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GHRH-stimulated pituitary small extracellular vesicles inhibit hepatocyte proliferation and IGF-1 expression by its cargo miR-375-3p

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Abstract

Small extracellular vesicles (sEV) have emerged as a novel mode of intercellular material transport and information transmission. It has been suggested hormones may regulate the production and function of sEV. However, the specific impact of growth hormone-releasing hormone (GHRH) on pituitary sEV production and the role of sEV in the regulation of the GHRH-GH-IGF axis has not been previously reported. The results of the present study demonstrated that GHRH increased the production of pituitary sEV by promoting the expression of Rab27a. More importantly, GHRH induced alterations in protein and miRNA levels within GH3-sEV components. Notably, GH3-sEV with GHRH treatment exhibited the enhanced ability to impede BRL 3A cell proliferation and the expression of IGF-1. Conclusively, for the first time, we corroborate the influence of GHRH on pituitary sEV, thereby presenting novel evidence for how sEV participates in the balance of the GHRH-GH-IGF axis. Importantly, this study provides new insight into a novel balance mechanism mediated by sEV within the endocrine system.

Keywords GHRH, Pituitary small extracellular vesicles, Hepatocyte, miR-375-3p, IGF-1

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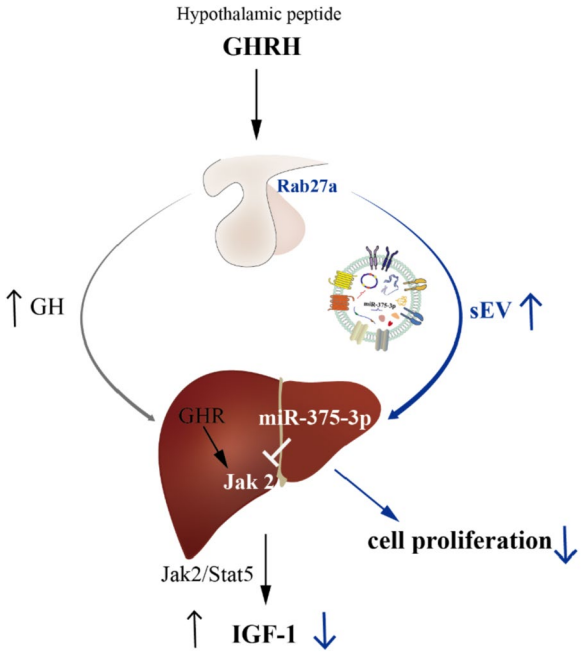
Graphical Abstract



Introduction

Small extracellular vesicles (sEV) are heterogeneous nanoscale vesicles secreted by almost all cells in the body [1], displaying a characteristic cup-shaped structure under transmission electron microscopy. Initially, sEV were considered as cellular metabolic byproducts or cellular "garbage". However, researchers have now recognized their significance as crucial cellular modulators in recent years [2–4]. Importantly, they mediate cell communication, which is a ubiquitous mechanism for intercellular interactions [5]. It has been discovered that sEV could serve as a carrier for a diverse array of substances, including proteins, lipids, and messenger RNA (mRNA), non-coding RNA (ncRNA), such as microRNA (miRNA) [6–9]. Furthermore, they are increasingly recognized for their regulatory function in the transportation of functional miRNA to target cells [10–14]. A substantial body of research now indicates that sEV play a widespread role in substance transport, information transmission, and immune response regulation [2, 4, 7].

Interestingly, while hormones and sEV are chemically distinct, it appears that they play pivotal roles in intercellular information transmission [15]. Moreover, numerous studies indicate that hormones can influence the secretion and functionality of sEV [16–18]. Furthermore, a recent study has shown that adenoma pituitary sEV may serve as nonhormonal pituitary-derived messengers mediating cellular signaling [19]. These limited studies hint sEV may be a regulator in endocrine network.

The pituitary gland, a central gland of the endocrine system within the animal body and human being, participates in regulating multiple physiological processes [20–22]. It has been well known the hypothalamic-pituitary-liver (GHRH-GH-IGF) axis plays an important role in regulating animal growth and development. Moreover, homeostasis of this axis is of particular importance physiologically. As well known, GH is both positively and negatively regulated to maintain its homeostasis. GH is regulated by the antagonistic actions of GHRH and somatostatin [23, 24], and the negative feedback of GH release through IGF-1 also play an important role [25, 26].

Up to now, researches have focused on exploring sEV from different types of pituitary adenoma [19, 27–29]. Our preliminary research has already demonstrated that the swine anterior pituitary can produce and release sEV, and contains functional non-coding RNA [30]. As a novel potential pathway for endocrine and paracrine signaling, the understand of sEV in the physiologically normal pituitary remains rare. As we all know, the growth hormone-releasing hormone (GHRH), a hypothalamic hormone,

positively affects growth hormone (GH) synthesis and secretion. Nevertheless, there is a dearth of reported studies exploring the influence of GHRH on pituitary sEV.

In this study, we aimed to investigate the effects of GHRH on the secretion and functionality of pituitary sEV, along with the associated mechanisms. We examined the changes in the sEV composition induced by GHRH, and the changes of their impact on IGF-1 and hepatocyte proliferation. The findings of this study provide valuable insights into the interplay between hormones and sEV, highlighting the role of pituitary sEV as a novel regulatory factor in the Hypothalamic-pituitary-liver axis.

Materials and methods

Cell culture and GHRH treatment

The rat pituitary cell line GH3 (ATCC) was cultured in F12 (Gibco, USA) medium supplemented with 2.5% fetal bovine serum (FBS) (Gibco USA), 15% horse serum (Hyclone, China) and 1% penicillin/ streptomycin (Gibco USA). The cell line of rat liver cells (BRL 3A) was cultured in in DMEM (Gibco, USA) medium supplemented with 10% FBS (Gibco USA) and 1% penicillin/streptomycin (Gibco USA). Porcine primary pituitary cell culture was performed as described in the previous studies [31–34]. Before treatment, the cells were starved in serum-free medium overnight. During treatment with GHRH, the cell culture medium was replaced with a fresh EV-depleted serum medium. EV-depleted serum was prepared by subjecting serum to ultracentrifugation (Optima XE, Beckman Coulter) at 120,000×*g* for 18 h at 4 °C according to the literature [35].

sEV isolation and analysis

sEV from cell culture supernatant (50 mL per group) were isolated using a differential ultracentrifugation method as described [36–38]. The obtained pellet was resuspended in PBS and stored at −80 °C. We assessed sEV protein concentration using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The morphology of sEV was examined with the transmission electron microscope. A drop of sEV suspension (about 10 μL) was fixed on a formvar-coated copper grid for 2 min, washed briefly in ultrapure water, negatively stained with 1% uranyl acetate, and observed by transmission electron microscopy (TEM; JEM-2000EX; Jeol, Tokyo, Japan) at an acceleration voltage of 80 kV. Size distribution, concentration, and intensity of sEV were analyzed by a NanoSight LM10 instrument (NanoSight, Amesbury, UK). The sEV markersCD63 and TSG101 were used as positive control whereas the endoplasmic reticulum protein Calnexin was used as a negative control in Western blot analysis.

