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Dysregulation of autophagy in melanocytes contributes to hypopigmented macules in tuberous sclerosis complex

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ABSTRACT

Background: Tuberous sclerosis complex (*TSC*) gene mutations lead to constitutive activation of the mammalian target of rapamycin (mTOR) pathway, resulting in a broad range of symptoms. Hypopigmented macules are the earliest sign. Although we have already confirmed that topical rapamycin treatment (an mTOR inhibitor) protects patients with TSC against macular hypopigmentation, the pathogenesis of such lesions remains poorly understood.
Objective: Recently emerging evidence supports a role for autophagy in skin pigmentation. Herein, we investigated the impact of autophagic dysregulation on TSC-associated hypopigmentation.
Methods: Skin samples from 10 patients with TSC, each bearing characteristic hypopigmented macules, and 6 healthy donors were subjected to immunohistochemical and electron microscopic analyses. In addition, *TSC2*-knockdown (KD) was investigated in human epidermal melanocytes by melanin content examination, real-time PCR, western blotting analyses, and intracellular immunofluorescence staining.
Results: Activation of the mTOR signaling pathway decreased melanocytic pigmentation in hypopigmented macules of patients with TSC and in *TSC2*-KD melanocytes. In addition, LC3 expression (a marker of autophagy) and autophagosome counts increased, whereas, intracellular accumulation of autophagic degradative substrates (p62 and ubiquitinated proteins) was evident in *TSC2*-KD melanocytes. Furthermore, depigmentation in *TSC2*-KD melanocytes was accelerated by inhibiting autophagy (*ATG7*-KD or bafilomycin A1-pretreatment) and was completely reversed by induction of autophagy via mTOR-dependent (rapamycin) or mTOR-independent (SMER28) exposure. Finally, dysregulation of autophagy, marked by increased LC3 expression and accumulation of ubiquitinated proteins, was also observed in melanocytes of TSC-related hypopigmented macules.
Conclusion: Our data demonstrate that melanocytes of patients with TSC display autophagic dysregulation, which thereby reduced pigmentation, serving as the basis for the hypomelanotic macules characteristic of TSC.

1. Introduction

The tuberous sclerosis complex (TSC) was initially described about 150 years ago as an autosomal dominant genetic disorder marked by progressive involvement of multiple systems. TSC results from mutations of either the *TSC1* or *TSC2* gene [1,2]. In patients afflicted, uncontrolled protein synthesis and cell growth due to constitutive mammalian target of rapamycin (mTOR) activation promote benign tumor proliferation at various sites [3,4]; and some non-tumorous symptoms, such as epilepsy and hypopigmented macules, are also observed [5,6]. The development of hypopigmented macules at birth or in early infancy is one of the hallmarks of TSC, upon which early diagnosis hinges [5,6].

Hyperactivation of mTOR is generally recognized as the mechanism for symptoms of TSC [7,8], and mTOR activation brought on by *TSC* gene inactivation is known to disrupt melanogenesis [9]. Other reports have also indicated that mTOR activation plays a negative role in regulating melanogenesis of mouse B16 melanoma cells [10–13]. Furthermore, Ho et al. and Hah et al. have documented that exposure of MNT-1 melanoma cells to

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rapamycin upregulates microphthalmia-associated transcription factor (MITF), a master regulator of melanogenesis [14,15]. Published studies from our group likewise have shown that use of topical rapamycin (an mTOR inhibitor) yields substantial improvement in the hypopigmented macules of patients with TSC [16,17], thus, implicating mTOR hyperactivation in the tell-tale hypopigmentation of TSC. Nevertheless, the pathogenesis of hypopigmented macules in this setting has yet to be fully elucidated.

Recent genetic studies revealed that an array of autophagy-related genes, including WD repeat domain, phosphoinositide-interacting protein 1 (WIPI1), and ATG6, modulates melanogenesis [18,19]. Moreover, autophagy is thought to affect skin color via regulation of melanin degradation in keratinocytes [20], and there is increasing evidence that autophagy is indeed involved in the regulation of pigmentation [21–23].

Macroautophagy (herein referred to as autophagy) is a highly regulated process that involves the formation of autophagosomes to capture and transport cytoplasmic components to lysosomes, for removing damaged organelles and degrading long-lived or aggregate-prone proteins. Furthermore, the activation of mTOR inhibits autophagy at an early step in autophagosome formation [24,25]. Neuronal autophagic dysfunction due to aberrant mTOR signaling also contributes to deficits in pruning of synaptic connections [26] and epileptogenesis [27] characteristic of TSC and other neurologic disorders [28]. Such findings are not surprising, given that both melanocytes and neural cells share a neural crest origin [29].

On the basis, we were compelled to examine whether autophagy, which is not only a downstream component of the mTOR pathway but is also critical in intracellular protein quality control [30], is altered in the hypopigmented macules of patients with TSC. In particular, we explored whether dysregulation of autophagy is sufficient to cause hypopigmentation and whether autophagic activation serves to reverse the pigmentatary void in TSC-model melanocytes.

2. Results

2.1. Increased mTOR activity and reduced melanocytic pigment content in hypopigmented cutaneous macules of patients with TSC

In a previous study, we confirmed the presence of melanocytes within hypomelanotic macules of patients with TSC; and although melanin granule levels were decreased, they increased after topical sirolimus (rapamycin) application [16,17]. For this study, we sampled the skin of healthy donors (n = 6) and hypomelanotic macules of patients with TSC (n = 10), using immunofluorescent staining to detect the melanocyte-specific protein, Pmel17. As with our previous report, no between-group differences in melanocytic position or number of basilar melanocytes were evident (Fig. 1A). In addition, the basilar melanocyte distribution and the flanking of epidermal keratinocytes in each group were similar. Ultrastructural details of these skin specimens were also examined under an electron microscope, mirroring the results of our previous report [17]. Numerous stage-IV mature melanosomes were present in both melanocytes and adjacent keratinocytes of healthy donor skin, whereas, those lesions related to TSC were grossly non-pigmented and virtually devoid of visible melanosomes, whether in melanocytes or adjacent keratinocytes (Fig. 1B). These results suggest that in patients with TSC, the number of melanocytes compared favorably with those of healthy skin, but mature melanosomes in melanocytes and keratinocytes were clearly diminished, thus corroborating an observation made by Jimbow et al. in the 1970s and data was reported in 2015 [17,31]. To check for mTOR hyperactivation (induced by *TSC* gene mutation) in melanocytes of TSC-related hypopigmented macules, we measured the expression level of phosphorylated S6 ribosomal protein (p-S6), a commonly used determinant of mTOR activity, obtaining healthy donor skin for comparison. Melanocytes in macular lesions of patients with TSC showed high expression levels of p-S6 (Fig. 1C), implicating mTOR-hyperactivation in the dearth of pigmentation found.

2.2. Reduced pigmentation in siRNA-mediated knockdown of TSC2 in cultured human epidermal melanocytes

To further investigate the mechanism involved in hypomelanotic macules of patients with TSC, *in vitro* experiments were performed, engaging specific siRNAs to knock down *TSC2* in cultured human epidermal melanocytes. This is an established method for generating TSC model cells [17]. Optimal transfection efficiency was then gauged, using a siRNA specific for *TSC2* to transfect primary melanocytes at various concentrations (range, 20–100 nM) and evaluating *TSC2* mRNA expression levels 24 h thereafter (Fig. 2A). Significant inhibition of *TSC2* gene expression was achieved across the entire concentration gradient (Fig. 2A), with no detectable effects on cell viability were detected at concentrations from 20 to 40 nM, as tested by MTT assay 96 h after transfection (Fig. 2B). However, to balance transfection efficiency and cell viability, 30 nM siRNA was deemed most suitable, silencing the target gene to 30% residual expression with no apparent viability loss. Furthermore, *TSC2* mRNA expression levels (Fig. 2C) and expression levels of tuberin protein (the product of *TSC2*) (Fig. 2D) were measured in melanocytes 12–96 h after transfection by real-time RT-PCR and western blot analysis, respectively, registering a sustainable *TSC2* knockdown effect (Fig. 2C, D).

