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Original Article

Exosomal miRNA derived from keratinocytes regulates pigmentation in melanocytes

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ABSTRACT

Background: Pigmentation is controlled by complex mechanisms. Evidence suggests that miRNAs can regulate pigmentation. However, the mechanism has not been fully elucidated. Objective In this study, we revealed a novel mechanism that regulates pigmentation involving exosomes, miRNAs and the crosstalk between keratinocytes and melanocytes.
Methods: The expression and localization of exosome specific marker TSG101 in keratinocytes and melanocytes; Changes of melanin content in melanocytes after co-culture of exosome and melanocytes; Expression changes of target gene TYR and its related genes and inhibitory effect of miR-330-5p on pigmentation were studied by using various molecular biological techniques.
Results: In this experiment, we used miR-330-5p in keratinocytes to verify the effect of keratinocyte derived exosome on melanocyte pigmentation. First, we found that keratinocytes secrete exosomes carrying miR-330-5p; moreover, greater miR-330-5p expression was found in exosomes derived from keratinocytes that overexpressed miR-330-5p. Second, we found that exosomes derived from keratinocytes with overexpression of miR-330-5p caused a significant increase in miR-330-5p in melanocytes. Finally, exosomes derived from keratinocytes that overexpressed miR-330-5p induced a significant decrease in the production of melanin and expression of TYR in melanocytes. Meanwhile, we overexpressed miR-330-5p in melanocytes, which also proved the inhibitory effect of miR-330-5p on pigmentation.
Conclusion: These findings suggest that keratinocytes crosstalk with melanocytes in the epidermal melanin unit via exosomal miRNAs. These studies reveal an important role of exosomes in melanocyte pigmentation, which opens a new pathway of melanogenesis.

1. Introduction

Coat color is an important economic trait of woolers. Pigmentation is a complex process: melanocytes produce eumelanin and pheomelanin and then form melanosomes, which are transferred into keratinocytes to become visible pigmentation [1]. Thus, melanocytes and their peripheral keratinocytes are functionally connected and form the epidermal melanin unit in the epidermis of the skin [2]. These two kinds of cells highly interact and communicate with each other, but the mechanism has not been fully explored.

Pigmentation is the core process of coat color formation, which is regulated by signalling pathways including α-MSH/ASP, Wnt, SCF/c-Kit or EDN pathways [3]. We have found that many growth factors and genes regulate melanogenesis. For example, we found that ocular albinism type 1 protein (OA1) and glycoprotein nonmetastatic melanoma protein b (GPNMB) regulate melanogenesis in mouse melanocytes; the expression of slc45a2 and endothelin 3 are also related to sheep coat colors [4–7]; cyclin-dependent kinase 5 could regulate melanogenesis and epidermal structures [8]; and FGF21 regulates the pigmentation of alpaca melanocytes

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by the ERK1/2 pathway [9]. Moreover, IGF1 can modulate alpaca melanogenesis by the cAMP pathway [10]. We also studied the role of HGF/c-Met signalling in alpaca coat color formation [11]. However, more studies are needed on the mechanism controlling the cross talk between keratinocytes and melanocytes.

Exosomes are a type of 40–100 nanometer biological membrane structure [12] and are a popular type of carrier of intercellular communication. Exosomes contain numerous biologically active substances, such as proteins, mRNAs, microRNAs (miRNAs), cytokines, transcription factors, etc. [13]. It has been reported that exosomes secreted by some cells can be loaded with miRNAs and then transported to the adjacent cells or target cells to suppress target gene expression [14]. Whether exosomes play a role in pigmentation is unknown.

miRNAs are single chains of non-coding RNA that regulate gene expression at the post-transcriptional level [15]. They also interact with mRNAs by degrading target mRNAs or inhibiting target mRNA translation [16]. More evidence shows that miRNAs play a critical role in many biological functions [17], including pigmentation and mammalian coat color formation. We detected the differential expression of miRNAs in the skin of different coat colors of animals through deep sequencing analysis [18,19] and found that Lpa-miR-nov-66 targets CDK5, miR-411a-3p targets IGF1R and miR-25 targets MITF to regulate melanogenesis in alpaca melanocytes [20–22]. Similarly, in mouse skin melanocytes, we found that miR-21a-5p targets SOX5 through the MITF pathway, and miR-27a-3p regulates Wnt3a to modulate melanogenesis [23,24]. Transgenic mice overexpressing miR-137 can change coat color, and miR-137 could inhibit the pigmentation of mouse skin melanocytes by the SCF/c-Kit pathway [25]. In contrast, miR-488 could degrade POMC mRNA in keratinocytes to modulate mice coat color by the α-MSH/MC1R pathway [26]. Although many miRNAs have been shown to play critical roles in skin biology and hair cycle regulation, we are interested in miR-330-5p. Because our previously miRNAs array also identified significant differential expression of miR-330-5p in the skin of different coat colors of animals [18,19]. Other report indicated that keratinocytes produce miR-330-5p [27] and miR-330-5p downregulated the expression of TYR that plays a key role in pigmentation [28,29]. Whether and how miR-330-5p regulates TYR in melanocytes to regulate pigmentation is not clear. We found that exosomes derived from keratinocytes carry miR-330-5p-targeting TYR to suppress melanocyte pigmentation. Our results also discovered a new mechanism related to the crosstalk between keratinocytes and melanocytes.