Cell transfection

According to the sequence of Rab 27a mRNA, three target Rab27a mRNA siRNA with different sites were designed to avoid the off-target effect. The target siRNAs and control siRNA (NC) were purchased from and synthesized by the GenePharma company. GH3 cells were transfected with NC or siRab27a using Lipofectamine 2000 (Invitrogen, USA).

Cell viability assay

Cell proliferation was evaluated using Cell counting kit-8 (CCK8; EZBioscience, EZB-CK8) method, 5-ethynyl-2′-deoxy uridine (EdU; Beyotime Biotechnology, C0071S) incorporation assay. Firstly, the rate of cell proliferation was determined with CCK-8 kit according to the manufacturer's instructions. The number of viable cells was assessed by measuring the absorbance at 450 nm using a Synergy 2 Multi-Mode Reader (Bio Tek Instruments, Inc., Winooski, VT, USA). Secondly, DNA synthesis was examined with EdU incorporation assay to evaluate cell proliferation. The EdU positive cells were counted and normalized by the total number of Hoechst 33,342 stained cells.

Quantitative real-time PCR analysis

Total RNA and miRNA were extracted from cells and sEV using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of RNA and miRNA were measured by NanoDrop 2000 (Wilmington, DE, USA). The cDNAs were obtained by Color Reverse Transcription Kit (with gDNA remover) (EZBioscience, Roseville, CA, USA). All qPCR experiments were performed on a real-time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and specific primers of the genes of interest, and amplification efficiencies were checked by standard curves. Gene expression was quantified by the comparative cycle threshold (Ct) method. The target gene expression was normalized to those of β-actin, while the relative miRNA levels were normalized to the U6 or cel-miR-39 in sEV and calculated as $2-(\Delta\Delta CT)$.

Western Blot

Total protein of indicated tissues, cells or sEV was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1 mM phenyl methane sulfonyl fluoride (PMSF). The protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham,

MA, USA) according to the manufacturer's instructions. Equal amounts of total protein were separated by SDS-PAGE and transferred to a PVDF membrane in a tris–glycine methanol buffer. Primary antibodies used include anti-CD63 (Sangon Biotech), anti-TSG101 (Zen Biotech), Calnexin (Sangon Biotech), anti-PCNA (Zen Biotech), anti-GH (Santa Cruz), anti-Rab27a (Sangon Biotech), anti-IGF-1 (Zen Biotech), anti-Jak2 (Zen Biotech), anti-Stat5 (Zen Biotech), anti-p-Stat5 (Zen Biotech) and anti-Tubulin (Bioworld). Membranes were washed and incubated with either anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase (HRP) for 30 min to 1 h at room temperature. The membranes were incubated with ImmobilonTM Western Chemiluminescent HPR Substrate (Millipore, Burlington, WA, USA) and scanned with a FlourChem M Fluorescent Western Imaging System (Protein Simple, Santa Clara, CA, USA).

ELISA

The concentrations of GH and IGF-1 were measured with ELISA kits purchased from Nanjing Jiancheng Bioengineering Institute. ELISA was performed according to the manufacturer's instructions. Color alterations in the wells were read using the 96-well microplate reader (BioTek Instruments).

Label-free quantitative proteomic analysis

Data-Dependent Acquisition Lable-free quantitative proteomic analysis was carried out by Lianchuan Biotechnology Company (Hangzhou, China). Briefly, the samples were homogenized using a tissue lyser (60 Hz, 2 min) followed by 15 min centrifugation (20,000*g*, 4 °C) and harvest the supernatant. After quality control of protein extraction, trypsin was added and the samples were incubated at 37 °C. The enzymatically digested peptides were desalted and dried peptide fractions were redissolved in pure water and stored at − 20 °C. Using Reversed-Phase High-Pressure Liquid Chromatography (HPLC) fractionation to obtain dried peptide samples. Then the samples redissolved with 0.1% FA followed by 10 min centrifugation (20,000×*g*) and supernatant was collected and injected into a self-loading C18 column. Separation was performed by Thermo Scientific EASY-nLC™ 1200 system at a flow rate of 300 nL/min. MaxQuant (version 2.1.4.0) software was used to analyze the DDA label-free MS/MS data and statistical analysis was performed in R (version 4.0.0). The raw protein intensity will be normalized by method "medium", Hierarchical clustering was performed using pheatmap package. Principal component analysis (PCA) was performed using metaX package. T test was used for statistical differential analysis and a cut of p-value < = 0.05 and fold change > = 1.2 was used to select statistically differential expressed proteins.

Statistics analysis

All experimental results are presented as the mean ± S.E.M. Student's t-test was used for two group comparison, and one-way ANOVA was used among more than two groups. $p < 0.05$ was considered as statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$.

Results

GHRH promotes the produce of sEV from GH3 cells

GH3 pituitary cells were used to investigate the effect of GHRH on pituitary sEV production. GH3-sEV was harvested by ultracentrifugation conducted as described in the methods guided by MISEV2018 [39, 36, 40]. Transmission electron microscopic (TEM) images showed that GH3-sEV had classic cup-shaped morphology (Fig. 1A). The distribution curve of the particle size of GH3-sEV was determined by nanoparticle tracking analysis (NTA) (Fig. 1B). Western Blot revealed that GH3-sEV was highly positive for sEV-associated protein makers CD63 and TSG101, but negative for Calnexin (Fig. 1C). All of these indicates the features of sEV.

We then added different concentration gradients of GHRH to the culture medium of GH3 cells. The results showed that 40 nM GHRH could promoted GH synthesis and secretion (Fig. 2A). At the same, NTA showed that the concentration (particles/ml) and intensity (a.u.) of GH3-sEV with GHRH treatment were significantly increased compared with the control group (Fig. 2B). Furthermore, GHRH could also elevate the level of CD63 in sEV of the same protein amount (Fig. 2C). These data suggest that GHRH promotes the production of sEV from GH3 cells.

