Article

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Proprotein Convertase Furin Regulates Melanogenesis via the Notch Signaling Pathway

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Background: Furin is a calcium-dependent serine protease found in almost all mammals. It plays an important role in embryogenesis, tissue homeostasis, tumors pathogenesis, viral infectious diseases, and neurodegenerative diseases. However, whether furin directly regulates melanin synthesis and transport has rarely been evaluated yet. The present study aimed to investigate furin potential function and mechanisms in melanogenesis.

Methods: Short hairpin RNAs targeting *furin* gene (sh-furin RNAs) were used to inhibit *furin* gene expression in human melanoma cell line MNT-1 cells. Then, intracellular melanin content was measured using a sodium hydroxide method. Extracellular melanin content was measured determining cell culture medium absorbance at 450 nm. Levodopa (L-DOPA) oxidation rate was measured to assess the tyrosinase activity. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting (WB) were performed to measure melanogenesis-related genes and Notch pathway-related genes expression levels. Human primary melanocytes (MCs) were extracted from foreskin tissues and were stimulated with a furin inhibitor. Then, the extracellular and intracellular melanin content, tyrosinase activity and molecules related to melanogenesis and the Notch pathway expression were measured in MCs with or without a furin inhibitor. Additionally, morpholino technology was used to inhibit furin in zebrafish. Zebrafish pigmentary phenotypes in the control group and furin inhibition group were observed with a stereo microscope. Then, MCs number in the tail and head of the zebrafish were counted using Image J software (version 1.53t, National Institute of Health, Bethesda, MD, USA). Meanwhile, melanin content, tyrosinase activity, and molecules related to melanogenesis and the Notch pathway expression levels were measured. Subsequently, valproic acid (VPA), a Notch pathway agonist, was used in MNT-1 melanoma cells treated with or without sh-furin lentiviral vectors for rescue experiments.

Results: Furin inhibition enhanced intracellular and extracellular melanin content, and cellular tyrosinase activity in MNT-1 cells and MCs. Additionally, furin inhibition increased melanin synthesis-associated and transport-associated proteins expression levels while inhibiting Notch pathway-relevant proteins. After using VPA to activate the Notch pathway in MNT-1 cells transfected with a sh-furin RNA, the biological effects resulting from furin knockdown were reversed. In addition, the results of *in vivo* experiments using morpholino to knock down *furin* gene in zebrafish further confirmed that furin knockdown regulated melanogenesis and impaired the Notch pathway.

Conclusions: This study clarified that furin affected the synthesis and transport of melanin via Notch pathway. Notch pathway may be a potential therapeutic target for pigmented skin diseases.

Keywords: furin; melanogenesis; melanin transport; Notch pathway; zebrafish

Introduction

Melanin is a well-known biopolymer generated from tyrosine. Its principal functions include skin tone maintenance and protection from ultraviolet radiation damage [1,2]. Melanin synthesis and storage occur in the melanosomes, which are characteristic cyst-like organelles of melanocytes (MCs) [3]. MCs produce melanin and then transfer it from the dendrites to adjacent keratinocytes in the metabolic process known as melanogenesis [4]. Aberrant melanin production or dysfunction of melanin transport can lead to skin pigmentary disorders (freckles, melasma, vitiligo, *et al.*) [5]. Importantly, pigmentary disorders may pose cosmetic concerns to patients and impair their social life, leading to psychosocial distress, affecting their quality of life [6]. Therefore, more research on melanogenesis mechanisms and regulation is essential to treat pigmentary disorders.

Melanin synthesis and transport are complex biological processes regulated by multiple factors. Tyrosinase (*TYR*), tyrosinase-related protein-1 (*TYRP-1*), and tyrosinase-related protein-2 (*TYRP-2*) are three essential enzymatic proteins that regulate melanogenesis process [7]. Their expression and activity can reflect melanin production levels and be used as biological markers for melanin production [8,9]. Microphthalmia-associated transcription factor (*MITF*) is the most important melanogenesis regulator [10]. It affects melanin production and transport by directly regulating melanin synthesis-related enzymes (*TYR*, *TYRP-1* and *TYRP-2*) and melanosome-related proteins expression [11]. Moreover, *MITF* is also involved in the survival and proliferation of MCs [12]. Fascin 1 (FSCN1) is one of the important molecules that can boost MCs migration and proliferation [13]. Myosin Va (Myo5a), Ras-associated protein Rab-27A (Rab27a), and Melanophilin (Mlph) can form a complex and this ternary complex can regulate melanosome motility [14]. Therefore, proteins Myo5a and Rab27a can be used as markers for melanin transport. It is worth mentioning, fibrous lumenal striations are pre-melanosomes morphological hallmarks and can serve as the place for deposition and melanin condensation [15]. While pre-melanosome protein 17 (*Pmel*17) is a chief determinant in fibrous lumenal striations biogenesis [15]. Hence, *Pmel*17 can be used as a molecular marker for melanosome formation.

Furin, a member of the subtilisin/Kexin family of proprotein convertases, is widely expressed in animals and plays a significant role in embryonic development, homeostasis, and various diseases [16,17]. To date, most studies on furin have focused on tumor progression [18,19], viral protein processing [20], cardiovascular diseases [21, 22], and neurodegenerative and neuropsychiatric diseases [16,23]. Only few studies have focused on the fact that furin may be involved in melanin production. A study in 2003 found that furin's cleavage on *Pmel*17 was vital for the melanosomes intraluminal fibrils development [24]. Another study has shown that furin participated in promelanin concentrating hormone cleavage process in cells lacking of regulated secretory pathway [25]. *In vitro* and *in vivo* data revealed that additional proprotein convertases (PC), such as furin convertase may also process proopiomelanocortin (POMC) [26], whose products include α-melanocyte-stimulating hormone (α-MSH) [27]. Moreover, α-MSH was proven to be an important factor to regulate melanogenesis [27]. Nevertheless, whether furin has a direct function in melanin synthesis and transport in MCs is still unclear.

Herein, this study provided the first description of furin's direct impact on melanin synthesis and transport.

Materials and Methods

Reagents and Antibodies

Hexa-D-arginine (D6R) (Cat. No. HY-P1028, 99.57% purity), valproic acid (VPA) (Cat. No. HY-10585, $\geq$98.0% purity), and levodopa (L-DOPA) (Cat. No. HY-N0304, 99.98% purity) were purchased from MedChemExpress (MCE) (Monmouth Junction, NJ, USA). Antibodies against *MITF* (12590S, 1:4000), *Rab27A* (69295S, 1:1000), Myo5a (3402S, 1:1000), FSCN1 (99978S, 1:1000), *Notch-1* (4380T, 1:1000), *Notch-2* (5732T, 1:1000), *Notch-3* (5276T, 1:1000), *Jagged-1* (2620T, 1:1000), *Jagged-2* (2210T, 1:1000), delta-like ligand 1 (*DLL-1*) (2588T, 1:1000), delta-like ligand (*DLL-4*) (2589T, 1:1000), hairy and enhancer of split-1 (*Hes-1*) (11988S, 1:1000), Cleaved *Notch-1* (N1ICD) (4147S, 1:1000), and β-*actin* (4970S, 1:2000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against furin (MA534677, 1:1000) was obtained from Invitrogen (Waltham, MA, USA). Antibody against delta-like ligand 3 (*DLL-3*) (25535-1-AP, 1:1000) was obtained from proteintech (Rosemont, IL, USA). Finally, antibodies against *TYR* (ab170905, 1:4000), *TYRP-1* (ab235447, 1:8000), and *TYRP-2* (ab221144, 1:4000) were obtained from Abcam (Cambridge, UK).

