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Inhibition of Melanogenesis by the Antidiabetic Metformin

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Several reports have demonstrated the inhibitory effect of metformin, a widely used drug in the treatment of type 2 diabetes, on the proliferation of many cancers including melanoma. Recently, it has been shown that metformin is able to modulate the cAMP level in the liver. As cAMP has a crucial role in melanin synthesis and skin pigmentation, we investigated the effect of metformin on melanogenesis both *in vitro* and *in vivo*. We showed that metformin led to reduced melanin content in melanoma cells and in normal human melanocytes by decreasing cAMP accumulation and cAMP-responsive element–binding protein phosphorylation. This inhibitory effect is correlated with decreased expression of master genes of melanogenesis, microphthalmia-associated transcription factor, tyrosinase, dopachrome tautomerase, and tyrosinase-related protein 1. Furthermore, we demonstrated that the antimelanogenic effect of metformin is independent of the AMPK pathway. Interestingly, topical application of metformin induced tail whitening in mice. Finally, we confirmed the antimelanogenic effect of metformin on reconstituted human epidermis and on human skin biopsies. These data emphasize the depigmenting effect of metformin and suggest a clinical strategy for using metformin in the topical treatment of hyperpigmentation disorders.

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INTRODUCTION

Melanocytes are specialized cells located at the basal layer of the epidermis that produce and transfer melanin pigments to the surrounding keratinocytes. Melanin is principally responsible for skin color and has an important role in the prevention of sun-induced skin injury. Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1), and dopachrome tautomerase (DCT), are involved in this enzymatic process that converts tyrosine to melanin pigments (Kobayashi *et al.*, 1994; Passeron *et al.*, 2005).

Melanin synthesis is stimulated by different factors including UV irradiation, alpha-melanocyte-stimulating hormone (α-MSH), or cAMP-elevating agents such as forskolin (Yamaguchi and Hearing, 2009). These stimuli activate the cAMP pathway in which the microphthalmia-associated transcription factor (MITF) is the most important target. Indeed, binding of α-MSH to melanocortin-1 receptor leads to adenylate cyclase increase, elevation of intracellular cAMP, and activation of protein kinase A (PKA). cAMP-activated PKA is then transferred to the nucleus and it phosphorylates its principal substrate cAMP-responsive element–binding protein (CREB). Phosphorylated CREB upregulates the transcription of genes whose promoters have a cAMP-response element sequence, and thus induces the expression of MITF, the master regulator in the transcription of genes involved in melanin synthesis such as tyrosinase, TRP1, and DCT (Bertolotto *et al.*, 1996; Busca and Ballotti, 2000). Deregulation of this signaling pathway may cause pigmentation disorders.

There are numerous causes of hyperpigmentation, such as postinflammatory etiologies, hormonally mediated factors, cosmetics, drug-induced causes, and UV radiation in addition to systemic conditions such as Addison's disease and Wilson's disease. These pigmentation disorders have traditionally been a relatively difficult condition to treat, and currently used depigmentating agents have little effect with sometimes severe side effects (Solano *et al.*, 2006).

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Abbreviations: AMPK, AMP-activated protein kinase; cAMP, cyclic 5′ adenosine monophosphate; CREB, cAMP-responsive element binding; DCT, dopachrome tautomerase; FSK, forskolin; MC1-R, melanocortin-1 receptor; Met, metformin; MITF, microphthalmia-associated transcription factor; α-MSH, alpha-melanocyte-stimulating hormone; NHM, normal human melanocyte; PKA, protein kinase A; PVDF, polyvinylidene difluoride membrane; RHE, reconstructed human epidermis; siRNA, small interfering RNA; TRP1, tyrosinase-related protein 1

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The oral antidiabetic drug metformin belongs to the family of biguanide drugs and is the most widely used antidiabetic drug in the world. This drug has been shown to inhibit the energy-sensitive AMP-activated protein kinase (AMPK)-mammalian target of rapamycin signaling pathway that leads to reduced protein synthesis and cell proliferation. Recent studies indicate that metformin can potentially be used as an efficient anticancer drug in various tumors (Ben Sahra *et al.*, 2010; Pollak, 2010). In addition, metformin was reported by several groups, including ours, to inhibit the proliferation and invasion of melanoma cells (Woodard and Platanias, 2010; Janjetovic *et al.*, 2011; Niehr *et al.*, 2011; Tomic *et al.*, 2011; Cerezo *et al.*, 2013). Interestingly, our previous work has shown that metformin was nontoxic on normal cells such as melanocytes, keratinocytes, or fibroblasts (Tomic *et al.*, 2011 and unpublished results).

As it was recently shown that metformin decreases the cAMP level (Miller *et al.*, 2013) and that cAMP has a crucial role in melanin synthesis and skin pigmentation, we investigated the effect of metformin on pigmentation *in vitro* and *in vivo*.

RESULTS

Metformin inhibits the synthesis and secretion of melanin by melanoma cells in a time- and a dose-dependent manner

To assess the effect of metformin on melanogenesis, B16 cells were stimulated by propigmenting agents α-MSH and forskolin in the presence or absence of metformin. Visual evaluation revealed a remarkable reduction in the melanin content noticeable from 48 hours of treatment in cells stimulated with forskolin but not in basal conditions (Figure 1a). Forskolin and α-MSH induced a high increase in melanin content in melanoma cells after 72 hours of treatment (Figure 1b). However, metformin significantly decreased the melanin synthesis in basal, forskolin, and α-MSH conditions (Figure 1b). As expected, melanin dosage indicated that both forskolin and α-MSH stimulated melanin secretion in culture medium after 72 hours of treatment (Figure 1c). This increase was abolished by metformin treatment.