GHRH may regulate the production of pituitary sEV via Rab27a

We next explore the potential underlying mechanism for GHRH to promote sEV production. It has been confirmed that Rab27a, a Rab family member, plays a central role in sEV secretion [41, 42]. Interestingly, our results showed that Rab27a expression was profoundly increased by GHRH (Fig. 3A). To further verify whether the process of GHRH-promoted pituitary sEV production is regulated by Rab27a, we transfected siRab27a to GH3 cells. As expected, one of three designed candidate siRab27a, siRab27a-1, significantly decreased the mRNA and

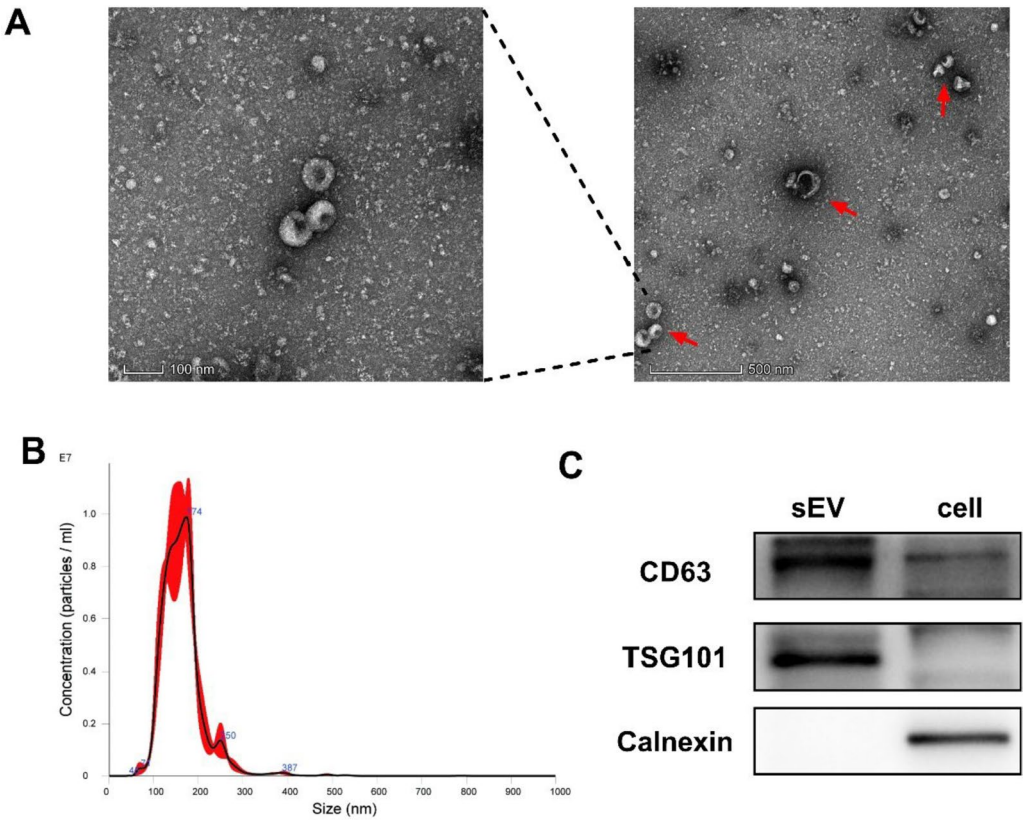


Fig. 1 Identification of GH3 sEV. **A** Analysis of GH3-sEV by transmission electron microscopy. **B** Nanoparticle tracking analysis (NTA) of size distribution of isolated GH3-Sev. **C** Western Blot analysis showing the biomarkers of sEV including CD63 and TSG101. Calnexin was used as a negative control

protein expression of Rab27a (Fig. 3B, C). NTA showed that the concentration and intensity of GH3-sEV was significantly decreased after Rab27a suppression (Fig. 3D). Interestingly, the inclusion of siRab27a reversed the facilitation of GHRH on the concentration and intensity of GH3-sEV (Fig. 3E), and the expression of Rab27a in GH3 cells as well (Fig. 3F).

Additionally, we further validated the above results in primary pituitary cells. Swine anterior pituitary primary cells were isolated as described previously [31, 32, 43],and were identified by cell morphology (Fig. 4A) and immunofluorescence staining with antibodies against the pituitary-specific marker Pit-1 (Fig. 4B). Then sEV was harvested by ultracentrifugation conducted as above, and was characterized by TEM (Fig. 4C), NAT (Fig. 4D) and western blotting analysis (Fig. 4E). We further compared the production of sEV from swine anterior pituitary primary cells and the expression of Rab27a in cells with GHRH treatment combining with siRab27a-1, and the results are exactly the same with that of GH3 cells (Fig. 4F, G). Taken together, these results show that GHRH is able to promote pituitary sEV production via Rab27a.

GHRH-treated GH3-sEV inhibits cell proliferation and IGF-1 expression more robustly in BRL 3A cells

To further reveal if GHRH treatment change the effect of GH3-sEV on hepatocyte and IGF-1, GH3 sEV and GHRH treated GH3-sEV were added to the culture medium of BRL 3A cells. The cell proliferation was evaluated with CCK8 kits and EdU staining, further confirmed by the detection of PCNA mRNA and protein expression. Interestingly, GHRH-treated GH3-derived sEV significantly inhibited BRL 3A cell proliferation compared with GH3-sEV (Fig. 5A–D). At the same time, GHRH-treated GH3-sEV obviously decreased the protein levels of IGF-1 within cells as well as in supernatant (Fig. 5D, E). Moreover, we obtained the same results using sEV from swine primary pituitary cells (Supplement Figure 1). These results suggest that GHRH-treated GH3-sEV inhibits cell proliferation and IGF-1 expression more robustly than GH3-sEV in BRL 3A cells.

GHRH alters the composition of GH3-sEV

Protein and miRNA are important functional molecules in sEV. We hypothesize that GHRH treatment alter the components of the GH3-sEV. To investigate potential