Cell Culture

Human melanoma cell line MNT-1 cells were obtained from American Type Culture Collection (ATCC) (CRL-3450, Manassas, VA, USA). MNT-1 cell line was validated with short tandem repeat (STR) analysis (intraspecies) by ATCC. No mycoplasma contamination was determined by Hoechst DNA stain (indirect) method, agar culture method and polymerase chain reaction (PCR)-based assay in MNT-1 melanoma cells. MNT-1 cells were cultured in accordance with a standard procedure. Human skin MCs were extracted from foreskin tissues as previously described with a minor modification [28]. MCs were identified by morphological observation method and confirmed by detecting specific markers of MCs (such as *TYR*, *TYRP-1* and *TYRP-2*). MCs were cultured in melanocyte medium MelM (2201, Sciencell, Carlsbad, CA, USA). Cells were cultured in a 5% CO_2 incubator at 37 °C. Cells were collected after treatment with different concentrations of D6R or VPA for RNA extraction or protein extraction, melanin content and tyrosinase activity.

Cell Infection

Three short hairpin RNA (shRNA) vectors targeting human *furin* gene (gene ID: 5045) were designed based on the gene sequence to avoid an off-target effect (sh-furin-1, 5′-GGACTTGGCAGGCAATTATGA-3′; Sh-furin-2, 5′- GCTCACCCTGTCCTATAATCG-3′; Sh-furin-3, 5′-GGTTTAATGACTGGGCCTTCA-3′). A scrambled shRNA sequence was used as negative control

(5′-GCACTATAGCACCGAGAATGA-3′). All lentiviral shRNAs were purchased from GenePharma Biotechnology Company (20210310, Shanghai, China). Then, lentiviruses were used to infect MNT-1 cells following manufacturer's standard procedure. Afterward, 1 μg/mL puromycin was used to select cells positively transfected with lentivirus particles.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were harvested and then split using the total RNA extraction reagent TRIZOL (15596026, Invitrogen, Waltham, MA, USA) to extract total cellular RNA. Following HiScript III RT SuperMix for qPCR kit instructions (+gDNA wiper) (R323-01, Vazyme, Nanjing, China), cDNA (complementary deoxyribonucleic acid) was prepared. PCR system was prepared using the ChamQ Universal SYBR (sensitive fluorescent DNA binding stain) qPCR Master Mix Kit (Q711-02, Vazyme, Nanjing, China). In **Supplementary Table 1**, sequences of primers used in the *in vitro* experiments are listed. Differences in target molecules mRNA (messenger ribonucleic acid) levels were detected using the LightCycler 480 qRT-PCR detection system (05015243001, Roche, Basel, Switzerland). PCR amplification conditions were performed according to the kit's instructions: 30 seconds at 95 °C; 40 cycles of 10 seconds at 95 °C and 30 seconds at 60 °C; 15 seconds at 95 °C, 60 seconds at 60 °C and 15 seconds at 95 °C. Relative target genes mRNA expression levels were normalized to β-*actin*. The $2^{-\Delta\Delta Ct}$ method was used to assess the fold difference between the two groups.

Protein Extraction and Western Blot (WB)

Radio-immunoprecipitation assay buffer (P0013B, Beyotime, Shanghai, China) and protease inhibitors (P8340, Sigma-Aldrich, St. Louis, MO, USA) were added to cell pellets to lyse cells for total protein extraction. Then, protein quantification was conducted using a bicinchoninic acid kit (P0012, Beyotime, Shanghai, China). Proteins of equal rank were loaded into SmartPAGE™ Precast Protein Gel (SLE001 and SLE019, smart-Lifesciences, Changzhou, China). Then, the proteins were transferred onto a polyvinylidene difluoride membrane (IPVH00010, Millipore, Billerica, MA, USA) using an electrophoretic transfer system (1703930, Bio-Rad, Hercules, CA, USA). After the membrane was immersed in Every Blot Blocking Buffer (12010947, Bio-Rad, Hercules, CA, USA) in a shaker for 5–15 minutes to block nonspecific binding sites, incubation with primary antibodies overnight in a 4 °C refrigerator was conducted. Subsequently, the membrane was incubated in a shaker with secondary antibodies for 1–2 hours at 20–30 °C. Next, the membrane was visualized and determined by enhanced chemiluminescence substrates (1705062, Bio-Rad, Hercules, CA, USA), Image Lab software (version 5.2.1, Bio-Rad, Hercules, CA, USA), and Image J (version 1.53t, National Institute of Health, Bethesda, MD, USA). β-*actin* was used as a reference to normalize target proteins.

Intracellular Melanin Content Assay

Sodium hydroxide (NaOH) method was applied to assess intracellular melanin content [29]. Cells were harvested at the same number (MNT-1 cells: 5×10^5 cells; MCs: 2×10^5 cells). After washing cell pellets in phosphate-buffered saline (PBS) twice, cell pellets were dissolved in 200 μL NaOH solution (1 mmol/L, containing 10% dimethylsulfoxide) and incubated at 37 °C for 2 hours. After 10 minutes of centrifuging at 12,000 g, supernatant absorbance at 450 nm was determined with a spectrophotometer.

Extracellular Melanin Content Assay

MNT-1 cells, with or without sh-furin RNAs (short hairpin RNAs targeting furin) transfection, were cultivated in 6-well plates in a 5% CO_2 incubator for 48 hours at 37 °C (5×10^5 cells/well). Then, cell culture medium was changed with a fresh culture medium after incubation at 37 °C for 48 hours, and the MNT-1 cells were cultured for another 48 hours.

MCs were cultivated in 12-well plates for 24 hours (2×10^5 cells/well) at 37 °C. After changing the culture medium with a fresh culture medium containing D6R (0, 0.1, 0.2, 0.4 mg/L), the MCs were cultivated for another 72 hours.

MNT-1 cells with or without sh-furin RNAs transfection were cultivated in the 6-well plates for 24 hours (5×10^5 cells/well). Then, the cell culture medium was replaced with a fresh culture medium containing VPA (0, 0.1, 0.2, and 0.4 mmol/L (mM)). Successively, MNT-1 cells were cultured for another 72 hours.

Finally, the cell culture medium was collected. For extracellular melanin content, the cell culture conditional medium was shortly centrifuged and transferred into 96-well plates, where it was directly detected at 450 nm using a spectrophotometer [29].

