Phosphorylation of MITF by AKT affects its downstream targets and causes TP53-dependent cell senescence

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Microphthalmia-associated transcription factor (MITF) plays a crucial role in the melanogenesis and proliferation of melanocytes that is dependent on its abundance and modification. Here, we report that epidermal growth factor (EGF) induces senescence and cyclin-dependent kinase inhibitor 1A (CDKN1A) expression that is related to MITF. We found that MITF could bind TP53 to regulate CDKN1A. Furthermore, the interaction between MITF and TP53 is dependent on AKT activity. We found that AKT phosphorylates MITF at S510. Phosphorylated MITF S510 enhances its affinity to TP53 and promotes CDKN1A expression. Meanwhile, the unphosphorlyative MITF promotes TYR expression. The levels of p-MITF-S510 are low in 90% human melanoma samples. Thus the level of p-MITF-S510 could be a possible diagnostic marker for melanoma. Our findings reveal a mechanism for regulating MITF functions in response to EGF stimulation and suggest a possible implementation for preventing the over proliferation of melanoma cells.

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1. Introduction

Melanoma arises from melanocytes, and it is one of the most treatment-resistant and notoriously aggressive human cancers (Soengas and Lowe, 2003). MITF is crucial for the growth, differentiation and pigment production of melanoma cells (Yajima et al., 2011). In a portion of human melanomas, MITF is an amplified oncogene, and it also has an oncogenic element in human clear cell sarcoma (Levy et al., 2006). Melanogenesis is controlled by the three genes, tyrosinase-related protein 1 (TYRP-1), tyrosinase-related protein 2 (TYRP-2) and TYR, which are all downstream of MITF (Bentley et al., 1994). Among them, TYR is a rate-limiting enzyme in the process of melanin production (Korner and Pawelek, 1982). MITF was identified, via integrative genomic analyses, as a line-edge survival oncogene that was amplified in malignant melanoma (Garraway et al., 2005). Moreover, MITF activates CDKN1A expression through cooperation with retinoblastoma 1 (RB1) to regulate cell proliferation (Carreira et al., 2005a).

The PI3K/AKT signaling pathway plays a central role in regulating the proliferation and survival of both normal and melanoma cells (Palmieri et al., 2015). However, activated AKT induces proliferation arrest and senescence in several cell lines (Astle et al., 2012). It has been reported that glycogen synthase kinase 3 beta (GSK3B), a direct target of AKT, phosphorylates MITF at S298 and enhances the expression of TYR (Takeda et al., 2000a). Many drugs that affect PI3K/AKT activity, such as bicalitin and sphingosylphosphorylcholine, are involved in the process of degradation of MITF (Jeong et al., 2015; Kim et al., 2010).

TP53 is a tumor suppressor that controls the cell cycle by regulating CDKN1A, cyclin-dependent kinase inhibitor 1B (CDKN1B), and cyclin-dependent kinase inhibitor 2A (CDKN2A) transcription (Biegaj et al., 2014). Too much or too little MITF causes cellular senescence in many types of melanoma cells (Carreira et al., 2005a; Strub et al., 2011). These reports indicate that regulation of cell
proliferation may be associated with MITF and TP53 in melanoma cells. Here, we reported that AKT phosphorylates MITF at S510, which mediates the cellular distribution and degradation of MITF. Unphosphorylatable MITF enhances TYR expression. The phosphorylation of MITF promotes its association with ID4 and inhibits the expression of TYR. However, phosphorylated MITF induces CDKN1A expression and senescence in the presence of TP53 instead of ID4 binding. MITF and p-AKT are inversely correlated in human melanoma.

2. Material and methods

2.1. Antibodies and reagents

The anti-HA, anti-MITF, anti-AKT, anti-p-AKT substrate antibody, anti-AKT1, anti-p-AKT-S473, anti-p-p44/42 MAPK, anti-p44/42 MAPK, anti-Pten, and anti-EGF Receptor were from Cell Signaling Technology. The anti-β-tubulin was from Santa Cruz Biotech. Total S T phosphorylation antibody was from BD Bioscience. The anti-MITF, anti-TP53, anti-TYR, anti-CDKN1A, anti-Lamin B, anti-alpha-tubulin, and anti-GSK3B antibodies were from AB Clonal Technology. PD98059, Wortmannin, PI103, and Ly294002 were from Selleck Chemicals. AKT1 was from Sigma-Aldrich.