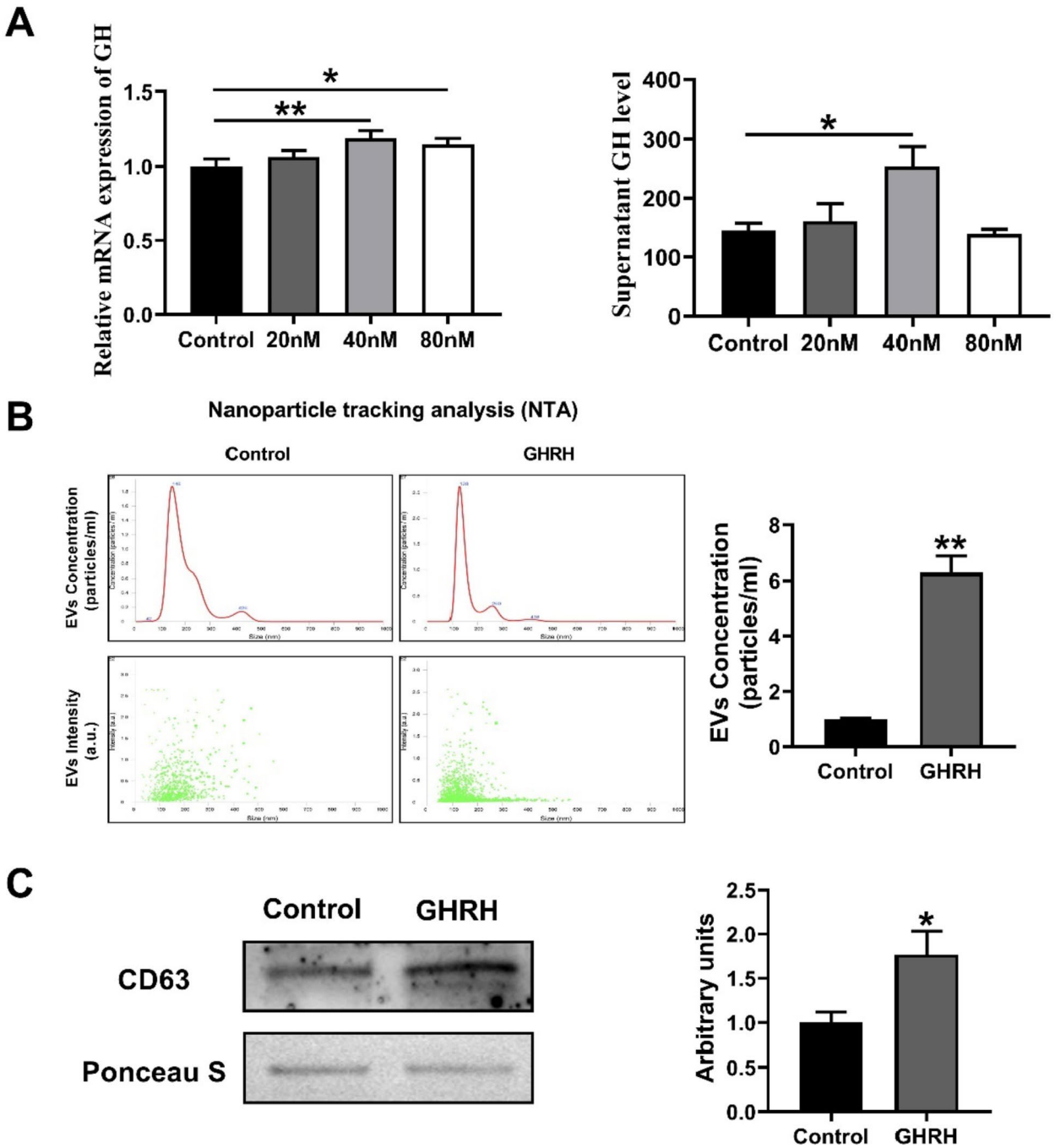


Fig. 2 GHRH promotes the produce of sEV from GH3 cells. **A** Effect of different doses of GHRH on GH mRNA and GH secretion after 12 h by qRT-PCR and ELISA, respectively. **B** GH3-sEV isolated from the control and GHRH-treated group were investigated by NTA for concentration (particles/ml) and intensity (a.u.) (n = 3). **C** CD63 protein level under 40 nM GHRH stimulation, Ponceau S was applied to represent the amounts of loaded total protein (n = 4). (*p < 0.05, **p < 0.01)

protein disparities between GH3-sEV generated with and without GHRH treatment, label-free quantitative analysis was performed, followed by bioinformatics analysis to probe the potential functional role of identified proteins. The EggNOG analysis revealed that most of these proteins function in intracellular signaling, metabolism and transport processes (Fig. 6A). Additionally, Reactome pathway analysis indicated that proteins with GH3-sEV are enriched in signaling, immune system, metabolism, cell cycle, small molecule transport pathways (Fig. 6B).

