Excessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly

Ju Hun Oh

Department of Medical Science

The Graduate School, Yonsei University

Excessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly

Directed by Professor Eun Jig Lee

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Ju Hun Oh

June 2022

This certifies that the Doctoral Dissertation of Ju Hun Oh is approved.

Thesis Supervisor: Eun Jig Lee

Thesis Committee Member #1: Young Suk Jo

Thesis Committee Member #2: Cheol Ryong Ku

Thesis Committee Member #3: Sang Ouk Chin

Thesis Committee Member #4: Ja Seung Koo

The Graduate School
Yonsei University

June 2022

ACKNOWLEDGEMENTS

This work would not have been possible without the support of my dissertation advisor Prof. Eun Jig Lee. His gift for conceptualization, enduring encouragement, and his practical advice have been an innumerable source of support for me during this process.

I would also like to thank the rest of my thesis committee, including Prof. Young Suk Jo, Prof. Cheol Ryong Ku, Prof. Sang ouk Chin, and Prof. Ja Seung Koo for their advice and feedback. Their varied perspectives have helped me to strengthen my work.

My sincere thanks also go to my immediate supervisor Dr. Chan Woo Kang for supporting and teaching me.

I am grateful to all of those with whom I have had the pleasure to work during this and other related projects including Dr. Yoon Hee Cho, Prof. Da Ham Kim, Dr. Yang Jong Lee, Eun Kyeong Wang, Bao Yaru, and SooHyun Lee.

Nobody has been more important to me in the pursuit of this project than my mother. I would like to thank my mother, whose love and guidance are with me in whatever I pursue. She is the one and only supporter for me.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Excessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly

Ju Hun Oh

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Eun Jig Lee)

Acromegaly is a disease with consistently high growth hormone (GH) levels in the circulation, caused by a GH-secreting tumor of the pituitary gland. In previous epidemiological studies, patients with acromegaly showed increased tumor development and metastasis, including thyroid and breast cancers. Conversely, according to the results of a follow-up study of patients with Laron syndrome, who have mutations in the growth hormone receptor (GHR), it is known that people with mutations in GHR do not develop cancer. To date, however, little is known about the precise mechanistic involvement of GH in tumor development and metastasis.

Here, we showed that excessive serum GH in acromegaly promoted breast cancer aggressiveness and metastasis, while GH antagonists prevented breast tumor progression. Excess GH acted as an antioxidant, increasing the expression of antioxidant genes involved in tumor metastasis. Particularly, growth hormone increases TCF-20 gene expression, which activates the transcription of Nrf2 and Nrf2 related genes and increases tumor metastatic phenotype. Modulating the expression of TCF-20 and growth hormone receptor normalize excessive GH-induced tumor progression and metastasis. We conclude that excessive GH acts as antioxidants and stimulates early tumor progression and metastasis by reducing oxidative stress. Excessive GH stimulates antioxidant related genes, especially TCF-20, dependent tumor

progression and metastasis.

Key words: Growth hormone, IGF-1, Breast cancer, Acromegaly, Metastasis

Excessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly

Ju Hun Oh

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Eun Jig Lee)

I. INTRODUCTION

The pituitary gland, known as “mater gland”, plays a central role in hormone secretion, including secretion of the growth hormone (GH). This gland is the central endocrine organ that controls feedback regulation of the body's hormone system [1]. GH is a peptide hormone that is mainly secreted by somatotropic cells within the anterior pituitary gland. This anabolic hormone is involved in many biological anabolic processes, including insulin-like growth factor-1 (IGF-1) secretion, muscle and bone growth, and glucose and fat metabolism [2]. GH is secreted in a pulsatile manner, which is regulated by hypothalamus stimuli, growth hormone-releasing hormones, and growth hormone-inhibiting hormones [3]. Dysregulation of GH secretion leads to GH excess or deficiency, causing several diseases.

Acromegaly is characterized by chronic GH and IGF-1 hypersecretions. It is primarily caused by a GH-secreting pituitary adenoma, a benign tumor composed of somatotroph cells of the anterior pituitary [4,5]. Pituitary gland tumors are the most common cause of head and neck tumors in adults after their 20s, accounting for 15% of all head and neck tumors [6,7]. Excess GH causes

progressive somatic disfigurement and a wide range of systemic manifestations, such as excessive growth in the bones and soft tissues in the body, contributing to abnormal height and exaggerated bone structure [8].

Previous epidemiological studies have shown the incidence of cancer, including thyroid and breast cancer, increases in patients with acromegaly [9-11]. Conversely, a follow-up study of patients with Laron syndrome, who have mutations in the GH receptor (GHR), characterized by a lack of IGF-1 production in response to GH, revealed that people with GHR mutations did not develop cancer [12].

Excessive GH secretion is involved in breast cancer development. According to a recent meta-analysis of 5 million women, a 10-cm increase in height increased the risk of hormone-dependent breast cancer by 17% [13,14]. The process by which cancer cells spread from the site of origin to other organs through the blood and lymphatic vessels and form a new colony is called metastasis [15]. Notably, autocrine GH and growth hormone receptors (GHR) are expressed by most breast cancers, with the highest expression in metastatic breast cancers [16]. Autocrine human GH could facilitate the epithelial-to-mesenchymal transition (EMT), a prerequisite of metastasis, in epithelioid breast cancer cells, resulting in a metastatic phenotype [17]. Autocrine GH is an oncogenic factor that promotes breast cancer stem cells [18].

Increasing evidences have shown that excess GH might play a pivotal role in the progression of tumor seeding, migration, and invasion of breast cancer cells by influencing tumor angiogenesis, stemness, and chemoresistance[15]. However, the precise mechanism of action of GH in breast

cancer metastasis has not been defined. To address this issue, the present study investigated the mechanism of GH signaling in breast cancer metastasis, in an acromegaly mouse model.

II. MATERIALS AND METHODS

1. Animal study

An animal model that was used in this study was previously established mouse model in our laboratory, which expressed phenotype of GH secreting pituitary adenoma [10]. Somatotroph specific aryl hydrocarbon receptor interacting protein (AIP) knockout (sAIPKO) mouse model was made using rGHp-Cre$^{tg/+}$; Aip$^{lox/lox}$ mice [19]. Aip$^{lox/lox}$ mice was used as controls. All animals in this study were over 24 weeks old female because most sAIPKO mice showed high blood levels of GH as it aged. Mice were maintained under controlled conditions (12h light: 12h darkness cycle, 21 ℃), and were given free access to laboratory chow and tap water. All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol and institutional guidelines for the proper and humane use of animals.

2. Orthotopic injection of breast cancer cells

Orthotopic injection of breast cancer cells into the mammary fat pad was performed and modified according to the previous study [20,21].

A. Preparation of cells

On the day of operation, wash the py230 cells derived from C57BL/6 once with phosphate buffered saline (PBS) and trypsinize the cells. Quench the trypsin by adding 10 ml serum containing DMEM media. Centrifuge the cells at 1,300 rpm for 5 min at RT to remove serum by resuspending the cells in serum-free media. Centrifuge the cells again to remove remainder serum completely. Resuspend the cells in media. And then, count the cells and calculate the number of cells. Use 500,000 cells/mouse in 150μl. Resuspend the cells in Matrigel. Keep them on ice.

