GnRH participates in the self-renewal of A549-derived lung cancer stem-like cells through upregulation of the JNK signaling pathway

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Received November 1, 2014; Accepted February 3, 2015

DOI: 10.3892/or.2015.3956

Abstract. Lung cancer is the leading cause of cancer-related mortality in humans. Exploration of the mechanisms underlying the self-renewal and stemness maintenance of cancer stem-like cells (CSLCs) will open new avenues in lung cancer diagnosis and therapy. Here, we isolated and identified a subpopulation of lung cancer stem-like cells (LCSLCs) from non-small cell lung carcinoma (NSCLC) A549 cells with features including self-renewal capacity *in vitro*, elevated tumorigenic activity *in vivo*, and high expression of stemness markers CD44, CD133, aldehyde dehydrogenase 1 (ALDH1) and Sox2, using a serum-free suspension sphere-forming culture method. We then found a higher expression level of gonadotropin-releasing hormone (GnRH) in the LCSLCs using a microarray assay, suggesting that GnRH may play a role in the self-renewal capacity and stemness maintenance in lung cancer cells. In addition, the suppression of GnRH capacity negatively regulated self-renewal and stemness maintenance in the LCSLCs. Overexpression of GnRH promoted stemness properties of A549-derived LCSLCs, indicating that GnRH expression is essential for the self-renewal and stemness maintenance in LCSLCs. Moreover, further investigations demonstrated that the promotion of GnRH functions of self-renewal and stemness maintenance in LCSLCs was associated with the JNK signaling pathway. Therefore, our results showed that GnRH participates in the self-renewal capacity and stemness maintenance of LCSLCs by upregulating the JNK signaling pathway, and GnRH may be useful as an alternative LCSLC therapy.

Introduction

Lung cancer is one of the most common causes of death from cancer worldwide. Non-small cell lung cancer (NSCLC), which accounts for ~80% of lung cancers, is associated with a poor 5-year survival in developing countries (less than 9%) and in the USA (not more than 15%) owing to its aggressiveness (1,2). Thus, it is necessary to explore novel therapeutic approaches for improving the quality of life of patients with lung cancer including NSCLC.

Accumulating evidence has emerged that a small population of cells possesses tumor-initiating activity in lung cancer and these cells are termed 'cancer stem-like cells (CSLCs)' (3,4), which possess a variety of unique biologic properties including self-renewal, expression of specific biological markers and stem cell genes, and the use of common signaling pathways and the stem cell niche (5-7). CSLCs differ from normal stem cells in their tumorigenic capacities for cancer initiation, recurrence, metastasis, and therapy resistance. Although the existence of CSLCs in human lung cancer has been previously studied, the regulation of self-renewal and stemness maintenance in lung cancer stem-like cells (LCSLCs) in the initiation of lung cancer remains unclear (8-10).

Gonadotropin-releasing hormone (GnRH) is synthesized in a small subset of neurons in the septal-preoptic-hypothalamic region (11,12). These neurons secrete the neurohormone into the hypophysial portal circulation, through which it reaches the anterior pituitary to stimulate the synthesis/release of the two gonadotropins that, in turn, regulate gonadal gender steroid production (13). In addition, GnRH and its receptor were also found to be expressed in several types of cancer tissues, either related (prostate, breast, ovarian and endometrial cancers) or unrelated (melanoma, glioblastoma, lung and pancreatic cancers) to the reproductive system, indicating that the expression of GnRH may be linked to tumor progression. Recent studies indicate that GnRH agonists have strong anti-tumor activity, such as anti-proliferation, anti-metastasis and anti-angiogenesis (14-16). These opposite biological effects of GnRH-R activation seem to be linked to specific intracellular signaling cascades that are coupled to this receptor in the different tissues (17,18). Therefore, GnRH has been considered a promising candidate for novel molecular-targeted strategies for the treatment of tumors.