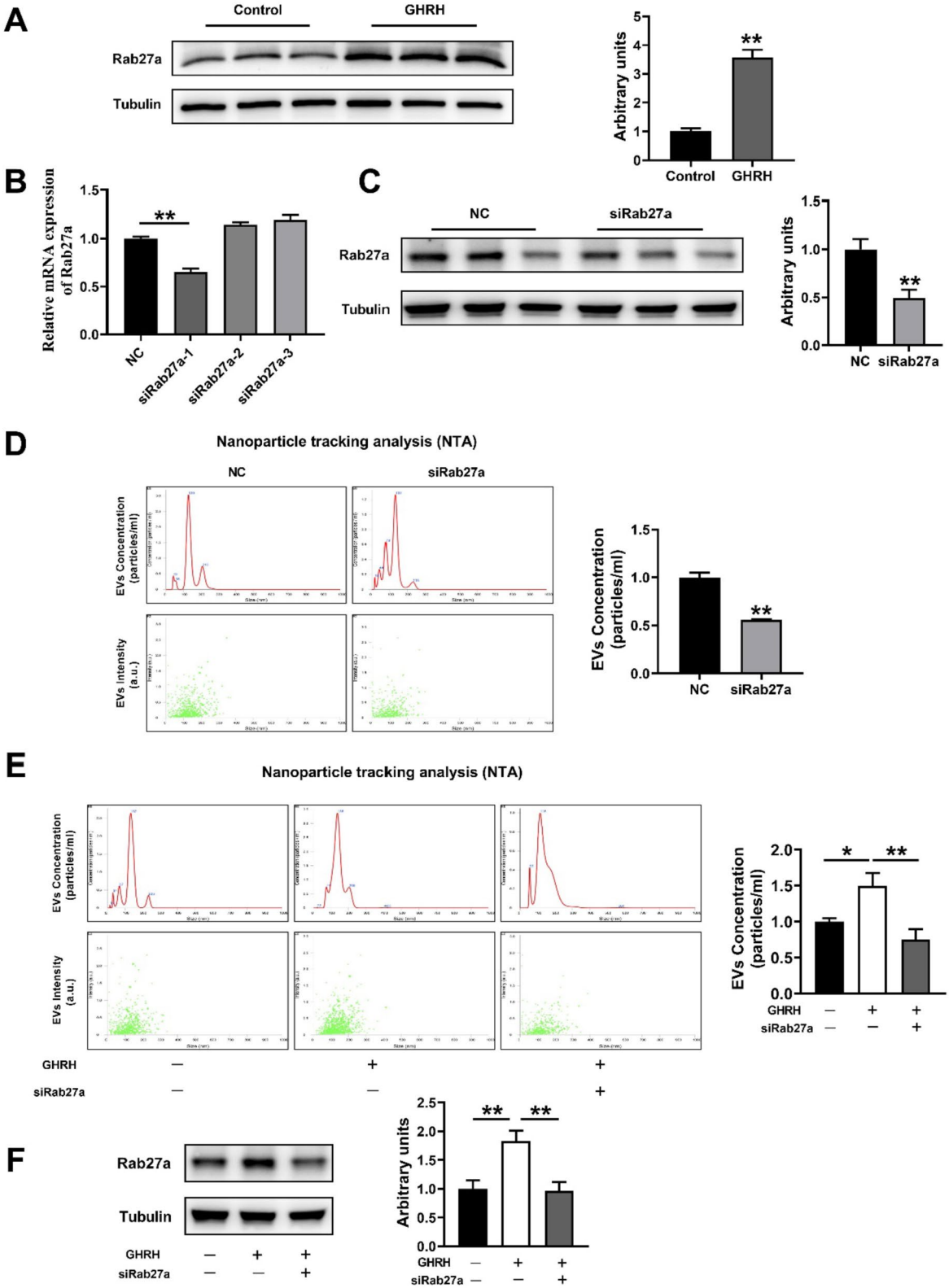


Fig. 3 GHRH increased the sEV production from GH3 via Rab27a. **A** The effect of GHRH on the Rab27a protein of GH3 cells. **B** The expression of Rab27a mRNA after transfection with siRab27a 1–3. **C** The expression of Rab27a protein after transfection with siRab27a-1. **D** NTA analysis for sEV concentration and intensity of the NC and siRab27a group. **E** NTA analysis for sEV concentration and intensity of NC, GHRH and GHRH + siRab27a-1 groups, and **F** The expression of Rab27a protein

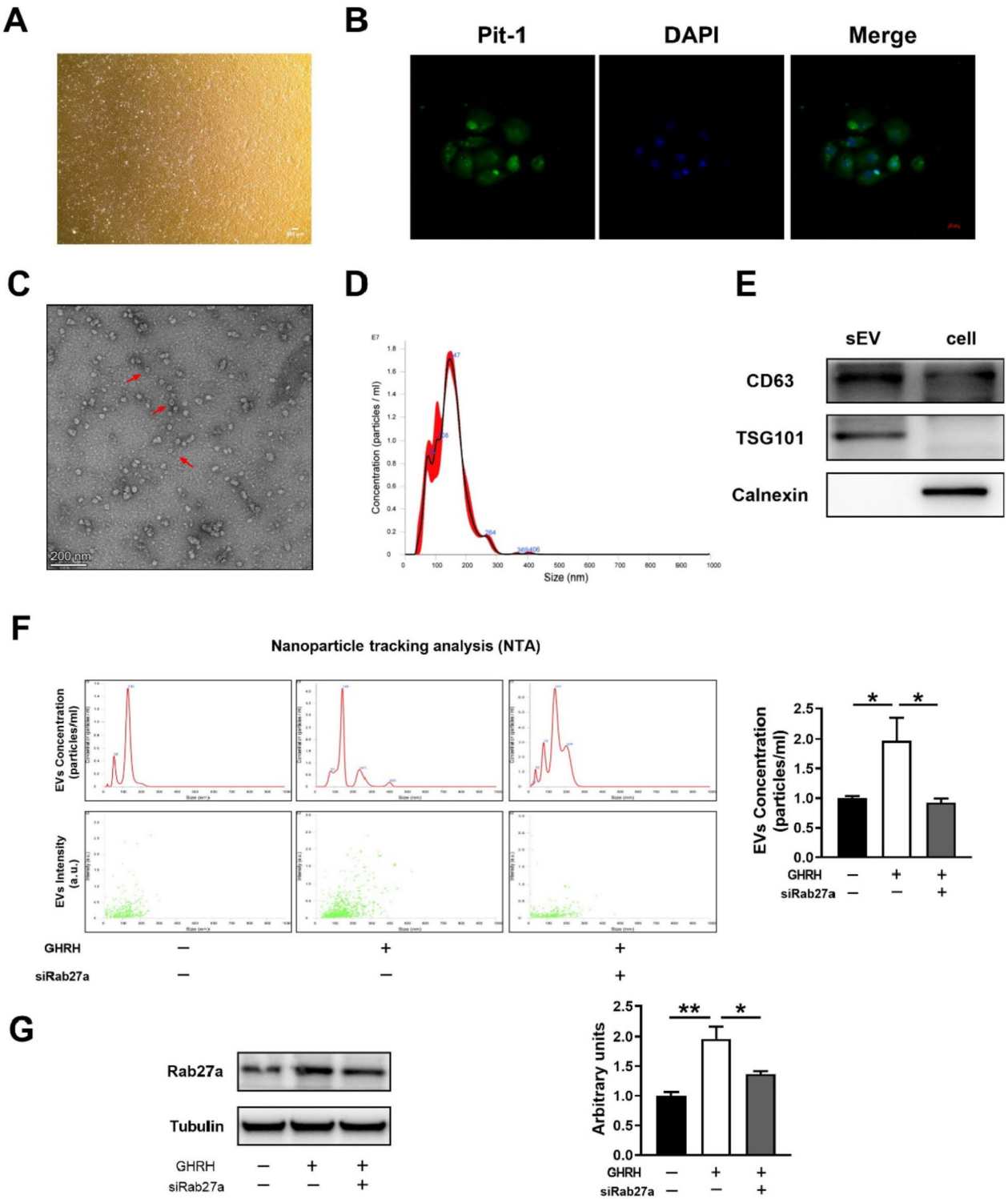


Fig. 4 GHRH increased the sEV production from swine primary pituitary cell via Rab27a. **A** Cell morphology of primary swine pituitary cell. **B** Pit1 immunofluorescence staining. **C** Image of transmission electron microscopy. **D** NTA analysis for sEV derived from primary swine pituitary cell. **E** Western Blot analysis showing the positive biomarkers of sEV including CD63 and TSG101, negative biomarker Calnexin. **F** NTA analysis for concentration and intensity, and **G** The expression of Rab27a protein in primary swine pituitary cell

