Uncoupling of ER/Mitochondrial Oxidative Stress in mTORC1 Hyperactivation-Associated Skin Hypopigmentation

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Accumulating evidence has described the involvement of mTORC1 signaling in pigmentation regulation; however, the precise mechanism is not fully understood. Here, we generated mice with conditional deletion of the mTORC1 suppressor *Tsc2* in melanocytes. It resulted in constitutive hyperactivation of mTORC1 and reduced skin pigmentation. Mechanistically, neither the number of melanocytes nor the expression of melanogenesis-related enzymes was decreased; however, endoplasmic reticulum and mitochondrial oxidative stress and lower melanization in melanosomes were observed. By contrast, abrogation of mTORC1 by rapamycin completely reversed the reduced pigmentation, and alleviation of endoplasmic reticulum stress by SMER28 or 4-phenylbutyrate (PBA) or alleviation of mitochondrial oxidative stress by administration of adenosine triphosphate partially reversed the reduced pigmentation in these mice. In addition, we showed that these mechanisms were involved in reduced pigmentation of *TSC2* small interfering RNA-transfected cultured human primary melanocytes and skin lesions of patients with the *TSC* gene mutation.

INTRODUCTION

The mammalian serine/threonine kinase mTOR was implicated in the regulation of pigmentation (Busca et al., 1996; Jeong et al., 2011; Ohguchi et al., 2005; Tsao et al., 2016). mTOR is found in two functionally and structurally distinct complexes named mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin, but mTORC2 is not inhibited by rapamycin. mTORC1 controls protein synthesis, ribosome biogenesis, cell growth, and autophagy. mTORC2 regulates cellular metabolism and the cytoskeleton (Saxton and Sabatini, 2017). mTORC1 activation, via *TSC* gene inactivation, disrupted pigmentation (Cao et al., 2017). Furthermore, rapamycin, an inhibitor of mTORC1, was reported to up-regulate melanogenesis in MNT-1 melanoma cells (Hah et al., 2012; Ho et al., 2011). We reported that rapamycin was effective for treatment of hypopigmented macules of patients with tuberous sclerosis complex (TSC) (Wataya-Kaneda et al., 2012, 2015), which supported a role for mTORC1 in pigmentation.

The mTORC1 pathway has been shown to control protein synthesis, cell growth, proliferation, cell metabolism, insulin resistance, autophagy, and stress responses (Laplante and Sabatini, 2009, 2012). Dysregulation of the mTOR pathway has been implicated in various diseases (Guertin and Sabatini, 2007; Saxton and Sabatini, 2017; Wullschleger et al., 2006). The mTOR pathway has attracted broad scientific and clinical interest. Besides, both the PI3K-mTOR and Ras-MAPK-ERK pathways are well known as the cell's chief mechanisms for controlling growth, survival, and metabolism in response to extracellular cues (Mendoza et al., 2011). The Ras-MAPK-ERK signaling pathway is known to inhibit melanogenesis by phosphorylating MITF and posttranscriptionally modulating the activity of MITF in melanocytes (Lin and Fisher, 2007). However, little is known about the mechanism of mTOR in the regulation of skin pigmentation.

In human cells, mTORC1 is activated by amino acids, insulin, growth factors, and stress mediators like starvation and hypoxia (Saxton and Sabatini, 2017); mTORC1 is negatively regulated by a heterodimeric complex consisting of two protein molecules: TSC1 (or hamartin) and TSC2 (or tuberin) (Kwiatkowski, 2003b). Inactivation of either *TSC1* or *TSC2* induces hyperactivation of mTOR. Furthermore, mutations in *TSC1* or *TSC2* cause the TSC disorder (Crino et al., 2006; van Slegtenhorst et al., 1997; Wienecke et al., 1995). TSC is a multisystemic disease characterized by formation of benign tumors in multiple organs and hypopigmented macules on the skin that occur along with seizures or autism (Ess, 2009). Hyperactivation of mTOR is thought to be the most likely trigger for lesion formation in TSC patients (Crino et al., 2006). In TSC, uncontrolled protein synthesis and cell growth from constitutive mTOR activation contribute to formation of benign tumors in multiple systems (Inoki et al., 2006; Kenerson et al., 2002). By contrast, in the melanocyte lineage in TSC, tumor formation has not been observed; however, hypopigmented macules arise at birth or in early

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infancy (Gold and Freeman, 1965; Jimbow, 1997). TSC was initially described approximately 150 years ago, and the molecular mechanisms underlying formation of these hypopigmented macules still are not fully understood. Based on our previous studies that showed topical treatment with rapamycin-reversed hypopigmentation in patients with TSC, we hypothesized that mutated *TSC1*- or *TSC2*-induced hyperactivation of mTORC1 leads to hypopigmented macules in TSC.

Studies of *Tsc*/mTORC1 regulation of pigmentation in vivo have been limited by the lack of an animal model, because homogeneous *Tsc* knockout is embryonically lethal and *Tsc* heterozygotes do not develop skin abnormalities (Kobayashi et al., 1999). To investigate whether *Tsc*/mTORC1 signaling in melanocytes plays a role in the regulation of pigmentation, we generated conditional *Tsc2*-knockout mice. As expected, deletion of *Tsc2* caused reduced skin pigmentation due to elevated mTORC1 activity in melanocytes. This mouse model provides a pathogenic mechanism for mTORC1 hyperactivation-mediated hypopigmentation.