Next, the impact of *TSC2* knockdown (KD) on pigmentation was confirmed, again using cultured human epidermal melanocytes. Similar to our previous study results [17], cells harboring *TSC2* siRNA were markedly less pigmented at 7 d after transfection than cells exposed to control siRNA (Fig. 2E). The melanin content of culture medium and cell lysates was also significantly suppressed by *TSC2* knockdown (Fig. 2F). Altogether, these findings imply that *TSC2* inactivation inhibits pigmentation in melanocytes, reaffirming observations made on hypopigmented macules in patients with TSC.

2.3. Dysregulation of autophagy in siRNA-mediated knockdown of TSC2 in cultured human epidermal melanocytes

To explore the role of autophagy in hypopigmented *TSC2*-KD melanocytes, we monitored levels of the autophagic marker, LC3B [32], using both intracellular immunofluorescent staining (Fig. 3A) and western blot analysis (Fig. 3C). Following efficient *TSC2* depletion through specific siRNA treatment, mTOR signaling was activated, as indicated by increased phosphorylation of the Ser-235/256 site on S6 (p-S6) in western blot analysis (Fig. 3C) and by intracellular immunofluorescent staining (Fig. 3A, red arrows). *TSC2*-KD Cells, presenting with higher levels of p-S6, also showed decreased cellular pigmentation (Fig. 3A, red arrows). Expression of LC3B was markedly increased in *TSC2*-KD melanocytes, confirmed by intracellular immunofluorescent staining (Fig. 3A, red arrows) and western blot analysis (Fig. 3C). Interestingly, p62 expression and ubiquitinated proteins levels, both substrates for autophagic degradation [33,34], were also increased (Fig. 3C). To confirm whether the impaired autophagic influx was involved, autophagosomes were directly assessed in anti-p62 immunostained electron microscopy (immuno-EM) preparations (Fig. 3D), appearing as double swelled membranes and p62-positive dots

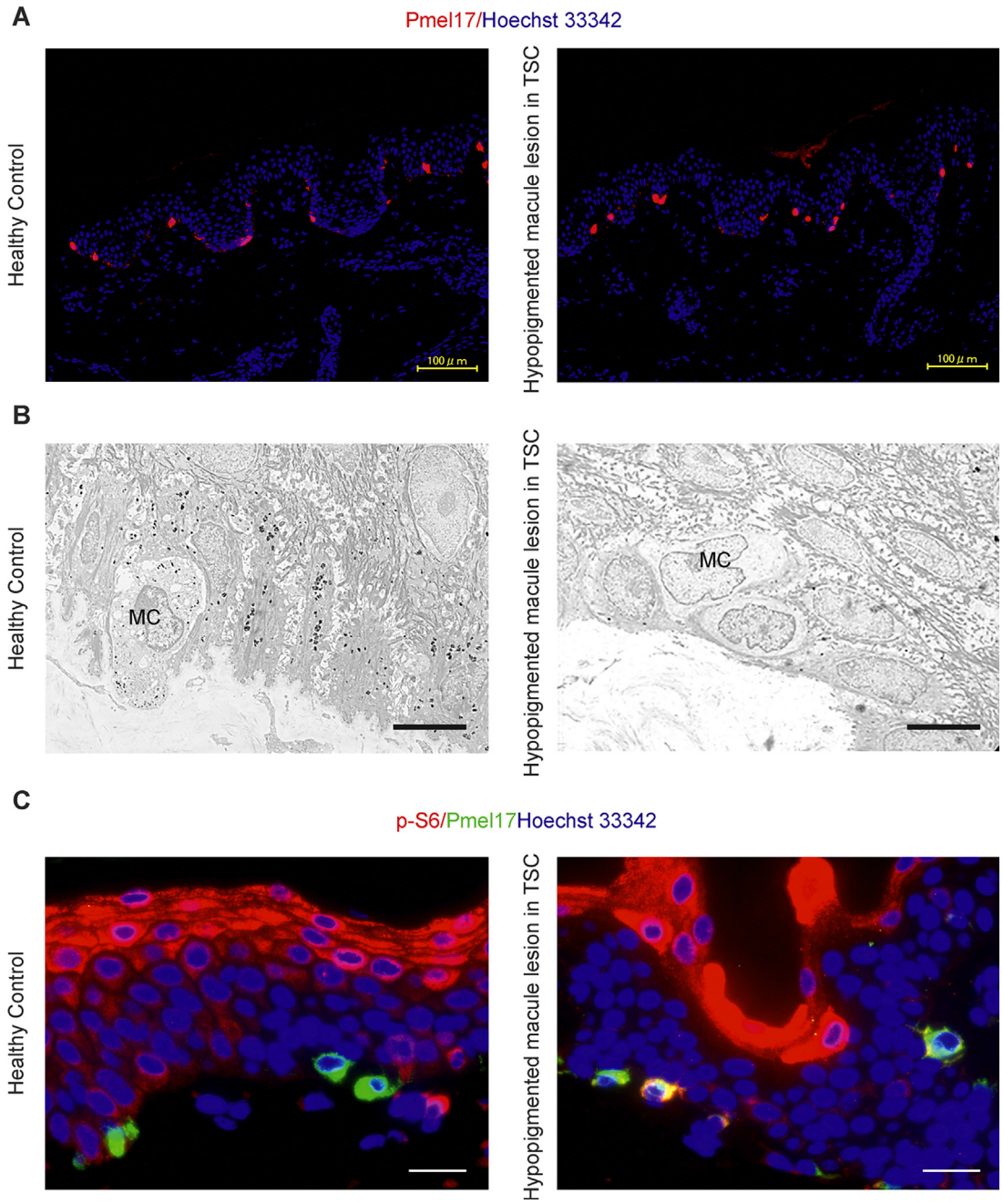

Fig. 1. Increased mTOR activity and reduced melanocytic pigmentation in hypopigmented macules of patients with TSC. Skin biopsy specimens from healthy donor (left panels) and hypopigmented macular lesion of patient with TSC (right panels) in representative sections: (A) Anti-Pmel17 immunofluorescenct stain (red) and Hoechst 33342 nuclear counterstain (blue) (yellow bar = 100 μm); (B) Ultra-thin electron microscopic sections (melanocytes = MC; black bar = 200 nm); and (C) Immunohistochemical stain (anti-p-S6, red; anti-Pmel17, green), with Hoechst 33342 nuclear counterstain (blue) (white bar = 5 μm).

[35]. Compared with control siRNA-transfected melanocytes, fewer melanosomes and more autophagosomes were observed (Fig. 3D, F). By further examination, both the early/initial autophagic vacuole (AVi) and late/degradative autophagic vacuole (AVd) (defined by morphological criteria in the previous report [36]) were increased significantly in *TSC2*-KD melanocytes (Fig. 3E, G), indicating autophagic impairment. It suggested that the basal level of autophagy in *TSC2*-KD melanocytes was still inadequate. Thus, autophagic dysregulation is implicit in *TSC2*-KD melanocytic dysfunction.

2.4. Autophagic inhibition accelerates reduced pigmentation of cultured human epidermal melanocytes

To confirm that dysregulated autophagy affects pigmentation in melanocytes, pigmentation was directly evaluated in autophagy-deficient melanocytes. Cultured human epidermal melanocytes were transfected with *ATG7* siRNA to knock down a gene essential for autophagosome formation and thus, inhibit autophagy. Western blot analysis confirmed that *ATG7* KD was efficient, successfully inhibiting autophagy and leading to decreased LC3 expression (Fig. 4C). This corroborated previously reported results

of *ATG5* KD in Melan-a cells [23] and *ATG18/WIPI1* KD in MNT cells [15]. After 7 days, cells transfected with *ATG7* siRNA were markedly less pigmented than control siRNA-transfected cells (Fig. 4A). The melanin content of culture medium and cell lysates was significantly suppressed by *ATG7* KD (Fig. 4B). Consistent with other studies underscoring the role of autophagy in pigmentation [19,23], evidence here indicates that loss of basal autophagy in melanocytes by knockout of a key autophagic gene (*ATG7*) results in a depigmentation phenotype, thereby supporting the contributory nature of insufficient autophagy in depigmentation of melanocytes.