The mouse has long served as a model organism in the fields of experimental and biomedical sciences, and is invaluable in the study of many aspects of biology. When the sequencing of the mouse genome was initially completed, the researchers analyzed 96% of the mouse genome sequence, in which 99% of the genes could be found homologous in the human genome sequence. It is confirmed that mice and humans are highly homologous at the gene level [30–32]. So this experiment was carried out with mouse keratinocytes and melanocytes.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal anti-Tyrosinase (ab180753; 1:500), rabbit monoclonal anti-TRP1 (ab178676; 1:500), and rabbit polyclonal anti-TRP2 (ab74073; 1:500) were purchased from Abcam. Rabbit polyclonal anti-TSG101 (Proteintech, 14497-1-AP, 1:500), rabbit polyclonal anti-CD9 (20579-1-AP, 1:500), mouse monoclonal anti-β-actin (60008-1-lg, 1:1000), Alexa Fluor 488-conjugated goat anti-rabbit IgG (SA00006-2, 1:200), and Alexa Fluor 594-conjugated goat anti-rabbit IgG (SA00006-4, 1:200) were obtained from Proteintech. Goat anti-rabbit IgG (CW0156S, 1:10000) and goat anti-mouse IgG (CW0102S, 1:10000) were from Cwbio.

2.2. Cell culture and transfection of miR-330-5p

Mouse keratinocytes and melanocytes were obtained from ScienCell Company. Cell cultures were performed in the laboratories of alpaca biology, College of Animal Science and Technology, Shanxi Agricultural University, China. Keratinocytes and melanocytes were maintained in keratinocyte medium and melanocyte medium, respectively, (ScienCell) supplemented with 10% fetal bovine serum (FBS, ScienCell) and 1% streptomycin and penicillin (ScienCell).

Melanocytes were transfected with miR-330-5p, miR-Negative Control and inhibitor- miR-330-5p using Lipofectamine 2000 Transfection Reagent (Thermo Fisher). Keratinocytes were transfected with miR-330-5p, and 48 h later, their conditional culture medium was used to isolate exosomes.

2.3. Exosome isolation and preparation

Keratinocytes transfected with miR-330-5p/miR-NC were maintained in keratinocyte medium (ScienCell) without FBS. Following two days of incubation, exosomes were isolated from the medium of the keratinocyte cultures by ultracentrifugation. Briefly, media was centrifuged at 800*g* for 10 min (4°C) to remove debris. Then, the supernatant was centrifuged at 16,000*g* for 60 min (4°C) to remove debris again. In addition, the exosomes were collected from the supernatant by centrifugation at 120,000*g*

Table 1
Primers used in this study.

Genes	Premier sequence/(5′→3)	Temperature/°C
TYR	F: AGCCTGTGCCTCCTCTAA	58
	R: AGGAACCTCTGCCTGAAA	
TYRP1	F: CGATACCCTGGGAACACT	60
	R: TACACGGACCTCCAAGCA	
TYRP2	F: CCAACGCTGATTAGTCGGA	58
	R: GAAGAAGGGAGGGCTGTCA	
MITF	F: AGGACCTTGAAAACCGACAG	58
miR-330-5p	R: GTGGATGGGATAAGGGAAAG	60
URP	TTGCTGACAGGATGCAGAAACACTCCAGC	60
U6	TGGGTCTCTGGGCCTGTGTCG	
	TGGTGTCGTGGAGTCG	
	F: CTCGCTTCGGCAGCACA	
	R: AACGCTTCACGAATTTGCGT	
β-actin	F: TTGCTGACAGGATGCAGAAG	58
	R: ACATCTGCTGGAAGGTGGAC	