RESULTS
Generation of mice deficient for Tsc2 in melanocytes
To activate mTORC1 signaling in melanocytes and address the potential role of mTORC1 signaling in the pigmentation of skin melanocytes, we generated mice with conditional deletion of *Tsc2* in melanocytes. As shown in Figure 1a and b, mice containing a floxed *Tsc2* allele were crossed with transgenic mice expressing Cre under the control of the melanocyte-specific *Mitf* promoter (i.e., MITF-M), which is abundant and specific in neural crest-derived melanocytes (Tachibana, 2000). The resultant Cre-mediated deletion of *Tsc2* exons 3, 4, and 5 inactivated expression of Tsc2. $Tsc2^{flox/flox}Mitf$::Cre mice, referred to as mcTsc2$^{ko/ko}$, were used for experiments, and $Tsc2^{+/+}Mitf$::Cre littermates, referred to as mcTsc2$^{+/+}$, were used as controls. In C57BL6 mice, epidermal melanocytes are mainly distributed in the tail, ear, and sole skin (Reynolds, 1954); therefore, mouse tail skin was analyzed in this study.

Epidermal melanocytes were isolated from mouse tail skin and cultured. In Western blot analyses of lysates from these cells, the product of the *Tsc2* gene, tuberin, was readily detected in control wild-type melanocytes but was absent in melanocytes from mcTsc2$^{ko/ko}$, which indicated successful [Q9] deletion of the *Tsc2* gene from melanocytes (Figure 1c). We further investigated the expression of downstream targets of [Q10] mTORC1 (phosphorylated [p-]S6 and p-4EBP1) for whether such phosphorylation suggests mTORC1 activation (Kwiatkowski et al., 2002; Onda et al., 2002). Consistent with loss of Tsc2, the expression of p-S6 and p-4EBP1 were markedly increased in mcTsc2$^{ko/ko}$ melanocyte lysates (Figure 1c), which suggested that mTORC1 was hyperactivated. Furthermore, mouse tail skin samples were examined via immunohistochemistry staining with anti-Pmel17 (melanocyte marker) and anti-p-S6 antibodies. Increased expression of p-S6 was observed in melanocytes from mcTsc2$^{ko/ko}$ tail skin compared with littermate controls (Figure 1e). These results showed that the deletion of *Tsc2* in melanocytes resulted in activation of mTORC1 signaling.

mcTsc2$^{ko/ko}$ mice exhibited reduced skin pigmentation
Four weeks after birth, mcTsc2$^{ko/ko}$ mice exhibited profoundly lighter tail skin pigmentation than their mcTsc2$^{+/+}$ littermates (Figure 1d). Skin sections from mcTsc2$^{ko/ko}$ mice had a markedly reduced number of scattered pigment granules in the epidermis compared with control mice (Figure 1e, left panel). Double staining for p-S6 and Pmel17 showed that epidermal melanocytes in tail skin were present but that mTORC1/S6 signaling was constitutively activated in mcTsc2$^{ko/ko}$ mice (Figure 1e, right panels). These observations indicated that melanocyte-specific deletion of *Tsc2* induced mTORC1 hyperactivation and resulted in reduced skin pigmentation.

Deletion of Tsc2 disrupted melanosome maturation in melanocytes
There was less pigmentation in tail skin from mcTsc2$^{ko/ko}$ mice than that from littermate controls, which was confirmed again via hematoxylin staining (Figure 2a) and bright field microscopy (Figure 2b, left panel). Decreased melanin is known to result from decreased numbers or absence of melanocytes in the epidermis or normal melanocyte numbers with little or no melanin production. Therefore, to determine whether less pigmentation resulted from reduced numbers of melanocytes, immunofluorescence staining was used to label skin sections using an antibody against the melanocyte-specific marker Pmel17. The numbers of epidermal melanocytes were similar between the mcTsc2$^{ko/ko}$ and control mice (Figure 2b). This result suggested that melanocyte dysfunction was the major reason for the observed tail skin phenotype in mcTsc2$^{ko/ko}$ mice.

To clarify the mechanisms involved in regulation of melanocyte function by Tsc2/mTORC1, transmission electron microscopy using ultrathin skin sections was performed. Marked reduction of mature melanosomes in melanocytes was detected in Tsc2$^{ko/ko}$ mice (Figure 2c) compared with samples from littermate controls. Moreover, the number of mature melanosomes was decreased; however, the number of immature melanosomes (indicated by the red arrows in Figure 2d) was greatly increased in melanocytes from mcTsc2$^{ko/ko}$ mice.

Next, to confirm whether the enzyme for melanin synthesis was correctly transported into melanosomes, immunoelectron microscopy was performed (Figure 2e). Spherical organelles without melanin deposition and a fibrillar internal matrix but with strong expression of Hmb45 indicate premelanosomes; organelles with melanin deposition are mature melanosomes (Raposo et al., 2001). However, we have also found many organelles without melanin deposition but with a fibrillar internal matrix and strong expression of Hmb45 and Tyrp1 (Figure 2e) or Hmb45 and tyrosinase (data not shown); we named this *premature melanosomes*. We found that premature melanosomes were increased but that mature melanosomes were decreased significantly in melanocytes from mcTsc2$^{ko/ko}$ mouse tail skin (Figure 2e, right panel; *P* < 0.01) compared with melanocytes from littermate controls. This suggests that the melanogenesis-related enzyme was successfully transported into melanosomes but that melanin synthesis in these melanosomes was disrupted in melanocytes of mcTsc2$^{ko/ko}$ mice. These results show that