To further confirm whether inhibition of autophagy accelerates depigmentation in *TSC2*-KD melanocytes, these cells were co-transfected with *TSC2* and *ATG7* siRNA, pretreating some prior to *TSC2* siRNA transfection with bafilomycin A1 (an inhibitor of vacuolar H^+-ATPase) to exaggerate autophagic inhibition. Compared with *TSC2*-KD melanocytes, *ATG7* co-transfected and bafilomycin A1-pretreated *TSC2*-KD melanocytes were far less pigmented (Fig. 4A), and then melanin content of culture medium and cell lysates was suppressed even more (Fig. 4B). Similarly, the increased expression of p62 and ubiquitinated proteins in *TSC2*-KD melanocytes was exacerbated by such ancillary autophagic inhibition (Fig. 4C). These results suggest that autophagy function is critical in maintaining pigmentation in *TSC2*-KD melanocytes.

2.5. Both mTOR-dependent and mTOR-independent autophagic enhancers abrogate pigmentation decline in TSC2-KD melanocytes

Autophagy was subsequently induced in *TSC2*-KD melanocytes to confirm attenuation of otherwise downregulated pigmentation in these cells, using either rapamycin (an mTOR-dependent inducer known to inhibit mTOR activation and activate autophagy in mammalian cells) [37] or SMER28 (an mTOR-independent inducer of autophagy) [38]. Cells harboring *TSC2* siRNA were markedly less pigmented than control siRNA-treated cells. However, both rapamycin and SMER28 brought great improvement, bolstering melanocytic pigmentation (Fig. 5A). The quantifiable melanin content of *TSC2*-KD melanocytes in both culture

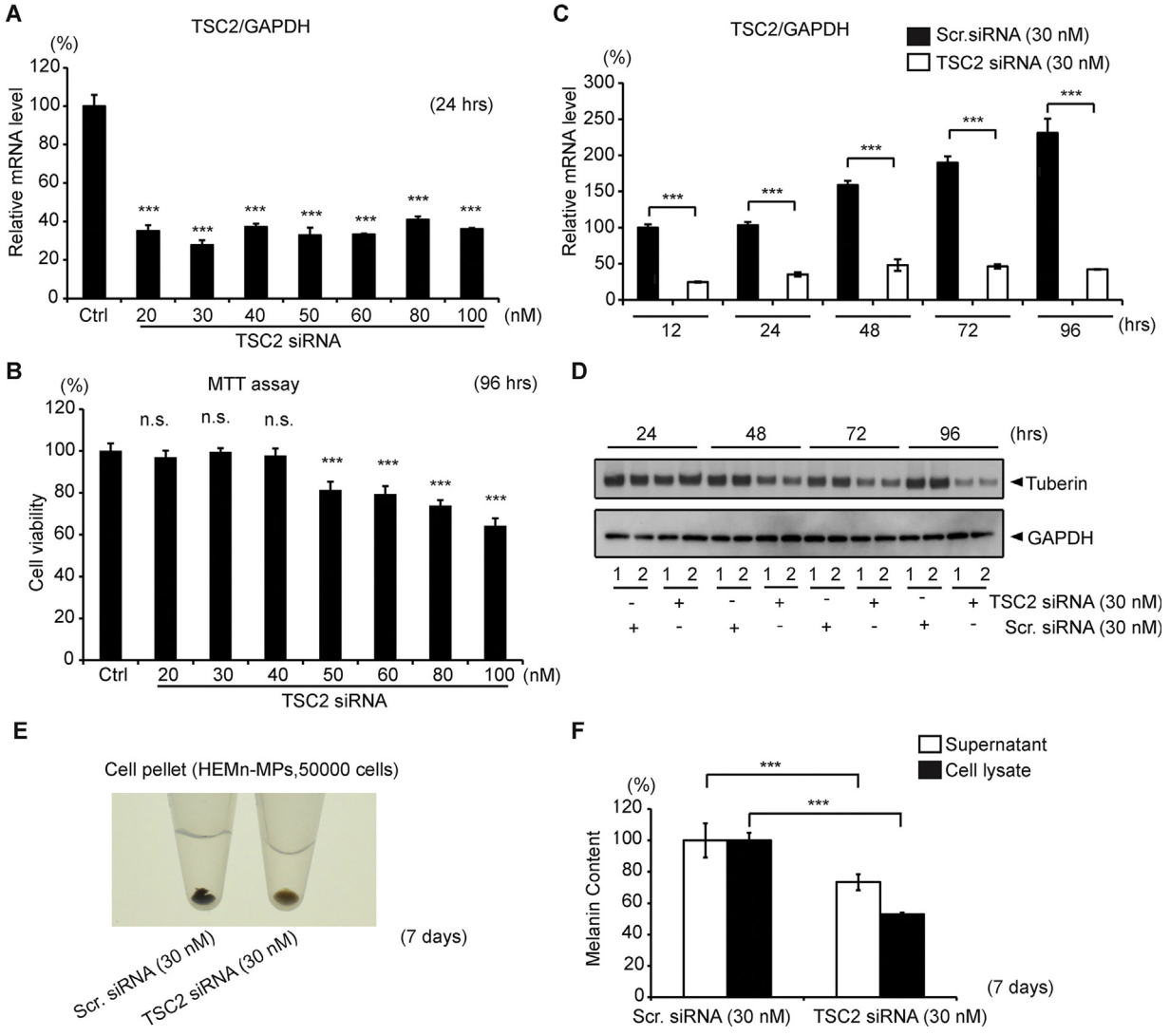

Fig. 2. Reduced pigmentation in siRNA-mediated knockdown of *TSC2* in cultured human epidermal melanocytes: (A) *TSC2* mRNA expression after 24 h of *TSC2*-specific or control (Scr) siRNA treatment as indicated; (B) Viability of cultured human epidermal melanocytes assessed by MTT colorimetric assay 96 h after specific or control siRNA at treatment as indicated; (C) *TSC2* mRNA expression after specified treatment (30-nM specific or control siRNA); (D) TSC2 protein levels after specified treatment (30-nM specific or control siRNA; western blot), GAPDH used as loading control, harvesting cells 7 days after transfection, and photographing cell pellets; (E) melanin content of culture medium and cell pellets assayed; and (F) Data shown as mean ± SD. n.s., no significance; *** $p < 0.01$.

medium and cell lysates was also significantly increased by these agents (Fig. 5B), as was LC3II expression (Fig. 5C). Increased expression levels of p62 and ubiquitinated proteins in *TSC2*-KD melanocytes were reversed as well through rapamycin and SMER28 autophagic induction (Fig. 5C). Hence, just as constitutive mTOR activation induces autophagic dysfunction and in turn reduces pigmentation in *TSC2*-KD melanocytes, both mTOR-dependent and mTOR-independent autophagic enhancers serve to mitigate such depigmentation.

2.6. Dysregulated autophagy also a melanocytic feature in hypopigmented macules of patients with TSC

Finally, skin samples from healthy control subjects and from hypopigmented macular lesions of patients with TSC were stained for the melanocyte-specific protein, Pmel17 (green), and LC3B or ubiquitin (red) to confirm autophagic dysregulation in hypomelanotic macules of patients with TSC. In Pmel17-positive cells, not only expression of LC3B, but also ubiquitin expression was markedly increased in TSC-related hypopigmented macules, relative to healthy control references (Fig. 6A, B). In addition, fewer melanin granules appeared in the epidermis of lesions from patients with TSC, compared with healthy controls (Fig. 6A, B, bright field). These results suggest dysregulation of autophagy reduces melanocytic pigmentation in the context of TSC.