B. Orthotopic injection

40-week-old female mice were anesthetized using 3% inhalant isoflurane. Fix the mouse on a heating pad. Shaved around the fourth nipple region and clean the shaved area by using the ethanol cotton swab. Make a small incision between the fourth nipple and the midline with a scissor and make a pocket by inserting the cotton swab moistened with PBS pH 7.4. Expose the mammary fat pad and squeeze the fat pad with tweezer to expose the fat pad to perform injection easily. Homogenize the cell mixture by pipetting up and down. Py230 cells can then be injected using an insulin syringe with a volume $\leq$120 μL. Successful injection is confirmed by the swelling of the tissue. Suture the incision site. All procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Yonsei university college of medicine.

C. Tumor volume measurement

The length, width, and depth of the tumor mass were measured every 2 days using calipers, and tumor volume was calculated as: tumor volume = 0.5236 X length X width X depth (mm3).

3. In vivo Bioluminescent Imaging

A. CMV-firefly-2A-GFP-Puro Transduction in Py230

Pre-made lentivirus CMV-firefly-2A-GFP-Puro (Cat# LYP020; Gen Target Inc, San Diego, CA, USA) transduction was conducted according to manufacturer's instructions. Briefly, seed cells in complete medium at the 50%~75% confluent and incubate overnight. The next day, thaw the pre-made lentiviral stock at room temperature and add the 50ul of virus stock to obtain the desired MOI. After 72 hours (no need to change medium), visualize positive transduction rate by fluorescence microscopy. Sort transduced cells by FACS

and select for antibiotic resistance.

B. Bioluminescence Imaging (BLI)

After tumor injection, BLI was performed every week. On the day of imaging, mice were intraperitoneally injected with D-luciferin (150mg/kg) and were placed in a light-tight mouse imaging chamber following anesthesia. Twenty minutes after injection, each animal was imaged alone in supine and prone positions with an exposure time of 10 min for each position weekly for 6 weeks by using IVIS Spectrum (Xenogen, Alameda, CA, USA). Regions of interest (ROIs) were analyzed, and total quantification of bioluminescence was quantified using Living Image® (Xenogen) software. Photons detected from breast cancer models were converted to average radiance (photon/sec/cm2/sr). Average radiance values are quantitative data obtained from region of intensity (ROI) where photons emitted by bioluminescent cells of assigned rectangular area over the whole body of each mouse. Both luminescence and image data were analyzed using Living Image software [22].

4. Transwell Matrigel invasion assay

Breast cancer cells were seed to Matrigel-coated transwell insert and add 0.75 mL of chemoattractant to the lower wells. Incubate cell invasion chambers overnight in a humidified tissue culture incubator. After incubation, non-invading cells were removed from the upper side of Matrigel-coated side by scrubbing. The invaded cells to the lower surface of the Matrigel were stained with 0.1% crystal violet and rinsed with distilled water. The dried Matrigel was placed on a slide, added with a drop of immersion oil, and then covered with a coverslip.

5. Migration assay

Py230 cells were seed at 1 x 10^6 cells/well in 6-well plate for 100%

confluence in 24h. In a sterile environment, use a 200 μl pipette tip to press firmly against the top of the tissue culture plate and swiftly make a vertical wound down through the cell monolayer. Carefully aspirate the media and cell debris and slowly add enough culture media against the well wall to cover the well. To take a snapshot picture and to check for wound closure.

6. RT^2 Profiler PCR Arrays

The amplified cDNA was used on the real-time RT2 Profiler PCR Array (QIAGEN, Cat. no. PAMM-028Z) in combination with RT2 SYBR®Green qPCR Mastermix (Cat. no. 330529). Each array plate contained one set of 96 wells for testing. Genomic DNA contamination, reverse transcription, and positive PCR controls were included in each 96-well set on each plate. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the assay reference gene. CT values were derived to an Excel file to build a table of CT values, which is then uploaded onto the data analysis web portal at http://www.qiagen.com/geneglobe. Samples contained controls and test groups. CT values were normalized based on Automatic selection from full panel of reference genes [23].

7. Luciferase assay

The putative SPBP binding sites were analyzed in the 2 kb upstream sequences of Nrf2 gene using PROMO [24]. Three putative binding sites on Nrf2 were amplified from the genomic DNA of breast cancer cell line using conventional PCR. Deletion mutants were constructed from the PCR products by an overlap extension PCR method [25]. The WT and deletion mutant constructs were cloned into pNL4 vector. For dual luciferase assays, each cloned vector was co-transfected with pRL-TK in HEK293T (2×10^5) cells and MDA-MB-231(5×10^5) using Lipofectamine® LTX with Plus™ Reagent (Invitrogen®, Carlsbad, CA, USA). Forty-eight hours after transfection,

luciferase activity was measured from the cell lysates using a dual-luciferase reporter assay system (Promega; Madison, WI, USA) using luminometer (EG & G Berthold, Bad Wildbad, Germany).

8. Cell culture and reagent

Mouse triple negative breast cancer cell line Py230 was purchased from the American Type Culture Collection and cultured in DMEM replenished with 10% fetal bovine serum (FBS; Hyclone Co., Logan, UT, USA), 1% Pen/Strep (Hyclone Co., Logan, UT, USA), and 0.1% MITO+ Serum Extender (Corning #355006). Py230 cells will not maintain property without MITO+ Serum Extender. Human breast cancer cell lines Hs578T and MDA-MB-231 were purchased from the American Type Culture Collection and cultured in DMEM replenished with 10% FBS (Hyclone Co., Logan, UT, USA), 1% Pen/Strep (Hyclone Co., Logan, UT, USA). Cells were grown in a culture incubator that maintained the atmospheric condition of 37°C and 5% CO2. Media was changed within 3 days and the cell line was split with trypsin/EDTA every week.

Recombinant human GH (Bio Vision or R&D Systems) was reconstituted in culture medium containing 0.1% BSA. Cells were placed in culture medium free of serum containing 0.1% BSA, GH was added, and cells were harvested 24 h later. If treatment was extended to 48 h, GH was added daily [26]. Antibodies against p-STAT5, STAT5, HO-1, Histone H3 were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin, GHR were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

9. siRNA transfection

siRNA targeting mouse Ghr (UAU AAU UUC UGU UUA CUG C), mouse TCF-20 (UAU UUA UAU AUA UAU AUU C), human GHR (UAU AAU UUC UGU UUA CUG C), and human TCF-20 (UAU ACU GCA UCA CAU

GCU GAG AAG G) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Py230 cells and Hs578T cells were seed at 0.8 x 10^6 cells/well in 6-well plate 24hr before transfection. siRNAs were transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA) in Opti-MEM media (Thermo Fisher Scientific, Waltham, MA, USA) for 16hr. Media was changed into culture media on the next day and incubated for additional 24h before analysis.

10. RNA isolation, quantitative Real-Time PCR

Total RNA was isolated using NucleoZOL (Macherey-Nagel, Duren, Germany) followed by reverse transcription (ReverTra Ace-α; TOYOBO, Osaka, Japan) according to the manufacturers' instructions. Resulting cDNA was subjected to quantitative real-time PCR. Quantitative real-time PCR was conformed with Power SYBR®Green Master Mix (4367659; Thermo Fisher Scientific, Waltham, MA, USA) using the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturers' instructions.