2.2. Cell culture and transfection

The A375, 293A, 293T, 293FT, B16, A2059, HeLa and U87 cell lines were purchased from ATCC and cultured in DMEM medium with 10% FBS and P/S in a humidified incubator at 37 °C with a 5% CO2 atmosphere. For transient expression experiments, cells were transfected with recombinant DNA using polyethyleneimine. For stable cells lines were selected by puromycin or hygromycin B, respectively.

2.3. DNA constructs and RNA interference

The human genes with HA, mGST, Myc and Flag tags were inserted into pRK5 using the indicated restriction enzymes. The TYR promoter in pGL3 was a gift from Prof. Colin R. Goding (Oxford University, UK). The shRNA sequences for knockdown of mouse Mitf, Pten and human AKT1 are listed as below: shMitf: TAAATCT- TAAATCTACACCT; shPten: TTATATCCCTTCCTTTGGG; shAKT1: CACAATAGCCATGCTGCTCAT. The sgRNA of Pten (AGATCGT- TACGAGAACAAtt and the sgRNA of TP53 (TGCCACGACCT CCTACAC) were cloned into the modified CRISPR lentivirus vector.

3. Methods

The Immunoprecipitation and Western blotting and Immunohistochemical staining were processed as described in (Liu et al., 2008). The CHIP assay procedure was performed as previously reported by Dr. Saramäki (Saramäki et al., 2006). The AKT kinase assay was performed as previously reported (Hu et al., 2005). The cellular fraction and luciferase assay and GST-tag proteins purification and senescence β-galactosidase staining and MTT assay were procedure following manufacturer’s manual. The p-MITF S510 antibody was generated and purified by AB Technology.

3.1. Statistical analysis

Statistical significance was measured via the unpaired and two-tailed Student’s test and is presented as follows: *p < 0.05, **p < 0.01 and ***p < 0.001. All error bars indicate the SEM.

4. Results

4.1. AKT regulates cell proliferation and melanogenesis in melanoma cells

The senescence or cell proliferation arrest of melanoma cells can be induced by EGF stimulation (Leikam et al., 2014). Here, we found that both EGF stimulation and lentiviral infection with epidermal growth factor receptor (EGFR) can induce CDKN1A expression and reduce TYR in melanoma cells (Fig. 1A, B). To confirm these results, we inhibited AKT. The abundance of CDKN1A in AKTI-treated B16 cells is less than that of cells treated with EGF. However, the abundance of TYR in AKT inhibitor-treated B16 cells is higher than that of cells treated with EGF (Fig. 1C). The MITT assay revealed that EGF stimulation prevents the proliferation of B16 cells (Fig. 1D). After treatment with EGF, the cells exhibit senescence and relatively high beta-Gal activity (Fig. 1E). AKT is associated with cell senescence (Astle et al., 2012). We hypothesized that MITF is a substrate of AKT. To confirm our hypothesis, we detected total phosphorylation of MITF after inhibition or stimulation of AKT. We found that phosphorylation of MITF depends on AKT activity (Fig. 1F). These results suggest that MITF is a target of AKT.

4.2. MITF is phosphorylated by AKT at serine 510

To assess whether MITF could be phosphorylated by AKT, we performed in vitro and in vivo kinase assays. GST-MITF or GST only was cotransfected with HA-AKT-CA (constitutively active AKT). A p-AKT–substrate antibody was used to detect MITF phosphorylation. A clear band was observed in the GST–MITF panel, but no signal was detected for GST only (Fig. 2A). Similar results were obtained by cotransfecting GST–MITF with HA–AKT–CA or HA vector as shown in Fig. 2B. The phosphorylation of MITF was reduced by PI3 K and AKT inhibitors even with EGF stimulation. However, MITF phosphorylation was not suppressed by an ERK inhibitor (Fig. 2C). These data indicated that AKT phosphorylates MITF.