3. Discussion

In our previous clinical study, rapamycin treatment improved hypopigmented macules in TSC [16,17]. Recent data also indicates

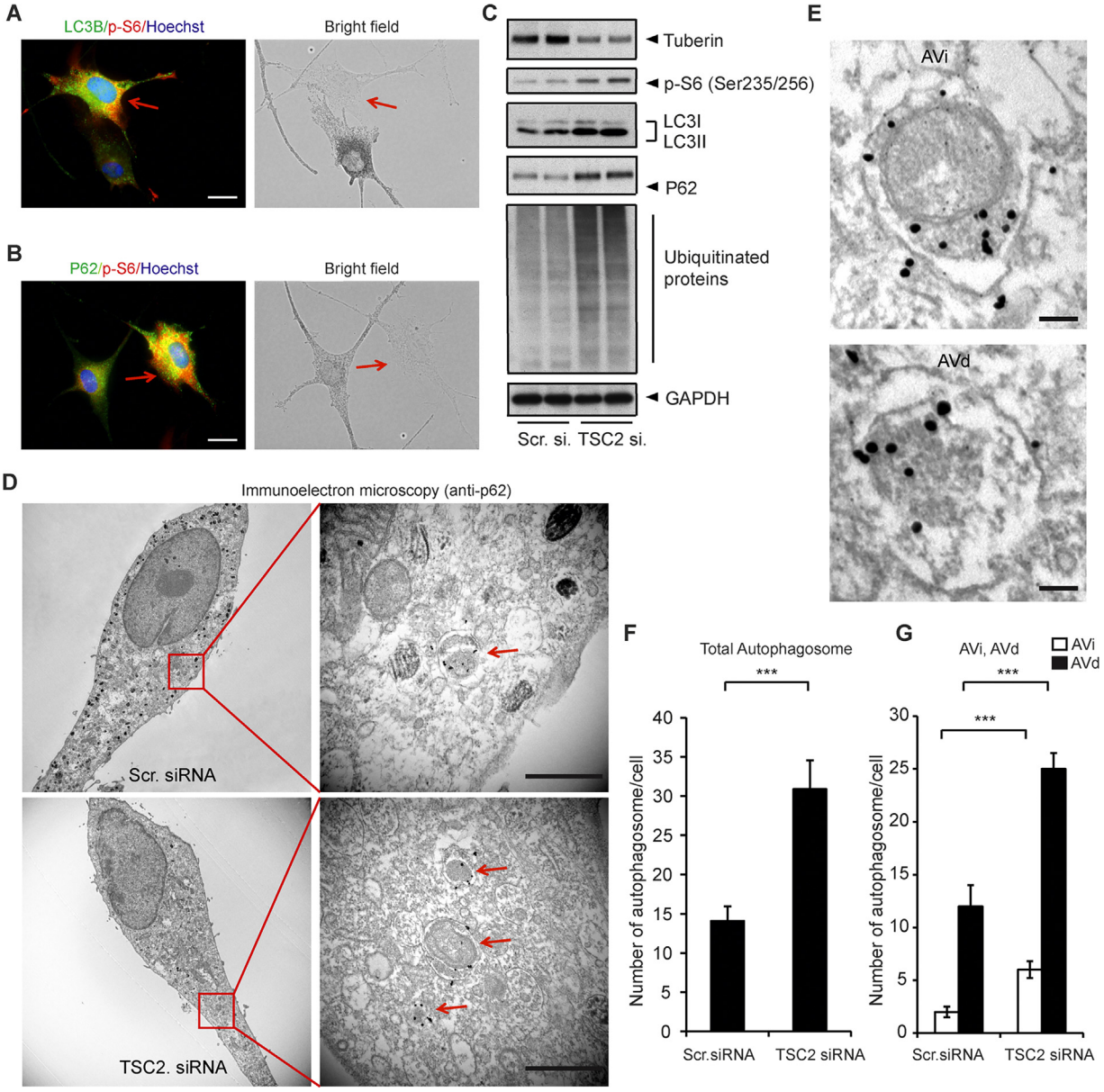

Fig. 3. Dysregulation of autophagy in siRNA-mediated knockdown of *TSC2* in cultured human epidermal melanocytes: Cells transfected (5-day control siRNA or *TSC2* siRNA treatment), then anti-LC3 B (green) (A) or anti-P62 (green) (B) antibody applied; anti-p-S6 staining (red) and Hoechst 3342 nuclear counterstain (blue) by confocal fluorescence and bright-field microscopy; (C) After 5-day transfection, western blot analysis of collected cells (antibodies indicated); (D) Transfected cells (7-day *TSC2* siRNA treatment) fixed and examined by immuno-EM (high-power ultrastructural detail in boxed area; black bar = 500 nM); and (E) Typical autophagosome, AVi, early/initial autophagic vacuole; AVd, late/degradative autophagic vacuole; black bar = 50 nM; Counts for control siRNA-transfected and *TSC2* siRNA-transfected melanocytes (n = 10) and autophagosomal totals for each melanocyte presented (F, G). All data expressed as mean ± SD. *** $p < 0.01$.

that activation of mTOR through *TSC* gene inactivation will disrupt melanogenesis [9]. Still, the precise mechanism by which mTOR hyperactivation results in hypopigmentation is not fully understood.

Findings of the present investigation indicate that both autophagic deficiency and dysregulation of autophagy significantly reduce melanocytic pigmentation, corroborating past reports of links between autophagy and melanogenesis. In an RNAi-based screening assay conducted elsewhere, downregulation of *WIPI1* (a human homologue of *ATG18*) limited the accumulation of MITF in MNT-1 cells, suppressing its target genes and interrupting melanosome maturation [15,18]. Furthermore, mice deficient in genes regulating the formation of autophagosomes (*BECN1*) [39] or genes that putatively control autophagosomal turnover (*VAC14*) [40] have borne an association with decreased pigmentation; and other reagents known to suppress autophagosomal turnover equally inclined to inhibit pigment production in skin equivalents [41].

The present study likewise revealed a distinct reduction in the pigmentation of cultured *TSC2*-silenced human primary melanocytes upon dysregulation of autophagy. Insufficient autophagy was evident as well in melanocytes found in hypopigmented macules of patients with TSC, showing increased intracellular accumulation of autophagic degradation substrates (p62 and ubiquitinated proteins). Moreover, induction of autophagy by rapamycin or SMER28 attenuated such accumulation, reversing the depigmentation of *TSC2*-KD melanocytes.

Autophagy is considered an evolutionary mechanism, serving to degrade and recycle damaged cellular proteins and subcellular organelles. It is a dynamic process under the control of various regulators, including both mTOR and alternative mTOR-independent pathways [42]. Given that mTOR is known to inhibit autophagy [24], our focus was on the impact of autophagy in melanocytes of hypopigmented macular skin lesions seen in patients with TSC. Unexpectedly, we found that the basal level of melanocytic autophagy in this disorder exceeded that of healthy donor melanocytes, conceivably reflecting a substantial induction of mTOR-independent autophagy. However, such regulation still appeared insufficient, considering the abundance of autophagic degradation substrates that lingered in these cells.

Functionally, mTOR serves to promote protein translation [43] while suppressing autophagy [24,25]. Therefore, a surplus of protein and autophagic deficiency are the consequences of melanocytic mTOR hyperactivation in the context of TSC. Because melanocytes typically display low-level proliferative activity, a protein overload of this kind is not compensated through cell division. The role of autophagy in balancing melanocytic protein synthesis and protein degradation is thus both critical [30,44]. Thus, in mTOR-hyperactivated melanocytes, there may be an imbalance between protein synthesis and protein degradation, and this imbalance can trigger huge intracellular stress, and eventually ER and mitochondrial stress might be induced in melanocytes. Besides, energy is critical in regulating melanosomal transporters, such as tyrosine and cysteine transporters, as well as proton pumps