In the present study, we found that GnRH is associated with the regulation of self-renewal and stemness maintenance in LCSLCs. Firstly, an *in vitro* sphere culture system was established to isolate and enrich A549 LCSLCs. We then determined the expression levels of tumorigenesis-related genes by microarray analysis in the LCSLCs. Compared with

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Key words: gonadotropin-releasing hormone, lung cancer stem-like cells, self-renewal, lung cancer, JNK

the adherent monolayer A549 cells, we found that the GnRH expression level was much higher in the LCSLCs, suggesting that GnRH may play a role in the regulation of self-renewal and stemness maintenance in LCSLCs. Further examinations indicated that GnRH expression was essential for the self-renewal and stemness maintenance by knockdown or overexpression of GnRH expression in the A549 LCSLCs. Based on our findings, GnRH and its receptor may represent an effective molecular target for the development of innovative tumor-targeted anticancer drugs directly or specifically for LCSLCs.

Materials and methods

Ethics statement. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Huazhong University of Science and Technology. The animal procedures were approved by the Committee on the Ethics of Animal Experiments of Huazhong University of Science and Technology.

Cell culture and transfection. Human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA), 100 U/ml penicillin and streptomycin (both from Invitrogen, Carlsbad, CA, USA) in a humidified 37˚C incubator with a 5% CO_2 atmosphere. The cells stably overexpressing GnRH were transfected with GnRH recombinant vector pCDNA3.1(+)-GnRH by using Lipofectamine 2000™ and selected with 500 μg/ml G418 (both from Invitrogen). After 72 h, the cells were analyzed by quantitative real-time PCR (qRT-PCR) or western blot analysis.

Isolation of the A549 lung sphere cells. The isolation of A549 sphere cells was modified using a previously reported protocol (3,19). To obtain sphere cultures, cells were plated at a density of $2x10^4$ cells/well in 24-well ultra-low plates (Corning Inc., Corning, NY, USA), supplemented with commercial hormone mix B27 (Gibco), 20 ng/ml EGF (Sigma-Aldrich, St. Louis, MO, USA), 10 ng/ml bFGF (Upstate, Lake Placid, NY, USA), 0.4% bovine serum albumin (BSA; Gibco), 4 μg/ml insulin (Sigma-Aldrich), 100 U/ml penicillin and streptomycin at 37˚C. After being cultured for 7 days, the lung cancer spheres were collected, dissociated into a single-cell suspension and resuspended in fresh medium for serial subcultivation every 7 days. Adherent monolayer A549 cells were used as the controls.

Colony-formation assay. For colony formation, the adherent monolayer A549 and the A549 sphere cells were dissociated into a single-cell suspension. The cells were plated into 100-mm dishes at a density of $2x10^3$ cells/well in DMEM with 10% FBS. The plates were further incubated for 2 weeks at 37˚C with 5% CO_2 until colonies were visible. The colonies were stained with 0.01% crystal violet and counted using inverted microscopy. The dimension of each colony was measured with Image J software (NIH, Bethesda, MD, USA).

Microarray and data analysis. Human genome microarray analysis was conducted by CapitalBio Corporation. Genes were determined to be differentially expressed when logarithmic gene expression ratios were >5-fold different and P-values <0.05. For data validation, the mRNA levels and the expression of the genes of interest were confirmed by qRT-PCR and western blot analysis.

qRT-PCR and western blotting. Total RNA was extracted from the tumor cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. qRT-PCR was performed using the SYBR PrimeScript RT-PCR kit (Takara, Japan) on a Rotor-Gene 6000 real-time genetic analyzer (Corbett Life Science, USA) according to the manufacturer's instructions. The GnRH forward primer was 5'atgaagccaattca aaaact3', and the reverse primer was 5'ttaaatcttcttctgccca3'. The PCR protocol included a denaturation program (95˚C for 2 min), followed by 40 cycles of amplification and quantification program (95˚C for 5 sec, 55-57˚C for 30 sec) and a melting curve program (55-95˚C, with a 0.5˚C increment each cycle). Each sample was replicated 3 times.