Based on the literature, AKT recognition motif is (R/K)XX(R/K)XX(S/T), where X is any amino acid. Several conserved AKT substrate motifs were found in MITF. We have identified that TFEB, a MITF family member, could be phosphorylated by AKT at S467 (data not shown). We aligned the amino acid sequences of the four families of the MITF gene family. A conservative AKT substrate motif can be found in the C-terminal of this family (Fig. 2D). Alanine scanning was used to accurately identify the phosphorylation site, and S510 of MITF was found to be a precise site (Fig. 2E). The in vitro kinase assays were performed to determine whether MITF is the direct target of AKT and whether S510 is the phosphorylation site. Using the p-AKT-substrate antibody and autoradiography of γ-32P-ATP, the results showed that AKT could phosphorylate MITF in vitro but not the MITF S510a mutant (Fig. 2F, G). MITF WT and S510A were transfected into HEK293T cells and then treated with or without EGF. The pull-down was determined using the p-AKT substrate antibody. No signal was detected in the S510A pull-down even with EGF treatment. This suggests that S510 is a phosphorylation site of AKT in vivo (Fig. 2H). To confirm this, we generated a specific antibody that was tested by recognizing the exogenous GST-MITF WT but not GST-MITF S510A (Fig. 2I). p-MITF was detectable with this antibody after EGF stimulation in B16 cells but not in PI3K and AKT inhibitor–treated cells (Fig. 2J). Similar results were observed in A2059 cells (Fig. 2K). Pten is upstream of AKT and inhibits the PI3K/AKT pathway. When we knockdown Pten, endogenous phosphorylation of MITF is augmented (Fig. 2L). We next asked where phosphorylation of MITF by AKT occurs. To answer this question, we cotransfected GST-MITF with NLS-AKT or Myr-AKT, and the cell lysate was determined using the p-MITF antibody.
The result showed that only Myr-AKT can phosphorylate MITF (Fig. S1A). Then AKT phosphorylates MITF in cytoplasm. All these results suggest that MITF is directly phosphorylated by AKT.

4.3. TYR and CDKN1A are regulated by phosphorylation of MITF

TYR and CDKN1A are the two most important genes downstream of MITF (Carreira et al. 2005a). We have already observed that the levels of TYR and CDKN1A are altered with EGF treatment. MITF is also phosphorylated by AKT; therefore, we speculated whether TYR and CDKN1A expression levels are affected by MITF phosphorylation. To determine the effect of MITF phosphorylation on TYR expression, we knocked down endogenous MITF by targeting its 3′ UTR, which will not affect exogenous MITF expression with only CDS. Additionally, we rescued the expression of MITF WT, S510A, S510D mutants by transfection. Western blotting revealed that the unphosphorylated mutant S510A enhanced TYR expression compared to MITF WT; however, the phosphorylated mimic S510D reduced TYR expression (Fig. 3A). We analyzed whether the expression of TYR corresponded to the activation of the PI3K/AKT signaling pathway. Both EGF and insulin stimulation activated the PI3K/AKT pathway and decreased TYR expression compared to PI3K/AKT inhibition (Fig. 3B). The above results were verified by a subsequent luciferase assay, and the ability to suppress TYR expression was demonstrated by either the S510D mutant or by simulations with EGF/insulin (Fig. 3C,D). The S510A mutant or AKT inhibition increased luciferase readout compared to its counterparts (Fig. 3C,E).

To explore whether CDKN1A expression is also regulated by MITF phosphorylation, we overexpressed MITF variants in HeLa cells. Overexpression of the S510D mutant increased CDKN1A compared to its counterparts (Fig. 3F). This result was confirmed by luciferase assay (Fig. 3G). The U87 cells were infected by the indicated exogenous lentivirus expressing MITF variants. CDKN1A was increased by MITF S510D infection (Fig. 3H). These results demonstrated that phosphorylation of S510 can regulate the two most important genes downstream of MITF.