Tyrosinase Activity Assay

L-DOPA oxidation rate was measured to assess tyrosinase activity as previous described with a minor modification [30,31]. In brief, the same number of cells were collected, and then washed with PBS. Secondarily, cell pellets were lysed with 200 μL of PBS containing 1% Triton X-100, and after that, they were frozen for 30 minutes in a –80 °C freezer. Then, cell pellets were melted at 37 °C and the deposit was separated by a centrifugation at 10,000 g for 5 minutes at 4 °C. Then, the supernatant was added to the 96-well plates and treated with 10 μL L-DOPA solution (1 mg/mL) for 2 hours at 37 °C. To assess the intracellular tyrosinase activity, solution absorbance at 490 nm was de-

tected by spectrophotometers. Tyrosinase activity was calculated as:

$(\%) = (OD490_{treatment\ group} - OD490_{blank\ group})/(OD490_{control\ group} - OD490_{blank\ group})$.

Zebrafish Experiments

Wild-type line AB strain zebrafish were provided by the company EzeRinka Biotechnology Co., Ltd. (EZSJ001, Nanjing, China). All the wild-type AB strain zebrafish were naturally paired, and healthy zebrafish embryos were collected 30 minutes after spawning. Subsequently, zebrafish embryos in the 1-cell stage were selected for morpholino injection. Under a stereo microscope (SZX16, Olympus, Tokyo, Japan), the zebrafish embryos in 1-cell stage were injected with 1 nL of control morpholino (Ctrl-MO) (6 μg/μL) or furin morpholino (furin-MO) (6 μg/μL). The morpholino sequences used in the experiments are as follows: Furin-MO: 5′-GAGGGACTCACAATCTGTTTCTCAT-3′; The Ctrl-MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′. To assess furin effect on pigment production in zebrafish, after morpholino treatment at 24, 48, 72, and 96 hours post fertilization (hpf), photographs were taken under a stereo microscope (SZX7, Olympus, Tokyo, Japan) to observe the pigmentary zebrafish phenotype. Then, the number of MCs in the tail and head of the zebrafish at 48 hpf and 72 hpf were counted using Image J software (version 1.53t, National Institute of Health, Bethesda, MD, USA). Meanwhile, zebrafish tissues were extracted at 96 hpf for melanin content, tyrosinase activity, and qRT-PCR, as previously described. The primers for zebrafish experiments are exhibited in **Supplementary Table 2**.

Statistical Analysis

Experimental results were all executed in triplicate or more. GraphPad Prism software (version 9.0.0, GraphPad Software Inc., Boston, CA, USA) was used for statistical analysis. All data were transformed into bar graphs and analyzed. Differences between groups were assessed with a student's *T*-test. Statistical difference was defined as a p value < 0.05.

Results

Furin Inhibition Increased Extracellular and Intracellular Melanin Content of MNT-1 Melanoma Cells and MCs

In order to investigate whether furin affected melanin synthesis and transport, three sh-RNA vectors were designed to knock down the *furin* gene in MNT-1 melanoma cells. As shown in Fig. 1A, MNT-1 cells transfection with sh-furin-1 and sh-furin-3 RNA resulted in about a 70% decrease in furin's mRNA level. Similarly, MNT-1 cells transfection with sh-furin-1 and sh-furin-2 RNA significantly dropped furin protein level, while MNT-1 cells transfection with sh-furin-3 RNA had no effect on the protein level (Fig. 1B). Therefore, sh-furin-1 RNA was used for subsequent experiments.

Hereafter, the effects of using sh-furin-1 lentiviral vectors to infect MNT-1 cells on melanin production was evaluated. Intracellular melanin content measurement was conducted using cell pellet extracts, while extracellular melanin content was assessed by measuring cell culture medium absorbance. An shRNA with a scrambled sequence was used as a negative control (NC). Cells infected with sh-furin lentiviral vectors had an increase intracellular melanin content (332.2% $\pm$ 7.3%, $p < 0.0001$) (Fig. 1C) and extracellular melanin in the culture medium (146.2% $\pm$ 7.3%, $p < 0.0001$) (Fig. 1D) compared to NC group. These results showed that furin knockdown accelerated intracellular melanin production and increased melanin secretion from cells to culture medium in MNT-1 cells.

Subsequently, different D6R concentrations (a stable furin inhibitor [32]), were applied to treat MCs for 72 hours. Overall, 0.1, 0.2, and 0.4 mg/L of D6R enhanced intracellular melanin content (240.7%, 223.0%, and 257.2%, respectively) (Fig. 1E) and secretion of melanin in MCs (138.4%, 151.1%, and 144.1%, respectively) (Fig. 1F).

Furin Inhibition Increased Tyrosinase Activity and Expression Levels of Melanin Synthesis-Relevant and Transport-Relevant Genes

Whether furin regulated melanin synthesis by modulating tyrosinase activity was further evaluated. As shown in Fig. 2A, cellular tyrosinase activity was up-regulated by 290.1% in MNT-1 cells transfected with sh-furin RNA compared to the NC group ($p < 0.0001$). Similarly, after MCs treatment with D6R (0, 0.1, 0.2, and 0.4 mg/L), MCs tyrosinase activity increased by 234.7%, 185.8%, and 87.5%, respectively, compared to the control group (Fig. 2B).

Next, Melanin synthesis-relevant and transport-relevant molecules expression levels in MNT-1 cells infected with sh-furin RNA and MCs treated with 0.1 mg/L D6R were detected. As expected, we observed that furin knockdown increased melanogenesis-relevant genes mRNA and protein levels (*MITF*, *TYR*, and *TYRP-2*) (Fig. 2C,D), melanin transport-related genes expression (Rab27a and Myo5a) (Fig. 2E,F), and *TYRP-1* protein levels (Fig. 2D) in MNT-1 cells. However, FSCN1 protein level in MNT-1 cells infected with sh-furin RNA was reduced, which indicated that furin may affect MCs migration (Fig. 2F). Interestingly, furin knockdown decreased Peml mRNA level (Fig. 2C), which was consistent with the previous study [24] and suggested that furin may influence melanosomes biogenesis. The change in melanogenesis-related and melanin transport-associated genes protein expression level in MCs with 0.1 mg/L D6R treatment was the same as in MNT-1 cells infected with sh-furin RNA (Fig. 2G,H).