To analyze the protein expression levels, the cells were washed by PBS 3 times and then lysed [1% Triton X-100 in PBS buffer with protease inhibitor (Roche, Indianapolis, IN, USA)] for 30 min on ice. After incubation, the cell lysates were centrifuged at 16,000 x g for 10 min. The supernatant was mixed with 5X SDS sample buffer and boiled for 10 min, and electrophoresed at 80 V through a 10% polyacrylamide-SDS denaturing gel. Separated proteins were electrotransferred to nitrocellulose, and the expression of GnRH, CD44, CD133, aldehyde dehydrogenase 1 (ALDH1), Sox2, JNK1, c-Jun or GAPDH protein was detected with the desired antibodies including mouse anti-GnRH monoclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-CD44 monoclonal antibody (1:1,000; Proteintech, Inc., Chicago, IL, USA), mouse anti-CD133 monoclonal antibody (1:200; Miltenyi Biotec, Germany), mouse anti-ALDH1 monoclonal antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Sox2 polyclonal antibody (1:1,000) and rabbit anti-JNK1 polyclonal antibody (1:1,000) (both from Abcam, Cambridge, UK), rabbit anti-phospho-c-Jun (Ser73) polyclonal antibody (1:2,000; Cell Signaling Technology) and mouse anti-GAPDH monoclonal antibody (1:3,000; GenScript Corp., Piscataway, NJ, USA), respectively. The signals were detected using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA).

Immunofluorescence microscopy. The cells were seeded on a sterile coverslip and washed with PBS 48 h later, and then fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 30 min. The fixed cells were washed with PBST (1X PBS containing 0.5% Triton X-100) and incubated with 2% BSA in PBST (blocking buffer), and then incubated with anti-GnRH antibody (1:200) in blocking buffer at 4˚C overnight. After 4 washes with PBST at room temperature for 15 min each, the cells were incubated with FITC-conjugated goat anti-mouse IgG (1:300; Jackson ImmunoResearch, West Grove, PA, USA). The cells were then washed 4 times, followed by staining with propidium iodide (PI; Sigma-Aldrich) to visualize the nuclear DNA, before being mounted in mounting medium

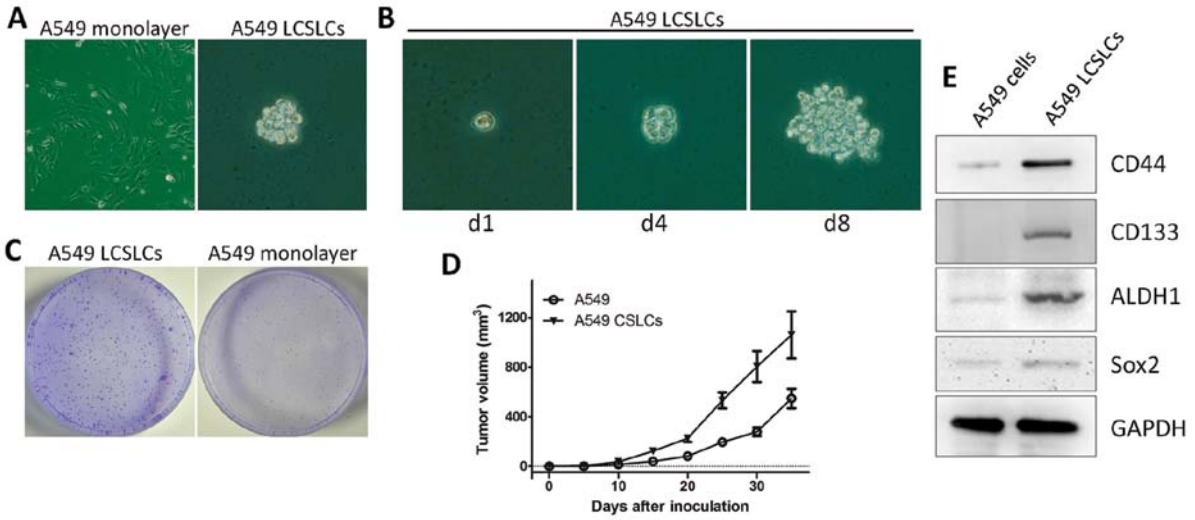

Figure 1. Identification of LCSLCs from lung cancer A549 cells. (A) Adherent monolayer A549 cells and sphere A549 cells. (B) A single cell of the A549 LCSLCs formed a new sphere. (C) Colony formation assay of monolayer A549 cells or A549 LCSLCs. (D) Quantitative analysis of the xenograft tumors formed by A549 monolayer cells or A549 LCSLCs. (E) The CD44, CD133, ALDH1 and Sox2 protein expression levels in the A549 monolayer cells or A549 LCSLCs. LCSLCs, lung cancer stem-like cells; ALDH1, aldehyde dehydrogenase 1.

Table I. Xenotransplantation of human lung cancer A549 cells and A549 LCSLCs into BALB/C immunodeficient mice.