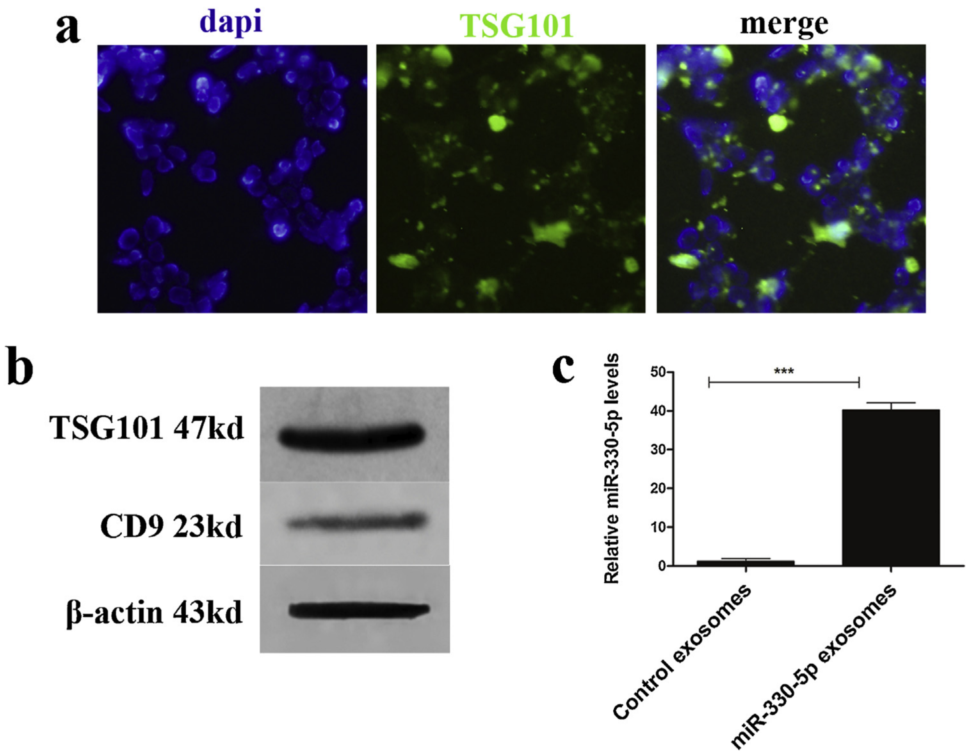

Fig. 1. Characteristics of exosomes and relative miR-330-5p levels in exosomes. (a) Keratinocytes were immunostained with anti-TSG101 (green) and DAPI (blue). (b) Western blot analysis of isolated exosomes using exosomal markers. (c) Relative miR-330-5p mRNA in exosomes derived from control or overexpressed miR-330-5p keratinocytes. Exosomes that were secreted from keratinocytes transfected with miR-NC were used as controls.

for 180 min (4°C). The pelleted exosomes were re-suspended in PBS (pH 7.2–7.4) and stocked at -80°C.

2.4. Fluorescence in situ hybridization

The cells on coverslips (about 70% confluency) were fixed in 4% paraformaldehyde for 10 min. The coverslips were washed in PBS and incubated in pepsin diluted with 3% citric acid for 20 min. After washing with distilled water three times, the coverslips were incubated with prehybridization solution for 4 h. Then, the coverslips were incubated in hybridization solution overnight at 42 °C without light. On the following day, the coverslips were washed with gradient saline-sodium citrate (SSC) buffer four times (10 min each). All coverslips were examined by a fluorescence microscope (Leica DM LB 2).

2.5. RNA extraction and real-time PCR analysis

Total RNA was isolated from the control and treated cells (mouse melanocytes and keratinocytes) using the Trizol LS Reagent (Takara). The total RNA of exosomes was isolated using the RNeasy Mini kit (Qiagen). The same amount of cDNA was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara) and random primers. In addition, cDNA of microRNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara) and miR-330-5p stem-loop and U6Fw primers.

Real-time PCR was carried out with Stratagene Mx3005p, using the SYBR Premix Ex Taq II (Takara). Target gene expression was normalized in relation to the expression of the endogenous controls, β-actin (for mRNA) and U6 (for microRNA). All primer sequences are shown in Table 1. The $\triangle$Ct method was used to quantitate gene expression and was expressed relative to the control/untreated samples. Each experiment was performed three times with 4 replicates per group.

2.6. Western blot analysis

Total protein was extracted from exosomes, melanocytes or keratinocytes using RIPA Lysis Buffer (Beyotime, China), according to the manufacturer's instructions. The protein concentrations were measured by a BCA kit (Applygen). The protein samples (100 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline-Tween (TBST) for 1 h. Then, the membranes were respectively incubated with antibodies in TBST overnight at 4 °C. β-actin was used as a loading control. After washing with TBST three times (10 min each), the HRP-conjugated secondary antibody was used. Finally, immunoreactive signals were developed using ECL Western blot detection reagents (Cwbio).

2.7. Melanin content measurement

Melanocytes treated with exosomes or transfected with miR-330-5p after 56 h were collected and washed in PBS 3 times. Cell suspensions were counted with a cell counter (Bio-Rad). The cells were centrifuged at 1000 rpm for 10 min at 4°C, and the pelleted-cells were incubated at 85°C for 10 min with 1 mL of 1 mol/L NaOH. Melanin content was measured for OD at 475 nm.

2.8. Immunofluorescence staining

The cells on coverslips (about 70% confluency) were fixed in 4% paraformaldehyde for 20 min at room temperature. The coverslips were washed in PBS and incubated in 3% hydrogen peroxide (15 min, 37 °C). After washing with PBS three times, the coverslips were blocked with 5% bovine serum albumin (BSA). The coverslips were incubated in TSG101 (diluted 1:50 in PBS) overnight at 4 °C. In