4.4. Phosphorylation of MITF by AKT induces its degradation

It has been reported that phosphorylation influences the stability of proteins (Swaney et al., 2013). Therefore, we first examined the stability of MITF after its phosphorylation. The MITF WT and its variants were transected into HEK293T cells. Then, the cells
Fig. 2. AKT phosphorylates MITF at S510 in vivo and in vitro.
(A) MITF is phosphorylated by AKT. The indicated plasmids were transiently transfected into HEK293FT cells, followed by glutathione bead pull-down and IB. The phosphorylation of MITF was determined using p-AKT substrate antibody. (B) GST-MITF was transfected with HA-AKT-CA or HA Vector. The thiolactylation bead pull-down was immunoblotted with p-AKT substrate antibody. (C) Schematic alignment of the protein sequences of MITF family proteins. (F) Ala scanning showed that serine 510 of MITF is the only phosphorylation site. (J) WT and S510A were transfected into HEK293FT cells, and cell lysates were analyzed by IB using the p-MITF S510 specific antibody. (K) Identification of endogenous phosphorylation of S510 in melanoma cells. B16 or A2059 cells were pretreated with indicated inhibitors and stimulated by EGF, followed by IB analysis with the indicated antibodies. (L) A2059 cells were infected with control or sgPTEN lentivirus. Then, cell lysates were harvested for IB analysis using the indicated antibodies.
were treated with cycloheximide (CHX) for the indicated times. The cell lysate was immunoblotted with the indicated antibody. MITF S510D degraded much faster than WT and S510A (Fig. 4A, Fig. S1B). These data revealed that phosphorylation at S510 caused MITF instability. We speculated whether this instability was due to increased ubiquitination of MITF. We transfected the GST-MITF WT, S510A and S510D into HEK293T cells. The cell lysates were pulled down using glutathione beads and the pull-down complex was determined using an anti-ubiquitin antibody after MG132 treatment. The result showed that the intensity in the S510D panel was much higher than that of its counterparts (Fig. 4B). To confirm that its stability was correlated with the phosphorylation by AKT, we conducted a time-course treatment of the cells with or without AKT1 combined with CHX. p-MITF and total MITF were examined. The stability of MITF was augmented when the cells were treated with AKTI (Fig. 4C). Knockdown of PTEN also reduced MITF and TYR (Fig. 4D). These results indicate that AKT-mediated phosphorylation of MITF led to degradation through the proteasome pathway.

4.5. The cellular distribution of MITF is affected by phosphorylation

To determine whether phosphorylation of MITF influences its localization, we took images and separated the cellular fractions with different treatments and different variants transfections. The B16 or A2059 cells were treated with EGF or AKTI, then the cells were fractionized and MITF was examined. MITF was translocated to the nucleus from the cytoplasm when AKTI was inhibited (Fig. 4E; Fig. S1C). Images of exogenous GFP-MITF WT, S510A and S510D revealed that S510A was much more highly concentrated and brighter in the nucleus than WT and S510D. However, WT and S510D were distributed in both the cytoplasm and the nucleus, but S510D was much weaker (Fig. 4F). We have demonstrated that AKT inhibition or the S510A mutant induces nuclear translocalization.

4.6. p-MITF binds ID4 to inhibit TYR expression

We have demonstrated that the phosphorylation of MITF decreased TYR expression. We next wanted to dissect the molecular
mechanisms by which MITF phosphorylation regulates TYR expression. Inhibitor of DNA-binding/differentiation proteins are a class of proteins that bind to proteins that contain bHLH domains and inhibit the binding of bHLH to DNA and disrupt its transcriptional capabilities (Zebedee and Hara, 2001). The gene family consists of four members: ID1, ID2, ID3 and ID4. Here, we showed that the interaction between MITF and ID4 is the strongest in the ID gene family (Fig. 5A). Even S510D interacted more strongly with ID4 than S510A and WT (Fig. 5B). MITF lost its ability to transcriptionally activate TYR when it was bound to ID4, which was shown via luciferase assay (Fig. 5C). ID4 has the strongest inhibitory effect on S510D in terms of TYR (Fig. 5D, E). These data demonstrated that p-MITF inhibits TYR expression through binding to ID4.