Metformin inhibits melanogenesis gene expression in melanoma cells and in normal human melanocyte (NHM) cells

To determine the mechanism of action of metformin on melanin synthesis, we examined its effect on key proteins involved in melanogenesis, such as MITF, tyrosinase, TRP1, and DCT.

In B16 cells, treatment with forskolin or α-MSH significantly increased protein levels of MITF, tyrosinase, and TRP1 (Figure 2a). However, we did not observe a significant change in DCT protein expression. Treatment of cells with metformin significantly reduced the levels of MITF, tyrosinase, TRP1, and DCT. The effect is more pronounced when we cotreated melanoma cells either with forskolin or α-MSH and metformin (Figure 2a). In NHM cells, forskolin induced a slight increase in MITF level compared with control (Figure 2b). NHM cells contain high level of MITF, which can explain the absence of stimulation on MITF level when we treated with forskolin. Forskolin induced a significant increase of TRP1 and DCT (Figure 2b). Treatment of NHM cells with metformin decreased MITF, tyrosinase, and DCT levels in a dose-dependent manner (Figure 2b). Interestingly, cotreatment of NHM cells with both metformin and forskolin induced the extinction of MITF, tyrosinase, and DCT. A decrease in MITF and genes of melanogenesis was observed after 12 hours of metformin treatment both in B16 cells and in NHM cells (Supplementary Figure 3 online).

Metformin inhibits MITF promoter activity

As MITF, through the regulation of tyrosinase expression, is a key factor in melanogenesis, we explored the possibility that metformin regulates MITF expression through a transcriptional regulation. To verify this, a luciferase-based promoter assay

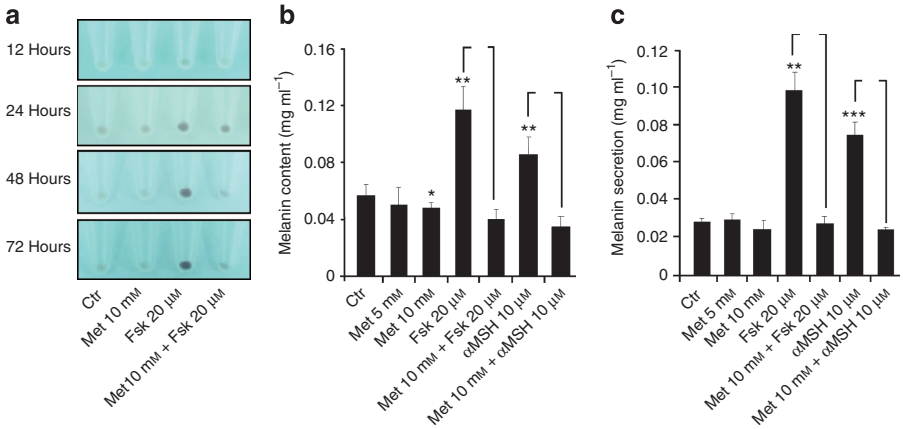

Figure 1. Antimelanogenic effect of metformin. B16-F10 cells at a density of 2.10^5 cells per well (six-well plate) were incubated overnight and were then treated with metformin 5 10 mM (Met), forskolin 20 μM (Fsk), alpha-melanocyte stimulating hormone (α-MSH) (10 μM), or a combination of these drugs, as indicated, for 12, 24, 48, or 72 hours. (**a**) Cell pellet images with or without the indicated treatment were taken using a digital camera. Melanin content (72 hours treatment) (**b**) and melanin secretion in culture medium (72 hours treatment) (**c**) were measured with synthetic melanin as a standard and then normalized to the protein fraction. The data show the mean ± SD of five separate experiments (*$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$) versus control.

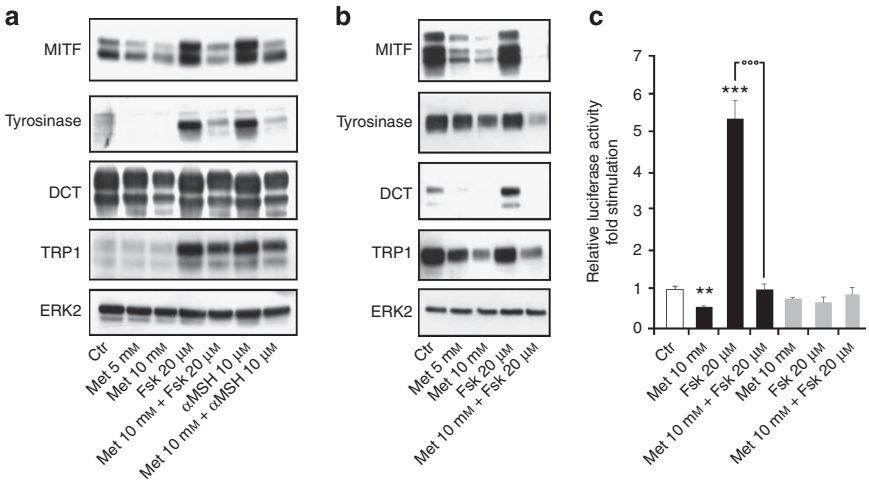

Figure 2. Metformin inhibits melanogenesis gene expression and microphthalmia-associated transcription factor (MITF) promoter activity. B16-F10 cells (**a**) or normal human melanocytes (NHMs) (**b**) were treated with metformin 5 10 mM (Met), forskolin 20 μM (Fsk), alpha-melanocyte stimulating hormone (α-MSH) (10 μM), or combinations of these drugs, as indicated, for 48 hours. Cell lysates were used for western blotting with indicated antibodies. (**c**) B16 cells were transiently transfected either with 0.5 mg of MITF promoter (p-MITF) (black bar) or pCMV–CREB133 (gray bar) (gift of Dr Mauviel Alain, Institut Curie, Orsay, France) encoding the MITF promoter containing a mutation in the cAMP-responsive element–binding protein (CREB)-binding site and 0.05 μg of pCMVβGal to control the variability in transfection efficiency. Cells were treated for 48 hours with different drugs as indicated. Luciferase activity was normalized to β-galactosidase activity and the results were expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are means ± SD of five experiments performed in triplicate (**$P \leqslant 0.01$, ***$P \leqslant 0.001$, versus control $^{\circ\circ\circ}P \leqslant 0.001$). DCT, dopachrome tautomerase; TRP1, tyrosinase-related protein 1.

