was observed, with a peak at 4 hours after the

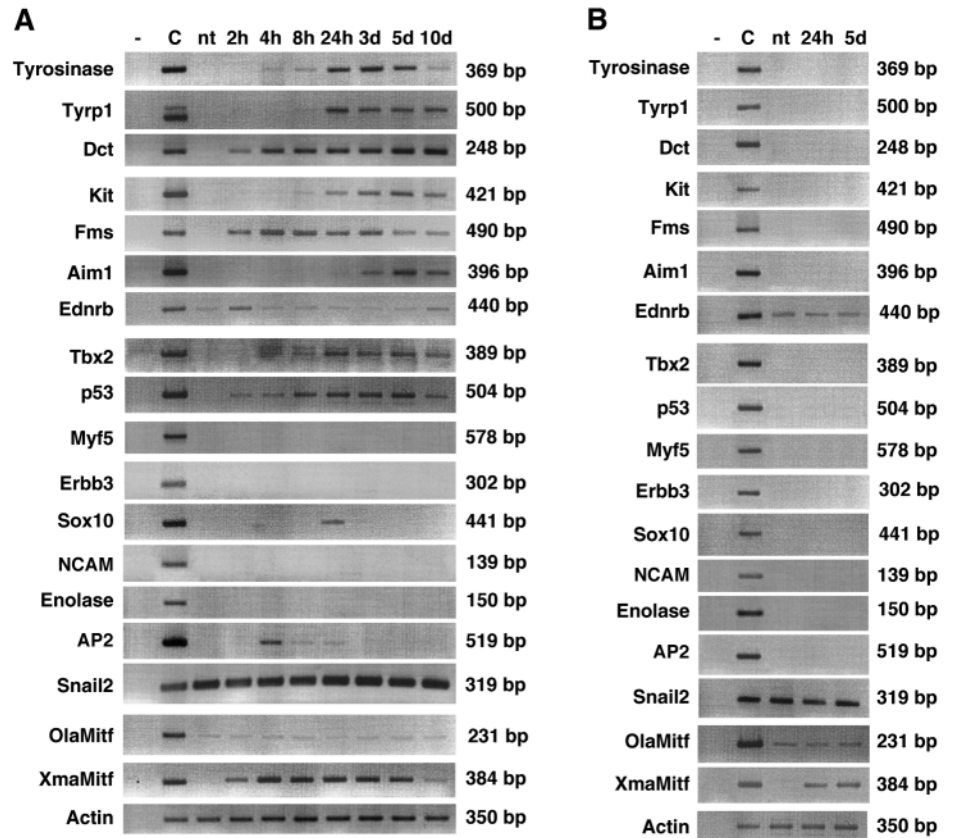


Fig. 4. Expression assays of MES-1 transfected cells. (A) Transfected with *XmaMitf*. (B) Transfected with the mutant *XmaMitf*. nt, non-transfected cells; -, no cDNA (negative control); C, positive control. cDNA from medaka skin probed for tyrosinase, Tyrp1, Dct, Kit, Fms, Aim1, Ednrb, Tbx2, P53, OlaMitf and Actin. cDNA from 5-day-old medaka embryos probed for ErbB3, Sox10, Ap2, Snail2, Ncam and enolase. cDNA from adult muscle for *Myf5* and for *XmaMitf*, MES-1 cells transfected with the *XmaMitf* construct.

transfection (Fig. 4A). However, this induction did not coincide with the transient expression of *Sox10* at 24 hours. For *Snail2* the non-transfected cells already expressed this gene and no change was observed after the transfection (Fig. 4A). All genes tested as markers for the *Mitf* induced differentiation were also analyzed in MES cells transfected with the mutant *Mitf* (Fig. 4B). No changes of expression were detected.

All genes were also tested in the *XmaMitf* transfected fibroblasts, where only in the case of tyrosinase, from 72 hours onwards a slight induction could be detected (Fig. 5). *Dct*, *Ednrb*, *P53* and *Snail2* were already expressed in the non-transfected cells. No change in expression was observed for *Dct* and *Snail2* (Fig. 5). In the case of *Ednrb*, a slight increase followed by a strong decrease below the initial level was detected (Fig. 5). An increase in the expression level of *P53* was observed from 24 hours on (Fig. 5). For the rest of the genes, expression was not detected at any time point. Thus, the effect of *Mitf* expression on the OLF cells seems to be much more limited than on the embryonic stem cells.