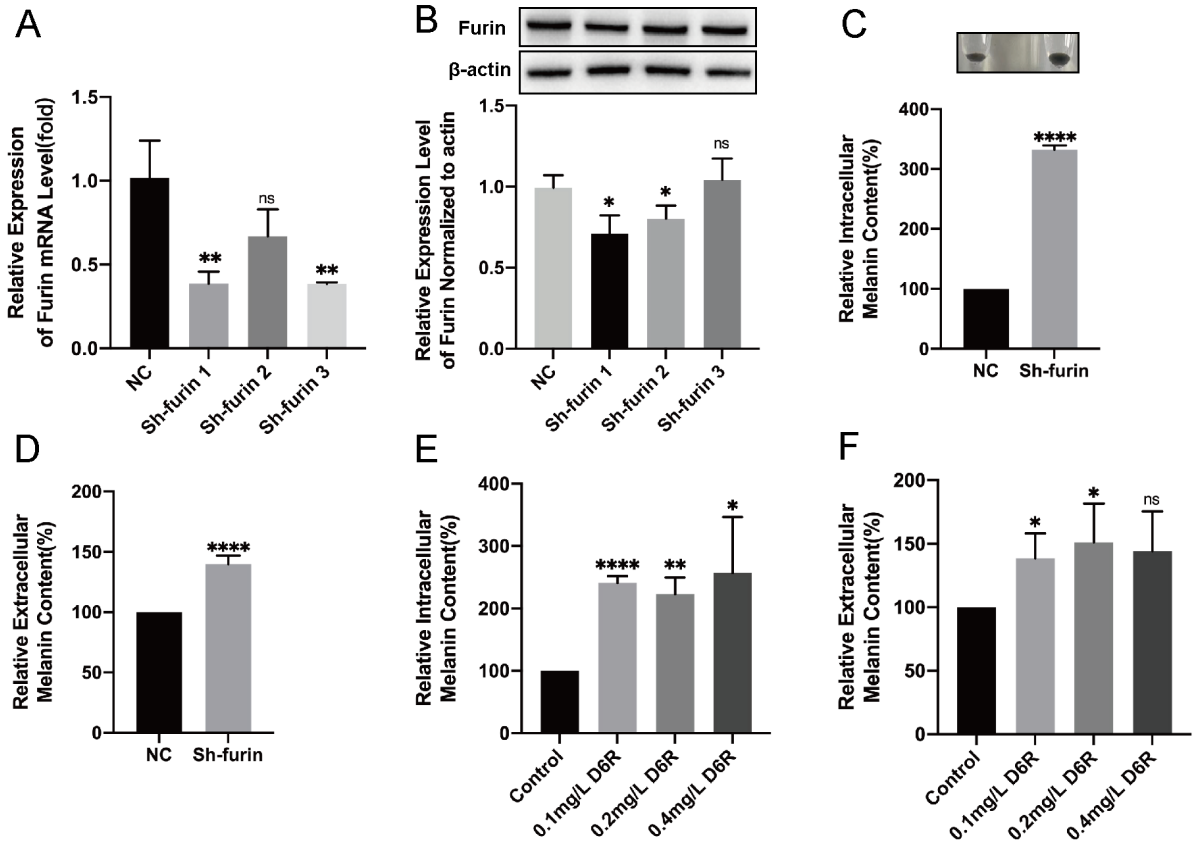

Fig. 1. Furin inhibition up-regulates melanogenesis and melanin transport. *Furin* gene was knocked down in MNT-1 melanoma cells using three short hairpin RNA (shRNA) vectors. (A) Furin mRNA and (B) furin protein expression levels were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot (WB) assays, respectively. (C) Intracellular melanin content and (D) extracellular melanin content in MNT-1 cells transfected with sh-furin RNA were determined and a corresponding panel showing cell pellets photos was created. (E) MCs intracellular melanin content and (F) extracellular melanin content were measured after treating MCs with different D6R concentrations (0, 0.1, 0.2 and 0.4 mg/L) for 72 hours. All results are displayed as mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ and ns no significance vs. negative control (NC) group or control group. N = 3.

In conclusion, these findings revealed that furin inhibition promoted melanin synthesis and transport by up-regulating tyrosinase activity and affecting melanogenesis-relevant molecules expression level.

Furin Inhibition is Accompanied by Inhibition of the Notch Signaling Pathway

Expression level of molecules involved in the Notch signaling pathways in MNT-1 cells transfected with sh-furin RNA was determined. Thus, qRT-PCR data demonstrated that furin knockdown led to an increase in ligand *Jagged-1* expression and a decrease of *DLL-1* and *DLL-3* (ligands of the Notch pathway) in MNT-1 cells (Fig. 3A). Ligands *Jagged-2* and *DLL-4* were not amplified, which may be due to their low expression level. Meanwhile, furin knockdown also decreased *Notch-1*, *Notch-2* (receptors of the Notch pathways), and target gene *Hes-1* mRNA levels (Fig. 3A,B). Nevertheless, no difference was observed in the *Notch-3* and *Notch-4* (receptors of the Notch pathway) mRNA levels (Fig. 3A). Additionally, furin knockdown also impaired the mRNA expression level of the mastermind-like (MAML) family, which is the co-activator of the Notch intracellular domain (NICD) (the active form of the Notch pathway) (Fig. 3B). WB analyses further revealed that *Notch-1*, *Notch-2*, *Notch-3*, *Jagged-1*, *Jagged-2*, *DLL-1*, *Notch-1* intracellular domain (N1ICD) and *Hes-1* protein levels decreased after furin knockdown (Fig. 3C,D). But *DLL-3* and *DLL-4* protein levels in MNT-1 cells transfected with sh-furin RNA did not differ significantly from the NC group (Fig. 3D).

Notch pathway-relevant protein expression in MCs treated with 0.1 mg/L D6R for 72 hours was also determined. Similarly, furin inhibition reduced *Notch-1*, *Jagged-1*, *Jagged-2*, *DLL-1*, N1ICD, and *Hes-1* (Fig. 3E), consistent with those in sh-furin MNT-1 cells. Taken together, these results disclosed that furin inhibition inhibited Notch pathway.