was used in B16 cells by using vector encoding wild-type MITF promoter (p-MITF), as described previously (Bertoloto *et al.*, 1996). As shown in Figure 2c, forskolin led to an approximately fivefold increase of MITF promoter activity. However, metformin significantly inhibited MITF promoter activity both in basal and in forskolin-stimulated conditions, demonstrating that metformin affects MITF transcription.

By using an MITF promoter containing a mutation in the CREB-binding site, we showed that neither forskolin nor metformin is able to modify CREB-mutated promoter activity (Figure 2c). All together, these results demonstrate that metformin is a specific inhibitor of MITF at the messenger RNA expression level.

Metformin inhibits melanogenesis by decreasing the production of cyclic AMP

cAMP-induced melanogenesis has been reported to be mediated by the activation of PKA and CREB phosphorylation. Treatment of B16 cells with forskolin or alpha-MSH during 24 hours increased PKA and CREB phosphorylation, whereas metformin induced a significant inhibition of protein phosphorylation in both basal and stimulated conditions (Figure 3a). No effect of metformin on PKA and CREB expressions has been observed.

The same effect of metformin on CREB phosphorylation was observed in NHM cells (Figure 3b). These results suggest that metformin exerts its depigmenting effects on melanoma cells and in melanocytes through the inhibition of CREB activation. This effect might be due to an increase in cAMP level. To verify this hypothesis, we measured the level of cAMP in response to metformin. Both forskolin and α-MSH increased cAMP in melanoma cells, with the maximum effect observed after 6 hours of treatment (Figure 3c). Metformin decreased cAMP accumulation in either forskolin- or α-MSH-stimulated conditions in a time-dependent manner, with the maximum effect observed after 48 hours of metformin treatment.

We next evaluated whether the diminution of the cAMP level in response to metformin is involved in MITF down-regulation. We used a membrane-permeable analog of cAMP: dibutyryl cAMP. Treatment of B16 cells with dibutyryl cAMP increases the levels of MITF protein and master genes of melanogenesis. In the presence of dibutyryl cAMP, metformin was unable to decrease MITF level as in control conditions (Figure 3d).

Metformin reduced MITF by an AMPK-independent pathway

The anticancer effects of metformin have been essentially attributed to its ability to activate the AMPK pathway. To determine whether AMPK has a role in melanogenesis inhibition by metformin, we abrogated AMPK activation using a dominant negative form of AMPK both in NHM cells (Figure 4a) and in Mel501 cells (Figure 4b). As expected, infection of AMPK-DN increases the total level of AMPK and inhibits partially (NHM) or totally (Mel501) the phosphorylation of AMPK and its downtream effector acetyl-CoA carboxylase in basal and stimulated conditions, indicating that the negative form of AMPK is expressed and is effective. In Mel501 cells, we observed that AICAR, a specific activator of AMPK, had no effect on MITF. Metformin decreased the protein levels of MITF and of key proteins involved in melanogenesis both in cells infected with control or AMPK-DN adenovirus. These results suggest that the AMPK pathway is not implicated in the antimelanogenic effect of metformin. Similar results were obtained when AMPK was knocked down