4.7. p-MITF interacts with TP53 to regulate CDKN1A expression

A high level of MITF in melanoma cells can induce the expression of CDKN1A, and MITF is associated with cell senescence in
4.8. Phosphorylation of MITF causes cell proliferation arrest and senescence

Previous data indicate that p-MITF prevents cell proliferation. We established inducible stable cell lines of the MITF variants. Cell viability was measured by the MTT assay. The results showed that all of the MITF variants could induce cell proliferation arrest; however, S510D had a stronger effect (Fig. 7A). When B16 cells were cotransfected with lentivirus expressing MITF variants and shMITF for 72 h and beta-Gal staining was performed, much more blue staining was observed in the S510D-infected cells. This indicated that S510D induced more cell senescence (Fig. 7B). Some researchers believe that cancer is a kind of parasitic organism; to survive, they are unscrupulous. Based on this opinion, we believe that melanoma is not excluded. To survive, they chose to weaken the activity of AKT. If this is the case, p-AKT and MITF should exhibit an inverse relationship. To determine whether this is the case, we performed immunohistochemical staining of p-AKT-S473 and MITF on melanoma slices from patients. We stained samples from 16 patients who had melanoma in their skin (Supplemental Table 1). The representative images are shown in Fig. 7C. We found that 90% of the melanoma samples showed an inverse relationship between p-AKT and MITF and positive relationship between p-AKT and p-MITF (Fig. 7C). In a small number of samples, such as sample number 15-24690, some regions showed a lower MITF/p-AKT ratio relative to the negative control (Fig. 7C). This result is consistent with our observation in cell lines. This indicates that in high number of MITF melanoma cells, the AKT activity will be reduced to avoid cell senescence through phosphorylation of MITF.

5. Discussion

We found that EGF could cause proliferation arrest and cell senescence of melanoma cells. The genes CDKN1A and TYR were controlled by AKT. AKT directly phosphorylates MITF at S510. The phosphorylation led to proteasome-mediated MITF degradation, which was concomitant with proliferation arrest and senescence. The unphosphorylative MITF translocates to the nucleus and promotes TYR expression. The phosphorylative mimic S510D localizes to both the cytoplasm and the nucleus and enhances CDKN1A expression. We also demonstrated that MITF S510D binds to ID4 to inhibit the transcription of TYR. In the presence of TP53, TP53 would compete with ID4 to bind MITF S510D. The p-MITF/TP53 complex binds to the CDKN1A promoter and transiently enhances CDKN1A expression in response to AKT activation. Our work explains the dual role of MITF in regulating melanoma progress (Fig. 7D).

It has been reported that phosphorylation of MITF at S73 by ERK also leads to CDKN1A expression and MITF instability (Kim et al., 2010; Wu et al., 2000a; Liu et al., 2010). P90-RSK1, which
Fig. 6. Phosphorylated MITF interacts with TP53 and induces CDKN1A expression.

(A) MITF binds TP53 in vitro. GST-MITF and HA-TP53 were transfected into HEK293FT cells and whole-cell lysates were subjected to GST pull-down and IB with the indicated antibodies. (B) Deletion of TP53 blocked the expression of CDKN1A. (C) The fragments of MITF were co-transfected with HA-TP53 into HEK293FT cells, followed by the IP and IB experiments. (D) MITF interacts with TP53 94–300. The diagram of TP53 fragments is shown in the top panel. (E) B16 cells were incubated with or without AKTI. The lysates were IPed with the MITF antibody and analyzed with the TP53 antibody. (F) MITF S510D strongly interacts with TP53. The indicated plasmids were transfected into HEK293 cells, followed by IP and IB analysis using the corresponding antibodies. (G) TP53 competes with ID4 in binding MITF. Myc-ID4 and mGST-MITF-S510D were co-transfected with or without HA-TP53 into HEK293FT cells, followed by downstream experiments and data analysis. (H) The CHIP assay was performed using the MITF antibody following the manufacturer’s instructions. PCR was performed using specific primers. (I) The indicated plasmids were transfected into MGH cells, then the cells were subjected to the subsequent procedures as described in Fig. 6H. The gray scale was calculated using Image J. Then, the data were subjected to analysis via Graphpad prism 5. Mean ± SEM. *p < 0.05.
is the direct target of BRAF-V600E, phosphorylates MITF at S409 and causes its degradation (Wu et al., 2000b). Our observations of phosphorylation at S510 of MITF are similar to the observations of phosphorylation of MITF at S73 and S409. Here, we found that AKT phosphorylates MITF at S510, which triggers its degradation. This phosphorylation leads to transient expression of its downstream target CDKN1A and reduces TYR transcription.

