MicroRNA-203 Regulates Melanosome Transport and Tyrosinase Expression in Melanoma Cells by Targeting *Kinesin Superfamily Protein 5b*

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MicroRNA (miR)-203 is known to be downregulated and to act as an anti-oncomir in melanoma cells. At present, we found that exogenous miR-203 increased pigmentation and protein expression levels of the melanoma antigen recognized by T cells (Melan-As/MART1s) and/or tyrosinase (TYR) in the human melanoma cells tested. Inversely, treatment with an inhibitor of miR-203 downregulated the expression level of TYR. The target gene of miR-203 involved in the mechanism was *kinesin superfamily protein 5b* (*kif5b*), which was revealed by gene silencing using short interfering RNA and luciferase activity assay. Furthermore, immunocytochemistry showed obvious accumulation of melanosomes around nuclei of human melanoma Mewo cells transfected with miR-203 or siR-*kif5b*. Importantly, treatment with the miR-203 inhibitor, but not miR-203, exhibited effects on human epidermal melanocytes isolated from lightly pigmented adult skin similar to those on melanoma cells. In addition, the data indicated that exogenous miR-203 also negatively regulated the cAMP response element–binding protein 1 (CREB1)/microphthalmia-associated transcription factor (MITF)/Rab27a pathway, which is one of the main pathways active in melanoma cells. In conclusion, our data indicated that anti-oncogenic miR-203 had a pivotal role in melanoma through reducing melanosome transport and promoting melanogenesis by targeting *kif5b* and through negative regulation of the CREB1/MITF/Rab27a pathway.

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INTRODUCTION

Mammalian epidermal melanocytes and melanoma cells, the latter originating from melanocytes, are specialized for the production of melanin pigment. Skin pigmentation results from a complex multistep process culminating in the distribution of melanin pigments throughout the various layers of the epidermis. The human body is protected from UVR by these pigments, and their excess production often induces hyperpigmentation. Melanosomes synthesize and preserve melanin pigments in melanocytes (Raposo and Marks, 2007). The melanosomes are mainly produced around the nucleus. Then, mature melanosomes are transported along microtubules and actin filaments, and finally arrive at the plasma membrane (Raposo and Marks, 2007). Kinesin superfamily proteins (KIFs) function as motors that move along microtubules and serve to carry cargoes such as membranous organelles, protein complexes, and mRNAs (Hirokawa *et al.*, 2009). For example, KIF5b contributes to the outward transport of melanosomes (Hara *et al.*, 2000). However, the precise mechanism underlying KIF-mediated melanosome transport via microtubules remains unclear. On the other hand, the key regulators of actin-based melanosome transport are myosin Va (Myo 5a), Rab27a, and Slac2-a (Ohbayashi and Fukuda, 2012).

Several important proteins contributing to melanogenesis have been clarified. Tyrosinase (TYR) is the most important enzyme of melanin synthesis, and its expression is induced by exposure to UVR as part of the pigmentation process. TYR acts with TYR-related protein 1 and dopachrome tautomerase to synthesize melanin (Costin *et al.*, 2005). The TYR gene family members such as TYR and TYR-related protein 1 contribute to the initiation of pigment synthesis in stage-3 melanosomes (Raposo and Marks, 2007). Another important factor involved in melanin synthesis is microphthalmia-associated transcription factor (MITF), which not only regulates the survival and proliferation of melanocytes but also promotes the transcription of several pigmentation genes (e.g., *TYR* and *TYRP1*) (Vachtenheim and Borovansky, 2010; Levy and Fisher, 2011). MITF also regulates, in a positive manner, the

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Abbreviations: CREB, cAMP response element–binding protein; HEMa-LP, human epidermal melanocytes isolated from lightly pigmented adult skin; KIF, kinesin superfamily protein; Melan-A, melanoma antigen recognized by T cell; miR/miRNA, microRNA; MITF, microphthalmia-associated transcription factor; siRNA, short interfering RNA; TYR, tyrosinase

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expression of Rab27a, which is a regulator of actin-based melanosome transport (Chiaverini *et al.*, 2008). On the other hand, melanoma antigen recognized by T cells (Melan-As/MART-1s) is a melanoma-specific antigen and also participates in melanosome biogenesis. In particular, it has been suggested that Melan-A has a role in early melanogenesis, because Melan-A is abundantly expressed in early melanosomes (De Maziere *et al.*, 2002; Basrur *et al.*, 2003).

MicroRNAs (miRNAs or miRs) have recently been identified as short (18–25 nucleotides), noncoding, small RNA molecules that suppress gene expression by binding to the complementary region of the 3′-untranslated region of their target mRNA (Cannell *et al.*, 2008; Sun *et al.*, 2008; Bartel, 2009). Over 1,300 miRNAs have been predicted to exist in humans (miRBase; http://www.mirbase.org/, accessed 1 December 2012). Furthermore, a recent report estimates that the number of recognized conserved miRNAs will increase with further study (Chiang *et al.*, 2010). Much experimental evidence has confirmed the role of miRNAs in the regulatory network of various physiological or pathophysiological processes such as development (Harfe, 2005), differentiation (Zhang *et al.*, 2009), cellular apoptosis (Lynam-Lennon *et al.*, 2009), cell proliferation (Kaddar *et al.*, 2009), and the development and progression of cancer (Cho, 2010, 2012; van Kempen *et al.*, 2012). Recently, Dynoodt *et al.* (2013) reported that miR-145 regulates melanosome transport and melanogenesis by targeting *myo 5a*. However, miRNA-associated mechanisms of melanosome transport and melanogenesis have mostly remained unknown.