11. Western blot

Total protein samples were isolated from frozen primary breast cancer and metastatic tumor and cultured cells using Cell lysis buffer (Cell signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Lysed on ice for 30 minutes and then centrifuged for 15 min at 13,000 rpm at 4°C. Protein samples were separated in a 10% SDS-polyacrylamide gel electrophoresis and transferred onto activated polyvinylidened difluoride (PVDF) membranes. Membranes were blocked in 5% skim milk, incubated with the specific primary antibodies at 1:1000 in TBST. After primary and secondary antibody incubation, the blot will be visualized using WESTSAVEup (west blotting substrate) and exposed to x-ray

film (Agfa HealthCare).

According to the manufacturer's instructions, nucleus and cytosol proteins were separated by using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Waltham, MA, USA).

12. GH and IGF-1 ELISA

Blood samples for GH and IGF-1 assays were collected at the time of 40 weeks and sacrifice after overnight fasting and serum and plasma sample was stored at -80°C. Mouse growth hormone ELISA kit 96-well plate (Millipore, Billerica, MA, USA) and Mouse IGF-1 ELISA kit (Abcam, Cambridge, UK) were used for and performed according to the manufacturer's instructions.

13. Immunohistochemistry

Mouse primary tumors and lung metastatic tumors were fixed for 7 days in 10% formalin. 5μm-thick tissue sections were cut from each block to perform either hematoxylin-eosin or immunostaining with TCF-20, Nrf2, Ho-1, Nqo-1, and GHR antibodies. After dewaxing and rehydrating paraffin sections and antigen retrieval by TRS low or High buffer for each antibody-conditions. After antigen retrieval, tissue sections were immunolabeled with primary antibodies used as follows: rabbit anti-TCF-20 (1:200, Novus Biologicals, CO, USA), mouse anti-GHR (1:250, Santa Cruz, CA, USA), mouse anti-NQO-1 (1:250, Santa Cruz, CA, USA), rabbit anti-Ho-1 (1:250, Cell signaling Technology, MA, USA). All slides were counterstained with hematoxylin.

III. RESULTS

1. Excess GH increased breast cancer growth in acromegaly mice

To define the impact of excess GH on breast cancer growth, the $Aip^{lox/lox}$; rGHp-$Cre^{tg/+}$ (Acro) mice and $Aip^{lox/lox}$ controls (Con) mice were used in a mouse model of Acromegaly. We also used the mouse breast cancer cell line Py230 of murine mammary tumor cells derived from a C57BL/6 female mouse. We injected orthotopically py230 cells into the fourth mammary gland of the animals (Figure 1A). The length, width, and depth of the tumor mass were measured every two days using calipers, and the tumor volume was calculated. The volume of breast cancer cells was 4- to 5- fold higher in Acro mice than in Con mice (Figure 1A, 1B). Serum GH levels were 3- to 4-fold higher in Acro mice, and tumor volume was positively correlated with serum GH levels (Figure 1C, 1D). Additionally, 50-week-old Acro mice showed tumor development in their mammary glands (Figure 1E).

These results indicated that excess GH signaling was strongly linked to breast tumor growth in animals with acromegaly.

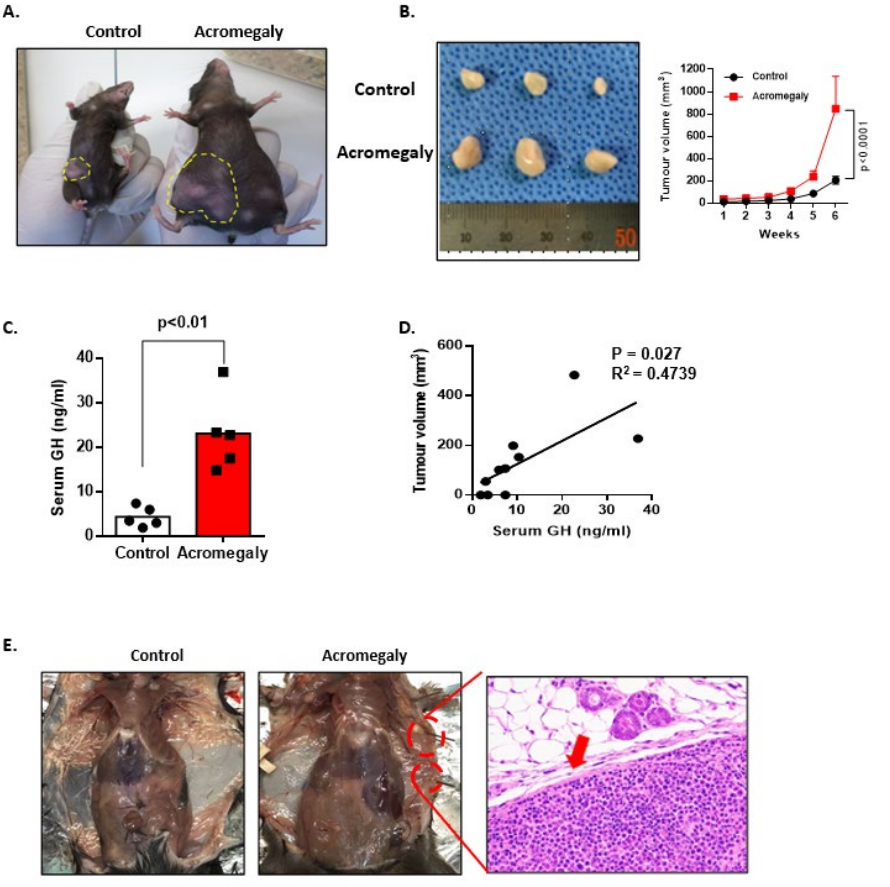


Figure 1. Excess growth hormone increases breast cancer growth in acromegaly mice. (A) Representative images of orthotopically injected breast tumor after 8 weeks. (B) Representative images of tumor volume and quantification of tumor volume. The length, width, and depth of the tumor mass were measured every 2 days using calipers, and tumor volume was calculated as: tumor volume = 0.5236 X length X width X depth (mm3). (C) GH concentration in serum of Con and Acro mice was evaluated using GH ELISA. (D) Linear progression between serum GH and tumor volume. (E) Representative images of breast tumor in 50-weeks Acro mice. Images were obtained at 200X magnification.

2. Suppression of GH signaling reduced tumor growth and aggressiveness

As excess GH promoted breast tumor growth, we investigated whether inhibition of GH signaling could protect Acro mice from tumor development. Py230 cells were engineered to stably and constitutively express luciferase by lentiviral-mediated gene transfer (Py230-Luc) (Figure 2A). Luciferase activity in Py230-Luc cells was confirmed by dose-dependent luciferin treatment (Figure 2B), and *in vitro* bioluminescence imaging (Figure 2C). Two weeks after Py230-Luc orthotopic injection, we measured tumor growth by bioluminescent in vivo imaging system (IVIS) (Figure 2D). After 4 weeks, when the primary tumor volume exceeded 100 mm^3, we subcutaneously injected a GH antagonist (Pegvisomant) in the peritumoral region. Inhibition of GH signaling by Pegvisomant significantly reduced tumor volume. Interestingly, some of the Pegvisomant-treated Acro-mice exhibited significant tumor regression in the mammary fat pad after 14 weeks. In contrast, vehicle-treated Acro mice exhibited gigantic tumor growth (~1,000 mm^3, Figure 2D).