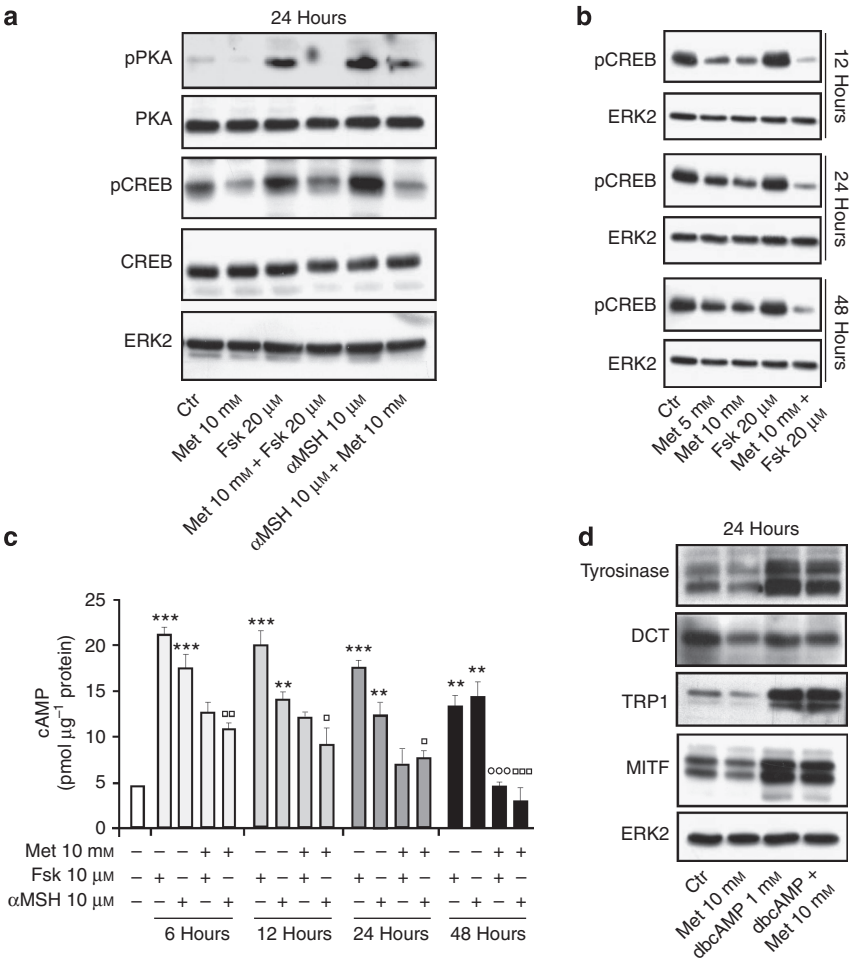

Figure 3. Metformin inhibits protein kinase A (PKA) phosphorylation, cAMP-responsive element–binding protein (CREB) phosphorylation, and cAMP accumulation. B16-F10 cells (**a**, **c**) or normal human melanocytes (**b**) were incubated with metformin 10 mM (Met), forskolin 20 μM (Fsk), alpha-melanocyte stimulating hormone (α-MSH) 10 μM, or a combination of these drugs at the indicated time. Cell lysates were then subjected either to western blotting with the indicated antibodies (**a**, **b**) or to total cAMP dosage according to the manufacturer's instructions (ArborAssays) (**c**); data are means ± SD of three independent experiments performed in triplicate (**$P \leqslant 0.01$, ***$P \leqslant 0.001$ versus control, $^{\circ\circ\circ}P \leqslant 0.001$, $^{\square}P \leqslant 0.05$, $^{\square\square}P \leqslant 0.01$, $^{\square\square\square}P \leqslant 0.001$). (**d**) B16-F10 cells were incubated with metformin 10 mM (Met), dibutyryl cAMP (dbcAMP) 1 mM, or a combination of these compounds, as indicated, for 24 hours. Cell lysates were used for western blotting with the indicated antibodies. ERK2 was used as a loading control.

by using small interfering RNA (siRNA) in Mel501 cells (Figure 4c).

Moreover, it has already been shown that LKB1 is the major kinase phosphorylating the AMPK under conditions of energy stress (Hardie *et al.*, 2012). We thus evaluated the effect of metformin on MITF protein level in G361 cells in which LKB1 is mutated. As shown in Figure 4d, metformin decreased the levels of MITF and the master genes of melanogenesis in G361 cells in basal and forskolin-stimulated conditions.

Metformin inhibits melanogenesis *in vivo*

To test whether the effects of metformin observed on melanogenesis *in vitro* were representative of its action *in vivo*, we examined the effect of metformin on tail pigmentation of C57BL/6J mice. To this end, C57BL/6J mice were treated daily by topical application of metformin (10 mM), forskolin (20 μM), or a combination during 8 weeks. As there is often a high variability of pigmentation on the tip of mouse tails, we chose them by taking into account the homogeneous pigmentation in this area (Figure 5a). We showed that daily topical application of forskolin had no effect on skin darkening (Figure 5b); this observation was confirmed by the absence of the effect of forskolin treatment on tail melanin content (Figure 5c). Nonetheless, forskolin induced a significant increase in DCT protein level and a slight increase in MITF and tyrosinase protein level (Figure 5d). Contrary to forskolin, daily topical application of metformin induced tail depigmentation in mice (Figure 5b). This effect correlates with a decrease in melanin content (Figure 5c) and with the level of master melanogenic genes (Figure 5d). Interestingly, the effect of metformin is more important when mice were cotreated with forskolin.

Finally, we tested the effect of metformin on human reconstituted epidermis. As presented in Figure 6a, direct