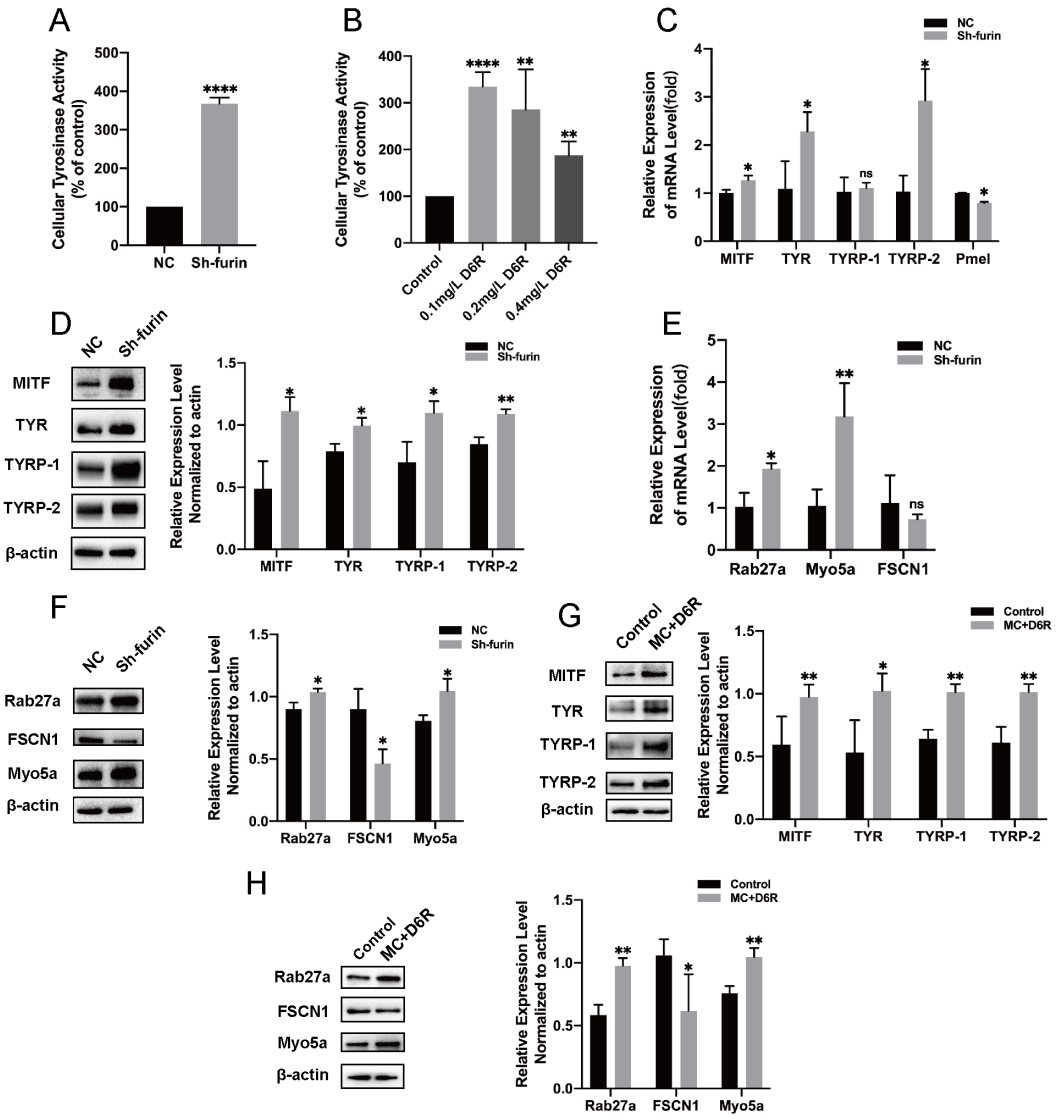

Fig. 2. Furin inhibition increases tyrosinase activity and affects melanogenesis-related and melanin transport-associated genes expression. (A) Tyrosinase activity in MNT-1 cells transfected with sh-furin RNA was measured. (B) Tyrosinase activity in MCs treated with different D6R concentrations was assessed. (C) mRNA and (D) protein expression levels of melanin synthesis-relevant molecules were determined in MNT-1 cells by qRT-PCR and WB assays, respectively. (E) mRNA and (F) protein expression levels of melanin transport-relevant molecules were detected in MNT-1 cells by qRT-PCR and WB assays, respectively. MCs cells were treated with 0.1 mg/L D6R for 72 hours, then melanin synthesis-relevant proteins (G) and (H) melanin transport-related proteins were determined by WB. All results are displayed as mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ and ns no significance vs. NC group or control group. N = 3.

Furin Affected Melanin Synthesis and Transport via the Notch Pathway

To further clarify whether furin inhibition-induced pigmentation depended on the Notch pathway, we applied VPA, which activate the Notch pathway [33], to treat MNT-1 cells transfected with sh-furin RNA. After treatment with VPA, N1ICD expression level was determined by WB assay in MNT-1 cells transfected with sh-furin RNA. As expected, the results suggested that 0.1, 0.2, and 0.4 mM VPA can activate the Notch signaling pathway, but N1ICD expression level did not increase with increasing concentration of VPA (Fig. 4A). Therefore, 0.1 mM VPA was selected for subsequent experiments. VPA reduced intracellular and extracellular melanin content and tyrosinase activity caused by furin knockdown to levels comparable to those in the NC group without VPA treatment (Fig. 4B–D). WB results showed that VPA increased N1ICD and *Hes-1* expression levels in MNT-1 cells transfected with sh-

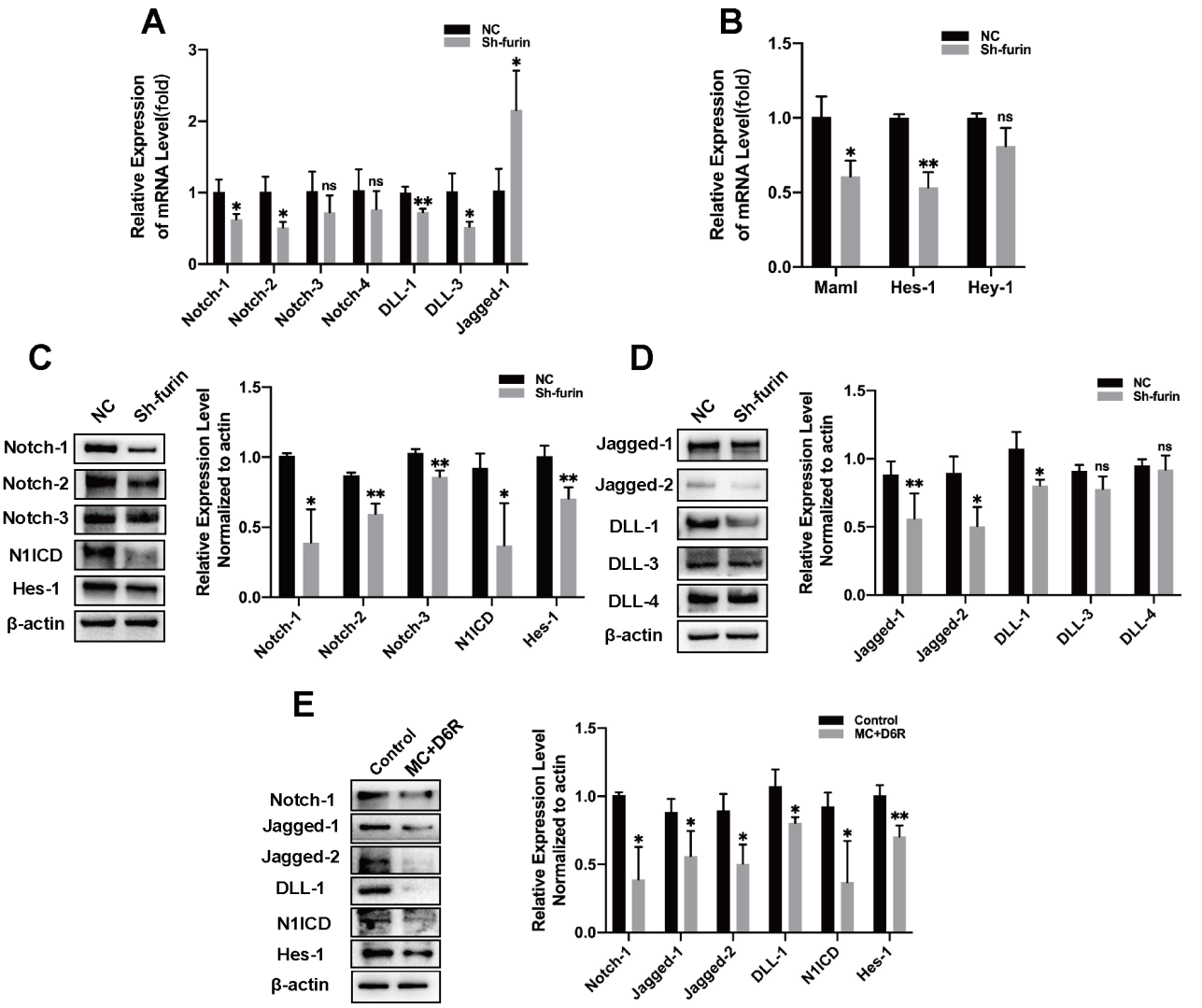

Fig. 3. Furin inhibition effect on Notch pathway. The mRNA expression of (A) receptors, ligands, and (B) Notch pathway downstream genes of MNT-1 cells were assessed by qRT-PCR. (C,D) Notch pathway-relevant proteins expression level in MNT-1 cells were determined by WB assays. (E) *Notch-1*, *Jagged-1*, *Jagged-2*, *DLL-1*, NICD, and *Hes-1* expression level were determined by WB assays in MCs treated with 0.1 mg/L D6R for 72 hours. All results are displayed as mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$ and ns no significance vs. control group. N = 3.