Cell type	Inoculum size	Tumor incidence
A549 cells	$1x10^4$	0/5
	$1x10^5$	0/5
	$1x10^6$	2/5
A549 LCSLCs	$1x10^4$	2/5
	$1x10^5$	5/5
	$1x10^6$	5/5

LCSLCs, lung cancer stem-like cells.

(Vector Laboratories, Burlingham, CA, USA), and sealed under a coverslip with nail polish. The prepared samples were observed and photographed under an Olympus FluoView FV1000 confocal microscope.

Xenograft tumor growth assay. The desired numbers of tumor cells were injected subcutaneously into the back of 4-week-old BALB/C-nude mice (n=5 each, Center of Experimental Animals, Huazhong University of Science and Technology, China). The mice were reared for 2 months, and the tumor growth and tumorigenic time were examined visually. At the end of the experiment, the mice were sacrificed and tumor xenografts were removed and measured. The tumor volume (TV) was calculated in accordance with the formula: TV (mm^3) = d^2 x D/2, where d and D represent the shortest and the longest diameter, respectively.

Statistical analysis. All experiments were carried out at least 3 times with triplicate samples. Data are presented as the mean ± SEM. Statistical analysis was conducted using SPSS13.0 software. A statistically significant difference was assessed by one-way ANOVA followed by multiple mean comparisons by the Student's t-test. A value of $P<0.05$ was considered to be statistically significant.

Results

LCSLCs derived from the A549 cell line display self-renewal capacity. In our study, we established a stable sphere culture system for isolating and enriching A549 sphere cells, which are regarded as LCSLCs, to elucidate their biological behaviors. As shown in Fig. 1, A549 cells that were cultured in the SFM with EGF and bFGF generated non-adherent, multicellular sphere LCSLCs (Fig. 1A). The result revealed that sphere LCSLCs exist in human lung cancer A549 cells. Moreover, it was found that single cells of the CSLCs from the lung cancer A549 cell line were able to form new spheres (Fig. 1B), suggesting that the A549 sphere has self-renewal capacity. We then assessed the self-renewal capacity of A549 spheres by colony formation assay. Compared with the A549 monolayer cells, the LCSLCs exhibited enhanced colony formation (Fig. 1C).

The self-renewal capacity of LCSLCs *in vivo* was revealed by examining the tumorigenic potential of A549 monolayer cells and A549 LCSLCs. We found that tumors derived from the A549 monolayer cells yielded much smaller sized tumors than those from the A549 LCSLCs (Fig. 1D). In addition, the xenograft tumor model in nude mice was also used to investigate the tumorigenicity of the A549 monolayer cells and A549 LCSLCs in BALB/C immunodeficient mice. The results indicate that $1x10^4$ A549 LCSLCs were enough to form tumors *in vivo*, whereas $1x10^6$ A549 monolayer cells were required to induce stable tumor formation (Table I).

LCSLCs derived from the A549 cell line overexpress stem cell markers. We next investigated the expression of cancer stem cell (CSC) biomarkers or stemness-related transcription factors, such as CD44, CD133, Sox2, ALDH1 and GAPDH in the A549 LCSLCs using western blotting. The results

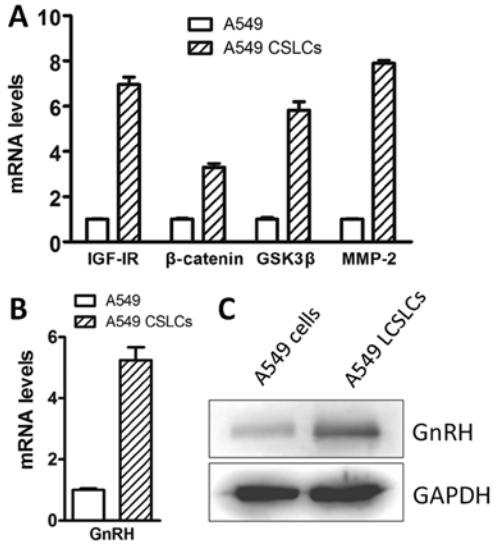

Figure 2. Upregulation of GnRH expression in the A549 LCSLCs. (A) The IGF-IR, β-catenin, GSK3β, MMP-2 and (B) GnRH mRNA levels in the A549 monolayer cells or A549 LCSLCs were examined by qRT-PCR. (C) The GnRH expression levels in the A549 monolayer cells or A549 LCSLCs were determined by western blotting. LCSLCs, lung cancer stem-like cells; GnRH, gonadotropin-releasing hormone; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time PCR.

demonstrated that, compared with the A549 monolayer cells, CD44, CD133, Sox2 and ALDH1 protein expression levels were apparently increased in the A549 LCSLCs (Fig. 1E), suggesting that the LCSLCs possess characteristics of CSCs.