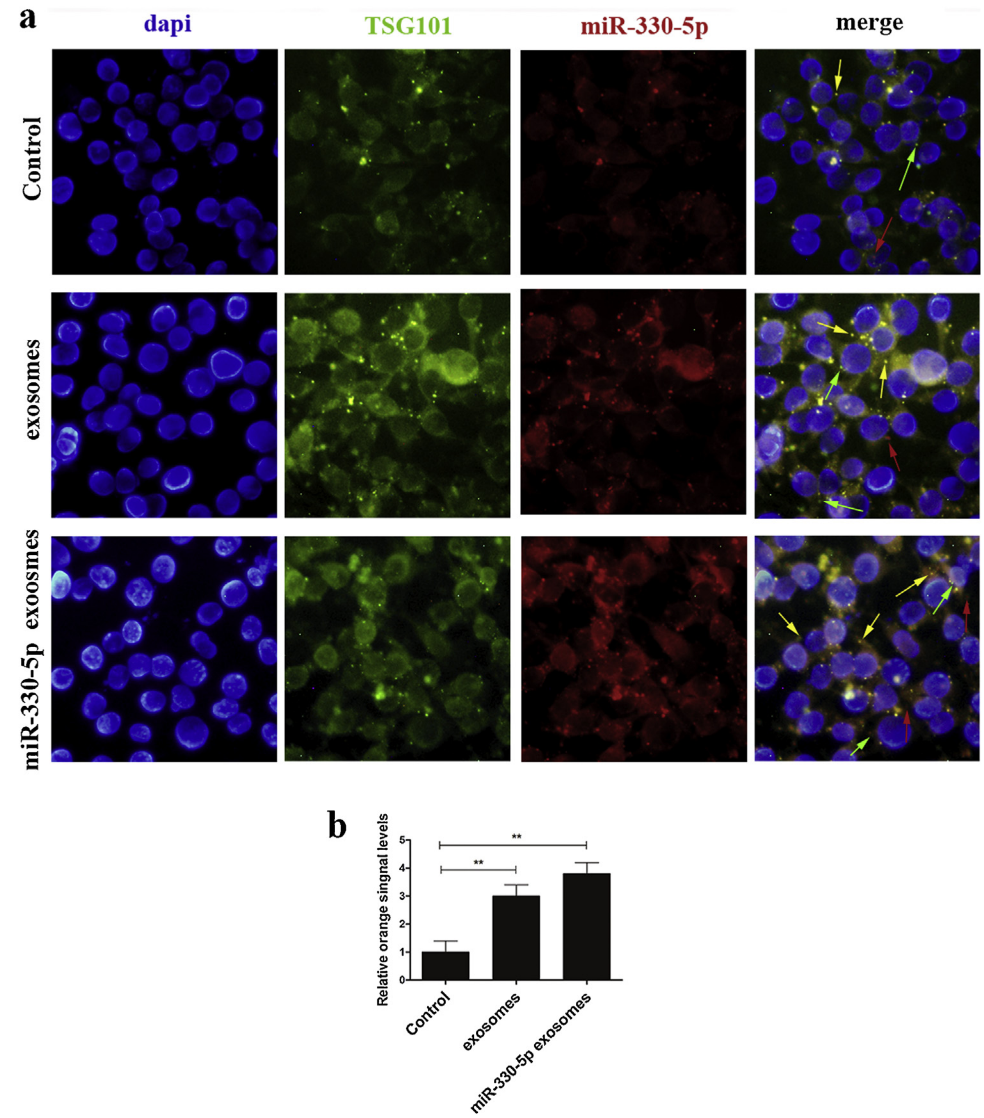

Fig. 2. The interaction between the exosomes derived from keratinocytes with melanocytes. (a) TSG101 (green), miR-330-5p (red) and DAPI (blue) fluorescence in situ hybridization of melanocytes after incubating with exosomes derived from keratinocytes. The results were observed as punctate structures in contact with melanocytes. In the merged column, the green dots indicate that the exosomes are not loaded with miR-330-5p, the orange dots represent the exosomes carrying miR-330-5p, and the red dots represent miR-330-5p. (b) The expression level of exosomes that carried miR-330-5p (orange spots) in these three groups. The melanocytes only had the same medium used as control.

the negative control, TSG101 was replaced with PBS. The coverslips were incubated in FITC-labeled goat anti-rabbit IgG (diluted 1:200 in PBS) for 1 h (37 °C) and washed with PBS six times. All coverslips were examined by a fluorescence microscope (Leica DM LB 2).

2.9. Statistical analysis

All experiments were repeated three times. Data are presented as the mean ± SD. Statistical analysis was conducted in the GraphPad Prism 5.0 software.

3. Results

3.1. Exosome isolation and overexpression of miR-330-5p in keratinocytes

We proposed that keratinocyte-secreted exosomes carry miR-330-5p. To test our hypothesis, we first immunostained keratinocytes with exosome marker, TSG101, and found that TSG101 was expressed in keratinocytes (Fig. 1a). We then isolated exosomes from mouse keratinocyte medium using differential centrifugation. The isolated exosomes were positive for exosomal markers TSG101 and CD9 (Fig. 1b).

To test whether the exosomes derived from keratinocytes carry miR-330-5p, we overexpressed miR-330-5p in keratinocytes, and 56 h later, the exosomes were isolated from the these cell culture medium. The relative expression of miR-330-5p in exosomes derived from keratinocytes transfected with miR-330-5p mimic was significantly higher than that of the control group (Fig. 1c). We concluded that keratinocytes express miR-330-5p, and they can be secreted to the medium via exosomes.