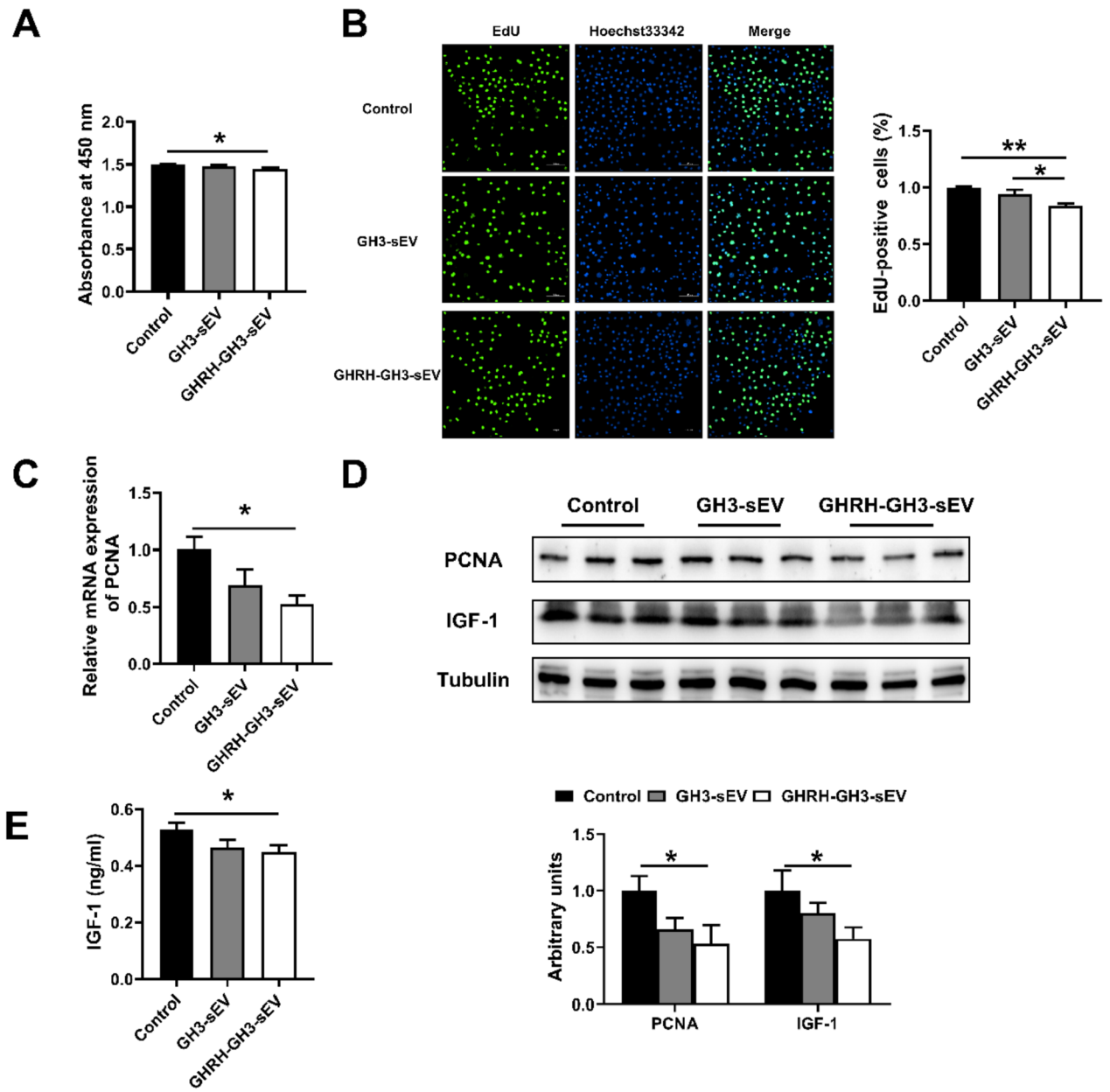


Fig. 5 GHRH-treated GH3-sEV inhibits cell proliferation and IGF-1 expression more robustly in BRL 3A cells. **A** The result of CCK8 kits. **B** The result of EdU-positive cells. **C** The expression of PCNA mRNA. **D** The levels of PCNA and IGF-1 protein in BRL 3A cells. **E** The levels of IGF-1 in cell supernatant

A total of 139 proteins were quantified, with 126 in the GH3-sEV group and 119 in the GHRH-GH3-sEV group. Among these, 10 proteins were up-regulated and 34 were down-regulated in the GHRH-GH3-sEV compared to GH3-sEV (Fig. 6C). It was discovered that the expression of proteins such as YWHAE, DCUN1D5, and MFGE8, which play roles in regulating the cell cycle, cell growth and the IGF transport pathway, were significantly down-regulated in GH3-sEV after GHRH treatment [44–48].

Additionally, prior RNA sequencing of GH3-sEV was conducted and the expression levels of the top 10 miRNAs were verified by using qRT-PCR (Fig. 6D). Subsequently, results showed a significant increase in the levels of miR-7a-5p, miR-7b, and miR-375-3p, and a significant decrease in the levels of let-7b-5p, let-7i-5p, and miR-30d-5p in GHRH-GH3-sEV relative