furin RNA (Fig. 4E). Interestingly, VPA further decreased *Notch-1* protein level of in MNT-1 cells transfected with sh-furin RNA (Fig. 4E). VPA reversed the increase of *MITF*, *TYR*, *TYRP-1*, *TYRP-2*, Rab27, and Myo5a caused by furin knockdown (Fig. 4F). In conclusion, furin knockdown induced melanin synthesis and transport via Notch pathway inhibition.

Furin Knockdown Inhibited Melanogenesis and Notch Signaling Pathway in Zebrafish

Furin impact on melanogenesis was also assessed in zebrafish. Interestingly, in contrast to the *in vitro* data, the zebrafish injected with morpholino targeting furin (referred to as the furin-MO group) had an impaired melanin phenotype at 48, 72, and 96 hpf compared to the control morpholino (referred to as the Ctrl-MO group) (Fig. 5A and **Supplementary Fig. 1**). After that, furin effect on the number of MCs in zebrafish larvae was tested. MCs in the tail and head at 48 hpf and the head at 72 hpf were reduced in the furin-MO group (Fig. 5B,C). We also found that melanin content decreased by 85.23 μg/mL (Fig. 5D), whereas tyrosinase activities decreased by 15.78% (Fig. 5E) in the furin-MO group at 96 hpf. Furthermore, furin knockdown in zebrafish resulted in a reduction of *MITF*, *TYR*, *TYRP-1a*, *TYRP-2*, *Pmel*, *Notch-1*, and *Hes-1* at 96 hpf mRNA levels (Fig. 5F).

Additionally, furin affected MCs migration. As shown in **Supplementary Video 1**, MCs migration to the tail was

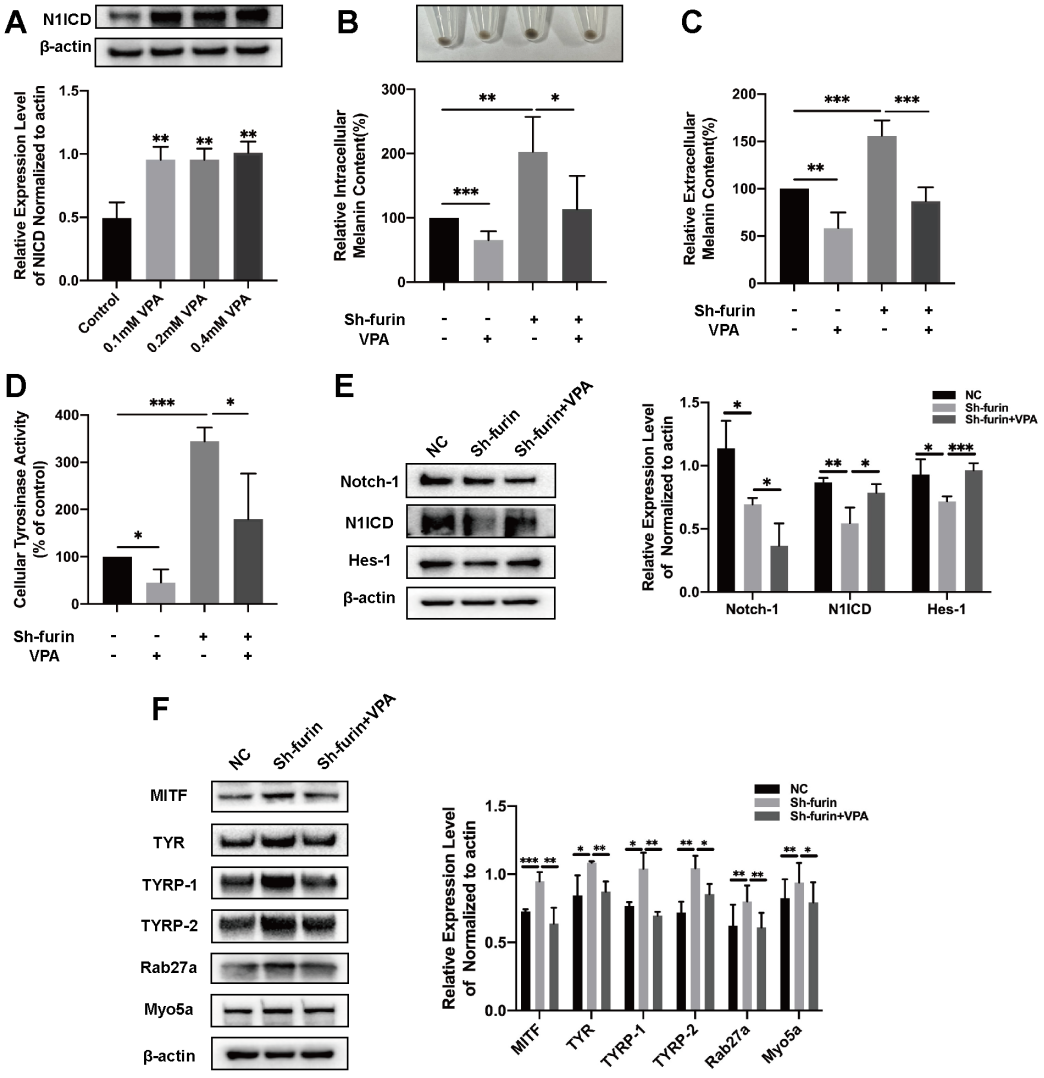

Fig. 4. Effects of valproic acid (VPA) treatment in MNT-1 cells transfected with sh-furin RNA on melanogenesis and the Notch pathway. (A) Expression levels of the Notch intracellular do-main (NICD) in MNT-1 cells treated with VPA (0, 0.1, 0.2, 0.4 mM), as determined by WB. (B) The intracellular melanin content, (C) extracellular melanin content, and (D) tyrosinase activity was detected by methods as described in MNT-1 cells transfected with sh-furin RNA or scramble sh-RNA, with or without 0.1 mM VPA treatment for 72 hours. (E) Expression levels of *Notch-1*, NICD, and *Hes-1* in MNT-1 cells infected with sh-furin RNA, with or without 0.1 mM VPA treatment compared to NC without VPA treatment. (F) Expression levels of melanin synthesis-related and transport-related proteins in MNT-1 cells infected with sh-furin RNA, with or without 0.1 mM VPA treatment compared to NC without VPA treatment. All results are displayed as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC group. N = 3.

smooth and continuous in Ctrl-MO zebrafish. But furin-MO zebrafish had disorganized pigment distribution and decreased migration ability (**Supplementary Video 2**). In brief, zebrafish experiments indicated that furin knockdown can inhibit the Notch signaling pathway and affect melanogenesis.