3.2. Exosomes interacting with melanocytes

Next, we tested the ability of exosomes to interact with melanocytes. We added exosomes derived from keratinocytes to melanocytes for 48 h. The fixed cells were processed for fluorescence in situ hybridization with fluorescein isothiocyanate

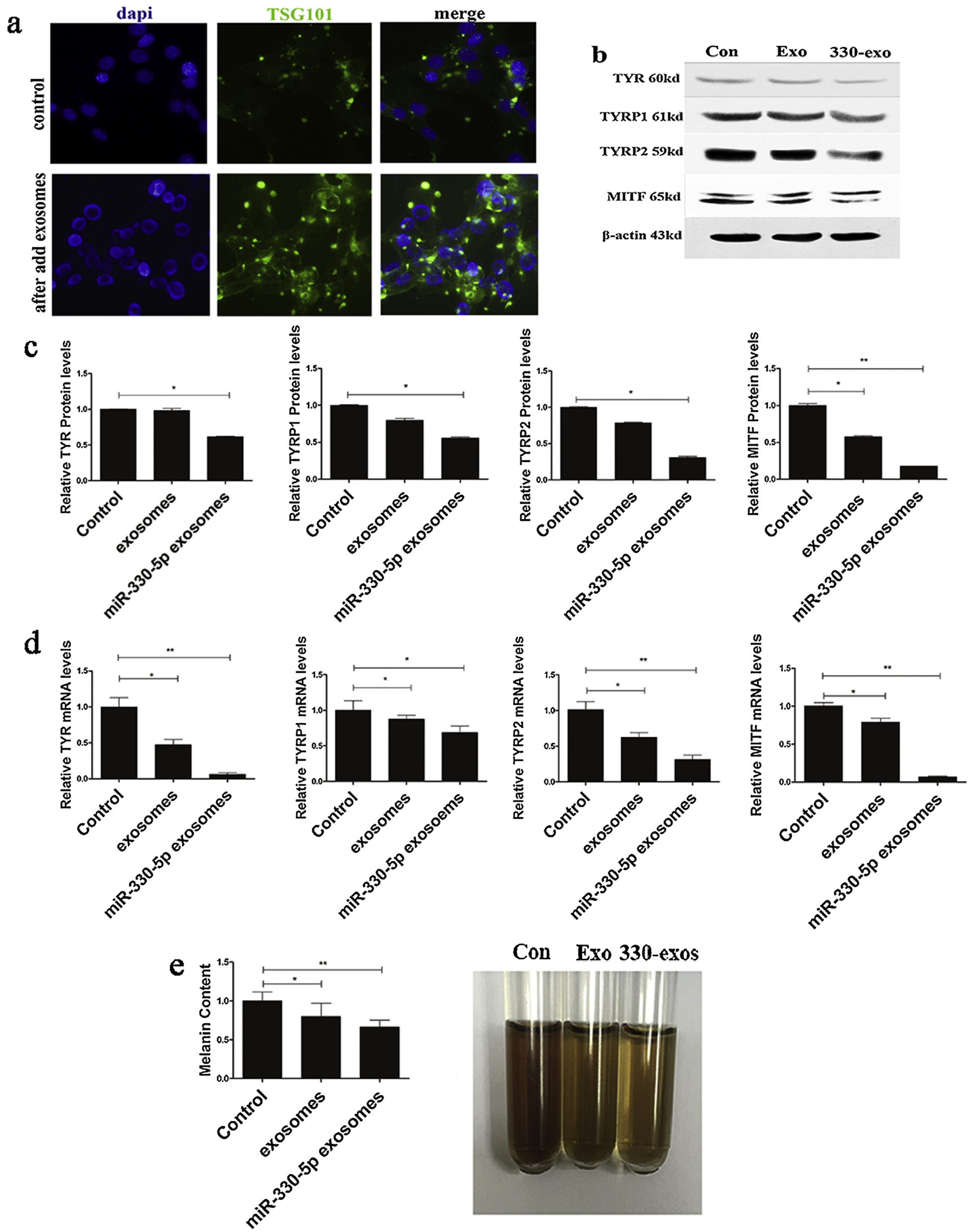

Fig. 3. (a) Exogenous exosomes can be expressed in melanocytes. Exosomes derived from keratinocytes with/without overexpression of miR-330-5p were incubated with melanocytes. These melanocytes were immunostained with TSG101 (green) and DAPI (blue). The melanocytes only had the same medium used as control. Exogenous exosomes regulate melanocyte pigmentation. (b–c) Proteins of TYR, TYRP1, TYRP2 and MITF were measured in melanocytes incubated with exosomes derived from keratinocytes with/without mir-330-5p for 56 h. (d) Relative mRNA expression of TYR, TYRP1, TYRP2 and MITF of melanocytes, same as the above treatment. (e) Intracellular melanin content analysis (optical density at 475 nm) of melanocytes incubated for 56 h with exosomes from keratinocytes (cultured only with medium and overexpressed miR-330-5p). The melanocytes only had the same medium used as control.