Taken together, these results indicate that *Mitf* expression is sufficient to initiate the complete differentiation from totipotent ES cells into the melanocyte lineage, yielding terminally functional differentiated cells expressing the specific genes of this cell type.

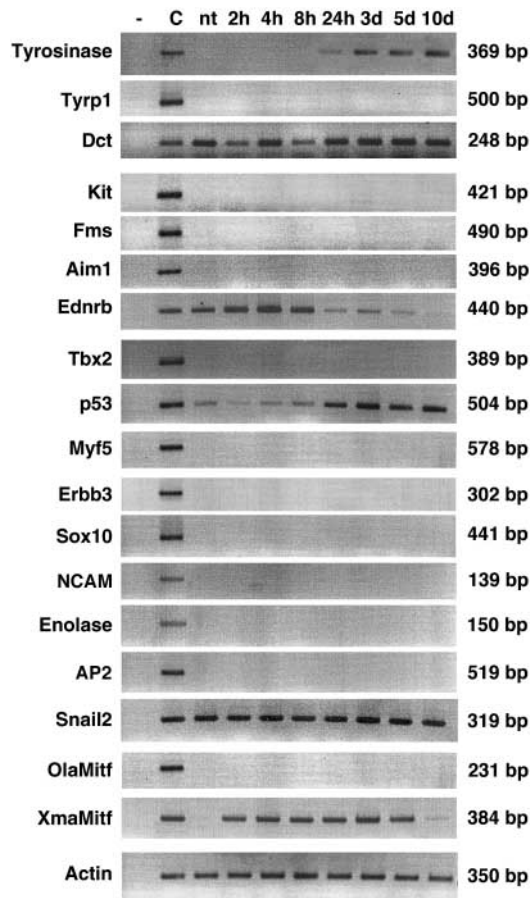


Fig. 5. Expression assays of OLF cells after transfection with *XmaMitf*. Controls are as for the MES cells (see Fig. 4).

Discussion

The importance of *Mitf* as a determining factor of melanocyte development was underlined when it was shown that expression of *Mitf* in mouse fibroblasts resulted in the adoption of some melanocyte specific traits (Tachibana et al., 1996). Similarly, overexpression of zebrafish *Mitf* resulted in ectopic melanised cells (Lister et al., 1999). Thus, it appeared that, in at least some cells, the expression of *Mitf* was sufficient to induce melanocyte-specific gene expression. In the present study, the starting point were pluripotent embryonic stem cells. We obtained completely morphologically differentiated melanocytes expressing all the specific markers tested: the melanogenic enzymes, a melanosomal structural protein, specific receptors and genes involved in the specification, commitment and survival of this cell type. These results indicate that *Mitf* can act as a master regulator of the melanocyte lineage, in that it is sufficient to direct differentiation of embryonic stem cells into melanocytes. The only other case so far reported of the transfection of a gene into ES cells and subsequent induction of a specific lineage is the case of GATA factors that directed mouse ES cells into the extra-embryonic endoderm lineage (Fujikura et al., 2002). In that case, the ES-like cells did not reach a terminal differentiation state indicating that these factors are responsible only for the first step of the differentiation process. By contrast,

Mitf expression was sufficient to induce and complete the entire differentiation process.

There was no induction of the endogenous *Mitf* gene in a kind of autoregulatory loop. This is consistent with the need for upstream factors in vivo that switch on *Mitf* at the right time during differentiation of neural crest cells towards melanocytes (Goding, 2000).

When *Mitf* was expressed in mouse fibroblasts (Tachibana et al., 1996) after antibiotic selection, only 2% of the transfected cells exhibited a melanocyte-like appearance: dendritic shape and melanosome-like structures but not melanin, explained by the finding that these contained a mutant *tyrosinase* gene. In that experiment, cells that had already differentiated were used, which are comparable to the OLF cells used in this study, where our results are similar. However, the fact that the morphology of the pigmented cells was not dendritic and that most of the specific markers were not induced, raises doubts if it is acceptable to consider the observed pigmented cells as true melanocytes or just as pigmented fibroblasts. The differences between both cell lines in terms of 'competence status' are probably responsible for the differences in the effect of *Mitf* expression. Only in the case of the ES-like cells does a true differentiation process seem to happen.