These results indicated that breast cancer aggressiveness could be induced by excessive serum GH and, conversely, alleviated by the suppression of excess GH signaling.

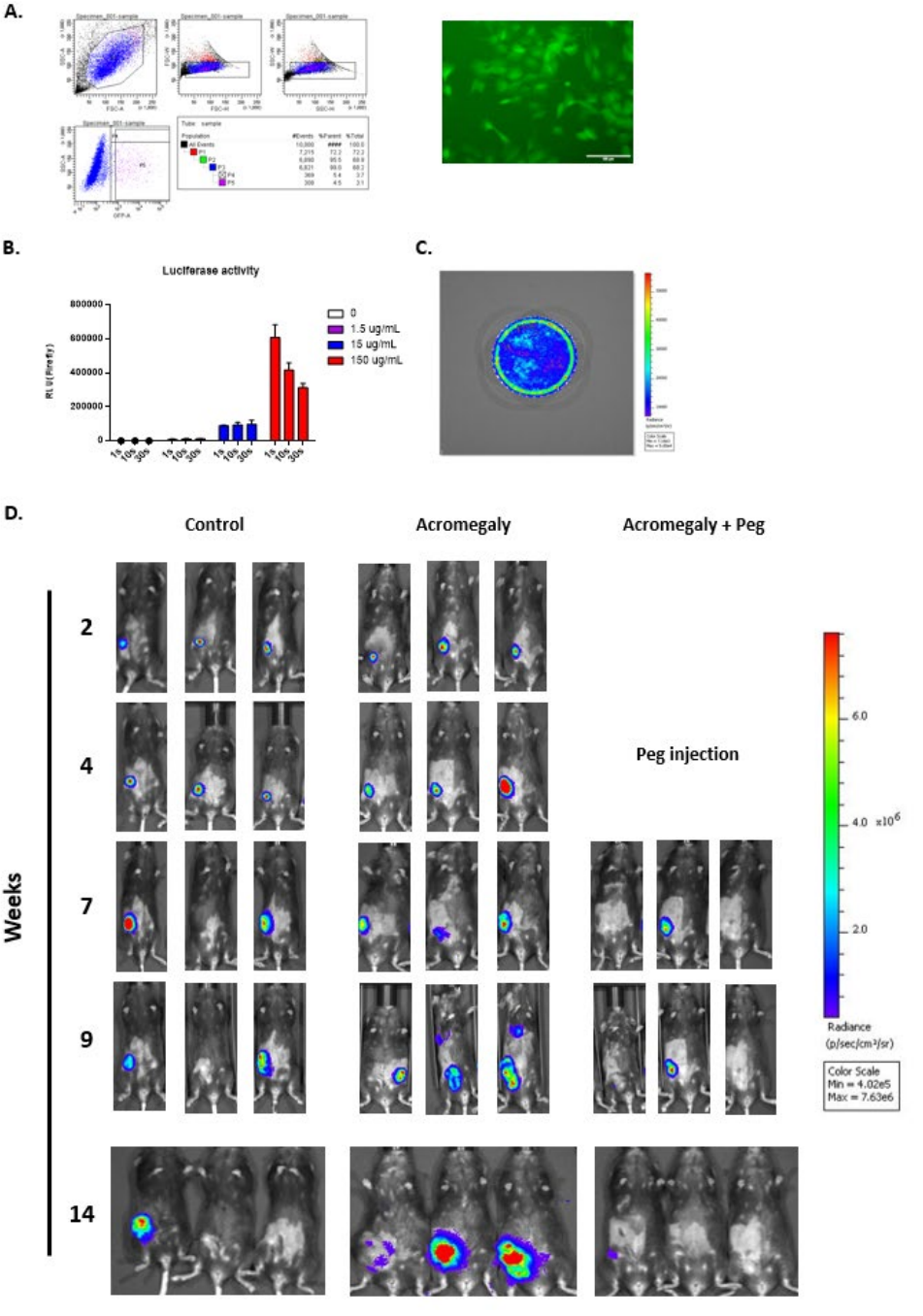


Figure 2. GH signaling suppression reduced tumor growth. (A) GFP-FACS sorting result of Py230-Luc and representative image of GFP fluorescent image of py230-Luc. (B) Luciferase activity confirmation by luciferin at day 14 after transfection. (C) *In vitro* bioluminescence image of py203-Luc cell at day 14 after transfection. (D) Con-mice and Acro mice were mammary fat pad-inoculated with 2 x 10^6 py230-Luc cells. 4 weeks later, Acro-mouse were treated with Pegvisomant (Peg) every day for 8 weeks. In vivo bioluminescence imaging of breast tumor after inoculation.

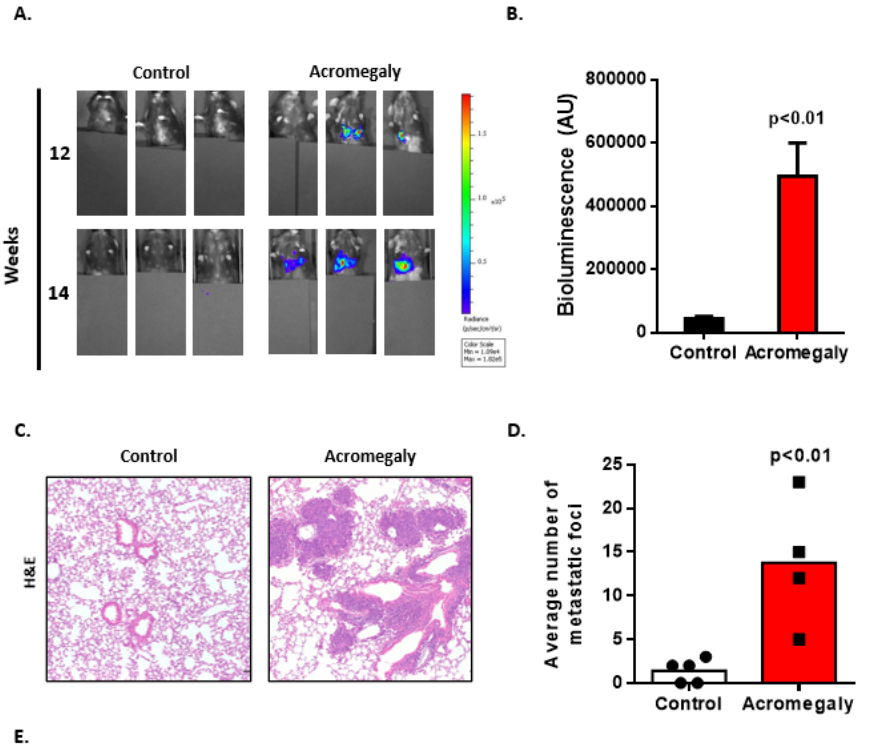
3. Excess GH signaling enhanced metastasis

Vehicle-treated Acro mice exhibited mild chest bioluminescence activity at week 9, compared to Con or Pegvisomant-treated mice. Thus, we speculated that excess GH might enhance tumor cell invasion and metastasis. To identify the role of excess GH in lung tumor invasion and metastasis, we measured lung-specific bioluminescence. Compared with the Con group, the Arco group showed significantly increased lung bioluminescence activity in the lungs after 12 weeks (Figure 3A, B). After 14 weeks, we had to sacrifice animals from the Acro-mice group because of breathing problems due to serious lung metastatic burden. In contrast, lung bioluminescence activity in Con mice was barely detectable (Figure 3A, B).