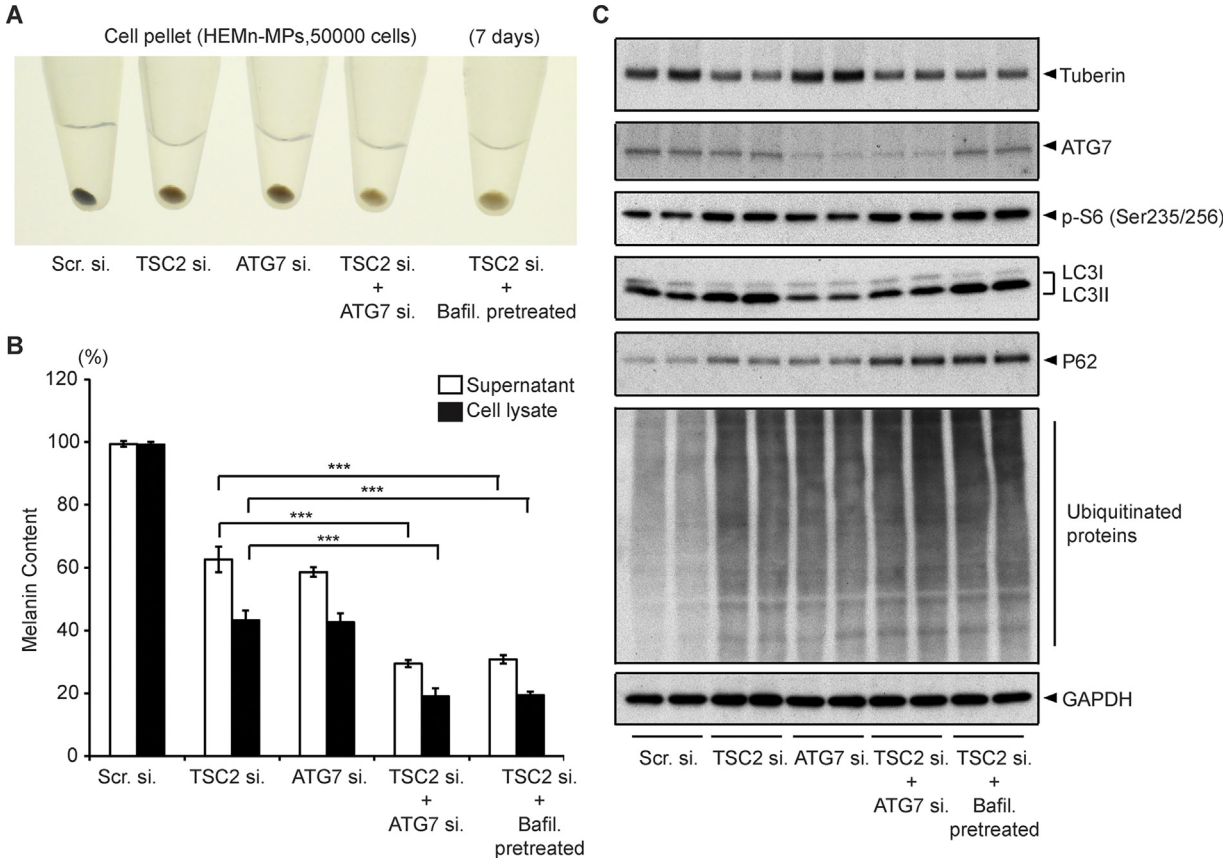

Fig. 4. Autophagic inhibition accelerates pigmentation reduction in cultured human epidermal melanocytes: (A) Cultured cells transfected with control (Scr), *ATG7*, or *TSC2* siRNA to inhibit autophagy and co-transfected (*TSC2* siRNA + *ATG7* siRNA) or bafilomycin A1 (100 nM, 3 h, 7 days) pretreated cells, with photograph of cell pellets; (B) Melanin content of culture medium and cell lysates assayed, expressing data as mean ± SD (*** $p < 0.01$); (C), 5 days after specified treatments, collected cells analyzed by western blot (antibodies as indicated).

that regulate the pH within melanosomes [45], which affects melanocyte function.

Nevertheless, the precise mechanism by which autophagic insufficiency leads to hypopigmentation is not entirely clear. Previous research has revealed that autophagy contributes to formation and maturation of melanosomes in cell cultures [19]. A very recent study based on melanocyte-specific *ATG7* inactivation in mice disclosed premature senescence and aberrant Nrf2 signaling in melanocytes, with cumulative oxidative stress ostensibly resulting in an autophagy-deficient melanocytic phenotype [46]. Despite these advances, the mechanism of autophagy-regulated pigmentation is still controversial. Dysregulated autophagy has been reported in neurons as well, mTOR inhibition by rapamycin accounting for improved neuronal symptoms in patients and in mouse model alike [47]. Because melanocytes and neurons share embryonic origin [48] and both cell types are affected in TSC [27], no doubt a similar mechanism is operant.

To date, we have demonstrated for the first time that insufficient autophagy leads to reduced pigmentation in *TSC2*-silenced melanocytes. Moreover, our efforts have revealed that dysregulated autophagy contributes to hypopigmentation in patients with TSC, in response to mTOR hyperactivation, and that enhancing both mTOR-dependent and -independent autophagy stands to improve depigmentation in TSC-model melanocytes.

In summary, our data suggest that insufficient autophagy is a likely contributor to epidermal pigmentation abnormalities, resulting in the hypomelanotic macules that are hallmarks of TSC. These findings provide new insight into the pathogenetic mechanisms of TSC and may have important therapeutic or preventive implications in the realm of hypopigmentation disorders.

4. Materials & methods

4.1. Human skin specimens

Paraffin-embedded tissue sections obtained from skin lesions of well-defined TSC patients with hypopigmented macules (n = 10), and samples from corresponding sites of healthy donors (n = 6) were examined. Written informed consent was obtained from all study participants prior to study inclusion. The protocol was approved by the ethics committee of Osaka University Faculty of Medicine (No. 10339).

4.2. Cell cultures

Normal human neonatal epidermal melanocytes from moderately pigmented donors (HEMn-MP) were purchased (Invitrogen [Thermo Fisher Scientific], Waltham, MA, USA) and cultured (37 °C, 5% CO2) in Medium 254 (M-254-500; Thermo Fisher) with 1% (v/v) Human Melanocyte Growth Supplement (Thermo Fisher). The cells were used at passage 6–8, seeding 6-well plates (5×10^5 cells/well)