We recently reported that miR-203 is downregulated and functions as an anti-oncomir in canine and human melanoma cells (Noguchi *et al.*, 2012, 2013b). In addition, miR-203 functions as a tumor suppressor in various kinds of cancer such as breast cancer, lung cancer, and squamous cell carcinoma (Takeshita *et al.*, 2012; Wang *et al.*, 2012; Jin *et al.*, 2013). Thus, its anti-oncogenic function has been a matter of focus. In this present study, we identified miR-203 as a regulator of melanosome transport and melanogenesis in melanoma cells. Furthermore, we comprehensively validated the mechanisms of melanosome transport and melanogenesis, which mechanisms involved a target gene of miR-203, i.e., KIF5b.

RESULTS

Exogenous miR-203 suppressed the growth of melanoma cells and regulated the pigmentation process

Exogenous miR-203 suppressed the growth of melanoma Mewo and A2058 cells (Figure 1a), in which miR-203 is downregulated compared with that in human normal epidermal melanocytes (Noguchi *et al.*, 2013b). Consistently, we found that transfection of Mewo cells with miR-203 resulted in hyperpimentation, as shown by Masson–Fontana ammoniacal silver staining (Figure 1b). Furthermore, western blotting analysis revealed that exogenous miR-203 increased the expression levels of Melan-A, HMB45, and TYR in Mewo cells (Figure 1c). Exogenous miR-203 also upregulated the TYR expression level in the amelanotic cell line A2058, in which no expression of Melan-A and HMB45 was observed (Figure 1c). These results led us to hypothesize that miR-203 contributed to melanogenesis and/or melanosome transport. Therefore, we focused on two KIFs, i.e., *kif2a* and *kif5b*, and *myo 5a* as potential target genes of miR-203 (http://www.targetscan.org/, accessed 5 August 2012). The transfection with miR-203 reduced the protein expression levels of KIF5b and KIF2a in both Mewo and A2058 cells (Figure 1c), as well as the mRNA expression levels of them in Mewo cells (Figure 1d). On the other hand, the expression level of Myo 5a in the Mewo cells was almost unchanged compared with that in the control (Figure 1c).

Transfection with an inhibitor of miR-203 showed effects opposite to those observed with exogenous miR-203

Transfection with an miR-203 inhibitor significantly enhanced the growth of Mewo and A2058 cells (Figure 2a). Furthermore, the expression levels of KIF2a and KIF5b were upregulated by this transfection (Figure 2b). On the contrary, the expression level of TYR was downregulated by it. Unexpectedly, in Mewo cells, the expression level of Melan-A was almost unchanged and that of HMB45 was markedly increased, even at 10 nM (Figure 2b). Hyperpigmentation was not grossly observed (data not shown).

miR-203 directly targeted *kif2a* and *kif5b*, but not *myo 5a*

Next, we performed luciferase activity assays for *kif2a*, *kif5b*, and *myo 5a* to validate whether miR-203 targeted these genes. As shown in Figure 2c, miR-203 did not affect the luciferase activity of pMIR-*myo 5a*-wild or -mutant. On the other hand, it did significantly suppress luciferase activities of pMIR-*kif2a*-wild and pMIR-*kif5b*-wild. In addition, mutation of the *kif5b* 3′-untranslated region-binding site (pMIR-*kif5b*-mut) markedly abolished the ability of miR-203 to regulate luciferase expression, and that of the *kif2a* one did so to a lesser degree. These results clearly demonstrate that miR-203 directly targeted *kif2a* and *kif5b*, but not *myo 5a*.

KIF5b regulated TYR expression level

Next, we examined the effect of silencing *kif2a* or *kif5b* by using short interfering RNA (siRNA) to validate the role of these genes in melanogenesis. The silencing of *kif2a* did not affect the cell growth of the melanoma cells tested (Figure 3a). On the other hand, *kif5b* silencing moderately, but significantly, suppressed that of Mewo cells at a concentration of 0.5 nM. Importantly, the knockdown of *kif5b* increased the TYR expression level in both Mewo and A2058 cells (Figure 3b). Knockdown of *kif5b* also increased the expression levels of Melan-A and HMB45 in Mewo cells, but not in A2058 cells, which did not express Melan-A and HMB45. *kif2a* silencing did not upregulate the expression levels of Melan-A and/or TYR in the cells tested (Figure 3b). However, unexpectedly, the HMB45 expression level in the Mewo cells was increased by *kif2a* silencing.

We also examined the expression levels of Akt and ERK1/2 as representative cell growth signaling pathways, because *kif5b* silencing showed a moderately suppressive effect on the growth of the Mewo cells. As a result, silencing of *kif2a* or *kif5b* upregulated the level of p-Akt and slightly