Next, metastatic lung foci were quantified by histological analysis using hematoxylin and eosin staining and Computed Tomography (CT) imaging of lung nodules. Acro-mice showed a significantly increase in the number of lung metastatic foci compared to Con-mice after 8 weeks (Figure 3C, D). By 16 weeks, the lung metastatic burden increased in Acro-mice along with tumor growth (Figure 3C~D, G).

Interestingly, lung metastases were more aggressive in Acro-mice harboring even smaller primary tumors than those in Con-mice primary tumors, in which lung metastasis was hardly confirmed (Figure 3E~F). We further measured the bioluminescence activity in other organs, where breast cancer mainly metastasized to, including the liver, brain, bone, and lymph nodes. No bioluminescence signals were observed in organs other than lungs (data not shown).

These results suggest that excess GH exposed breast tumor is more prone to develop lung metastasis.



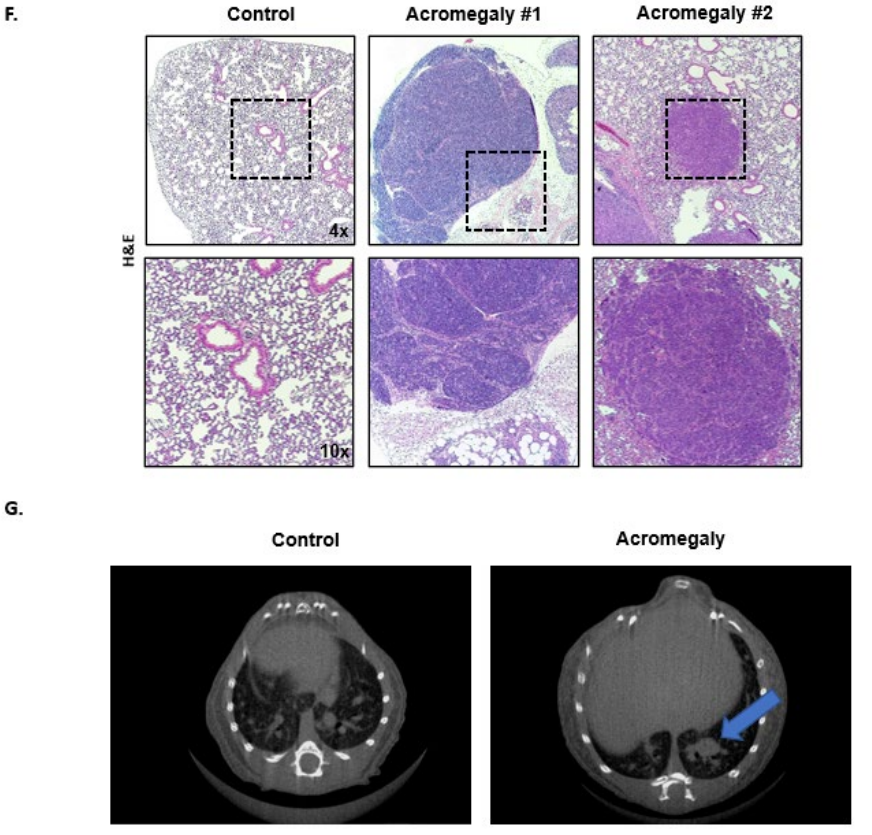


Figure 3. Excess GH stimulates lung metastasis. (A) In vivo bioluminescence images of lung metastasis from representative mice at 12 weeks and 14 weeks after inoculation with py230-Luc cells (n= 8 mice per group). (B) Quantification of lung metastasis. (C) Representative H&E staining of lung metastatic foci. (D) Quantification of H&E-stained metastatic foci area of lung lesions. (E, F) Representative larger primary tumor of Con-mice with no lung metastasis and smaller primary tumor of Acro-mouse with aggressive lung metastases and (F) representative H&E staining of those lungs. (G) Representative images of CT analysis of lung metastasis.

4. Exogenous GH induced breast cancer cell growth and invasiveness

To define the mechanisms of tumor growth and the pro-metastatic effects of excess GH, the effects of excess GH on colony formation, and invasiveness in breast cancer cell lines were examined.

Phosphorylation of the signal transducer and activator of transcription 5 (STAT5) is the initiating step for GH signaling [27]. GHR expression was confirmed in all human triple-negative breast cancer (TNBC) cell lines (MDA-MB-231, Hs578T, and HCC-1806) and the murine Py230 cells. Furthermore, exogenous GH treatment induced phosphorylation of STAT5 (Figure 4A) [28].

We performed proliferation assays. GH increases cell proliferation in Py230cells and Hs578T cells (Figure 4C). Also, in MDA MB 231 cell and Hs578T cells, GH treatment significantly increased tumor cell invasion and migration compared to the control cells (Figure 4D). As GHR was found to be expressed in breast cancer cell lines, we investigated whether modulating its expression would affect breast tumor growth. We used short interfering (si)RNAs to knock down GHR expression in Py230 cells and analyzed tumor growth and survival. When treated with exogenous GH, the colony formation of siGHR-treated py230 cells reduced than siCON-treated py230 cells (Figure 4E). In py230 cells, GH treatment significantly increased tumor cell migration compared to the control cells by dose-dependent manner (Figure 4F).

These results indicated that exogenous GH increases breast cancer cell growth and invasiveness.