Upregulation of GnRH expression in the A549 LCSLCs. After establishing the stable sphere culture system for enriching and isolating LCSLCs from A549 cells, we performed microarray analysis to further investigate the characteristics of the LCSLCs. The categories of the microarray data (P<0.001) were summarized on the basis of a gene ontology database (data not shown). Genes that were significantly altered in the LCSLCs were functionally categorized into signaling pathways in the KEGG database. Top 10 signaling pathways were correlated with the differentially expressed genes in the LCSLCs, such as IGF, phosphatidylinositol-3-kinase (PI3K)/AKT/GSK3β and Wnt/β-catenin pathways (Fig. 2A), which is consistent with previous reports (10,19-21). Intriguingly, the genes in the GnRH signaling pathway were significantly altered in the LCSLCs, and qRT-PCR and western blotting also confirmed that the mRNA and protein expression levels of GnRH expression were significantly upregulated in the LCSLCs (Fig. 2B and C), suggesting that GnRH may play a role in self-renewal and stemness maintenance of LCSLCs.

Suppression of GnRH capacity negatively regulates the self-renewal and cell stemness of the lung cancer cells. Since higher expression of GnRH was found in the LCSLCs, we next characterized the stemness-associated properties of the A549 cells and examined the biological functions of GnRH on these properties. We then blocked the effects of GnRH by the treatment of cetrorelix acetate, which is a man-made antagonist of GnRH (Selleck, Houston, TX, USA). The cetrorelix acetate was dissolved in sphere cell culture medium, and the concentration was 5 nM, which was modified according to a previous protocol (22). The sphere-forming assay was used to identify stem cells retrospectively based on the utility of the assay in evaluating self-renewal and differentiation at the single-cell level *in vitro*. In our LCSLC culture system, spheroid formation was observed on day 6, indicating that the A549 cells possessed a significant portion of LCSLCs. However, the cetrorelix acetate treatment in the A549 cells significantly inhibited spheroid formation (Fig. 3A). We also found that the treatment of cetrorelix acetate inhibited the cell proliferation of the A549 LCSLCs as shown by the colony-formation assay (Fig. 3B). Western blotting demonstrated that