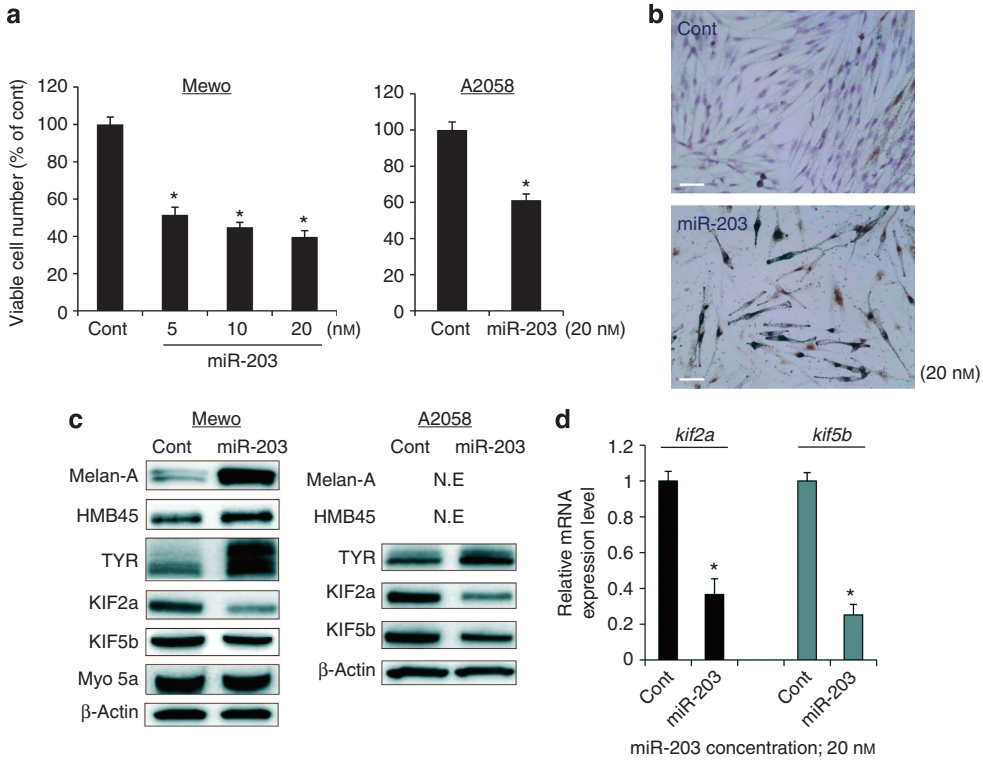

Figure 1. Exogenous microRNA (miR-203) suppressed the cell growth, induced hyperpigmentation, and downregulated the expression levels of kinesin superfamily proteins (KIFs). (**a**) Exogenous miR-203 suppressed the growth of melanoma cells. (**b**) Mewo cells stained with Masson–Fontana ammoniacal silver stain. Bar = 20 μm. (**c**) Expression levels of various proteins by western blotting. N.E, not expressed. (**d**) mRNA expression levels of *kif2a* and *kif5b* in Mewo cells were downregulated by exogenous miR-203. The cell count, protein extraction, and RNA extraction were performed at 96 (Mewo) or 72 (A2058) hours after the transfection. *$P<0.01$. A *P*-value was determined for the difference between the cells transfected with control miRNA and those transfected with miR-203. Data are expressed as the mean + SD ($n=3$). Cont, control.

downregulated that of p-ERK1/2 in Mewo cells, whereas the levels of Akt and ERK1/2 in these cells were almost unchanged (Figure 3b).

KIF5b regulated the intracellular distribution of melanosomes, and the distribution of KIF5b was consistent with that of the melanosomes

Immunofluorescence analysis showed that melanosome marker HMB45 obviously accumulated around the nuclei of Mewo cells transfected with miR-203 or siR-*kif5b*, indicating blockage of melanosome transport, whereas HMB45 was distributed diffusely in dendrites and around the nuclei in the cells transfected with control miRNA or siR-*kif2a* (Figure 4a and b). Furthermore, the intracellular distribution of HMB45 was consistent with that of KIF5b, but not with that of KIF2a (Figure 4b and c). Strong expression of KIF2a was observed in the nuclei (Figure 4b). These findings indicated that miR-203 reduced melanosome transport by negatively regulating the expression level of KIF5b.

miR-203 inhibitor enhanced the growth of HEMa-LP cells and contributed to diffuse distribution of melanosomes

The expression level of miR-203 in human normal epidermal melanocytes, human epidermal melanocytes isolated from lightly pigmented adult skin (HEMa-LP), was significantly upregulated compared with that in Mewo cells, but not compared with that in A2058 cells (Figure 5a). The expression level of KIF5b in HEMa-LP and melanoma cells appeared to be inversely correlated with that of miR-203 (Figure 5a and b). Consistent with the case for melanoma cells, the transfection with the miR-203 inhibitor enhanced the growth of HEMa-LP (Figure 5c), upregulating KIF5b expression and downregulating TYR expression (Figure 5d), although exogenous miR-203 had no effect.

We also examined the intracellular distribution of melanosomes in HEMa-LP using immunofluorescence analysis. As shown in Figure 5e, melanosomes tended to have accumulated around the nuclei in the cells treated with control miRNA or miR-203, whereas they were diffusely localized in the cells treated with the miR-203 inhibitor. However, the alteration of melanosome distribution was smaller than that in Mewo cells.

miR-203 regulated the expression levels of cAMP response element–binding protein 1, MITF, and Rab27a in Mewo cells

Our data indicated that exogenous miR-203 positively regulated the expression level of TYR. Thus, we also performed western blotting to examine the expression levels of p-cAMP response element–binding protein 1 (CREB1) and CREB1, the former of which is a transcription factor of the *mitf* gene.