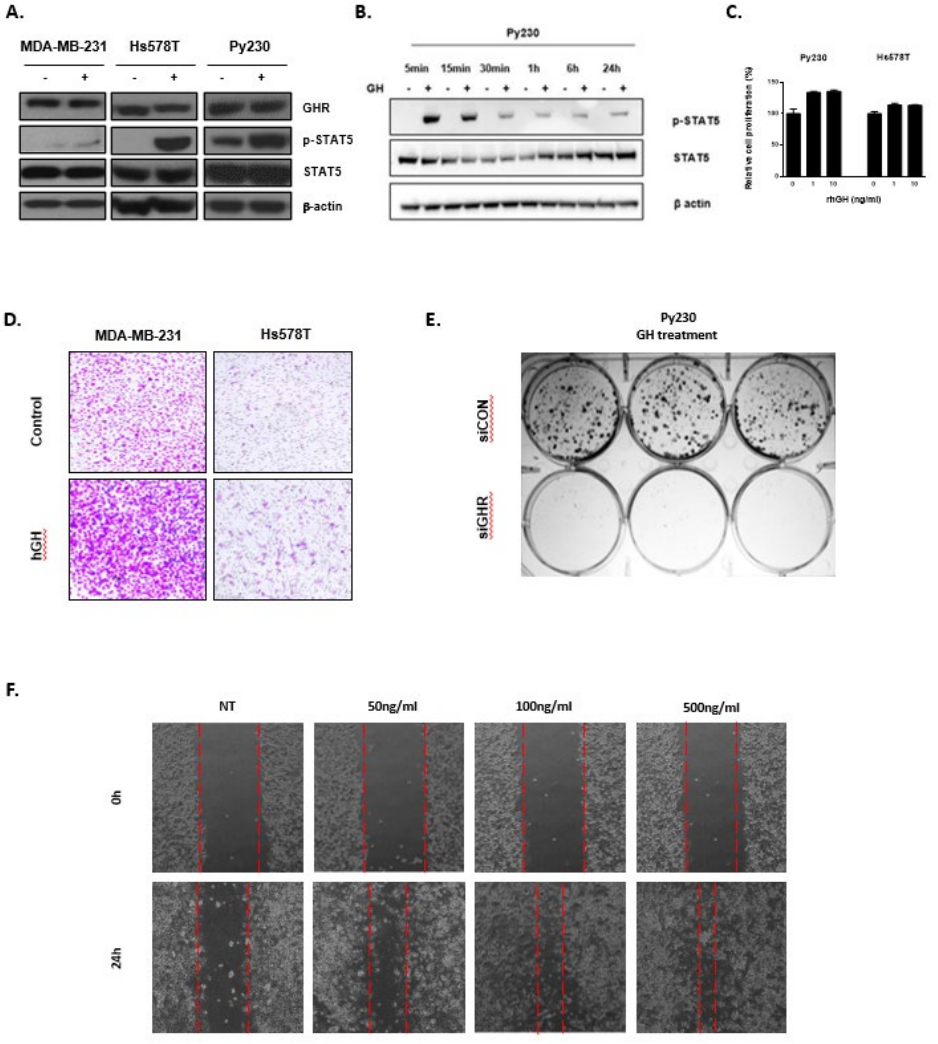


Figure 4. Exogenous GH increases breast cancer cell growth and invasiveness. (A) Western blot analysis of MDA-MB-231, Hs578T, and Py230 cells treated with GH showing amounts of GHR and STAT5. (B) Western blot analysis of Py230 cells treated with GH for indicated time showing amounts of STAT5. (C) Representative images of invasion assay in MDA-MB 231 cells and Hs578T treated with control or GH. (D) Colony formation assay in GH treated-Py230 cells transfected with siCON or siGHR.

5. GH enhanced antioxidant-related genes by upregulating TCF-20/Nrf2.

Previous studies have demonstrated that GH acted as a molecular component of the neoplastic growth by suppressing p53, reducing apoptosis in colon cancer cells and suppressing colon mucosal p53/p21 *in vivo* [26]. In addition, GH might induce EMT in various cancers through the activation or repression of EMT-related mediators, including known oncogenic mediators (cSRC and PTEN) and the canonical GH mediators (JAK2 and STAT5) [29]. However, most studies only demonstrated the role of autocrine GH in cancer cells, but not in conditions with excess endocrine GH, such as acromegaly.

To investigate the molecular mechanism of excess GH in breast cancer metastasis, we used a mouse tumor metastasis RT^2-profiler PCR array (RT^2 array) in primary tumors and lung tissues (Figure 5A). In line with previous reports, MMP9 and PTEN were also upregulated in primary tumors and lung metastatic lesions of Acro-mice compared to those of Con-mice. The most highly upregulated target in primary tumors and lung lesions of Acro mice compared to those of Con mice was TCF-20 (Figure 5A).

The transcriptional coregulator TCF-20 (also known as SPBP) is a 220 kDa multidomain nuclear protein, which is expressed in most cells and tissues. TCF-20, originally identified as a platelet-derived-growth-factor (PDGF) induced protein, is involved in transcriptional activation of MMP3 as well as metastasis by promoting EMT [30]. Recently, TCF-20 was shown to be a transcriptional coactivator of NRF2, which regulates the expression of autophagy receptor p62 [30] and Nrf2, (a master transcriptional regulator of the cell antioxidant program). Increasing evidence has demonstrated that persistent activation of Nrf2 promoted tumor metastasis [31-33]. However, whether the activation of the TCF-20/Nrf2 axis promotes tumor metastasis remains unclear. Since TCF-20 was increased in primary and metastatic tumors of mice with excess GH secretion, we examined the impact of the induction of TCF-20/Nrf2

activation by excess GH on tumor metastasis.

Consistent with the RT^2 array results, TCF-20 was confirmed to be upregulated in both primary tumors and lung metastatic lesions using qPCR analysis (Figure 5B).

To further confirm the relationship between excess GH and TCF-20 activation, we examined the mRNA expression of TCF-20, Nrf2, and target genes of Nrf2 in TNBC cell lines. The expression of TCF-20 and antioxidant -related genes (Nrf2, Ho-1, Bach1, and ARNT1) was upregulated in both GH-treated Hs578T cells (Figure 5C) and Py230 cells (Figure 5D). In line with Fig 5C and D, TCF-20 protein levels were increased by recombinant GH treatment in a dose-dependent manner (Figure 5E). To examine the nuclear expression of GH-induced Nrf2 nuclear translocation, we isolated cytoplasmic and nuclear proteins from Py230 cells. Interestingly, Nrf2 expression in the nuclear fraction as increased by GH treatment in Py230 cells. In addition, Ho-1, a target gene of Nrf2, increased in the cytoplasmic fraction following GH treatment in py230 cells. (Figure 5F).

These results indicated that excess GH acted as an antioxidant by increasing the expression of target genes of Nrf2 via TCF-20/Nrf2 signaling activation.