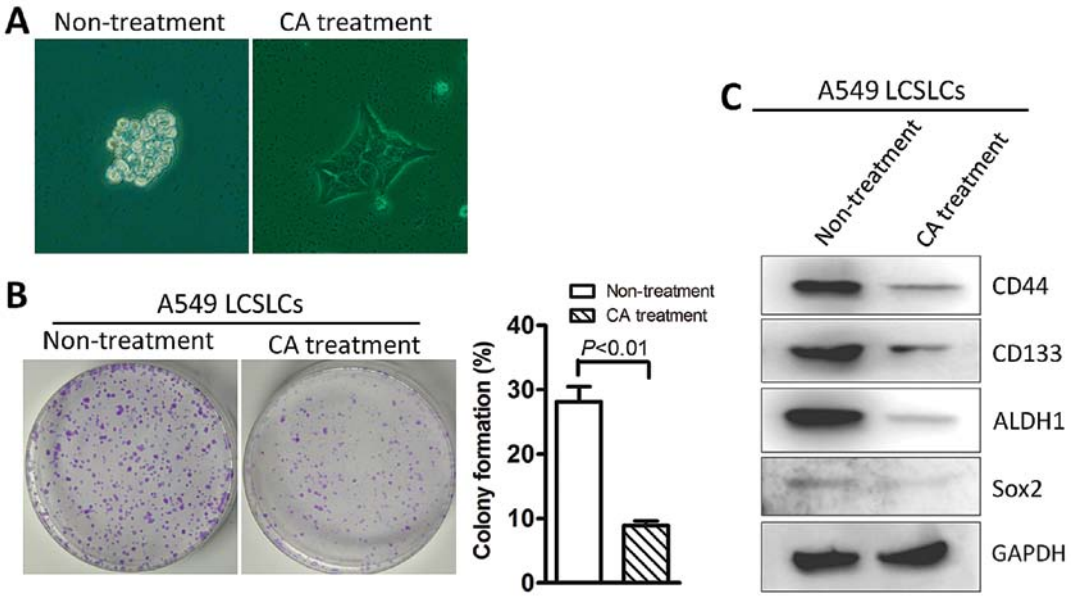

Figure 3. Inhibition of GnRH capacity downregulates self-renewal and cell-stemness in LCSLCs. (A) Spheroid formation and (B) colony formation assay of A549 LCSLCs with or without CA treatment in the sphere culture system. (C) The CD44, CD133, ALDH1 and Sox2 protein expression levels in the A549 LCSLCs with or without CA treatment. CA, cetrorelix acetate; LCSLCs, lung cancer stem-like cells; GnRH, gonadotropin-releasing hormone; ALDH1, aldehyde dehydrogenase 1.

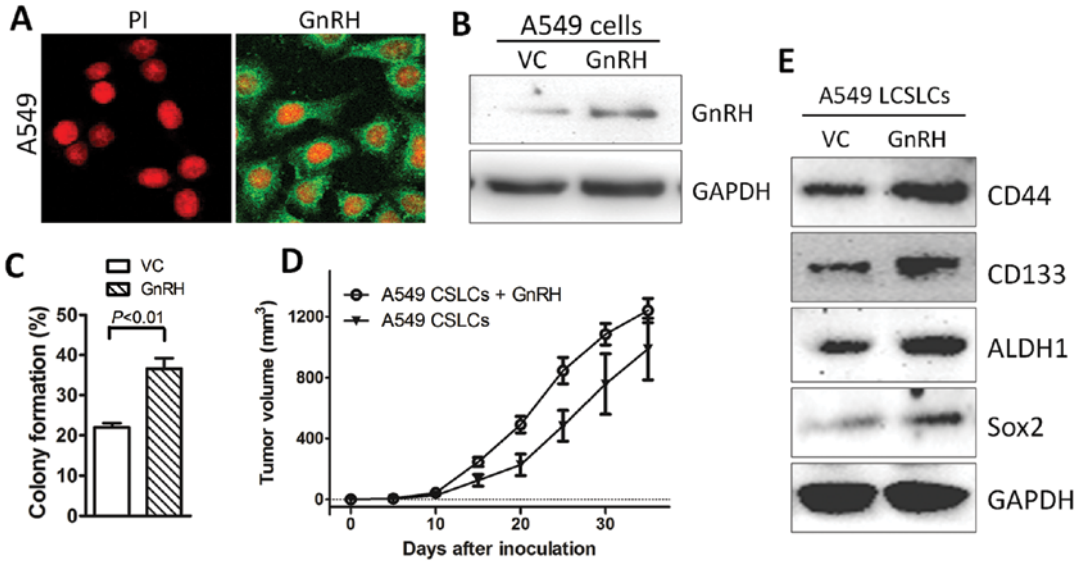

Figure 4. Excessive expression of GnRH induces stemness properties of A549 cells. (A) The distribution of overexpressed GnRH in A549 cells. (B) The GnRH protein expression levels were determined by western blotting in the GnRH-transfected A549 cells and the control. (C) Colony formation assay of GnRH-overexpressing A549 LCSLCs and the control. (D) Quantitative analysis of the xenograft tumors formed by the GnRH-overexpressing A549 LCSLCs and the control. (E) The CD44, CD133, ALDH1 and Sox2 protein expression levels in the GnRH-overexpressing A549 LCSLCs and the control. The A549 cells which transfected with pCDNA3.1(+) were regarded as the vehicle control (VC). LCSLCs, lung cancer stem-like cells; GnRH, gonadotropin-releasing hormone; ALDH1, aldehyde dehydrogenase 1.