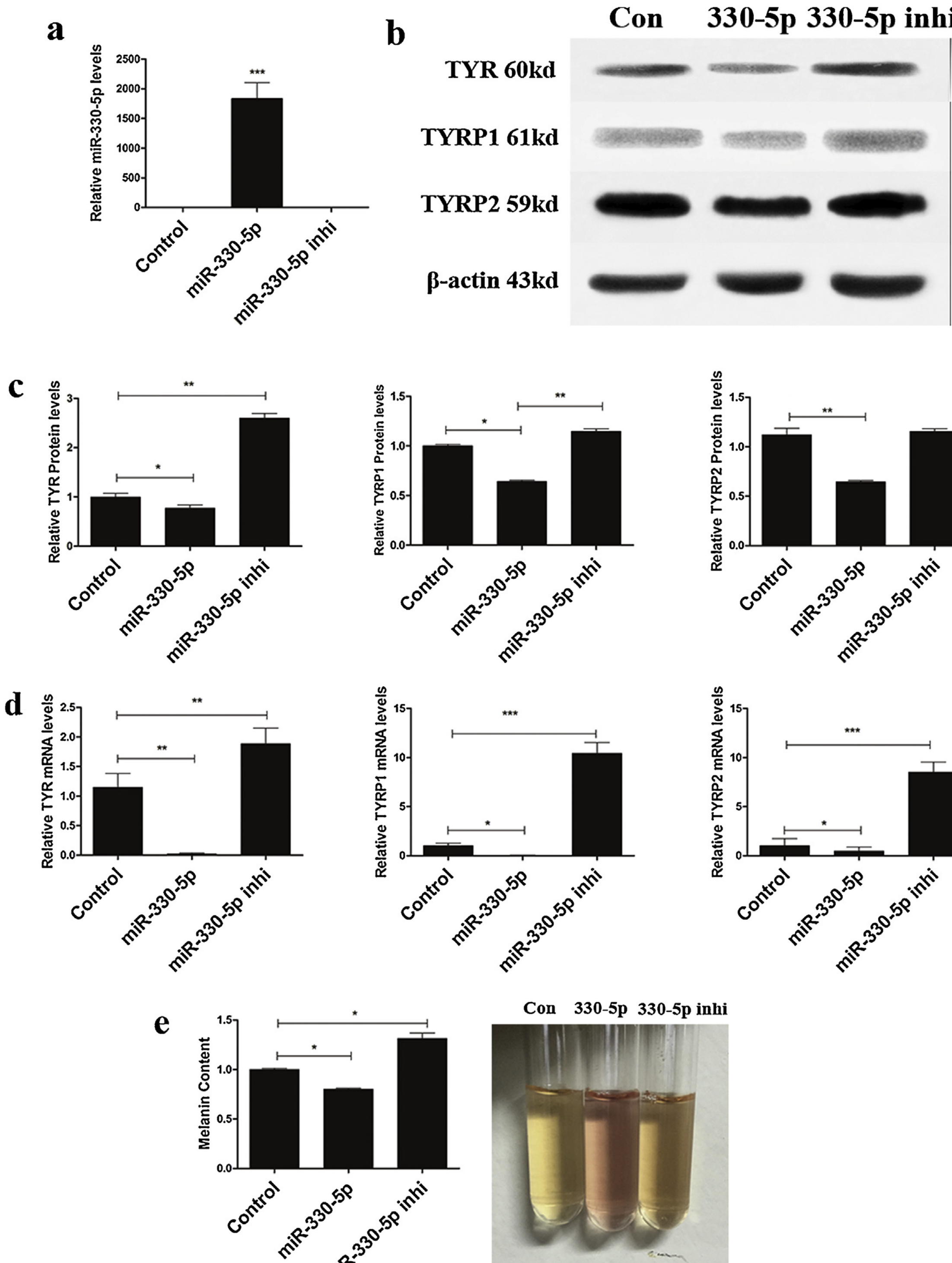

Fig. 4. The effect of miR-330-5p on melanocytes. (a) The mRNA expression of miR-330-5p in melanocytes of control, overexpressed miR-330-5p and inhibitor miR-330-5p groups. (b) Western blotting of TYR, TYRP1, and TYRP2 in cells transfected with labeled miRNAs. (c) Respectively, the levels of proteins in Fig. 1b were quantitated by densitometry and normalized to β-actin levels. (d) The relative mRNA expression of TYR, TYRP1 and TYRP2 in melanocytes transfected with miRNAs. (e) Intracellular melanin content of melanocytes after transfection of mimic miR-330-5p and inhibitor miR-330-5p. The melanocytes that were transfected with miR-NC were used as controls. Graphs represents the fold-induction upon miRNA transfection with control set at 1. Data are shown as mean +/- SD. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$.

(FITC)-labelled TSG101 probe (green) and Texas Red-labelled miR-330-5p probe (red). As shown in Fig. 2, the green fluorescence (exosomal marker, TSG101) became stronger after the exosomes were added to the melanocyte culture system, and the red fluorescence (miR-330-5p) was strongest when exosomes derived from keratinocytes with overexpressed miR-330-5p were added. These results suggest that exogenous exosomes could be transferred into melanocytes to release miR-330-5p. In addition, these results reinforce the mechanism of the crosstalk between keratinocytes and melanocytes via miR-330-5p.