In other studies on melanocyte differentiation, treatment with retinoic acid (Watabe et al., 2002), or seeding ES cells, melanocyte precursors or neural crest cells on a cell layer (Yamane et al., 1999) have been used as the inducing stimulus. These experiments led to melanocytes, but the mechanisms that triggered the differentiation process could not be elucidated. In addition, these experiments generated multiple cell lineages as well as melanocytes. In our case, the simple initiation of *Mitf* expression gives rise to terminally differentiated melanocytes from completely undifferentiated ES-like cells. Only rarely were very few neuron-shaped cells detected in the cultures. The absence of expression of the pan-neuronal markers NCAM and neuron-specific enolase indicates either that they were not completely differentiated or that they constitute only a negligible proportion of the whole cell population; no other cell types were observed at the microscopic level. We cannot, however, exclude the possibility that the transient induction of Sox10 expression is a sign of some early differentiation into the Schwann cell lineage.

A high percentage of the MES cells became melanocytes after *Mitf* expression. However, not every transfected cell developed into a melanocyte. This may be explained by the transient transfection experiment where usually transgene DNA amounts vary in different cells. It appears reasonable to assume that a threshold level of *Mitf* is necessary (J.B. and M.S., unpublished) to trigger the differentiation process.

Cellular motile activities of fish pigment cells underlie colour change and constitute crucially important strategies to avoid attack, obtain prey or for communication with conspecifics (Fujii, 2000). In our study, this ability of the differentiated cells to aggregate the pigment granules was used as a functional assay because it requires a whole signal transduction and cytoskeleton motility machinery (Uchida-Oka and Sugimoto, 2001). The clear response of the melanocytes to the treatment confirms their status as terminally differentiated and functional melanocytes.

Besides showing a typical melanocyte morphology, heavy

pigmentation and ability to aggregate the melanosomes, it was possible to analyse at the molecular level for changes in the expression profile. Genes were analysed that are known targets of Mitf as well as other melanocyte markers not directly linked to Mitf so far.

In the case of the tyrosinase gene family, it was already clear that Mitf binds and activates their promoters. The temporal pattern identifies *Dct* as the earliest induced gene. This is comparable to the *in vivo* situation in mouse embryos, where *Dct* is expressed early in the melanoblasts, whereas tyrosinase and *Tyrp1* are detected later (Steel et al., 1992). In zebrafish embryos, *dct* is also already expressed in melanoblasts (Kelsh et al., 2000). In humans, the level of activation of the *TYRP1* promoter is lower than that of the tyrosinase promoter (Fang et al., 2002). This may also be true in medaka and could explain why we can detect tyrosinase expression but not that of *Tyrp1* in the OLF cells. The presence of *Dct* even in the non-transfected fibroblasts is surprising but also in non-transfected mammalian fibroblasts (NIH/3T3 cells) *Dct* expression was reported (Tachibana et al., 1996). It is possible that this gene has a second, still unknown, function in fibroblasts.

Concerning the melanocyte-specific markers, it has been reported that in mammals *Aim1* is transcriptionally modulated by Mitf, although the mechanism of interaction is not yet clear (Du and Fisher, 2002). In the *Mitf* transfected MES cells, *Aim1* is induced as a rather late event. As *Aim1* is not induced in the *Mitf* transfected OLF cells, the interaction between Mitf and *Aim1* may require factor(s) which are not present in the OLF cells. For Mitf and Kit, complex interactions were shown: Mitf is needed for the maintenance of Kit expression in melanoblasts and Kit signalling modulates Mitf activity and stability in melanocyte cell lines (Hou et al., 2000). There is an E-box motif in the *Kit* promoter through which Mitf can activate *Kit* (Tsujimura et al., 1996). Thus, the observation of *Kit* upregulation in Mitf expressing MES cells may be due to a direct interaction of Mitf with the *Kit* promoter. Again, the lack of induction in the fibroblast cells suggests special requirements of this interaction. A connection between Mitf and Fms had been established in mammals, where Fms seems to act in the osteoclast lineage like Kit in the melanocyte lineage (Kawaguchi and Noda, 2000). In zebrafish an unexpected role has been described for Fms in pigment pattern development where Kit and Fms are required by two separate populations of pigment cells (Parichy et al., 2000; Parichy and Turner, 2003). At present, we cannot say if in MES cells Kit and Fms are expressed by different cells or simultaneously.