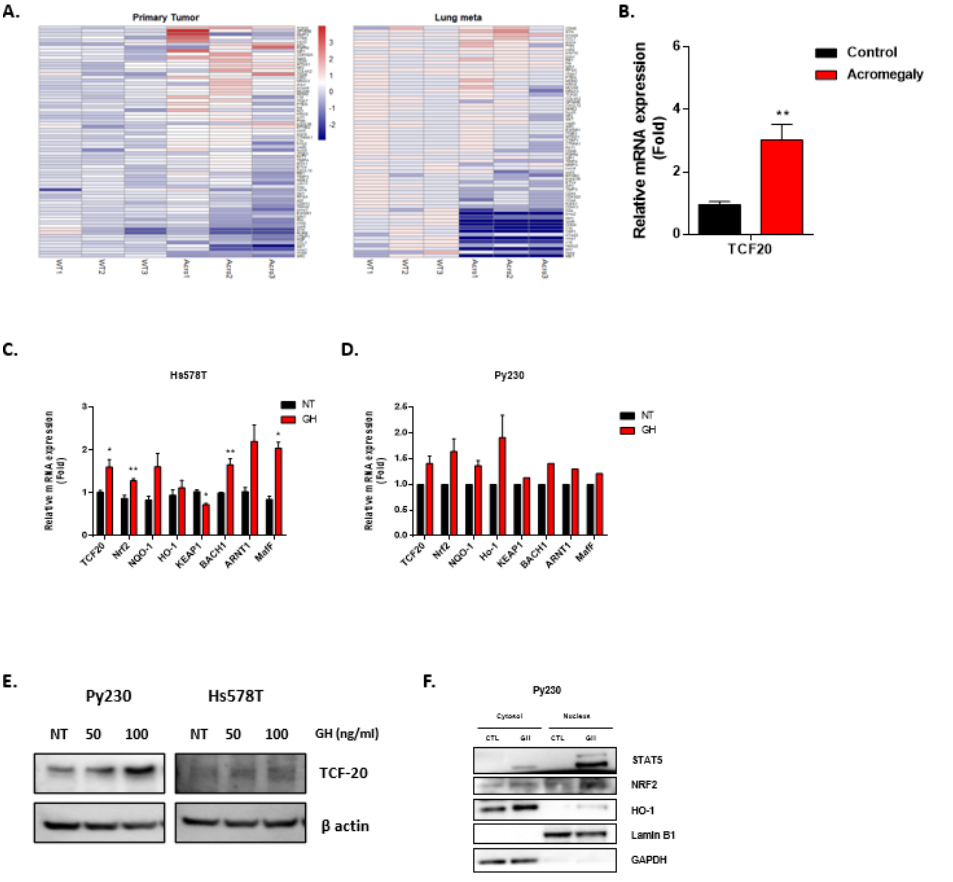


Figure 5. GH enhances antioxidant related genes by upregulating TCF-20/Nrf2. (A) Shown are heat maps for up-regulated genes related with metastasis in both primary tumors and lung metastatic tumors. (B) Real-time qPCR analysis of TCF-20 mRNA expression in Con-mice and Acro-mice confirmed transcriptomic data in (A). (C, D) Real-time qPCR analyses of TCF-20 and Nrf2 targeted genes in Hs578T cells (C) and Py230 cells (D) (n=4). Results represent the mean of at least three independent experiments. *$p<0.05$, **$p < 0.01$ vs. non-treated (NT) control. (E) Western blot analysis of Hs578T cells and Py230 cells treated with GH showing amounts of TCF-20. (F) Western blot analysis of cytosol and nucleus proteins of Py230 cells treated with GH. Showing amounts of GH targeted proteins and Nrf2 targeted proteins.

6. GHR and TCF-20 inhibition attenuated excess GH-induced breast cancer metastasis

Given the upregulation of antioxidant genes and TCF-20/Nrf2 activation upon GH treatment in TNBC cells, we hypothesized that the GH/TCF-20/Nrf2 axis was involved in tumor metastasis. To verify this hypothesis, we used GHR and TCF-20 siRNAs to knock down their expression in TNBC cells. Consistent with previous results, GH increased the expression of TCF-20/Nrf2 and its target genes in siCON group. In contrast, the upregulation of TCF-20/Nrf2 and its target genes was abolished by the introduction of siRNAs against GHR (siGHR) or TCF-20 (siTCF-20) in TNBC cells (Figure 6A-B). In line with the quantitative PCR results, GH treatment resulted significant STAT5 and Nrf2 nuclear translocation, along with cytosolic HO-1 protein expression in the siCON. In contrast, the effect of GH on Nrf2 translocation and HO-1 expression was decreased in both siGHR- and siTCF-20-treated TNBC cell (Figure 6C). We further explored consequences of inhibiting GH/TCF-20/Nrf2 signaling axis on TNBC metastatic feature and established stable GHR or TCF-20 knocked down TNBC cells.

Together, these results suggested that excess GH induced-TCF-20 activation could promotes breast cancer metastasis by upregulating Nrf2 and antioxidant related genes.

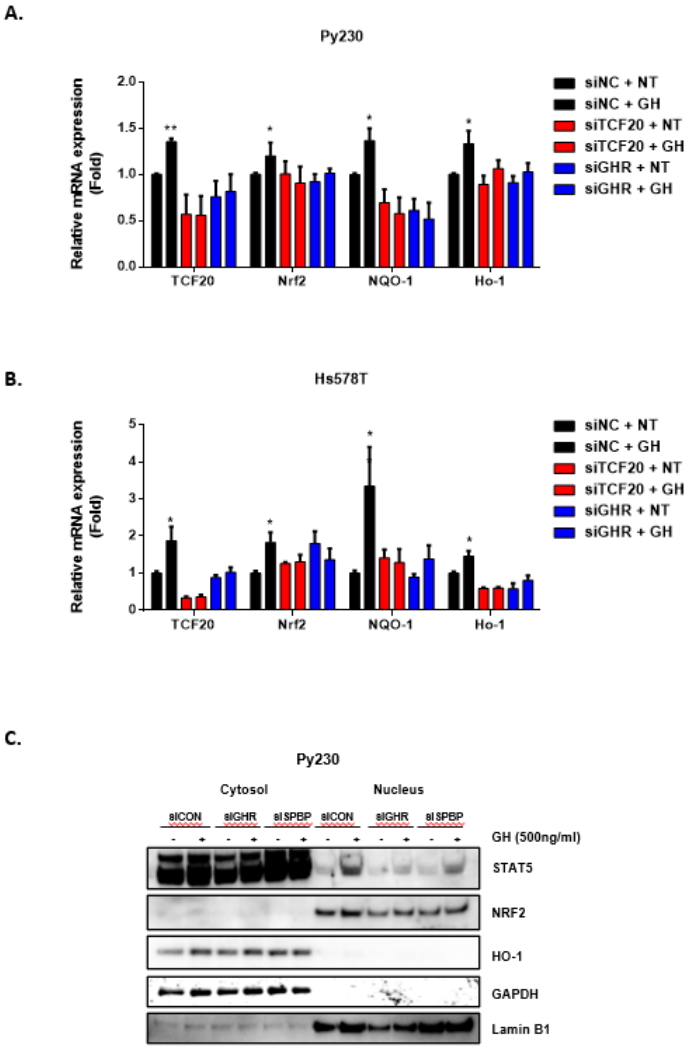


Figure 6. GHR and TCF-20 inhibition attenuates excessive GH-induced breast cancer metastasis. (A~B) Real-time qPCR analyses if TCF-20 and Nrf2 targeted genes in Py230 cells and Hs578T cells (n=3). Results represent the mean of at least three independent experiments. *p<0.05, **p < 0.01 vs. siCON transfected and non-treated control. (C) Western blot analysis of cytosol and nucleus proteins of siRNA transfected Py230 cells treated with GH. Showing amounts of GH targeted proteins and Nrf2 targeted proteins.

7. Excess GH induced TCF-20 and Nrf2 related proteins in lung-metastatic tumor burdens of Acromegaly mouse

As TCF-20 and Nrf2-related genes are involved in GH-induced breast cancer metastasis, we investigated whether TCF-20 and Nrf-2 related proteins are upregulated in lung-metastatic tumor burdens of Acromegaly mouse. Levels of TCF-20 and Nrf2 levels were confirmed by immunohistochemistry. Endogenous GHR proteins were expressed in lungs of all animals, but in metastatic tumors, GHR expression was highly expressed in Acro-mice (Figure 7A). Consistently, TCF-20 proteins and Nrf2-related proteins were upregulated in lung-metastatic tumor burdens of Acro-mics (Figure 7B~E). However, in the peg treated group, TCF-20 and Nrf2-related proteins did not increase (Figure 7B~E).

Together, these results suggest that excessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly by upregulating TCF-20 and Nrf2-related proteins.