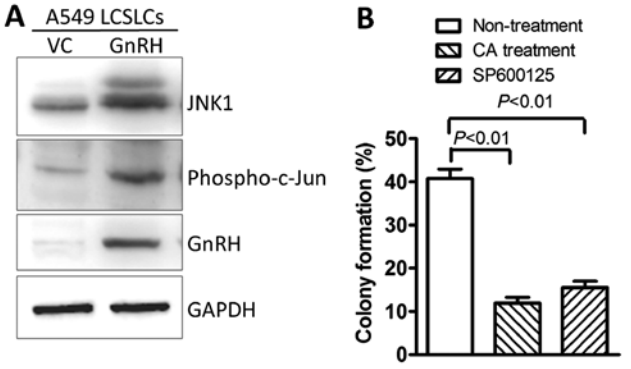

Figure 5. Overexpression of GnRH induces upregulation of the JNK signaling pathway. (A) Expression of JNK and phosphorylated c-Jun in the GnRH-overexpressing A549 LCSLCs and the control. (B) The inhibitory effect of JNK inhibitor (SP600125) and CA treatment on the colony formation of A549 LCSLCs. CA, cetrorelix acetate; LCSLCs, lung cancer stem-like cells.

the expression levels of CD44, CD133, Sox2 and ALDH1 were significantly decreased in the cetrorelix acetate-treated A549 cells in the sphere culture system, compared with the non-treated A549 cells, suggesting that GnRH expression was associated with the self-renewal capacity of the LCSLCs in the A549 cells (Fig. 3C).

Excessive expression of GnRH induces stemness properties in the A549 cells. We then analyzed the consequences of overexpression of GnRH protein by stably transfecting pCDNA3.1(+)-GnRH vector into the A549 cells. After examination using confocal microscopy, we found that most of the A549 cells expressed GnRH protein in the stable transfectants. In addition, at overexpression levels, GnRH was primarily localized in the cytoplasm in the A549 cells (Fig. 4A). Similarly, western blotting further confirmed the overexpression of GnRH in the A549 cells, indicating the stable expression of GnRH protein in this transfectant (Fig. 4B). Furthermore, overexpression of GnRH increased the colony formation in the LCSLCs (Fig. 4C), which was significantly decreased by cetrorelix acetate treatment (Fig. 3B), indicating a higher frequency of LCSLCs derived from the GnRH overexpressing spheres.

The role of GnRH in the self-renewal of LCSLCs *in vivo* was also revealed by examining the tumorigenic potentials of the GnRH overexpressing LCSLCs. We found that tumors derived from the LCSLCs with GnRH overexpression had larger sizes than those of the non-treated cells (Fig. 4D). Moreover, western blotting demonstrated higher expression of stemness markers or stemness-related transcription factors in the GnRH overexpressing LCSLCs (Fig. 4E), indicating that GnRH is a positive regulator of stemness maintenance in LCSLCs.

Promotion of GnRH function for self-renewal and stemness maintenance in the LCSLCs through the JNK pathway. To further elucidate the molecular mechanism of GnRH function in the self-renewal and stemness maintenance in LCSLCs, we further investigated the regulation of the signaling pathways, which may be related to stemness maintenance by GnRH expression in LCSLCs. After determining the expression of several genes, we found that GnRH overexpression regulated the JNK pathway in LCSLCs (Fig. 5A), which is consistent with the previous reports in other tumor tissue (23,24). Furthermore, we found that SP600125, a reversible JNK inhibitor, can significantly inhibit the colony formation capacity of GnRH overexpressing LCSLCs (Fig. 5B), suggesting that GnRH may be associated with the capacity of self-renewal and stemness maintenance through upregulation of the JNK signaling pathway in LCSLCs.

Discussion

CSLCs are a subpopulation of cancer cells, which possess unique survival capabilities and distinct stem cell properties (25), play a critical role in cancer initiation, progression, recurrence, and cancer therapeutic failure (3,26,27). The pathways that regulate self-renewal and cell fate in the CSC system are beginning to be elucidated. To date, little is known regarding the self-renewal and stemness maintenance, as well as the regulatory mechanisms of CSCs. In the present study, we found that GnRH was required for the self-renewal and stemness maintenance of lung cancer cells. The self-renewal capacity can be regulated by GnRH overexpression or inhibition in the LCSLCs. Moreover, we found that this inhibition of GnRH function for self-renewal and stemness maintenance was through the JNK signaling pathway.