Discussion

Furin is a calcium-dependent serine endoprotease mainly responsible for cleaving its substrates into their biologically active forms [34]. Up until now, furin has been shown to cleave more than 150 substrates with important biological functions [35,36], including two hormones that regulate melanogenesis [25,26]. However, furin potential role in melanin synthesis and transport process is not clear. Previous *in situ* and *in vitro* experiments confirmed

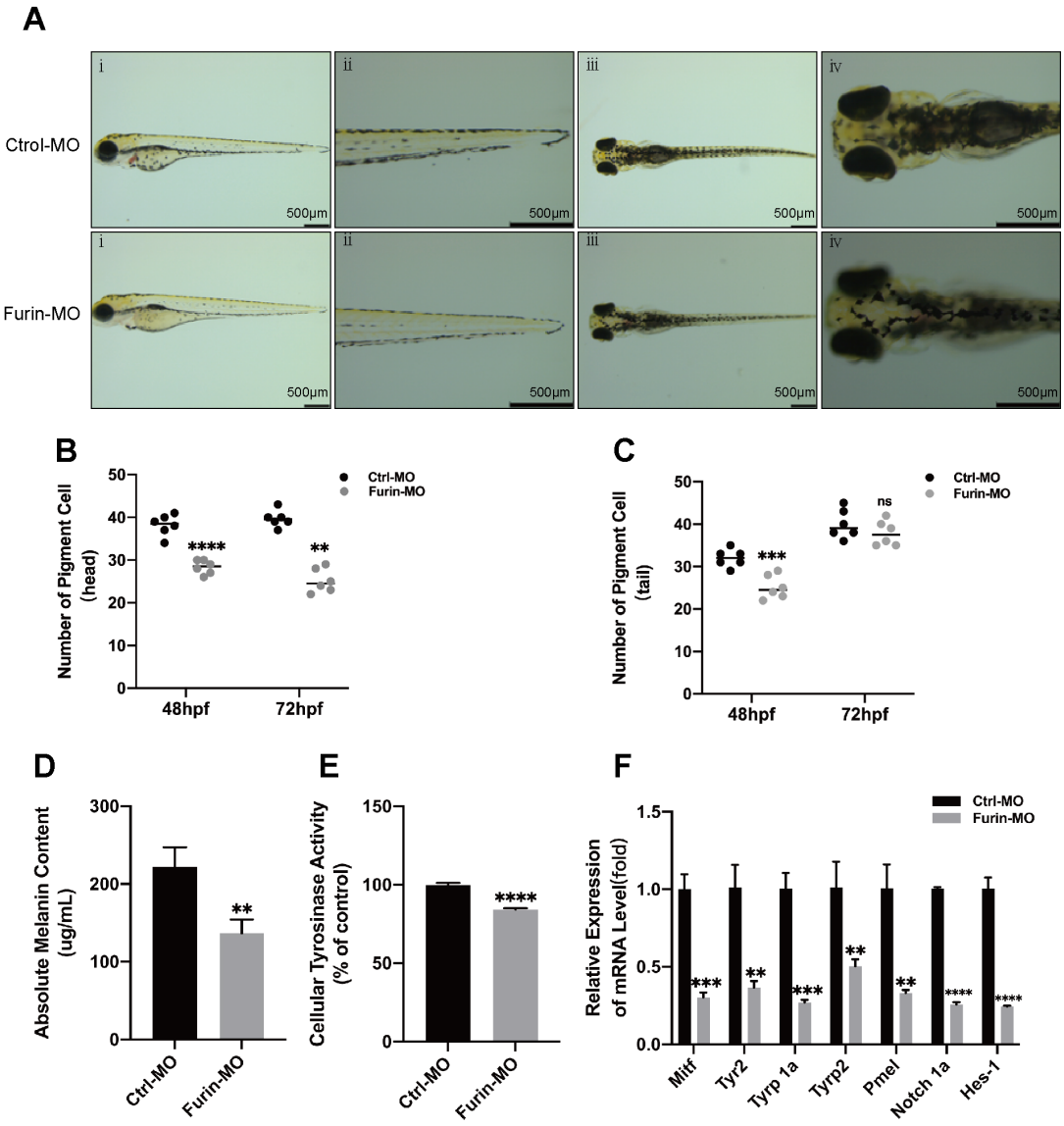

Fig. 5. Furin effect on pigmentation in zebrafish. (A) Representative photographs of zebrafish. Zebrafish embryos were injected with furin morpholino (furin-MO group) or control morpholino (Ctrl-MO group) for 96 hours post fertilization (hpf). Furin effect on the pigmentation in the lateral (i), tail (ii), back (iii), and head (iv) of zebrafish were observed under stereomicroscope. (B) The number of pigment cell of head and (C) tail was counted in zebrafish treated with furin-MO or Ctrl-MO for 48 hpf or 72 hpf. N = 6. (D) Absolute melanin content was measured in 96 hpf zebrafish. N = 3. (E) Tyrosinase activity was measured in 96 hpf zebrafish. N = 3. (F) *MITF*, *TYR*, *TYRP-1a*, *TYRP-2*, *Pmel*, *Notch-1* and *Hes-1* mRNA expression levels were determined by qRT-PCR. N = 3. All results are displayed as mean ± standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ and ns no significance vs. zebrafish in the Ctrl-MO group.

that the epidermal keratinocytes and MCs highly expressed furin [37]. Furin is required for *Pmel*17 cleavage [24], a type I integrin in MCs premelanosome matrix. Hence, we suspected that furin might regulate MCs bio-functions. Therefore, furin was inhibited in MNT-1 melanoma cells and MCs to observe the impact of furin on melanogenesis. As a result, it was observed that furin inhibition promoted melanin synthesis and extracellular melanin secretion in MNT-1 melanoma cells and MCs. *TYR* catalyzes L-tyrosine transformation into dopaquinone and is the most important rate-limiting enzyme in melanin synthesis [7]. *TYRP-1* and *TYRP-2* are responsible for turning dopachrome and DOPA into eumelanin [7]. These three enzymes are vital for melanin synthesis. In this study, we found that furin inhibition not only prompted *TYR* activity but also increased *TYR*, *TYRP-1*, and *TYRP-2* expression levels. Furin inhibition also increased *MITF* expression, a key transcription factor that regulates MCs' bio-functions [10]. Mean-

while, consistent with the previous finding [24], this study found that furin knockdown reduced *Pmel*17 expression level in MNT-1 melanoma cells. These results indicated that furin knockdown-induced increase in melanin synthesis was independent of melanosomes biogenesis. Besides, the melanin may be irregularly distributed in furin knockdown cells.