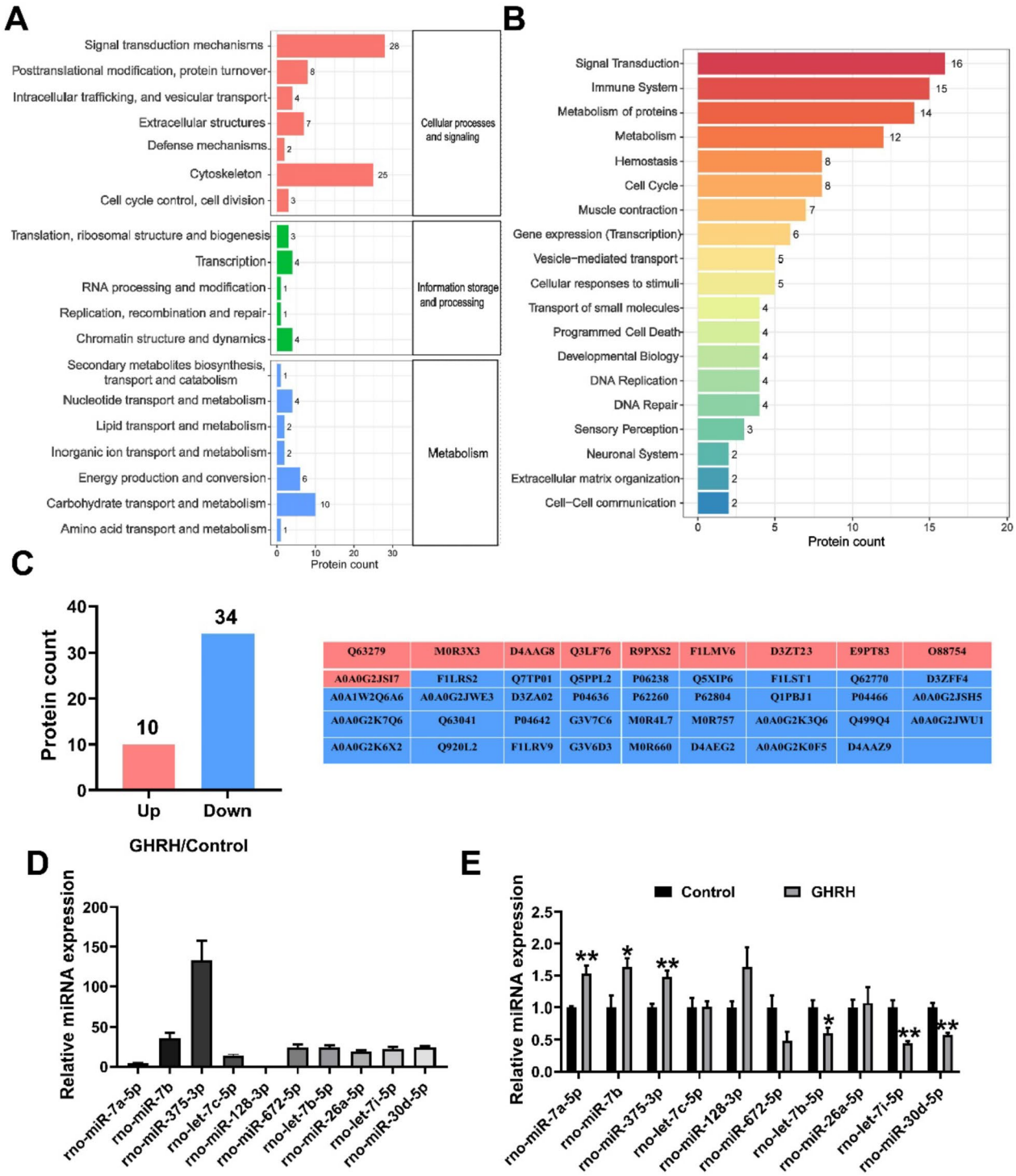


Fig. 6 GHRH alters the protein and miRNA composition of GH3-sEV. GO **A** EggNOG analysis. **B** Reactome analysis. **C** The protein changes between GHRH-GH3-sEV and GH3-sEV. **D** Identification of top 10 miRNAs in GH3-sEV. **E** The comparison of miRNAs in GHRH-GH3-sEV and GH3-sEV

to GH3-sEV (Fig. 6E). The results above suggest that GHRH induces alterations in the composition of GH3-sEV components, both protein and miRNA.

GHRH-GH3-sEV may regulate cell proliferation and IGF-1 via its increased miR-375-3p

To probe the possible mechanisms by which

GHRH-GH3-sEV influence BRL 3A cell proliferation and the expression of IGF-1, we assessed the changes in miRNA levels within the BRL 3A cells. The miRNA with the highest level in GH3-derived sEV was miR-375-3p, and more importantly, its level was significantly increased after GHRH treatment as shown (Fig. 6E). Interestingly, in sEV donor BRL 3A cells, the expression of miR-375-3p were also significantly higher in the GHRH-GH3-sEV treatment compared to the control and GH3-sEV treatment (Fig. 7A). Previous studies have indicated that miR-375-3p is involved in the regulation of cell proliferation [49, 50]. Based on these findings, it is proposed that miR-375-3p play a crucial regulatory role in this process.

To further confirm the function of miR-375-3p, we transfected BRL 3A cells with NC and miR-375-3p mimics and the efficiency of miR-375-3p transfection was confirmed by the dramatically upregulated miR-375-3p in BRL 3A cells (Fig. 7B). More importantly, miR-375-3p overexpression prominently inhibited proliferation of BRL 3A cells (Fig. 7C–F). At the same time, we found a marked reduction in the expression of IGF-1 protein in cells and supernatant (Fig. 7F–G).

Previous studies have shown that miR-375-3p directly targets the 3'UTR of *Jak2*, which involved in the regulation of IGF-1 expression through the Jak/Stat pathway [51–56]. Therefore, we examined the expression of Jak2 after transfection of miR-375-3p mimics. The results showed that JAK2 protein levels was significantly decreased and phosphorylation levels of Stat5 was also robustly inhibited (Fig. 7H). To determine whether GHRH-GH3-sEV regulate IGF-1 through Jak2/Stat5, we examined the change of the protein levels with different treatments. Interestingly, GHR-GH3-sEV attenuates the expression of Jak2 and p-Stat5 more robustly than GH3-sEV (Fig. 7I). The results above indicate that GHRH-GH3-sEV exhibits great efficacy in suppressing cellular proliferation and IGF-1 expression compared to GH3-sEV. This enhanced potency can be attributed to its ability to modulate the JaK2/Stat5 pathway via miR-375-3p.

Discussion

A growing body of research indicates that sEV can serve as a communicator to mediate material exchange and information transfer between cells and tissues [2–4], by carrying a variety of cargoes including proteins, lipids, nucleic acids, *ect* [7]. Interestingly, they exhibit heterogeneity because of their different sources and sorting mechanisms. Furthermore, sEV functions as significant signaling molecules in the endocrine system, which help maintain internal environment homeostasis in addition to hormones. For example, a recent investigation has revealed that hypothalamic neural stem/progenitor cells exhibit an endocrinological function by secreting extracellular vesicles miRNAs that contribute to the regulation of aging speed in the hypothalamus [57]. sEV derived from pancreatic islets play an important role in intercellular communication [58, 59]. Moreover, they also demonstrate a considerable contribution in maintaining reproductive system operation [60–62]. However, there are differences between sEV and hormones in terms of their biological constituents, biogenesis, and functional mechanisms [15]. In addition, the relationship between sEV and hormones is far from entirely clear. We summarize that on the one hand, hormones could affect the secretion of sEV and sEV also have potential to impact hormone secretion [17, 18, 63–65]; On the other hand, the combination of hormones and EVs has been shown to yield a more efficacious effect [66–71]. Balance in the hormone network plays an important role in maintaining homeostasis. There are both positive feedback and negative feedback regulation in the somatotropic axis signaling. GH is regulated by the antagonistic actions of GHRH and somatostatin, and the negative feedback of IGF-1 [23–26]. However, it is unclear whether and how there is positive or negative feedback between sEV and GH.

Our previous research has shown that sEV derived from swine anterior pituitary contain functional noncoding RNAs and they may participate in the cellular metabolic and biosynthetic process [30]. As we all know, GHRH is involved in regulating pituitary GH synthesis and release and the hypothalamus-pituitary-liver axis plays an important role in the growth regulation of animals. The question of whether GHRH affects the production of pituitary sEV and sEV function is of great interests.

If GHRH affect production of sEV of pituitary cells remains unexplored. The present study indicates that GHRH not only promotes the secretion of GH, but also facilitates the production of pituitary sEV. There are a number of studies reveals that Rab27a plays a very crucial

(See figure on next page.)

Fig. 7 GHRH-GH3-sEV may regulate cell proliferation and IGF-1 via its increased miR-375-3p. **A** The expression of miR-375-3p in BRL 3A cells in different treatments. **B** Relative expression of miR-375-3p following transfection with NC and miR-375-3p mimics. **C** The cell proliferation results detected with CCK8 kits and **D** EdU-positive assay. **E** The expression of PCNA and IGF-1 mRNA and **F** protein expression in BRL 3A cells. **G** The IGF-1 protein levels in the BRL 3A cells supernatant. **H** The effect of miR-375-3p mimics on Jak2/ Stat5 pathway proteins. **I** The effect of GHRH-GH3-sEV on Jak2/Stat5 pathway proteins