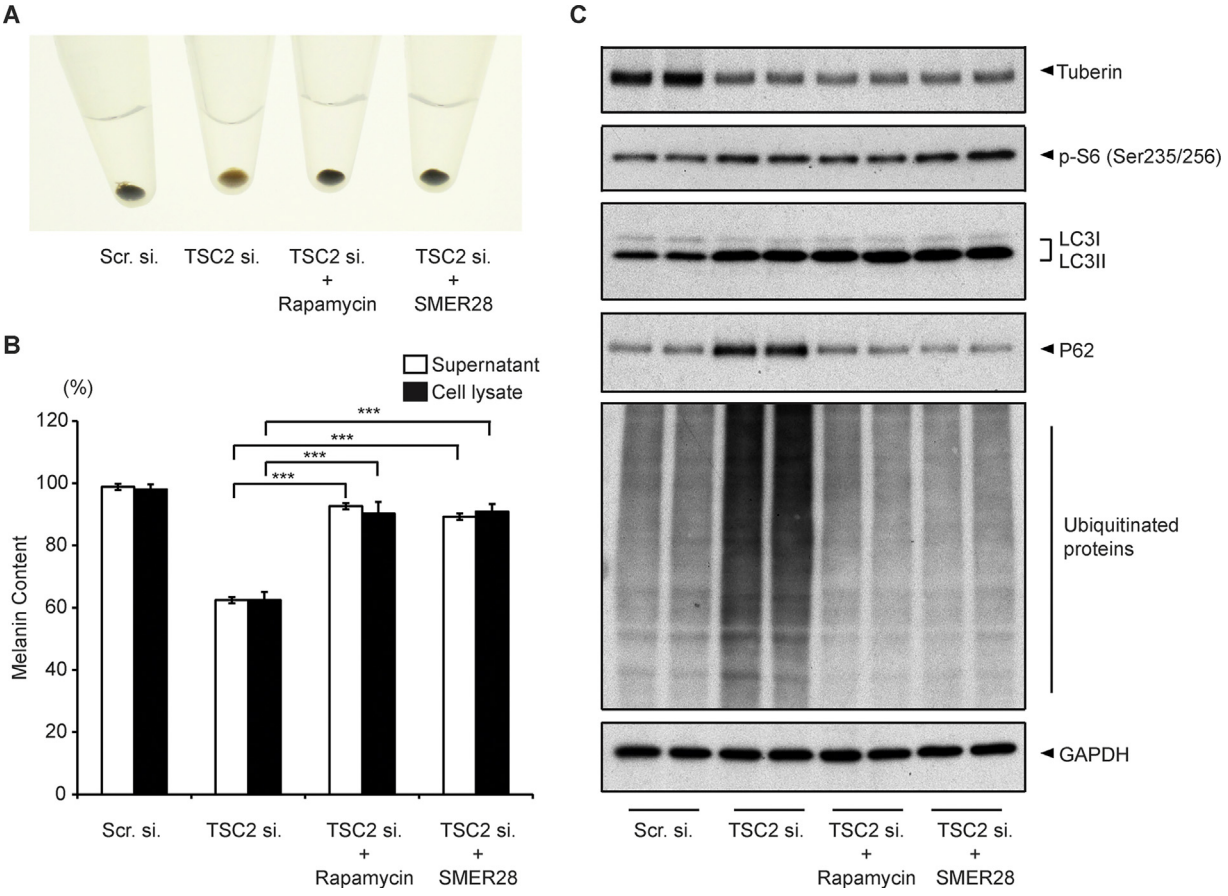

Fig. 5. mTOR-dependent and mTOR-independent autophagic enhancers abrogate reduced pigmentation in *TSC2*-KD melanocytes: (A) Cultured human epidermal melanocytes transfected (control [Scr] or *TSC2* siRNA) and treated (10 nM rapamycin or 50 μM SMER28) to induce autophagy, with photograph of cell pellets after 7 days; (B) Melanin content of culture medium and cell lysates assayed, expressing data as mean ± SD (*** $p < 0.01$); and (C) 5 days after specified treatments, collected cells analyzed by western blot (antibodies as indicated).

12 h before transfection. Treatment with 10 nM rapamycin (Calbiochem, San Diego, CA, USA), 50 μM SMER28 (Sigma, St Louis, MO, USA), or 100 nM bafilomycin A1 (Sigma) proceeded for periods indicated prior to extraction of RNA and protein.

4.3. RNA interference

For siRNA-mediated KD of *TSC2* or *ATG7*, HEMn-MPs were transfected with either target or control siRNA (Cosmo Bio Ltd, Tokyo, Japan), using a transfection reagent (LipofectamineRNAi MAX; Thermo Fisher) according to the manufacturer's instructions. For optimization, siRNA was diluted to final concentrations of 20, 30, 40, 50, 60, 80, and 100 nM. The functional assays were subsequently performed after validation of *TSC2* or *ATG7* KD in the absence of cellular viability effects. The sequences of siRNA targeting human *TSC2* were as follows: 5′-CGAACGAGGUGGUGUCCUATT-3′ (sense) and 5′-UAGGACACCAC-CUCGUUCGTT-3′ (antisense). The sequences of siRNA targeting human *ATG7* were as follows: 5′-GGAGUCACAGCUCUUCCUUTT-3′ (sense) and 5′-AAGGAAGAGCUGUGACUCCTT-3′ (antisense).

4.4. Cell viability assay

HEMn-MPs (1×10^4 cells/well) were cultured in 96-well flat-bottom tissue culture plates. After experimental treatments, cells were washed three times in cold PBS, and cell viability was evaluated (Cell Count Reagent SF colorimetric assay; Nacalai Tesque, Kyoto, Japan). Briefly, reagent (10 μl) was added to each well, and cells were incubated (2 h, 37 °C). Cell viability was determined colorimetrically, measuring OD_{450} via a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viable cells was calculated as follows: T/C × 100,

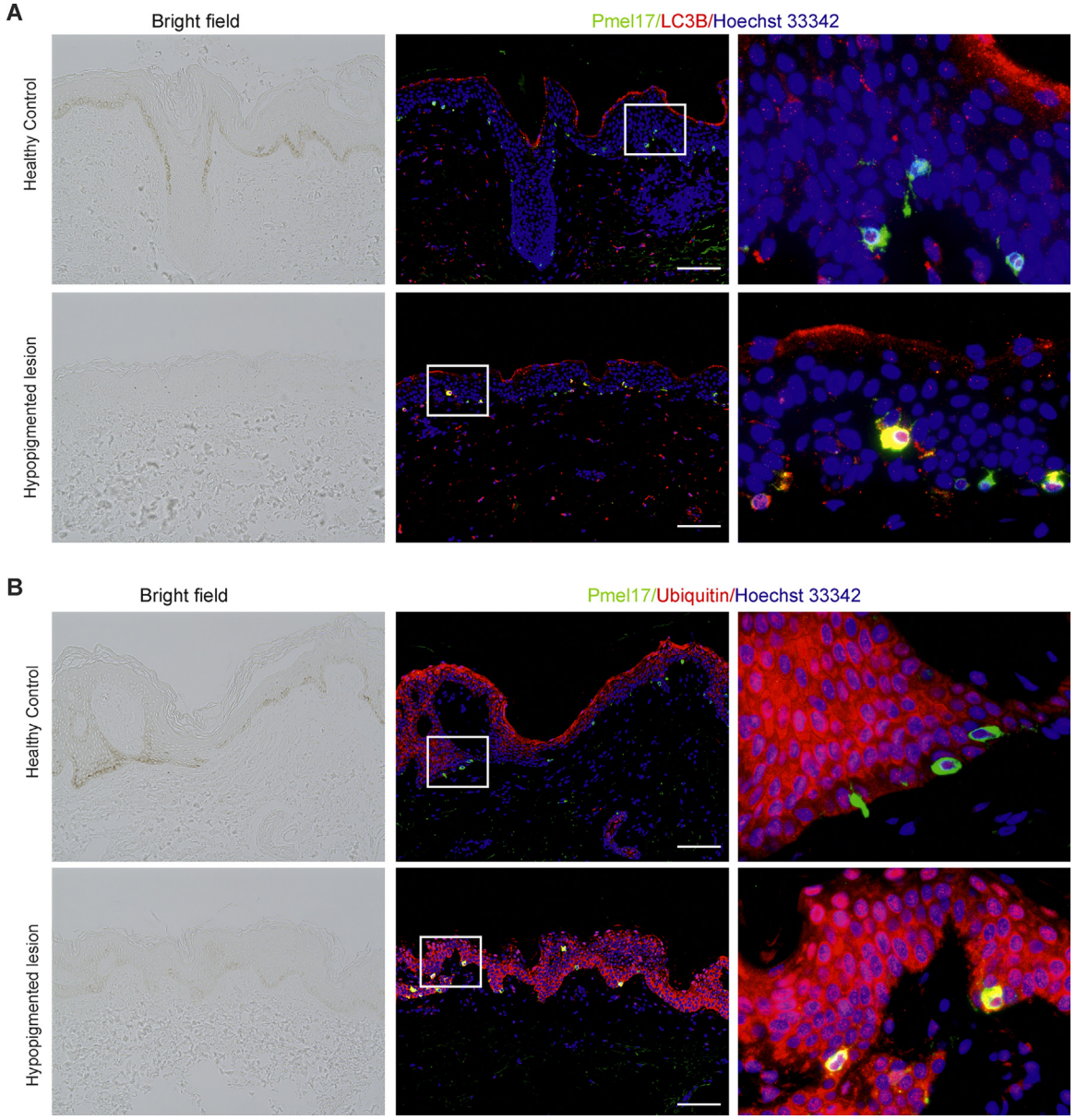

Fig. 6. Dysregulation of melanocytic autophagy in hypopigmented macular skin lesions of patients with TSC: Skin biopsy from healthy donor and hypopigmented macular lesion of patient with TSC investigated: Immunohistochemical staining, applying antibodies to (A) anti-Pmel17 antibody (green) anti LC3B antibody (red) or (B) anti-Ubiquitin antibody (red). Nuclei counterstained using Hoechst 33342 (blue). Bright-field microscopic image shown (left panel), with corresponding area on right (white bar = 100 μm).

where T is the mean OD_{450} of the treated group, and C is absorbance of the control group.