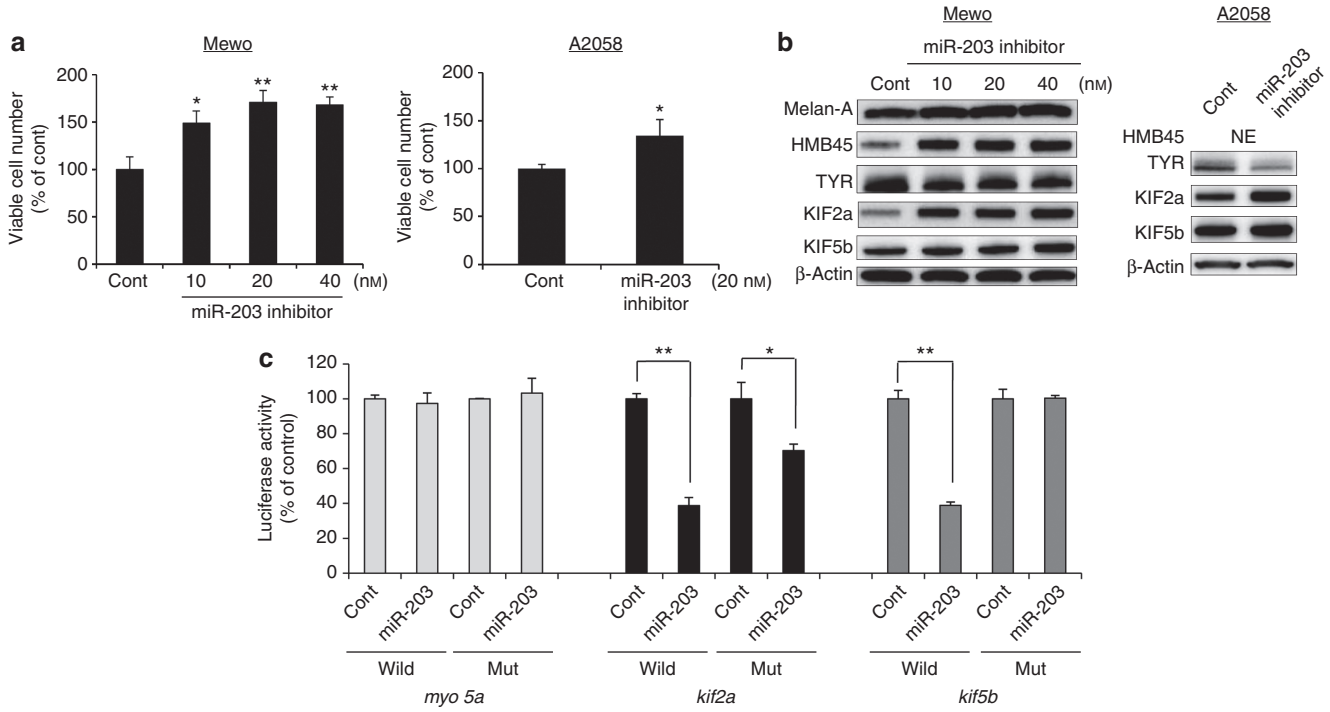

Figure 2. The effects of the transfection with microRNA (miR)-203 inhibitor on melanoma cells and luciferase assay for putative target genes of miR-203. (**a**) Transfection with miR-203 inhibitor enhanced the growth of melanoma cells. (**b**) Expression levels of various proteins by western blotting. NE, not expressed. The assays were performed at 96 (Mewo) or 72 (A2058) hours after the transfection. (**c**) Relative luciferase activities after co-transfection with control miR or miR-203 and each of the sensor vectors having the 3′-untranslated region of *myo 5a*, *kif2a*, or *kif5b*. $*P<0.05$ and $**P<0.01$. *P*-values were determined for differences between the cells transfected with control miR and those transfected with miR-203 inhibitor (**a**) or between the bracketed samples (**c**). Data are expressed as the mean + SD ($n=3$). Cont, control.

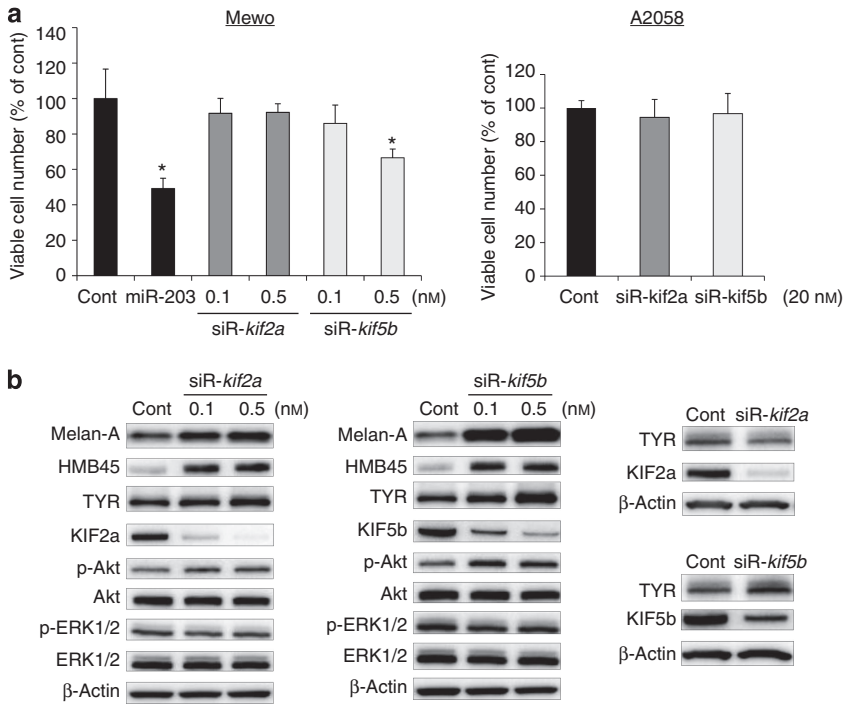

Figure 3. Knockdown of *kif5b* by using short interfering RNA (siRNA) upregulated the expression levels of Melan-A and TYR. (**a**) Comparison of the effects on the cell growth among the cells transfected with miR-203, siR-*kif2a*, or siR-*ki5b*. (**b**) Expression levels of various proteins by western blotting. The cell count and protein extraction were performed at 96 (Mewo, left and middle panels) or 72 (A2058, right panel) hours after the transfection. $*P<0.01$. A *P*-value was determined for the difference between the cells transfected with control miRNA and those transfected with miR-203 or each siRNA. Data are expressed as the mean + SD ($n=3$). Cont, control.