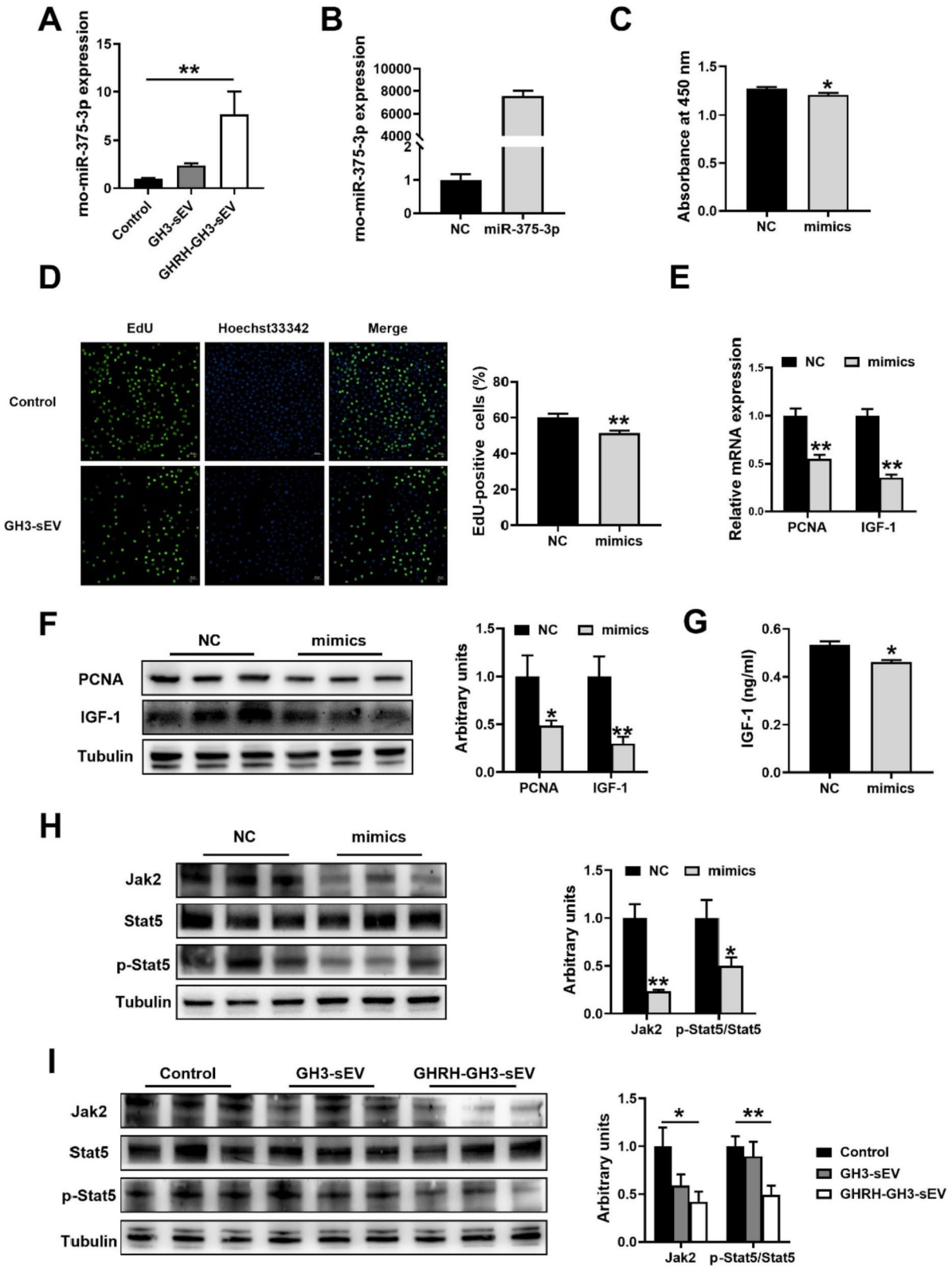


Fig. 7 (See legend on previous page.)

role in sEV secretion [41, 42], and it was further confirmed in our research both in GH3 cells and primary pituitary cells. It is clear that GHRH promotes sEV production by increasing Rab27a, but the precious regulation mechanism is waiting for further exploration. Our study firstly provides evidence that GHRH promotes the yield of pituitary sEV, which is a new potential way in its biological regulation, even a new model for other hormones.

If GHRH change composition and function of GH3 derived sEV is the next key interesting point. Our results showed that GHRH could cause components alterations in GH3-sEV, both protein and miRNA. A total of 139 proteins were quantified through Label-free quantitative proteomic analysis. It showed that 10 proteins were up-regulated and 34 were down-regulated in the GHRH-GH3-sEV, comparing to GH3-sEV. Moreover, the levels of miRNAs in GH3-sEV group were altered following stimulation with GHRH. Notably, in contrast to GH3-sEV, GHRH-GH3-sEV exhibited a more robust suppression on the proliferation of BRL 3A cells, and the expression of IGF-1 as well. Upon comparing these altered proteins, it was discovered that the expression of proteins such as YWHAE, DCUN1D5, and MFGE8, which play roles in regulating the cell cycle, cell growth and the IGF transport pathway [44–48], were significantly down-regulated after treatment with GHRH, which may partially account for the suppression of GHRH-GH3-sEV. More interestingly, the expression of miR-375-3p showed a significant increase in BRL 3A cells treated with GHRH-GH3-sEV. It has been reported that miR-375-3p targets *Jak2* 3'UTR, which is involved in regulating IGF-1 expression through the Jak/Stat pathway [51–56]. In the present study, we validated that GHRH-GH3-sEV is a negative factor to regulate the expression of IGF-1 through the Jak2/Stat5 pathway by a way of delivering more miR-375-3p to recipient cell. Collectively, it is illustrated, for the first time, that GHRH alters protein and miRNA in pituitary derived sEV and changes its function of the inhibition of cell proliferation and IGF-1 expression in BRL 3A through miR-375-3p, and some key proteins as well.

In conclusion, our findings empirically demonstrate the influence of GHRH on pituitary sEV production and its cargos, leading to more robust inhibition of liver cell proliferation and IGF-1 expression. More importantly, this inhibition is just the opposite to GH function. GHRH elevates GH secretion and simultaneously increased pituitary sEV production to balance GH function. Thus, it is deduced that pituitary sEV plays a negative role in GHRH-GH-IGF axis. To our knowledge, it is the first time to suggest the idea that sEV is a new balance mediator in hormone regulation network, providing a theoretical foundation for a homeostatic regulatory mechanism between sEV and hormones.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12951-024-02857-y.

Additional file 1.

Author contributions

Jiali Xiong: Writing-original draft, Methodology, Formal analysis, Data curation. Yuxuan Wang: Data curation, Validation, Supervision. Hailong Wang: Methodology, Supervision. Junyi Luo: Data curation, Project administration. Ting Chen: Methodology, Supervision. Jiajie Sun: Data curation, Conceptualization. Qianyun Xi: Conceptualization, Funding acquisition. Yongliang Zhang: Writing-review&editing, Funding acquisition, Supervision. All authors reviewed the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare no conflict of interest.

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