4.5. Melanin content assay

To determine melanin content, cells were dissolved in 200 μl of 1 N NaOH (30 min, 100 °C), and melanin released in cell suspensions was quantified, recording absorbance at 405 nm (as described previously) [49]. The calculated melanin content was corrected, based on cell count.

4.6. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated f pellets using a kit (Maxwell16 LEV simplyRNA Tissue Kit; Promega, Madison, WI, USA) following the manufacturer's instructions. RNA integrity was verified by gel electrophoresis. Total RNA (100 ng) was reverse-transcribed into first-strand cDNA (ReverTra Ace® qPCR RT Master Mix; TOYOBO, Osaka, Japan). The primers used for real-time PCR were as follows: *TSC2*, 5′-GAGAACCCGCTCAGCCCTTT-3′ (sense) and 5′-TGTGTTGGAGCGAGGCAGAG-3′ (antisense); and GAPDH, 5′-GACAGTCAGCCGCATCTTCT-3′ (sense) and 5′-GCGCCCAATACGACCAAATC-3′ (antisense). Each reaction was performed in triplicate.

4.7. Western blot analysis

Proteins from cell pellets were extracted, using 5 μg for western blot analysis (as described previousl) [50]. Primary antibodies and dilutions used were as follows: anti-Tuberin; anti-phospho-S6 (Ser235/256); anti-P62; anti-ubiquitin; anti-ATG7; and anti-GAPDH (Cell Signaling Technology, Beverly, MA, USA), 1:1000; and anti-LC3 (MBL, Nagoya, Japan), 1:1000. GAPDH was used as a loading control.

4.8. Immunohistochemistry

Skin samples were fixed in 10% formaldehyde for routine processing and paraffin embedding, then sectioned (4 μm) and subjected to immunofluorescence staining. Studies were performed using primary antibodies to Peml17 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-S6, p62, ubiquitin, and LC3 B (1:100; Cell Signaling Technology, Danvers, MA, USA) secondary antibodies (anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555; Thermo Fisher) [51]. Sections were ultimately counterstained (Hoechst 33342, 1:500: Thermo Fisher). The stained proteins were visualized by light microscope or under a confocal microscope (Biozero; Keyence Co, Osaka, Japan).

4.9. Immunofluorescent staining of cells

Cells (5×10^3 per chamber) were seeded into 8-chamber culture slides (Nunc Lab-Tek; Thermo Fisher). After indicated experiments, cells were washed in ice-cold PBS, fixed in methanol (5 min), incubated in primary antibody (p-S6, LC3B, or p62 [1:100]; Cell Signaling Technology), and incubated in secondary antibody (anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555; Thermo Fisher). A nuclear counterstain was then applied (Hoechst 33342, 1:500; Thermo Fisher). Samples were visualized under a confocal microscope (Biozero; Keyence Co).

4.10. Electron microscopy

Punch biopsies of skin (1 mm) and cells propagated in culture dishes were fixed, using 2.5% glutaraldehyde in 0.1 M PBS buffer (2 h, pH 7.4) or 0.5% glutaraldehyde in 0.1 M PBS buffer (15 min, pH 7.4), respectively. The samples were then washed three times in PBS and post-fixed (1 h) using 1% OsO_4 in PBS, then undergoing dehydration (graded ethanol solutions) and EPON embedding. Once the resin hardened, the plastic dish was removed from the EPON block. Ultra-thin sections (80 nM) were cut horizontal to bottom of dish, transferred to 200 mesh grids, and dual-stained (uranyl acetate and lead citrate) for EM examination (H-7650 transmission EM; Hitachi, Tokyo, Japan).

4.11. Pre-embedding immuno-EM

Pre-embedding immune-EM was performed as detailed previously [52] with slight modification. Briefly, cells were harvested, fixed in 4% paraformaldehyde (1 h). Cells were then permeabilized and blocked for 30 min, and exposed to primary antibodies and colloidal gold (1.4-nm diameter, Nanoprobes)-conjugated secondary antibodies overnight at 4 °C, respectively. The signal was intensified with a gold enhancement kit (GoldEnhance EM, Nanoprobes) for 5 min at room temperature. The specimens were post-fixed in 1% OsO4, then dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. Ultra-thin sections were collected and stained with uranyl acetate and lead citrate. Images were taken with a Hitachi H7650 electron microscope (Tokyo, Japan) that was equipped with an AMT CCD-based camera system.

4.12. Statistical analysis

All experiments were repeated at least three times, expressing data as means ± standard deviation (SD). Student's two-tailed *t*-test (Microsoft Excel; Microsoft Corp., Redmond, WA,USA) was used for comparisons between groups, setting statistical significance at $p < 0.05$.

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References

[1] M. van Slegtenhorst, R. de Hoogt, C. Hermans, M. Nellist, B. Janssen, S. Verhoef, et al., Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34, Science 277 (1997) 805–808.
[2] R. Wienecke, A. Konig, J.E. DeClue, Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity, J. Biol. Chem. 270 (1995) 16409–16414.
[3] H.L. Kenerson, L.D. Aicher, L.D. True, R.S. Yeung, Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors, Cancer Res. 62 (2002) 5645–5650.
[4] K. Inoki, H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, et al., TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth, Cell 126 (2006) 955–968.
[5] A.P. Gold, J.M. Freeman, Depigmented nevi the earliest sign of tuberous sclerosis, Pediatrics 35 (1965) 1003–1005.
[6] K. Jimbow, Tuberous sclerosis and guttate leukodermas, Semin. Cutan. Med. Surg. 16 (1997) 30–35.
[7] D.J. Kwiatkowski, Rhebbing up mTOR: new insights on TSC1 and TSC2: and the pathogenesis of tuberous sclerosis, Cancer Biol. Ther. 2 (2003) 471–476.
[8] K. Inoki, M.N. Corradetti, K.L. Guan, Dysregulation of the TSC-mTOR pathway in human disease, Nat. Genet. 37 (2005) 19–24.
[9] J. Cao, M.E. Tyburczy, J. Moss, T.N. Darling, H.R. Widlund, D.J. Kwiatkowski, Tuberous sclerosis complex inactivation disrupts melanogenesis via mTORC1 activation, J. Clin. Invest. 127 (2017) 349–364.