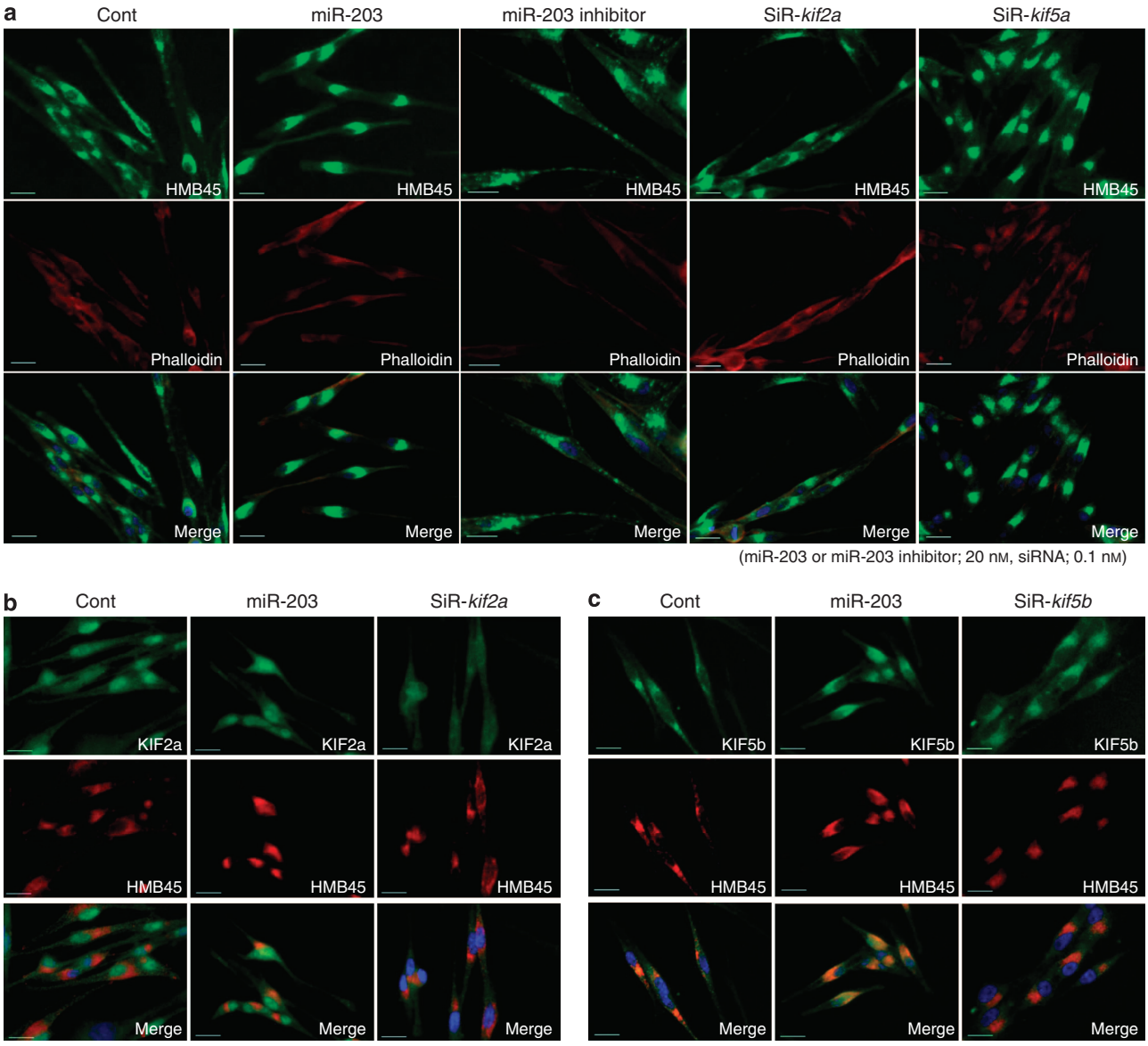

Figure 4. Exogenous microRNA (miR)-203 or *kif5b* silencing inhibited melanosome transport in Mewo cells. Immunostaining by the immunofluorescence method was performed 96 hours after transfection with control miRNA, miR-203, miR-203 inhibitor, or each short interfering RNA (siRNA). (**a**) HMB45 accumulated around the nuclei in the cells transfected with miR-203 or siR-*kif5b*. On the other hand, HMB45 was also observed in the peripheral region of the cells transfected with control miR, miR-203 inhibitor, or siR-*kif2a*. (**b**) Expression of KIF2a was mainly observed in the nuclei and was unrelated to that of HMB45. (**c**) The intracellular distribution of KIF5b and HMB45 was coincidental. Nuclei were counterstained in blue with Hoechst33342. Bar = 20 μm. Cont, control.

Its product, MITF, is one of the key regulators of melanogenesis and survival and proliferation of melanocytes. We also examined the expression of Rab27a, which is a downstream molecule of MITF and one of the key regulators of actin-based melanosome transport. Exogenous miR-203 downregulated the expression levels of CREB1, MITF, and Rab27a, although the level of p-CREB1 remained almost unchanged (Figure 6a). Inversely, the treatment with the miR-203 inhibitor increased the protein expression levels of these genes. On the other hand, the expression level of MITF was unchanged, and that of Rab27a was increased, by silencing of *kif2a* or *kif5b*.

DISCUSSION

We recently reported that exogenous miR-203 induces cellular senescence by targeting *E2F3* in melanoma cells (Noguchi *et al.*, 2012). In the current study, we found that treatment of Mewo cells with exogenous miR-203 resulted in hyperpigmentation of the cells. We unraveled part of the mechanisms of the pigmentation process and melanosome transport by transfecting melanoma cells with miR-203. As the results obviously showed, miR-203 contributed to melanogenesis by increasing the number of melanosomes through negative regulation of KIF5b expression. Our data also revealed that the mechanism for upregulation of TYR by