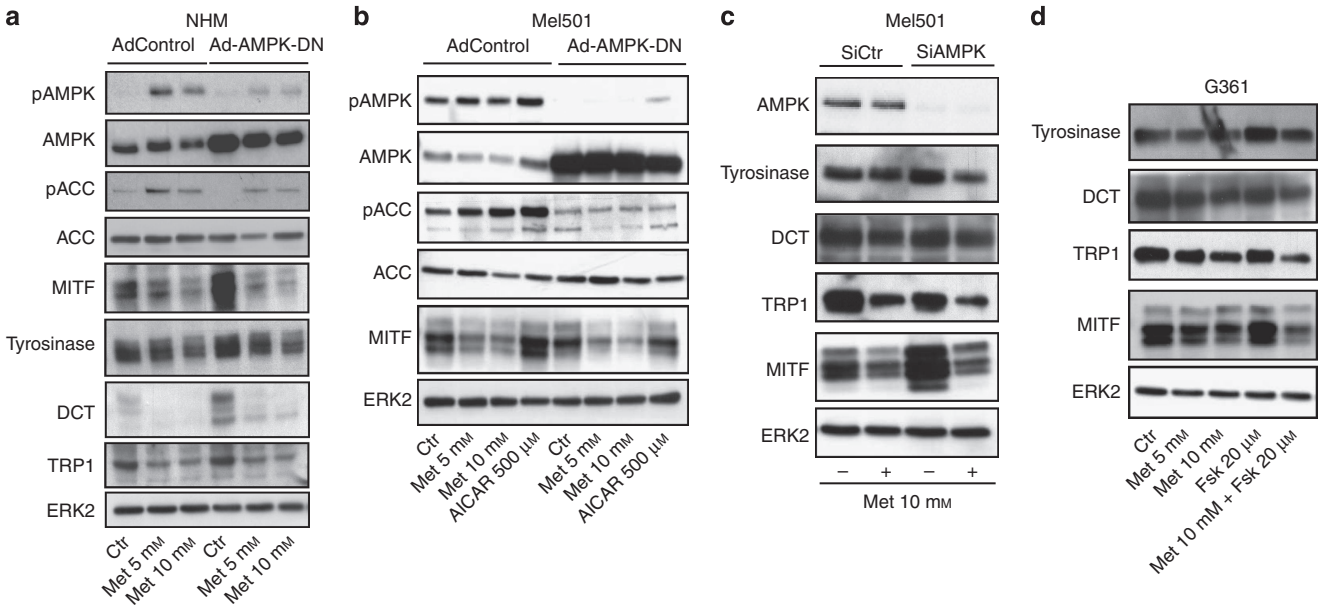

Figure 4. Metformin inhibits microphthalmia-associated transcription factor (MITF) by an AMP-activated protein kinase (AMPK)-independent pathway. Normal human melanocyte (NHM) (**a**) or Mel501 cells (**b**) were transfected either with Ad-AMPK-DN or AdControl. After 24 hours of transfection, the medium was changed and cells were either exposed or not exposed to metformin 5 and 10 mM or AICAR 500 µM for another 24 hours. (**c**) Mel501 cells transfected with 20 µM of either siRNA control (SiCtr) or Si-AMPK were either exposed or not exposed to metformin 10 mM for 24 hours. (**d**) G361 cells were incubated overnight and then treated with metformin 5 and 10 mM (Met), forskolin 20 µM (Fsk), or a combination of both for 24 hours. Cell lysates were used for western blotting with the indicated antibodies. ERK2 was used as a loading control. ACC, acetyl-CoA carboxylase; DCT, dopachrome tautomerase; TRP1, tyrosinase-related protein 1.

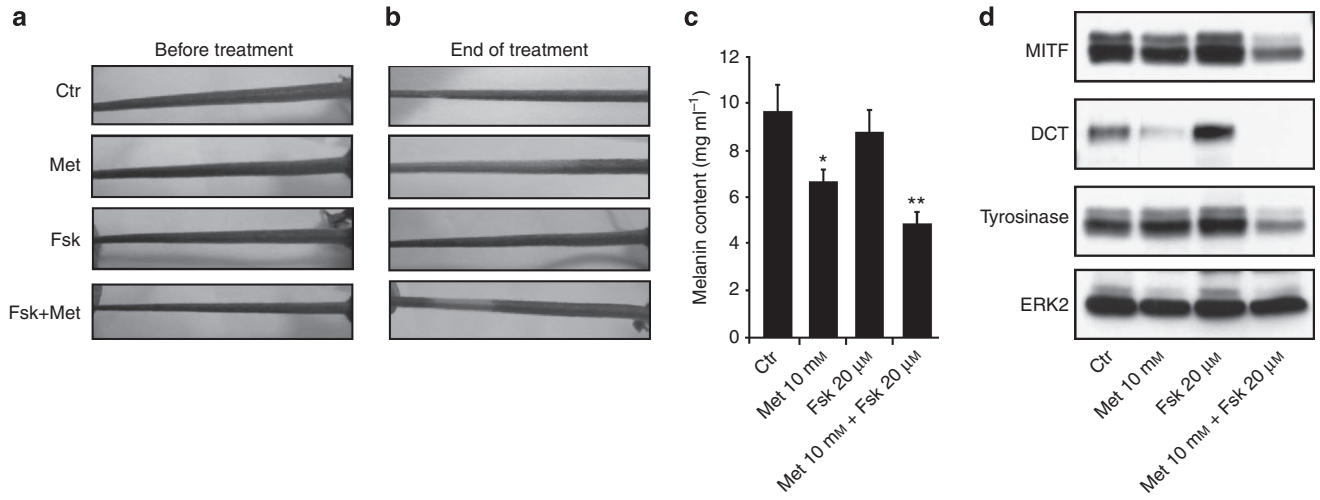

Figure 5. Metformin inhibits melanogenesis *in vivo*. C57BL/6J mice (six mice per group) were topically treated daily on the tail with 10 mM metformin, 20 µM forskolin, or a combination prepared in standard dermatologic vehicle consistent of 30% weight:volume solution of 70% ethanol, 30% propylene glycol during 8 weeks. Solvent control (Ctr) consisted of the same volume of 70% ethanol/30% propylene glycol. Mice tails were photographed using a digital camera before (**a**) and at the end of treatment (**b**). Animals were then killed and the skin was then removed from the tail and cut into two equal parts; the first part was used to evaluate melanin content (**c**) and the second part to study melanogenic protein by western blotting (**d**) (*$P \leqslant 0.05$; **$P \leqslant 0.01$ versus control). DCT, dopachrome tautomerase; MITF, microphthalmia-associated transcription factor.

photography of the well containing reconstructed human epidermis after 15 days of stimulation by forskolin and/or metformin indicated that forskolin increased melanin expression, whereas metformin inhibited its synthesis. As expected, western blot analysis (Figure 6b) indicated that forskolin increased the levels of melanogenesis proteins such as MITF and tyrosinase. In contrast, metformin abolished completely the expression of these proteins in basal or