[10] K. Ohguchi, Y. Banno, Y. Nakagawa, Y. Akao, Y. Nozawa, Negative regulation of melanogenesis by phospholipase D1 through mTOR/p70 S6 kinase 1 signaling in mouse B16 melanoma cells, J. Cell Physiol. 205 (2005) 444–451.
[11] H.S. Jeong, S.H. Lee, H.Y. Yun, K.J. Baek, N.S. Kwon, K.C. Park, et al., Involvement of mTOR signaling in sphingosylphosphorylcholine-induced hypopigmentation effects, J. Biomed. Sci. 18 (2011) 55.
[12] Y.T. Tsao, Y.F. Huang, C.Y. Kuo, Y.C. Lin, W.C. Chiang, W.K. Wang, et al., Hinokitiol inhibits melanogenesis via AKT/mTOR signaling in B16F10 mouse melanoma cells, Int. J. Mol. Sci. 17 (2016) 248.
[13] R. Busca, C. Bertolotto, J.P. Ortonne, R. Ballotti, Inhibition of the phosphatidylinositol 3-kinase/p70(S6)-kinase pathway induces B16 melanoma cell differentiation, J. Biol. Chem. 271 (1996) 31824–31830.
[14] Y.S. Hah, H.Y. Cho, T.Y. Lim, D.H. Park, H.M. Kim, J. Yoon, et al., Induction of melanogenesis by rapamycin in human MNT-1 melanoma cells, Ann. Dermatol. 24 (2012) 151–157.
[15] H. Ho, R. Kapadia, S. Al-Tahan, S. Ahmad, A.K. Ganesan, WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition, J. Biol. Chem. 286 (2011) 12509–12523.
[16] M. Wataya-Kaneda, M. Tanaka, A. Nakamura, S. Matsumoto, I. Katayama, A novel application of topical rapamycin formulation, an inhibitor of mTOR, for patients with hypomelanotic macules in tuberous sclerosis complex, Arch. Dermatol. 148 (2012) 138–139.
[17] M. Wataya-Kaneda, M. Tanaka, L. Yang, F. Yang, D. Tsuruta, A. Nakamura, et al., Clinical and histologic analysis of the efficacy of topical rapamycin therapy against hypomelanotic macules in tuberous sclerosis complex, JAMA Dermatol. 151 (2015) 722–730.
[18] A.K. Ganesan, H. Ho, B. Bodemann, S. Petersen, J. Aruri, S. Koshy, et al., Genome-wide siRNA-based functional genomics of pigmentation identifies novel genes and pathways that impact melanogenesis in human cells, PLoS Genet. 4 (2008) e1000298.
[19] H. Ho, A.K. Ganesan, The pleiotropic roles of autophagy regulators in melanogenesis, Pigment Cell Melanoma Res. 24 (2011) 595–604.
[20] D. Murase, A. Hachiya, K. Takano, R. Hicks, M.O. Visscher, T. Kitahara, et al., Autophagy has a significant role in determining skin color by regulating melanosome degradation in keratinocytes, J. Invest. Dermatol. 133 (2013) 2416–2424.
[21] E.S. Kim, Y.K. Jo, S.J. Park, H. Chang, J.H. Shin, E.S. Choi, et al., ARP101 inhibits alpha-MSH-stimulated melanogenesis by regulation of autophagy in melanocytes, FEBS Lett. 587 (2013) 3955–3960.
[22] E.S. Kim, H. Chang, H. Choi, J.H. Shin, S.J. Park, Y.K. Jo, et al., Autophagy induced by resveratrol suppresses alpha-MSH-induced melanogenesis, Exp. Dermatol. 23 (2014) 204–206.
[23] W.J. Yun, E.Y. Kim, J.E. Park, S.Y. Jo, S.H. Bang, E.J. Chang, et al., Microtubule-associated protein light chain 3 is involved in melanogenesis via regulation of MITF expression in melanocytes, Sci. Rep. 6 (2016) 19914.
[24] J. Kim, M. Kundu, B. Viollet, K.L. Guan, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell Biol. 13 (2011) 132-U171.
[25] C.H. Jung, S.H. Ro, J. Cao, N.M. Otto, D.H. Kim, mTOR regulation of autophagy, FEBS Lett. 584 (2010) 1287–1295.
[26] G. Tang, K. Gudsnuk, S.H. Kuo, M.L. Cotrina, G. Rosoklija, A. Sosunov, et al., Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits, Neuron 83 (2014) 1131–1143.
[27] J. McMahon, X. Huang, J. Yang, M. Komatsu, Z. Yue, J. Qian, et al., Impaired autophagy in neurons after disinhibition of mammalian target of rapamycin and its contribution to epileptogenesis, J. Neurosci. 32 (2012) 15704–15714.
[28] R.A. Nixon, The role of autophagy in neurodegenerative disease, Nat. Med. 19 (2013) 983–997.
[29] E. Dupin, N.M. Le Douarin, Development of melanocyte precursors from the vertebrate neural crest, Oncogene 22 (2003) 3016–3023.
[30] K.B. Kruse, J.L. Brodsky, A.A. McCracken, Autophagy: an ER protein quality control process, Autophagy 2 (2006) 135–137.
[31] K. Jimbow, T.B. Fitzpatrick, G. Szabo, Y. Hori, Congenital circumscribed hypomelanosis: a characterization based on electron microscopic study of tuberous sclerosis, nevus depigmentosus, and piebaldism, J. Invest. Dermatol. 64 (1975) 50–62.
[32] N. Mizushima, Methods for monitoring autophagy using GFP-LC3 transgenic mice, Methods Enzymol. 452 (2009) 13–23.
[33] M. Komatsu, Y. Ichimura, Physiological significance of selective degradation of p62 by autophagy, FEBS Lett. 584 (2010) 1374–1378.
[34] K. Takayama, A. Matsuura, E. Itakura, Dissection of ubiquitinated protein degradation by basal autophagy, FEBS Lett. 591 (2017) 1199–1211.
[35] C. Kishi-Itakura, I. Koyama-Honda, E. Itakura, N. Mizushima, Ultrastructural analysis of autophagosome organization using mammalian autophagy-deficient cells, J. Cell Sci. 127 (2014) 4089–4102.
[36] W. Liou, H.J. Geuze, M.J.H. Geelen, J.W. Slot, The autophagic and endocytic pathways converge at the nascent autophagic vacuoles, J. Cell Biol. 136 (1997) 61–70.
[37] B. Ravikumar, C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, et al., Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease, Nat. Genet. 36 (2004) 585–595.
[38] R.A. Floto, S. Sarkar, E.O. Perlstein, B. Kampmann, S.L. Schreiber, D.C. Rubinsztein, Small molecule enhancers of rapamycin-induced TOR inhibition promote autophagy, reduce toxicity in Huntington's disease models and enhance killing of mycobacteria by macrophages, Autophagy 3 (2007) 620–622.
[39] C.Y. Chow, Y. Zhang, J.J. Dowling, N. Jin, M. Adamska, K. Shiga, et al., Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J, Nature 448 (2007) 68–72.
[40] N. Jin, C.Y. Chow, L. Liu, S.N. Zolov, R. Bronson, M. Davisson, et al., VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P(2) in yeast and mouse, EMBO J. 27 (2008) 3221–3234.
[41] L. Ni-Komatsu, C. Tong, G. Chen, N. Brindzei, S.J. Orlow, Identification of quinolines that inhibit melanogenesis by altering tyrosinase family trafficking, Mol. Pharmacol. 74 (2008) 1576–1586.
[42] N. Mizushima, Autophagy: process and function, Genes Dev. 21 (2007) 2861–2873.
[43] M. Laplante, D.M. Sabatini, mTOR signaling at a glance, J. Cell Sci. 122 (2009) 3589–3594.
[44] N. Mizushima, The role of autophagy in quality control inside neural cells, Rinsho Shinkeigaku 46 (2006) 885–886.
[45] Y. Yamaguchi, M. Brenner, V.J. Hearing, The regulation of skin pigmentation, J. Biol. Chem. 282 (2007) 27557–27561.
[46] C.F. Zhang, F. Gruber, C. Ni, M. Mildner, U. Koenig, S. Karner, et al., Suppression of autophagy dysregulates the antioxidant response and causes premature senescence of melanocytes, J. Invest. Dermatol. 135 (2015) 1348–1357.
[47] Y.N. Rui, W. Le, Selective role of autophagy in neuronal function and neurodegenerative diseases, Neurosci. Bull. 31 (2015) 379–381.
[48] W.J. Pavan, D.W. Raible, Specification of neural crest into sensory neuron and melanocyte lineages, Dev. Biol. 366 (2012) 55–63.
[49] Y. Kotobuki, A. Tanemura, L.L. Yang, S. Itoi, M. Wataya-Kaneda, H. Murota, et al., Dysregulation of melanocyte function by Th17-related cytokines: significance of Th17 cell infiltration in autoimmune vitiligo vulgaris, Pigment Cell Melanoma Res. 25 (2012).
[50] L.L. Yang, H. Murota, S. Serada, M. Fujimoto, A. Kudo, T. Naka, et al., Histamine contributes to tissue remodeling via periostin expression, J. Invest. Dermatol. 134 (2014) 2105–2113.
[51] L. Yang, S. Serada, M. Fujimoto, M. Terao, Y. Kotobuki, S. Kitaba, et al., Periostin facilitates skin sclerosis via PI3K/Akt dependent mechanism in a mouse model of scleroderma, PLoS One 7 (2012) e41994.
[52] A. Yamamoto, R. Masaki, Pre-embedding nanogold silver and gold intensification, Methods Mol. Biol. 1474 (2016) 269–278.