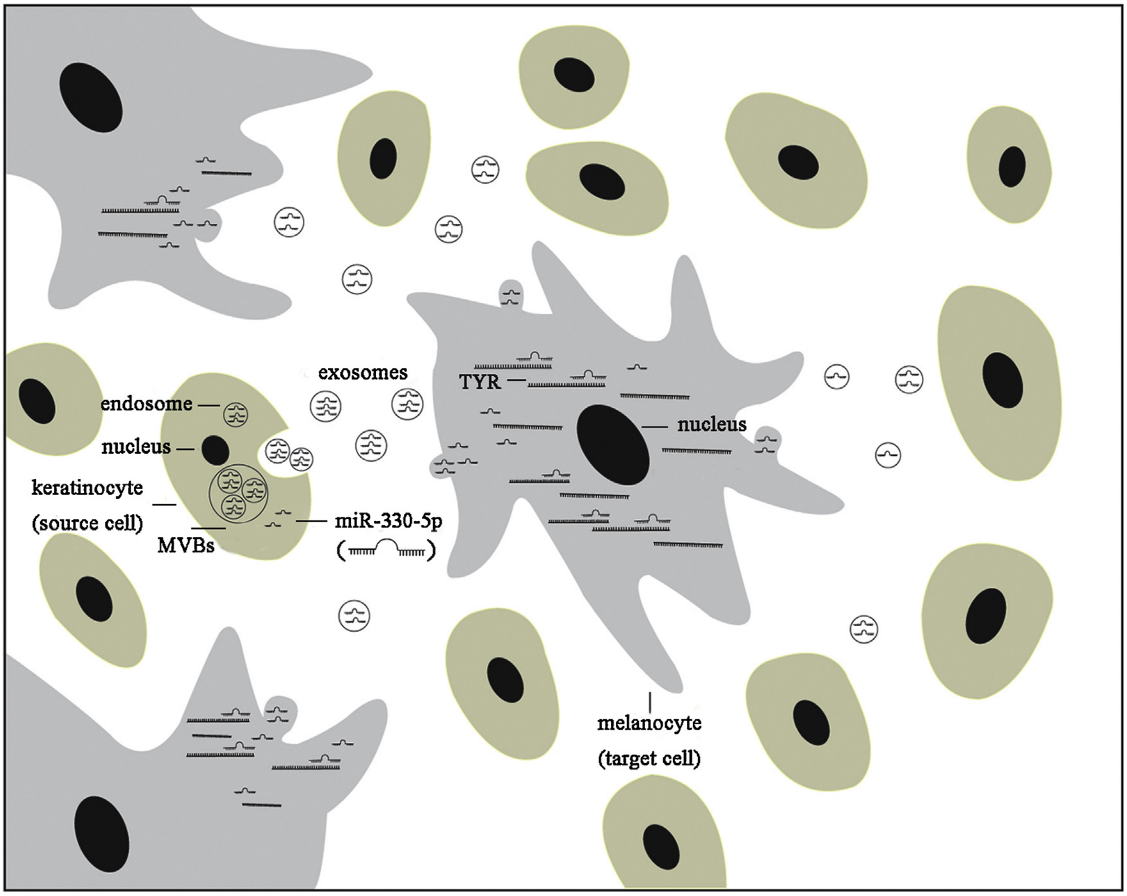

Fig. 5. The interaction of keratinocytes with melanocytes via exosomal miRNAs. The schematic diagram imitating the process of exosomes synthesis, transport, and release active substances into target cell. The endosomes of keratinocytes inward budding produces intraluminal vesicles that accumulate to form multivesicular bodies (MVBs). The MVBs fuse with the plasma membrane, release exosomes containing miR-330-5p into the extracellular space. The exosomes contact with melanocytes and release the active substances (e.g. miR-330-5p) into melanocytes. miR-330-5p regulates pigmentation by inhibiting TYR expression in melanocytes.

3.3. Exosomes carry miR-330-5p that regulate melanocyte pigmentation

The results in Fig. 2 indicate the interaction between exosomes and melanocytes. Next, we investigated whether miR-330-5p contained in the exosomes could mediate and affect melanin production and gene expression. To determine the effect of exosomes carrying miR-330-5p in melanocytes, we added exosomes into the melanocyte culture system. Immunofluorescence microscopy showed that the expression of TSG101 increased when exosomes were added into the melanocytes (Fig. 3a).

Tyrosinase family genes including TYR, TYRP1 and TYRP2, are very important in melanin synthesis [33]. It was reported that MITF regulates gene expression [34]. To evaluate whether TYR [28] is the target gene of miR-330-5p, we measured the expression of TYR, TYRP1, TYRP2 and MITF in melanocytes treated with different exosomes. We found that the expression levels of TYR, TYRP1, TYRP2 and MITF proteins and genes were downregulated in the exosomes overexpressed with the miR-330-5p group compared with the control (Fig. 3b–d). The melanin content in melanocytes was also decreased when exosomes that overexpressed miR-330-5p were added (Fig. 3e). These results reinforce a role of miR-330-5p in the downregulation of TYR in melanocytes, as reported. In summary, our results highlight that the exosomes secreted by keratinocytes can carry their miRNAs to regulate melanocyte pigmentation.