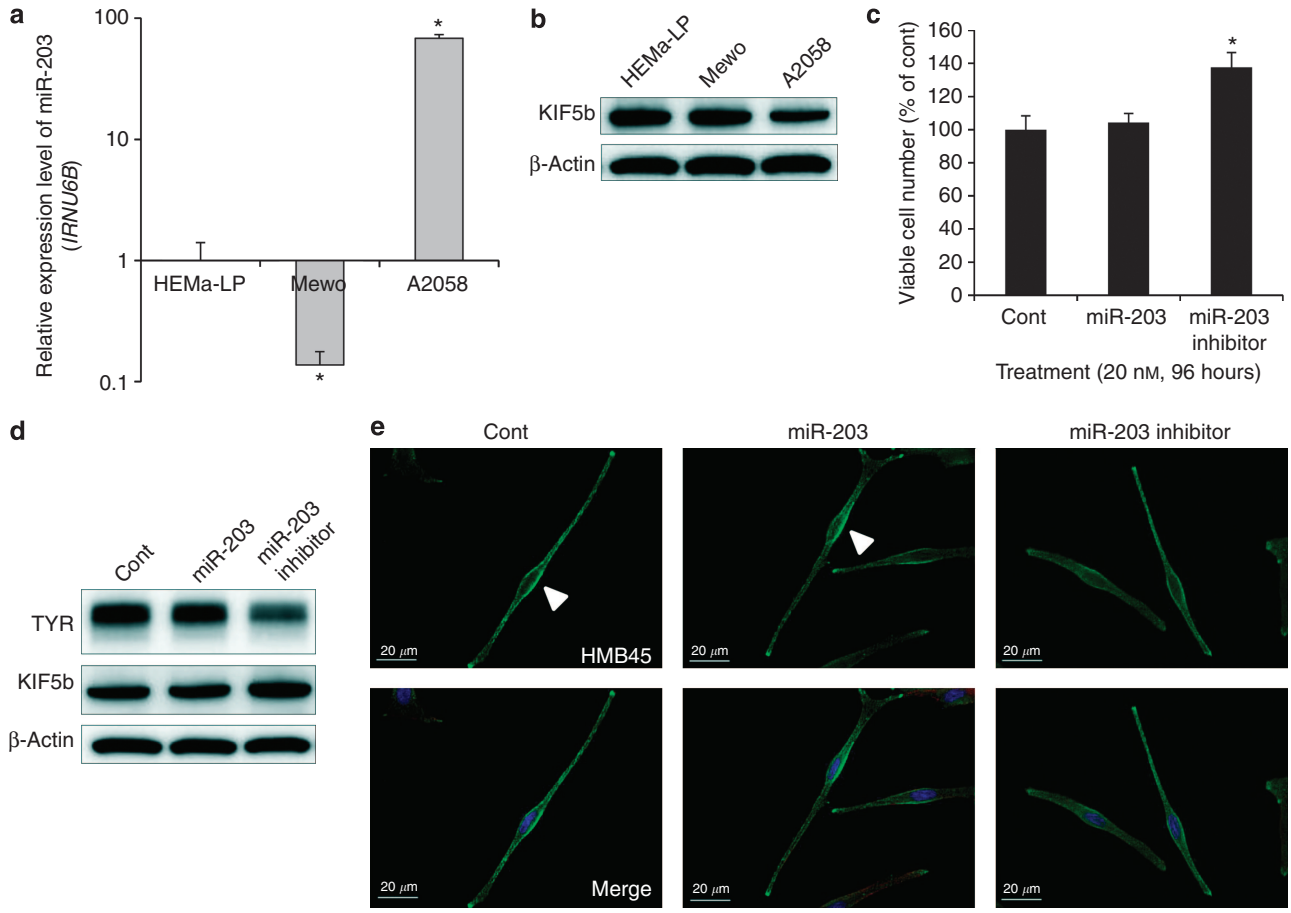

Figure 5. Effects of treatment with microRNA (miR)-203 or miR-203 inhibitor on HEMa-LP were consistent with those on melanoma cells. The expression level of miR-203 (**a**) and the protein expression level of KIF5b (**b**) in HEMa-LP and melanoma cell lines. (**c**) Transfection with miR-203 inhibitor enhanced the growth of HEMa-LP. (**d**) Effects of treatment with miR-203 or miR-203 inhibitor on TYR and KIF5b expression levels. (**e**) Intracellular distribution of melanosomes using immunofluorescence analysis. Bar = 20 μm. The arrowhead indicates accumulated melanosomes. The assays were performed at 96 hours after the transfection. *$P<0.01$. A *P*-value was determined for the difference between HEMa-LP and each melanoma cell line (**a**) or between the cells transfected with control miRNA and those transfected with miR-203 or miR-203 inhibitor (**c**). All calculated data are expressed as the mean + SD ($n=3$). Cont, control.

miR-203 was independent of MITF, because exogenous miR-203 downregulated the expression level of MITF. Furthermore, the downregulation of KIF5b by miR-203 resulted in the inhibition of melanosome transport. KIF5b belongs to the N-kinesin family and transports mature melanosomes toward the peripheral region along microtubules (Ohbayashi and Fukuda, 2012; Ohbayashi *et al.*, 2012). However, it has never been reported that KIFs regulate TYR expression. Therefore, a further detailed study is needed to reveal the mechanism by which KIF5b regulates TYR.

On the other hand, as was shown in Figures 2a and 3b, the transfection with the miR-203 inhibitor or siR-*kif2a* also increased the expression level of HMB45 without upregulating that of TYR. Therefore, we examined the time-dependent changes in the expression levels of various proteins in Mewo cells transfected with miR-203 or miR-203 inhibitor. The results shown in Supplementary Figure S1a and b online revealed that the expression levels of HMB45, Melan-A, and TYR in Mewo cells were transiently decreased after the transfection with control miRNA. Then, they were increased, consistent with the accelerating cell growth. The HMB45 expression level in the cells transfected with each miRNA was increased to almost the same level found at 0 hour after the transient decrease. On the other hand, the expression levels of Melan-A and TYR in the cells transfected with miR-203 were increased to more than twice the level at 0 hour after the transient reduction in spite of the arrested cell growth. The transient decrease in protein expression levels was not associated with the expression level of miR-203 (Supplementary Figure S1c online). These results indicate that upregulation of HMB45 was a nonspecific effect and not directly related to melanogenesis.