Aside from melanin production, melanosomes and melanin transfer from MCs to nearby keratinocytes play a role in skin pigmentation. Rab27a, Myo5a, and MAPL form a complex that enables melanin transfer from MCs to dendrites and neighboring cells [14]. FSCN1, an actin-bundling protein, has a clear role in MCs migration [13]. FSCN 1 deficiency could cause a defect in MCs migration and cell proliferation [13]. It was found that furin inhibition notably contributed to a rise in mRNA and protein levels of Rab27a and Myo5a in MNT-1 cells and MCs. Furin inhibition also led to an increase in melanin content in cell culture supernatants. Based on these results, furin inhibition was considered to promote melanin transport. Furthermore, furin inhibition also reduced FSCN1 expression level, which suggested that furin affected MCs migration.

However, research by Spencer JD *et al.* (2008) [37] found that furin significantly decreased in patients' epidermal with progressive vitiligo and increased in the actively re-pigmenting lesions of patients with vitiligo. After reducing H_2O_2 level with a narrowband ultraviolet radiation B (UVB)-activated pseudocatalase cream PC-KUS, furin expression level in nonlesional skin returned to normal levels [37]. As mentioned above, this finding seems to be contradictory to our results. This could be explained because vitiligo is an autoimmune skin disease targeting MCs and has a variety of underlying mechanisms [38]. It is speculated that MCs attack by autoreactive cytotoxic CD8+ T cells is believed to be the main factor responsible for the patches of depigmentation [39]. As a result, MCs injuries may obscure furin's role in MCs.

Notch signaling is highly conserved and serves as a critical regulator of cell fate and tissue formation during embryogenesis [40,41]. Furin is essential for Notch signaling pathway activation. Notch signaling pathway receptors experience several cleavages to function, and the cleavage at Furin cleavage site S1 cleavage mediated by furin is requisite for transporting receptors to cell membrane [42,43]. In line with previous studies [12,22], findings here showed that furin was required to activate Notch pathway. It was shown that furin inhibition down-regulated *Notch-1*, *Notch-2*, *Jagged-2*, *DLL-1*, Hes-1, and N1ICD expression. Furin inhibition also decreased *Notch-3* and *Jagged-1* protein expression and *DLL-3* mRNA expression but did not affect *DLL-4* and *Notch-4* expression. *Jagged-1* mRNA upregulation may be attributable to feedback from cells. Furthermore, *Notch-1* and *Hes-1* mRNA levels were reduced in Furin-MO zebrafish.

Prior research has indicated that Notch signaling pathway regulates MCs' development and pigmentation. Notch signaling is essential for melanoblasts and melanocyte stem cells (MSCs) survival via recombinant signal binding protein for immunoglobin kappa J region (RBP-J) and Hes1 [44–47]. Conditional ablation of *Notch-1* and/or *Notch-2* in mice resulted in a dose- and time-dependent hair graying and hair follicle MCs depletion, but had no effect on dermal or retinal MCs [47]. Likewise, Notch signaling pathway is critical for preventing MSCs and MCs differentiation before reaching the hair bulb and the correct MCs localization in the outer root sheath and hair matrix [44]. Moreover, genes associated with Notch signaling pathway, such as *POFUT-1*, *PSENEN*, *Nicastrin*, and *ADAM10*, have been identified as pathogenic genes for inherited reticular pigmentation illnesses [48–51]. Another research study discovered that Notch signaling can suppress *MITF* transcription [52]. This study further confirmed that Notch pathway is essential for melanin synthesis and transport. According to reports, histone deacetylase inhibitor VPA can stimulate *Notch-1* signaling [33]. Considering that *Notch-1* and *Notch-2* have mostly overlapping functions in pigmentation [47], VPA was used to activate *Notch-1* signaling for rescue experiments. Results showed that VPA could promote N1ICD expression level. But interestingly, *Notch-1*expression was further decreased after VPA application in MNT-1 cells transfected with sh-furin RNA. The mechanism of this effect is unclear, but we speculated that it may result from the transformation of *Notch-1* into the N1ICD induced by VPA. Furthermore, it was found that VPA could rescue the increase in melanin synthesis and secretion, tyrosinase activity, and proteins related to melanogenesis caused by furin knockdown. These results revealed that furin affected MCs biofunctions through the *Notch-1* pathway.

Finally, we confirmed furin effect on pigmentation in zebrafish. Contrary to *in vitro* experiments, furin-MO zebrafish had a decreased *MITF*, *TYR*, *TYRP-1*, and *TYRP-2* level, a lower melanin content, and a drop in tyrosinase activity compared to zebrafish Ctrl-MO group. Interestingly, similar to our results, previous research reported that knockdown of several genes related to Notch signaling pathway (*POFUT-1, PSENEN, Nicastrin,* and *ADAM10,* which are Dowling-Degos disease (DDD) and reticulate acropigmentation of Kitamura (RAPK) causative genes, respectively [51]) in zebrafish all resulted in a decrease in melanin [48–50]. These experiment results seemed to contrast with patients with DDD and RAPK clinical manifestations. Therefore, it was speculated that the difference between *in vivo* and *in vitro* experiments may result from the genetic differences between zebrafish and humans. But more *in vivo* experiments on other pigmented animal models are needed to confirm furin effect on melanogenesis.

Melanogenesis is a vital physiological activity that is regulated by a complicated network of signal pathways. Apart from the Notch pathway, several signaling pathways,

such as α-MSH-melanocortin 1 receptor (MC1R) pathway, Akt (PKB and protein kinase B) pathway, mitogen-activated protein kinases (MAPK) pathway, and wingless/integrated (Wnt) pathway, can encourage melanogenesis [53]. Liang *et al.* [54] discovered that furin might activate the downstream extracellular signal-regulated kinase (ERK)/MAPK and Akt signaling pathways. Moreover, furin inhibition can avoid cell migration by downregulating Wnt pathway [55]. Consequentially, furin may also influence melanogenesis via other conventional routes. In addtion, in a previous study, molecular docking results indicated that "melanin, eumelanin, L-dopaquinone and L-DOPA emphatically bind with the active site of furin protein" [56], and these indicated that furin directly acted by interacting with melanogenesis intermediates. Further research on furin function and specific mechanisms on important intermediates in melanin biosynthesis is needed. Furthermore, whether furin affected melanogenesis via other classical pathways warranted further exploration.

Conclusions

In conclusion, this study revealed that furin modulated melanogenesis physical process by affecting Notch pathway. It opens the possibility of targeting furin or the Notch pathway to treat pigmentary disorders.

Availability of Data and Materials

The data supporting the conclusions of this paper are included within the manuscript.

Author Contributions

CL and LL—designed the research study; LL—performed the research; WJ, YZ and JZ—provided help and advice on the melanin and WB experiments; LL and YG—analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. And this study protocol was approved by the Ethics Committee of the Hospital of Skin Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College (2022-KY-006/2022-KY-049).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202335175.15.

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