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, embryo 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 lineages 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-tetradecanoyl-phorbol-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 dorsal 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 consistent with being fully differentiated pigmented 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 medaka fibroblast-like cell line (Etoh et al., 1988). 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

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**Table 1. Primers and PCR conditions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer sequence (5’→3’)</th>
<th>T_a (°C)</th>
<th>Number of cycles</th>
<th>Product size</th>
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<tr>
<td>Tyrosinase</td>
<td>D29686</td>
<td>Tyra, AAGGAGTCTGCTGCAGGTTGG; Tyrc, TGTCAGCTGTGGTATGACGA</td>
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<td>369 bp</td>
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<tr>
<td>Trp1</td>
<td>AF072305</td>
<td>Trp1b, GATCAGGTTGATGGTTCGTT; Trp1c, GCCGGACAGTAGTCGATTGAG</td>
<td>58</td>
<td>35</td>
<td>500 bp</td>
</tr>
<tr>
<td>Dct</td>
<td>AV177491</td>
<td>Trp2d, GTGCGTGCTCCTAACCCTTAAGC; Trp2b, CCGGACTGTGAGGCTGCTG</td>
<td>60</td>
<td>35</td>
<td>248 bp</td>
</tr>
<tr>
<td>Ednrb</td>
<td>AB057354</td>
<td>Ednrb1, GACTGCAGATTCTACGGC; Ednrb2, CAGGTTGACCTGACGCCTC</td>
<td>51</td>
<td>40</td>
<td>440 bp</td>
</tr>
<tr>
<td>Fms</td>
<td>*</td>
<td>Fms1, TGAACGGATTTCCGACGAC; Fms2, TAGAATTGAGTGACACCG</td>
<td>56</td>
<td>35</td>
<td>490 bp</td>
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<tr>
<td>Kit</td>
<td>BJ01204</td>
<td>Kit5, GGAAGGCTGCACTGCTCAGT; Kit6, ATGCAGGTTGAGGCTTGCCTTTG</td>
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<td>421 bp</td>
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<tr>
<td>Aim1</td>
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<td>35</td>
<td>396 bp</td>
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<tr>
<td>Tbx2</td>
<td>AJ298301</td>
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<tr>
<td>P53</td>
<td>AF212997</td>
<td>P531, TGACTCGGCGGATCCAAATATTTT; P532, CCAGACGTCGCCTTGATTTAG</td>
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<tr>
<td>Myf5</td>
<td>BJ012067/</td>
<td>Olanmyf1, CTCTATTTGAGGACGATCTCTC; Olanmyf2, GGTCATGAGGTTGATACG</td>
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<tr>
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<td>Sox10</td>
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<td>NCAM</td>
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<td>NCAM-1, TGTTGCTGGGCTAAACAC; NCAM-2, GTTCTACATGACGATGTCCTT</td>
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<td>Enolase-1, GTTAGCCTATGCTCTTGTG; Enolase-2, CCACACTTCTTTATACCTT</td>
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<td>35</td>
<td>150 bp</td>
</tr>
<tr>
<td>AP2</td>
<td>BJ001734</td>
<td>AP2-1, GGAATAGGGAATGAGCAGG; AP2-2, GAGCCTGTGTGTTTGATGATA</td>
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<td>519 bp</td>
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<tr>
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<td>Snail2-1, TCACTGCTGCCACCTTCTCA; Snail2-2, TGAGCGGAGTGCATCTTTCA</td>
<td>51</td>
<td>35</td>
<td>319 bp</td>
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<tr>
<td>OlaMitf</td>
<td>GOLWno4, 461_k14.b1</td>
<td>OlaMitf-1, TCAGTCTGACTTTGTGATA; OlaMitf-2, TGTGTTGATTGAAAGCTTCTGAT</td>
<td>53</td>
<td>35</td>
<td>231 bp</td>
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<tr>
<td>XmaMitf</td>
<td>AF475090</td>
<td>MiEx4for, CTGATCTGAGCACACATCAGC; MiEx7rev, CCAGCCTTTTGATGCGCTG</td>
<td>64</td>
<td>35</td>
<td>384 bp</td>
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<tr>
<td>Actin</td>
<td>ST4888</td>
<td>Actin-1, TCAACACGCTCTTGCGACAG; Actin-2, CGAGCTCTATGCTTTCTTTCACG</td>
<td>60</td>
<td>30</td>
<td>349 bp</td>
</tr>
</tbody>
</table>

*Primers were derived from the zebrafish sequence AF240639.*
Mitf directs differentiation in ES-like cells

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 μg 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 ~0.1% of the transfected cells.

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

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 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.
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 melanosomal-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. 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.

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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 (Kelsch 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 (NH3/3T3 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 Trp1 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 becomes melanocytes recapitulate the expression of some genes 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 (Thissé 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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Mitf directs differentiation in ES-like cells

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