3.4. MiR-330-5p downregulates TYR in melanocytes

To test miR-330-5p plays a role in melanocytes, we transfected melanocytes with miR-330-5p mimics or miR-330-5p inhibitor using liposome transfection techniques. RNA and protein were analyzed in these melanocytes 56 h after transfection. In the miR-330-5p mimics group, the real-time PCR analysis showed that the relative mRNA expression of miR-330-5p was 1825-fold higher than that of the control or inhibitor groups (Fig. 4a), indicating that miR-330-5p was effectively transfected into melanocytes. Next, we measured the expression levels of TYR, TYRP1 and TYRP2, three key genes for melanin production. The results indicated that miR-330-5p significantly suppressed both the protein (Fig. 4b–c) and gene expression (Fig. 4d) of all three genes. In contrast, miR-330-5p inhibitor increased their expression vs. that of the control (Fig. 4b–d). Finally, we measured the melanin content in the cell culture medium and found that miR-330-5p significantly inhibited melanin content in melanocytes (Fig. 4e). We concluded that miR-330-5p suppresses TYR expression in melanocytes to suppress pigmentation.

4. Discussion

Coat color is determined by melanocyte stem cell growth, melanogenesis, the formation and transportation of melanosomes, keratinocytes receiving melanosomes and its distribution, etc. Our study demonstrated that in animal skin or hair follicles, keratinocytes and melanocytes can communicate with each other through exosomes and that exosomes transport specific miRNAs (miR-330-5p) into melanocytes to regulate pigmentation. These results were supported by reports from Kim, N.H. et al, and their results suggest that miR-675 derived from keratinocyte-secreted exosomes participate in H19-stimulated melanogenesis via the MITF signalling pathway [35]. Furthermore, there are more than 30 differentially expressed miRNAs of exosomes secreted by keratinocytes in blacks and Caucasians [2]. These studies in humans reinforce our view that the mouse keratinocyte secreted exosomes can establish contact with the surrounding cells in addition to soluble factors; exosomes can also selectively carry specific miRNAs to target melanocytes by altering gene expression and enzyme activity in regulating pigmentation. Our results also showed that MITF was decreased by adding exosomes to melanocytes (Fig. 3b–d). Since MITF can activate the tyrosinase promoter to control the expression of TYR and tyrosine-related proteins [36], whether exosomes target MITF via miRNAs or bioactive substances is unknown and needs further analysis and verification in the future. Tarafder showed that Rab11b coupled with exo/endocytosis affects melanin transportation between melanocytes and keratinocytes [37]. This process may also involve the function of exosomes. Recent studies have also shown that ultraviolet-B (UV-B) radiation of melanocytes activates nitric oxide, which acts on melanogenesis pathways through upregulating melanocortin-1 receptor [38], and the nitric oxide synthase in keratinocytes is involved in this process [39]. In addition, UV-B radiation can increase the content of miR-448 in keratinocytes and decrease melanin production by acting on melanocytes [26]. Thus, the external environment can affect pigmentation by influencing both melanocytes and keratinocytes. Under these conditions, melanocytes and keratinocytes are sensitive to UV radiation, and melanin can absorb UV radiation to protect human skin from DNA damage [40]. Such a function requires close cooperation and communication between keratinocytes and melanocytes.

Exosomes are important mediators of cell-to-cell communication, which are involved in the immune response, apoptosis, angiogenesis, inflammation and other biological processes. Cells secrete exosomes that present signal molecules to nearby or distant tissues or cells and play a regulatory role [41]. Chavez-Munoz demonstrated that keratinocytes can secrete exosomes to transport stratifin to stimulate the activity of MMP-1 in fibroblasts [42]. Joshua L's study showed that melanoma exosomes that depend on the establishment of angiogenesis contribute to the growth of tumors [43]. WNT5A, which is released from exosomes, could strengthen the invasion and metastasis ability of melanoma cells [44]. Some evidence has shown that exosomes carrying miRNAs can discriminate target mRNAs in the recipient cells [45]. In addition, when the expression of miRNAs in the cells changed, the corresponding miRNAs in the exosomes also showed the same trend [46]. Alessandra Lo Cicero's study indicated that human keratinocytes were irradiated by ultraviolet-B, and the content of miR-3196 in exosomes secreted by these keratinocytes were significantly different [2]. The data presented in the previous study showed that exosomes isolated from serum of active vitiligo patients differentially expressed 47 miRNAs, compared with healthy controls [47]. miR-222 [16], miR-125b [48], miRNA-17, miRNA-19a, miRNA-21, miRNA-126, and miRNA-149 [49] are differentially expressed in melanoma patients, compared with normal controls. Yoon C indicated that Burkitt's lymphoma exosomes can deliver miR-155 to retinal pigment epithelial cells [50]. Our results showed that exosomes derived from keratinocytes carried miR-330-5p and can affect pigmentation (Fig. 5). In conclusion, the emergence of the exosome field provides an interesting opportunity to further understand cell-to-cell communication in keratinocyte and melanocyte crosstalk.

Therefore, the transport of biological molecules via exosomes is a novel potential method for the regulation of pigmentation. This study will open a new horizon to explain the formation of coat color and provide a theoretical basis for obtaining more coat color resources in animal husbandry.

Conflicts of interest

The authors state no conflicts of interest.

Author contributions

Haidong Wang and Liping Zhang designed research; Ying Liu and Linli Xue analyzed data; Ying Liu, Linli Xue, Hang Gao, Lucheng Chang, Xiuju Yu, Zhiwei Zhu performed research; Ying Liu and Haidong Wang wrote the paper; Xiaoyan He and Jianjun Geng contributed new reagents or analytic tools; and Yanjun Dong and Hongquan Li developed software necessary to perform and record experiments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.02.001.

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