For *Ednrb*, a transient activation was reproducibly observed. However, the possible presence of two *Ednrb* orthologues in medaka (alike the situation in chicken and zebrafish) makes it difficult to infer the implication of this expression. *Ednrb* is expressed in neural crest cells before the onset of Mitf, suggesting that in these experiments *Ednrb* may be considered as a neural crest stage marker of the differentiation process rather than a melanocyte-specific gene. It has been proposed that *Ednrb* functions in neural crest suppressing the differentiation of early stage melanocytes and enteric neuron precursors, rendering them competent for migration and accessible to mitogenic influence of other factors (McCallion and Chakravarti, 2001; Shin et al., 1999). Thus, when terminal differentiation is initiated, it may be necessary to repress *Ednrb*

expression. In our experiments *Ednrb* expression returned to basal levels very rapidly, before expression of *tyrosinase* and *Tyrp1* started and any morphological signs of pigment cell differentiation was observed. Intriguingly, this transient activation followed by a downregulation was seen in both MES and OLF cell lines, after Mitf transfection.

The results obtained with *Tbx2* confirm a link with Mitf in medaka. This family of transcription factors is involved in the maintenance of cell identity. *Tbx2* is a known target of Mitf in mouse and it has been proposed that it may be the initiation of one of the pathways that Mitf uses to play its role in melanocyte development and survival (Carreira et al., 1998; Carreira et al., 2000).

Mouse ES cells express high levels of P53 and differentiation results in a reduction of the P53 levels (Sabapathy et al., 1997). By contrast, in medaka, P53 expression increases during development. In MES cells, expression is not detected (Chen et al., 2001). Our results agree with these previous data confirming P53 as a general marker of embryonic cell differentiation in medaka. Expression of P53 is also induced when the adult fibroblasts (OLF) are transfected, suggesting a link between Mitf and P53 that is still unclear. We observed an erratic expression of some genes that are expressed in neural crest cells *in vivo*. However, this erratic expression did not occur at exactly the same time. *Ednrb* was induced already at 2 hours, *AP2* at 4 hours and *Sox10* at 24 hours. Although we do not know the *in vivo* time course of gene expression in medaka neural crest cells, the expression profile of these three genes in the *Mitf* transfected ES cells is difficult to explain. For *Sox10* it is clear that *in vivo* it is required for *Mitf* expression (Dutton et al., 2001; Elworthy et al., 2003), but as yet there is no evidence for the opposite. The induction of *Sox10* was seen after some melanocyte specific genes already started to express. *Sox10* is also expressed in some melanocyte cell lines (Goding, 2000). The expression of the three genes might therefore be due to the transient presence of other cell types or their precursor stages. Alternatively, the expression may be due to the *in vitro* conditions. Another hypothesis could be that the ES cells on their enforced path to become melanocytes recapitulate the expression of some genes which *in vivo* are characteristic for the neural crest cell stage. Of course, a 'pure' neural crest-step cannot be expected as Mitf is downstream of the neural crest induction and is expressed only in a part of the neural crest derivatives. Our data do not allow us to define the mechanism that make the ES cells express these neural crest markers after Mitf expression. More experiments are necessary to clarify this point and methods have to be developed that allow the analysis of expression on the single cell level in the differentiating ES cells.

The wide use of *Snail2* as a neural crest marker and the recent study which strongly suggest that Mitf interacts with *Slug* (the closest *Snail2* homolog in mammals) (Sánchez-Martín et al., 2002), prompted us to look for the effect of Mitf expression on this gene. But surprisingly, *Snail2* is already present in the non-transfected MES and OLF cells, and its expression is not affected by Mitf. In zebrafish embryos, *Snail2* is expressed in gastrula stages; later, its expression is suppressed in some lineages but not in neural crest and its derivatives (Thisse et al., 1995). It has also been shown that *Snail2* controls the expression of genes involved in cell properties affecting the interactions of neighboring cells, such

as cell-cell adhesion (Thisse et al., 1995). Such interactions are probably very important in vitro and may explain its expression in both cell lines. Therefore Snail2 cannot be used in our differentiation system as a marker for neural crest as it is used in vivo. More experiments need to be carried out in order to clarify the role of this factor in these cell lines.

Mitf is the earliest known marker of commitment to the melanocyte lineage and most of the signalling molecules or transcription factors implicated genetically in melanocyte development affect either Mitf expression or its function (reviewed by Goding, 2000). Our data support a proposed role for Mitf as a master developmental regulator, the expression of which is sufficient to initiate and complete the differentiation of medaka pluripotent cells into melanocytes.

Many questions remain to be addressed, among them the role of the genes upstream of Mitf, the ability of other *Mitf* isoforms to direct differentiation of MES cells, and the molecular mechanisms of Mitf interaction with its target genes, direct or indirect. The fact that MES cells are embryonic stem cells and not embryonic carcinoma cell lines, and that the formation of embryoid bodies is not necessary after the transfection, makes the medaka ES cell system an elegant model that will help to achieve more insight into this and other differentiation pathways.

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