Tyrosinase is the limiting step for melanin synthesis, which is controlled by MITF (Slominski et al., 2004). Melanin is lost during PI3K/AKT activation (Lee et al., 2012; Song et al., 2015) and TYR is downregulated by EGF treatment (Shin et al., 2014).
GSK3β phosphorylates MITF at S298 and blocks its transcriptional activation of TYR (Takeda et al., 2000b; Khaled et al., 2002). More melanin was produced in B16 cells when AKT was inhibited (Hung et al., 2014). All of our findings are consistent with these results, and these data are related to MITF phosphorylation. We found MITF S510A could induce melanin production by upregulating TYR expression. Thus we demonstrated the insight mechanism that AKT regulates Melanin production. Unphosphorylative MITF is much more stable than the WT and phosphorylative forms. We found that phosphorylated MITF bound more ID4, which inhibits the transcriptional activation of TYR by MITF.

Cellular senescence is an irreversible proliferation arrest that is activated by various cellular and molecular stresses (Ben-Porath and Weinberg, 2004; Campisi, 2005). Senescence or proliferation arrest could be induced by oncogenes such as KRAS mutations in normal human and mouse cells in culture (Vicent et al., 2010). PI3K/AKT is also an inducer of senescence (Astle et al., 2012; Kennedy Ras et al., 2014; Sun et al., 2014). Consistent with previous reports, we observed cell proliferation arrest as a result of PI3K/AKT activation in B16 melanoma cells. CDKN1A is a predominant inducer of cell proliferation arrest and senescence (Kuilman et al., 2010). Here, we found that CDKN1A is dramatically upregulated with EGF stimulation. The S510D mutant also increased CDKN1A expression and senescence.

Normally, cell senescence is related to RB1 and TP53 (Benhamed et al., 2012; Qian and Chen, 2013; Chen et al., 2005). Indeed, the ability of MITF to regulate CDKN1A is likely to be highly complex, with many factors contributing directly or indirectly to the regulation of CDKN1A expression by MITF (Carreira et al., 2005b, 2006). However, we did not observe any differences in the interactions between WT, S510A, S510D and RB1 (data not shown). Meanwhile, MITF still upregulated CDKN1A even after knockdown of RB1. It is indicated that this phosphorylation regulating CDKN1A expression is not mediated by RB1. Actually, we discovered that p-MITF directly binds TP53 to regulate CDKN1A expression and that p-MITF does not bind ID4 in the presence of TP53.

In normal skin cells, p-AKT and MITF are moderately expressed (http://www.proteinatlas.org), and we believe that MITF is in a controlled transcriptional state to maintain normal physiological function. Here, we identified an important role of MITF in response to EGF stimulation and dissected the mechanism by which MITF regulates CDKN1A in human melanoma (Huang et al., 2014). Indeed, we observed that the interaction between MITF and p-AKT was an inverse relationship in patient samples. p-MITF-S510 is an alternative diagnostic marker for human melanoma. Our results help us to understand the multiple roles of MITF in melanoma and open up a new avenue for the treatment of melanoma.

Competing financial interests

The authors declare no competing financial interests.

Author contributions

Z. L. designed the experiments and wrote the manuscript; C. W. processed the experiments and wrote the manuscript; L. Z., Y. W. and S. G. provided the slices of human melanomas; Q. S. and H. W. completed experiments; X. F. classified the slices of human melanoma after staining; H. C. provided material and suggestions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2016.09.029.

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