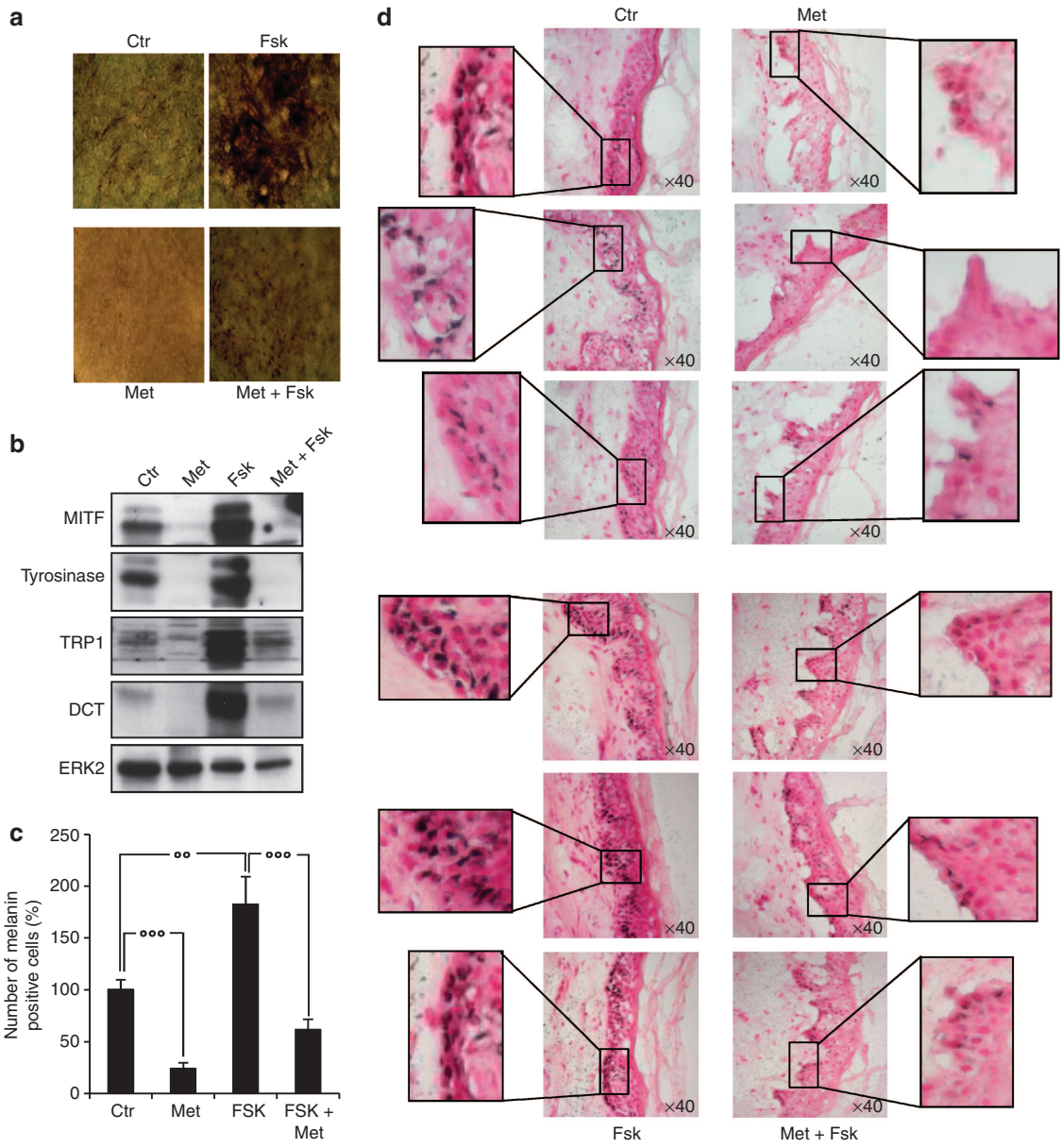

Figure 6. Metformin induces human skin depigmentation. Human skin punch biopsy or reconstructed human epidermis was incubated with metformin 10 mM (Met), forskolin 20 μM, (Fsk) or a combination of these drugs, as indicated, for 15 days. (**a**) Reconstructed human epidermis was photographed using a digital camera at the end of treatment. (**b**) Proteins were extracted and melanogenesis protein expression was evaluated by western blotting. (**c**) Melanin distribution in human skin punch biopsy was determined by Fontana–Masson staining. (**d**) Melanin-positive cell quantification in human skin punch biopsy was evaluated. Data are means ± SD of five independent experiments ($^{\circ\circ}P \leqslant 0.01$, $^{\circ\circ\circ}P \leqslant 0.001$ versus control). DCT, dopachrome tautomerase; MITF, microphthalmia-associated transcription factor; TRP1, tyrosinase-related protein 1.

forskolin-stimulated conditions. To confirm this, another series of experiments were conducted directly on human skin biopsies prepared from abdominoplasty. Biopsies were maintained in culture during 15 days and were stimulated for 2 days each by forskolin and/or metformin. Fontana–Masson staining was performed on paraffin-frozen skin sections to detect intracellular melanin (Figure 6c). Forskolin increases the number of cells that contains melanin, whereas metformin strongly decreases the number of melanin-positive cells in control and forskolin-treated conditions. Quantification of melanin-positive cells (Figure 6d) confirms the Fontana–masson staining observations.

These data confirmed the results that we obtained *in vitro* and demonstrated clearly that metformin has a depigmenting effect in intact cells, as well as in mice and humans.

DISCUSSION

Melanogenesis, a major function of differentiated melanocytes, has an important role in protecting skin from sun-related injuries and is principally responsible for skin color. Abnormal

accumulation of melanin pigments is responsible for pigmented disorders such as melasma and senile lentigo. Current treatment options are not fully efficient and in several cases induce side effects. Thus, it is imperative to identify agents with better efficacy and fewer side effects.