We also validated the effects of exogenous miR-203 or the inhibition of endogenous miR-203 expression on HEMa-LP cells (Figure 5). At first, we examined the expression level of miR-203 in HEMa-LP and the melanoma cells tested. As a result, unexpectedly, the expression level of miR-203 in HEMa-LP cells, procured from Invitrogen (Carlsbad, CA), was markedly lower than that in A2058 cells, inconsistent with the results obtained with human normal epidermal

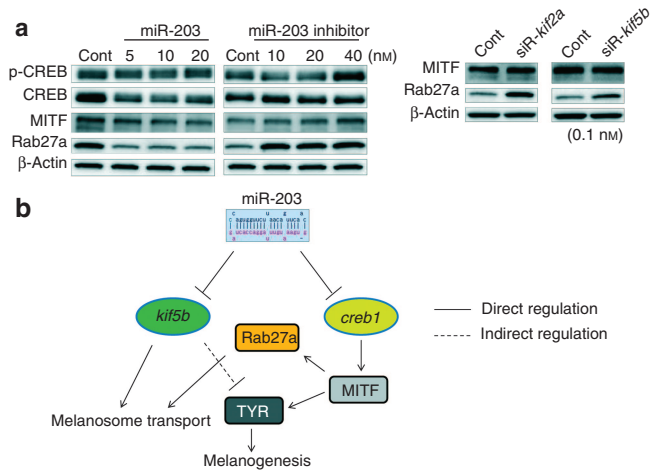

Figure 6. Exogenous microRNA (miR-203) downregulated the expression levels of CREB, MITF, and Rab27a in Mewo cells. (**a**) Exogenous miR-203 downregulated the expression levels of CREB, MITF, and Rab27a, whereas the MITF expression level remained almost unchanged. The Rab27a expression level was upregulated in the cells transfected with siR-*kif2a* or *kif5b*. Each short interfering RNA was used at a concentration of 0.1 nM. The protein extraction was performed at 96 hours after the transfection. (**b**) Flow diagram of the target genes of miR-203 and the related pathways of melanosome transport and melanogenesis. Cont, control.

melanocytes, HEMs, from ScienCell Research Laboratories (Carlsbad, CA) in our previous study (Noguchi *et al.*, 2013b). These results indicate that the characteristics of melanocytes, such as the amount of pigment and the passage number, affect the expression level of miR-203. Importantly, however, exogenous miR-203 did not affect the growth or biochemical phenotype of the HEMa-LP cells. These data indicate that the effects of exogenous miR-203 on normal melanocytes were smaller than those on melanoma cells.

In addition, we examined the effects of intratumoral injection of miR-203 into mice bearing xenografted Mewo cells. We determined that the injection dose of miR-203 was 0.3 nmol per administration, which was determined by the results found in our previous study (Noguchi *et al.*, 2013a). As shown in Supplementary Figure S2 online, the effects of miR-203 administration *in vivo* on the expression levels of TYR and KIF5b were mostly consistent with those obtained *in vitro*. However, no obvious tumor-suppressive effects were observed (data not shown), possibly caused by no significant repression of the expression levels of p-CREB and CREB (Supplementary Figure S2 online). Accordingly, to assess the tumor-suppressive effect of miR-203 *in vivo*, a higher dose of miRNA and extended experimental period might be explored in the future.

We validated the expression level of MITF in Mewo cells, because the TYR expression level was increased by transfection with miR-203 or siR-*kif5b*. Interestingly, the results showed that the MITF expression level was decreased by exogenous miR-203, whereas *kif5b* silencing did not affect it. These results suggest that the mechanism regulating TYR involved not only MITF but also KIF5b. We hypothesized that miR-203 targeted an upstream molecule of *mitf*. Based on the database (http://www.targetscan.org/, accessed 1 February 2013), we focused on *creb1* as a potential target gene of miR-203. CREB is activated by phophorylation at its Ser133, and the activated CREB increases *mitf* gene expression (Wan *et al.*, 2011). Our data indicated that miR-203 regulated MITF expression level by targeting *creb1*. Subsequently, Rab27a expression was suppressed through downregulation of MITF, because MITF regulates Rab27a and TYR (Chiaverini *et al.*, 2008). Thus, a further study should focus on the function of the miR-203/CREB1/MITF/Rab27a pathway in melanoma development and progression to elucidate the comprehensive role of miR-203 in melanoma.

In addition, we attempted to reveal the significance of KIF2a suppression by exogenous miR-203. KIF2a regulates neural migration by the uncontrolled elongation of axon collaterals (Homma *et al.*, 2003). Therefore, we performed an invasion assay by using matrigel invasion chambers (Becton and Dickinson, Franklin Lakes, NJ). The results shown in Supplementary Figure S3 online indicated that exogenous miR-203 significantly inhibited the migration of both melanoma cell lines tested and that this inhibition was partly associated with the suppression of KIF2a in Mewo cells. However, further comprehensive studies are needed to disclose the function of KIF2a in melanoma cells.

In conclusion, we showed that miR-203 directly regulated microtubule-based melanosome transport in a negative manner by targeting *kif5b* in melanoma cells. In addition, our data indicated that miR-203 regulated TYR expression without upregulating MITF expression and possibly regulated actin-based melanosome transport by suppressing Rab27a, a downstream molecule of CREB1. Altogether, our data indicate that miR-203 had a pivotal role in melanoma cells, reducing melanosome transport and promoting the pigmentation process (Figure 6b).

MATERIALS AND METHODS

Cell culture and cell viability

Human malignant melanoma cell lines A2058 and Mewo were purchased from Health Science Research Resources Bank (Osaka, Japan). HEMa-LPs were obtained from Invitrogen. The cells were maintained according to the manufacturer's protocol. The number of viable cells was determined by performing the trepan blue dye exclusion test.

Cell transfection with miRNA or siRNA

The cells were seeded into six-well plates at a concentration of 0.5×10^5 (Mewo and A2058) or 1.0×10^5 (HEMa-LP) cells per well the day before transfection. We used Pre-miR-203 (Applied Bios stems, Foster City, CA) as an miR-203 mimic and nirvana miRNA inhibitor for hsa-miR-203 as an miR-203 inhibitor (Applied Bios stems) for transfection into cells. Transfection was achieved by using cationic liposome's, Lipofectamine RNAiMAX (Invitrogen), at a concentration of 5–20 nM (miR-203) or 10–40 nM (miR-203 inhibitor), according to the manufacturer's Lipofection protocol. siRNAs for *kif2a* and *kif5b* were also used for transfection of the cells, 5′-UAAUCUGAAACACUGCAUGGCUCCG-3′ (siR-*kif2a*) and 5′-CAGAUCUCCGCUGUGAACUUCCUAA-3′ (siR-*kif5b*; Invitrogen). Pre-miR miRNA Precursor Molecules-Negative Control no. 2 (Applied Biosystems) was used as a nonspecific control miRNA.