In the last few years, it has been established that GnRH is expressed in a variety of human tumor tissues, such as ovary, endometrium, placenta, breast, prostate, and lung tumors (28,29). For example, in both the endometrium and the placenta, activation of locally expressed GnRH increases the activity of the urokinase type plasminogen activator and matrix metalloproteinase (MMP) system, suggesting the GnRH may reduce the migratory and the invasive behaviors of cancer cells by regulating MMP-2 expression/activity (30,31). In addition, GnRH angonists have been shown to exert strong anti-proliferative effects on human prostate, breast and ovarian cancer cells (32-34). Moreover, GnRH may exert antitumor activity not only by reducing cancer cell proliferation and metastasis, but also by affecting the process of angiogenesis. It has been found that GnRH analogs significantly decrease the synthesis and secretion of VEGF in melanoma cells (35). Based on these observations, tumor GnRH and its receptor are now considered a very promising candidate for novel molecular-targeted strategies for the treatment of tumors. However, limited data exist on the significance of GnRH expression in CSCs, and the association between GnRH and stemness maintenance in CSCs.

To investigate the functions of GnRH in CSCs, we firstly isolated the sphere subpopulation from human NCLSC A549 cells using a sphere-forming enrichment culture method with selective serum-free medium. The results demonstrated that the sphere cells could proliferate and form a new sphere, confirming that the sphere cell subpopulation possessed self-renewal capacity (Fig. 1). We then discovered that the expression levels of CSC biological markers, such as CD44, CD133 and ALDH1, and stemness-related transcription factors Sox2 in the LCSLCs, were much higher than that in the monolayer A549 cells. As known, CD44, CD133, ALDH1, and Sox2 are important for the sorting and identification of various types of CSCs, especially in lung cancer.

Although the functions of GnRH in various tumors have been well studied in recent years, the data available on the presence of GnRH in lung cancer are still limited (36). After microarray assay analysis, we found many alterations in gene expression levels in the A549 monolayer cells and LCSLCs. The higher expression level of GnRH in LCSLCs encouraged us to investigate the functions of GnRH in the regulation of stemness maintenance in LCSLCs (Fig. 2). Notably, both overexpression and inactivation of GnRH demonstrated that it is essential for the self-renewal and stemness maintenance *in vivo* and *in vitro* (Figs. 3 and 4). Regardless of the mechanisms, our results suggest that GnRH capacity regulates the stemness maintenance of LCSCs.

GnRH has been known to regulate different intracellular signaling cascades, such as MAPK (p38/MAPK, ERK1/2, and JNK), PI3K, and phosphotyrosine phosphatase (PTP) (23,37-39). In ovarian and endometrial cancer cells, the GnRH agonist triptorelin has been reported to increase JNK activity, leading to c-Jun binding to DNA and, ultimately, to an anti-proliferative effect (17,40). Moreover, JNK was shown to mediate the GnRH-stimulated invasive behavior of ovarian cancer cells by increasing the expression of MMP-2 and MMP-9 (24). Therefore, we hypothesized that the GnRH function of self-renewal and stemness maintenance in LCSLCs was through the JNK signaling pathway. Song *et al* provided evidence that the JNK/c-Jun signaling pathway mediates EPHA2-dependent tumor cell proliferation, motility, and cancer stem cell-like properties in NSCLCs (41). A recent study also demonstrated that JNK signaling is crucial for the maintenance of self-renewal and tumorigenicity of glioma stem-like cells and drug/IR resistance, and can be considered a promising target for eliminating stem-like cancer cells in glioma (42). Furthermore, in ovarian CSCs, it was found that JNK plays an essential role in the maintenance of the self-renewal and tumor-initiating capacity (43). As expected, our findings demonstrated similar results, that GnRH overexpression can induce the activation of the JNK signaling pathway in LCSLCs (Fig. 5), suggesting that the GnRH-mediated activation of the JNK pathway may be essential for self-renewal and stemness maintenance in LCSLCs.

Although our studies suggest that JNK signaling mediates GnRH-dependent stemness maintenance in LCSLCs, it cannot be excluded that other GnRH-regulated signaling pathways may also play a role in this process. It still remains largely unknown whether the precise role of GnRH in the self-renewal and stemness maintenance in LCSLCs is through regulation of the JNK pathway or through other signaling pathways. Therefore, the precise mechanisms and pathways, by which GnRH participates in the upregulation of the JNK pathway for self-renewal and stemness maintenance, require further investigation.

Acknowledgements

The present study was supported by grant WX13B02 from the Foundation of Health and the Family Planning Commission of Wuhan Municipality, China.

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