In humans and in mice, it is well established that cAMP has a critical role in the regulation of melanogenesis through the activation of the transcription factor CREB (Ao *et al.*, 1998; Busca and Ballotti, 2000). A recent study reported that metformin decreases cAMP accumulation in hepatocytes to antagonize glucagon action (Miller *et al.*, 2013). Then, the purpose of the present work was to investigate the inhibitory effects of metformin on melanin formation, which is closely related to pigmentation of the skin. Our results showed that metformin decreased melanin production and secretion in a time- and a dose-dependent manner in B16-F10 melanoma cells. This effect is due to a sharp decrease in cAMP accumulation especially in forskolin- or α-MSH-stimulated conditions, which lead to CREB deactivation and then to a decrease in master genes of melanogenesis, MITF, Tyrosinase, DCT, and TRP1. Furthermore, metformin inhibits MITF promoter activity, suggesting that it regulates MITF at the transcriptional level. In addition, MITF promoter containing a mutation in the CREB-binding site is not altered by metformin, which suggests that the CREB-responsive motifs are essential for the antimelanogenic effect of metformin.

We already showed that metformin induces cell cycle arrest of melanoma cells after 24 hours of treatment and apoptosis after 96 hours of treatment (Tomic *et al.*, 2011). In our study, metformin reduced MITF and melanogenic gene levels after 12 hours of treatment (Supplementary Figure 3 online). Moreover, metformin did not induce any change in the number of B16-F10 cells at the short treatment time, which suggests that the antimelanogenic effect of metformin in melanoma cells is not related to its effect on cell viability. These observations suggest that the effect of metformin on pigmentation was not due to a cytotoxic effect. The antimelanogenic effect of metformin on NHM cells confirmed this hypothesis. Indeed, we already showed that metformin had no effect on NHM viability (Tomic *et al.*, 2011). Here, we showed that metformin, as in melanoma cells, inhibited melanogenesis genes in a dose- and a time-dependent manner without affecting their viability, demonstrating clearly that the effects of metformin on cell viability and on pigmentation are independent.

Compelling evidence has been gathered indicating that metformin action is mainly mediated by AMPK activation (Zhou *et al.*, 2001; Shaw *et al.*, 2005). As metformin activated AMPK, we asked whether the kinase was required for the effects of metformin on melanin production.

In this study, inactivation of AMPK by an AMPK dominant negative construct or inhibition of AMPK expression by siRNA did not abrogate the inhibitory effect of metformin on MITF, indicating that metformin-inhibited melanogenesis seems to be independent of the AMPK pathway. These results are in accordance with recently published results demonstrating that the effects of metformin on glucagon metabolism are independent of the AMPK pathway (Miller *et al.*, 2013). However, expression of Ad-AMPK dominant negative mutant in NHM cells only partially diminished metformin-dependent activation of acetyl-CoA carboxylase, one of the major AMPK substrates. Therefore, we cannot exclude that the residual AMPK activation by metformin is sufficient to mediate metformin effect on MITF expression.

In agreement with a recent report (Borgdorff *et al.*, 2014), we observe that the inhibition of AMPK by either Ad-AMPK dominant negative mutant (Figure 4a) or Si-AMPK (Figure 4c) led to significant increases of basal MITF levels in these cells, suggesting also a role of AMPK in regulating MITF levels.

Taken together, our findings suggest to consider both AMPK-dependent and AMPK-independent in the control of MITF in response to metformin. Further, we demonstrate that metformin suppresses CREB-dependent MITF induction, which can affect the expression of melanogenic enzymes by inhibiting cAMP accumulation.

To test whether the effects of metformin on melanogenesis are representative of its action *in vivo*, we examined the effect of metformin on C57BL/6J mouse tail pigmentation. C57BL/6J mouse tails are highly pigmented, which can explain the lack of effect of forskolin; moreover, the fact that the concentration of forskolin that we used might not have been sufficient to induce a significant increase of melanogenesis in mouse tails cannot be excluded. We showed that, like *in vitro*, metformin is able to inhibit melanogenesis *in vivo*. Indeed, metformin induced progressive tail whitening noticeable after 8 weeks of topical treatment. Further, similar results were obtained on human reconstituted epidermis and in human skin biopsies.

These results confirm those obtained *in vitro* and suggest that metformin can be used in the treatment of skin hyperpigmentation. Our data are in accordance with a beneficial effect of metformin on the treatment of skin diseases, especially those associated with hyperinsulinaemia and hyperandrogenism, such as acne, hidradenitis suppurativa, acanthosis nigricans, and psoriasis (Ortonne and Passeron, 2005; Cardinali *et al.*, 2012). Interestingly, it is important to mention that no depigmenting effect has been reported in diabetic patients treated with metformin. Moreover, we already showed that systemic treatment of mice with metformin induced regression of melanoma tumors. However, we observed no change of skin pigmentation in metformin-treated mice compared with untreated mice, suggesting that the depigmenting effect of metformin is only observed when used topically.

This study reports the potent inhibitory effect of metformin on melanogenesis and demonstrates that metformin is a skin-whitening agent. The inhibitory effects of metformin were dose- and time-dependent and did not incur significant cytotoxicity. Considering our results, it is becoming clear that the antimelanogenic effects of metformin are due to the inhibition of cAMP/CREB/MITF axis, the most preponderant pathway involved in melanogenesis.

Taken together, these results prompted us to conclude that metformin, for which the safety has been validated in a number of studies, had a therapeutic potential in the treatment of hyperpigmentation disorders.

MATERIALS AND METHODS

Cell cultures

NHMs were obtained from the foreskins of Caucasian children, as described previously (Botton *et al.*, 2009).

B16-F10, Mel501, Skmel28, and G361 melanoma cells were cultured in DMEM, supplemented with 7% fetal bovine serum and penicillin/streptomycin (100 IU; 50 g ml^{-1}) and maintained in 5% CO_2 at 37 °C.