Western blotting

Total protein was extracted from whole cells by the procedure described previously (Noguchi *et al.*, 2011). Protein contents were measured with a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Ten micrograms of lysate protein for western blotting was separated by SDS-PAGE using polyacrylamide gels and electroblotted onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences, Boston, MA). Details of the method used after blotting were described earlier (Noguchi *et al.*, 2011). The antibodies used in this study were anti-human KIF2a rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, TX), anti-human KIF5b rabbit polyclonal antibody, anti-human TYR mouse mAb, anti-human HMB45 mouse mAb, anti-human MITF rabbit polyclonal antibody, anti-human Rab27a mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human Akt rabbit polyclonal antibody, anti-human phosphorylated-Akt (Ser473; p-Akt) rabbit polyclonal antibody, anti-human ERK1/2 rabbit mAb, anti-human phosphorylated-ERK1/2 (Thr202/Tyr204; p-ERK1/2) rabbit mAb, anti-human Myo 5a rabbit polyclonal antibody, anti-human CREB rabbit mAb, and anti-human phosphorylated-CREB (Ser133; p-CREB) rabbit mAb (Cell Signaling Technology, Danvers, MA), all of which were properly diluted with Tris-buffered saline and Tween 20 containing 2% BSA and 0.01% sodium azide. The loading control was prepared by re-incubating the same membrane with anti-human β-actin antibody (Sigma, St Louis, MO).

Masson–Fontana ammoniacal silver staining

For the detection of melanin pigment, Mewo cells were plated on 13-mm glass coverslips (Matsunami Glass Industry, Osaka, Japan) in 1.0 ml of culture medium plus 10% (w/v) fetal bovine serum per well of a six-well plate. At 96 hours after the transfection, cells were fixed with 100% methanol. Then, they were stained according to the Masson–Fontana ammoniacal silver staining method. Briefly, cells were incubated in ammoniacal silver solution overnight. After fixation in acid-fixing solution, they were stained with kernechtrot.

Quantitative reverse transcriptase PCR using real-time PCR

Total RNA was isolated from cells by the phenol/guanidium thiocyanate method with DNase I treatment. For determination of mRNA expression levels, total RNA was reverse transcribed with a PrimeScript RT Reagent Kit (TaKaRa, Otsu, Japan). Real-time PCR was then performed using SYBR *Premix Ex Taq* (TaKaRa). The relative expression level of mRNA was calculated by the ΔΔ*Ct* method. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

To determine the expression of miRNAs, we used TaqMan MicroRNA Assays (hsa-miR-203 and RNU6B; Applied Biosystems) to reverse transcribe the mature miRNA sequences to their cDNA. The PCR procedure was performed using real-time PCR according to our previous study (Noguchi *et al.*, 2013b). The relative expression level of miR-203 was calculated by the ΔΔ*Ct* method. *RNU6B* was used as an internal control.

Assay for luciferase activity

We constructed the sensor vector by joining the regions with a possible binding site from the 3′-untranslated region of human *kif2a*, *kif5b*, or *myo 5a* to a luciferase reporter pMIR-control vector (Applied Biosystems) to examine the target sequence recognized by miR-203. For amplification of those mRNAs, total RNA was reverse transcribed with a PrimeScript RT Reagent Kit (TaKaRa). The sequences of the primers used in this study were as follows: *kif2a*-sense-2361, 5′-TTCCGTGCACGTCTACAAGA-3′; *kif2a*-antisense-2728, 5′-CTGGCTCTGGATTGAGTCCA-3′; *kif5b*-sense-5283, 5′-CATGGGTATTGGTGCTGTGT-3′; *kif5b*-antisense-5711, 5′-CTCTTCTGTCACCTCATGTC-3′; *myo 5a*-sense-11424, 5′-CAG TGCTTGGCAGGTTAGTA-3′; and *myo 5a*-antisense-11681, 5′-TTTG CGCAACACTGTTAGCG-3′. In addition, to generate the sensor vectors with 2 or 3 mutations in the binding site for miR-203, we mutated seed regions from CATTTCA to CAACGCA (mut; PrimeSTAR Mutagenesis Basal Kit; TaKaRa). The sensor vectors with mutations were submitted to Life Science Research Center, Gifu University, for DNA sequencing. Mewo cells were seeded in 12-well plates at a concentration of 0.5×10^5 per well the day before the transfection. The sensor vector (concentration, 0.5 μg per well) and 20 nM miR-203 or nonspecific control miRNA were used for the co-transfection of the cells by using cationic liposomes Lipofectamine RNAiMAX. Forty-eight hours after the co-transfection, luciferase activities were measured by using a Dual-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Immunocytochemistry

Mewo cells were plated on 13-mm glass coverslips (Matsunami Glass Industry) in 1.0 ml of culture medium plus 10% (w/v) fetal bovine serum per well of a six-well plate, and HEMa-LP was added onto two-well chamber slides (Thermo Fisher Scientific, Rochester, NY). At 96 hours after the transfection, cells were immunostained with anti-HMB, anti-KIF2a, or anti-KIF5b antibody according to the immunofluorescence protocol of Cell Signaling Technology. The nuclei were stained with Hoechst33342, and for actin labeling the cells were incubated with the fluorescent F-actin probe Rhodamine Phalloidin (Cytoskeleton, Denver, CO). The cells were viewed with a BIOREVO fluorescence microscope (Keyence, Osaka, Japan).

Statistics

Each examination was performed in triplicate. All calculated data were compared by using Student's *t*-test. A *P*-value <0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare 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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