Reagents and antibodies

Metformin, forskolin, and MCDB153 medium were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Anti-MITF was obtained from Abcam (Cambridge, MA), and anti-ERK2 (D-2), anti-DCT, and anti-tyrosinase monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-CREB and anti-phospho-CREB, anti-phospho-PKA, anti-PKA, anti-phospho-acetyl-CoA carboxylase, and anti-acetyl-CoA carboxylase were purchased from Cell Signaling Technology (Beverly, MA).

Determination of melanin content

Melanin content was measured as previously described (Bellei *et al.*, 2010). Briefly, B16 cells ($2{,}5 \times 10^5$ cells per 35-mm well) were plated for 24 hours and then treated with various concentrations of different effectors. Cells were centrifuged and pellets were photographed and then solubilized in 100 µl of 1 M NaOH in 70 °C for over 2 hours to dissolve melanin, and the absorbance was measured spectrophotometrically at 405 nm by using a plate reader. Melanin production was calculated by normalizing the total melanin values with protein content. The results are expressed as fold of stimulation compared with control conditions.

Western blot assays

Cells were grown in six-well dishes with or without different effectors at the time indicated in the corresponding figure. Cells were then lysed and western blot analysis was performed as described (Tomic *et al.*, 2011).

The western blot assays were representative of at least three independent experiments.

cAMP immunoassay

The cAMP concentration was measured using a cAMP immunoassay kit (ArborAssays, Ann Arbor, MI). Briefly, B16 cells were lysed in 0.1 M HCl to inhibit the phosphodiesterase activity. The supernatants were collected, neutralized, and diluted, and then a fixed amount of cAMP conjugate was added to compete with the cAMP. Next, a substrate solution was added to the wells to determine the bound enzyme activity. The color development was stopped, and the absorbance at 420 nm was then read. The level of cAMP in the sample was determined based on a standard curve.

Transient transfection of siRNA

Briefly, a single pulse of 50 nmol l^{-1} of siRNA was administrated to the cells at 50% confluency by transfection with 5 µl of Lipofectamine RNAiMAX in Opti-MEM medium (Invitrogen, Washington, DC) for the time indicated in the figure legends. Control scrambled and AMPK-specific SiRNA were purchased from Invitrogen.

Luciferase reporter assays

B16-F10 cells were seeded in 24-well dishes, and transient transfections were performed the following day, as described previously (Bertolotto *et al.*, 1996). Briefly, cells were transiently transfected with 0.5 mg of MITF promoter (p-MITF) and 0.05 µg of pCMVβGal to control the variability in transfection efficiency. The transfection medium was changed 6 hours later with DMEM supplemented with 7% fetal bovine serum and cells were exposed to drugs. At 48 hours after transfection, soluble extracts were harvested in lysis buffer and assayed for luciferase and β-galactosidase activities. All transfections were repeated at least five times.

Infection of dominant-negative AMPK

Adenovirus encoding a dominant negative form of AMPK was a generous gift from Dr Foufelle (INSERM, UMR-S /872, Paris, France). An adenovirus whose expression cassette contains the major late promoter with no exogenous gene was used as the control (AdControl). NHM or Mel501 cells were seeded on 6-well plates to reach 90% confluence and then transfected either with a dominant negative form of AMPK or AdControl. After 24 hours of transfection, the medium was changed to DMEM with 7% fetal bovine serum, and cells were exposed to different drugs for another 24 hours.

In vivo experiments

Animal experiments were carried out in accordance with the Declaration of Helsinki Principles and were approved by a local ethics committee.

Drugs were prepared as a 30% weight:volume solution in a standard dermatologic vehicle of 70% ethanol and 30% propylene glycol (Sigma-Aldrich, St Louis, MO).

C57BL/6J mice of 8 weeks of age were used for this experiment. Preparations of agents were applied to the tail with a micropipette and then smoothened out over the tail with the side of a pipette tip so that a similar amount of solution would be applied to the entire tail. Animals were treated daily with 400 µl of different agents for 8 weeks. Animals were killed by CO_2 narcosis before tail skin sampling to evaluate melanin content and the melanogenic gene transcription.

Reconstructed human epidermis model

SkinEthic Reconstructed Human Epidermis (SkinEthic RHE) (SkinEthic, Lyon, France) consists of fully differentiated three-dimensional epidermal tissue grown from normal human keratinocytes for 17 days in a chemically defined medium at the air–liquid interface. The three-dimensional reconstructed epidermis was received on day 18, and preincubated for 24 hours in a growth culture medium (SkinEthic) at 37 °C, 5% CO2, according to SkinEthics procedures. SkinEthic RHE were treated with different drugs as indicated, and the medium was changed every 2 days. After 2 weeks of treatment, the RHE was separated from the polycarbonate support and stored at −80 °C before analysis.

Human skin punch biopsy

Normal human samples were obtained from an abdominoplasty surgery patient. The skin punch biopsy was obtained according to the standard procedure by using a 4-mm-round Visipunch instrument (Kai Medical, Tokyo, Japan) and then kept in complete DMEM 10% fetal bovine serum medium. Skin punches are typically between 2 and 5 mm in diameter and extend to a depth of ~4 mm. The skin cell

constructs were cultured for 15 days and the medium was changed every 2 days with the corresponding drugs. All human skin samples were donated with informed consent and with the approval of the CHU de Nice ethics committee following the Declaration of Helsinki protocols.

Fontana–Masson staining

A silver stain kit to stain human skin melanin was used according to the manufacturer's instructions (Interchim, Montluçon, France).

Statistical analysis

Data presented are mean ± SD of three independent experiments performed in triplicate. Statistical significance was assessed using the Student's *t*-test except for *in vivo* experiments, in which statistical significance was assessed using a two-tailed Wilcoxon rank sum test. A value of $P \leqslant 0.05$ was accepted as statistically significant when comparing experimental and control groups.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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