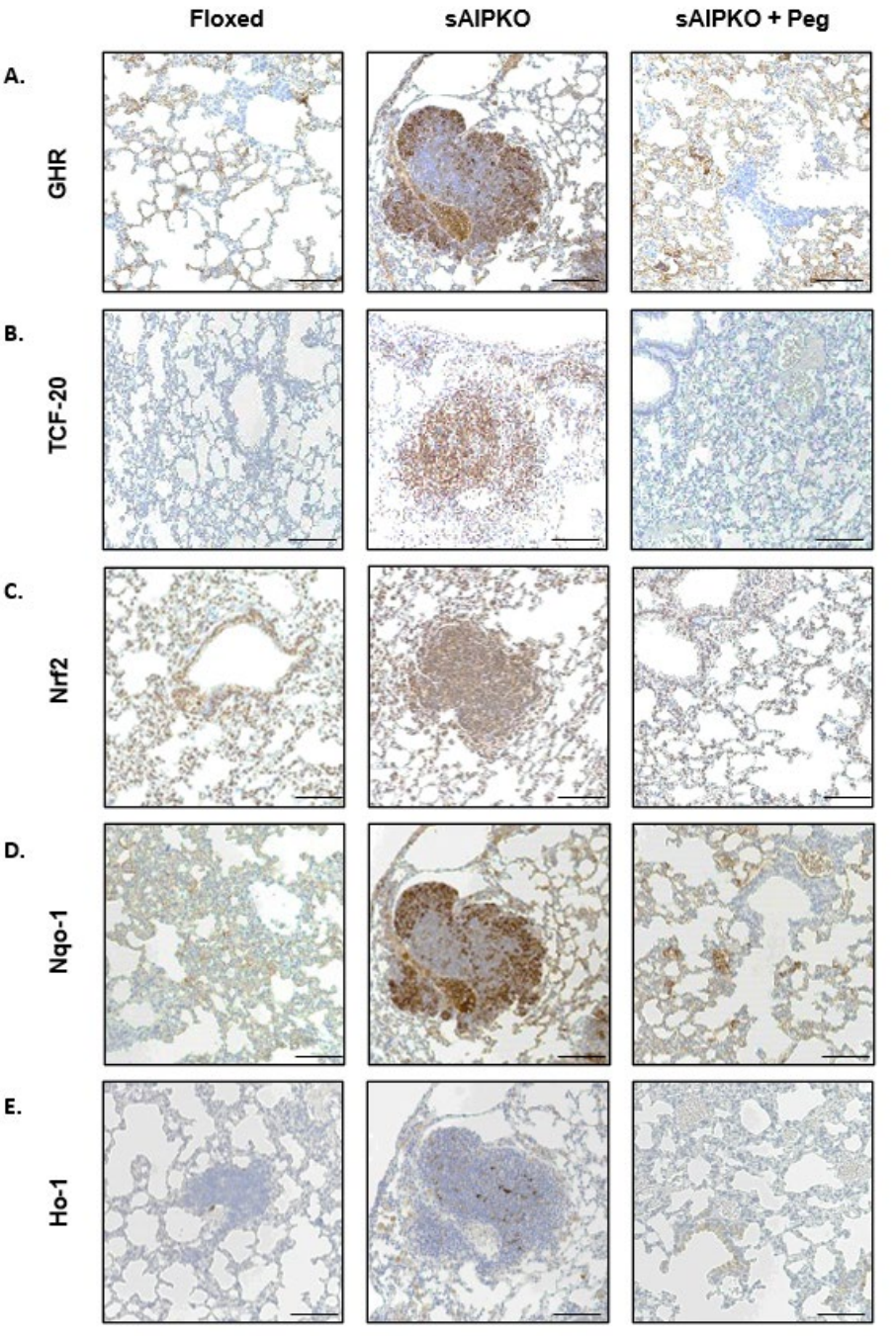


Figure 7. E**xcessive GH facilitates aggressiveness and metastasis of breast cancer in acromegaly by upregulating TCF-20 and Nrf2-related genes.** (A~D) Representative immunohistochemistry images of target proteins, (A) GHR, (B) TCF-20, (C) Nrf2 (D) NQO-1 (E) Ho-1 respectively. Images were obtained at 100X magnification. Scale Bar represent 50μm.

IV. DISCUSSION

The present study identified the excessive GH increased breast tumor development and lung metastasis. Mechanistically, excessive GH promoted breast tumor aggressiveness and metastasis in an acromegaly mouse model by activating the TCF-20/Nrf2 signaling. GH acts like antioxidant and activates TCF-20 and Nrf2, which is related with reducing oxidative stress. Excess GH-induced TCF-20 and Nrf2 increases breast tumor aggressiveness and lung metastasis. These results were consistent with previous reports that cancer development and metastasis can be stimulated by antioxidants, especially in lung cancer [31,33].

In previous studies, patients with acromegaly showed increased incidence of cancers, including thyroid and breast cancers. Conversely, in a follow-up study of patients with Laron syndrome, who have GHR mutations, people with inactivating GHR mutations did not develop cancer Furthermore, excessive GH secretion is involved in breast cancer development. According to a recent meta-analysis of 5 million women, a 10-cm increase in height elevated the risk of hormone-dependent breast cancer by 17% [14]. In the present study, we confirmed a similar trend in an animal model of acromegaly. When tumors were inoculated into Acro mice, tumor growth rate and aggressiveness were significantly increased compared to those in Con mice. Even at 50 weeks of age, mammary gland tumors were observed only in Acro mice (Figure 1E). Consistent with previous clinical studies, these results indicated that excessive GH signaling was strongly linked to breast tumor growth in animals with acromegaly.

Pegvisomant is a GHR antagonist for acromegaly treatment [11]. Pegvisomant inhibits the action of GH on GHR to reduce the production of IGF-1. Several studies have shown that Pegvisomant is an effective treatment of acromegaly as well as certain types of tumors, including endometrial and breast cancers, in

xenograft models [34]. However, the antitumor effect of this agent in an animal model of acromegaly has not been investigated. This study provided the first evidence that excessive GH stimulated tumor development and metastasis in an acromegaly animal model, which could be inhibited by Pegvisomant (Figure 2C). These results indicated that excessive GH signaling was required for tumor development and metastasis.

Metastasis requires at least six steps: detachment, intravasation, immune evasion, survival in circulation, extravasation, and colonization of a distant organ. Excessive GH increased the efficiency of several steps of metastasis. The induction of antioxidant-related genes by excessive GH was involved in all six steps of metastasis development in Acro mice with primary tumors transplanted into the mammary gland. In this context, immune invasion was likely a less important factor because the effect was cell-autonomous and was observed in immunocompetent mice (Figure 3A~D). In vivo results from orthotopic xenograft models suggested that excessive GH was necessary and sufficient for breast cancer development. In addition, we observed that some Con mice, with larger primary larger tumors than those of Acro mice, had no metastatic lung nodules (Figure 3F).

In line with in vivo metastatic features of Acro mice, the effect of excessive GH on colony formation, migration, and invasion in TNBC cell lines was evaluated. These results further suggested that excessive GH played a critical role in promoting breast cancer aggressiveness and metastasis.

Recent studies have provided compelling evidence that antioxidants, such as Nrf2 and Bach1, accelerated cancer progression and metastasis [31,33]. Antioxidants protect primary tumor cells from reactive oxidative species (ROS) and DNA damage [32,35]. Several studies have reported that GH reduced ROS and oxidative stress, preventing cell senescence [13,26,36]. In this study, excessive GH induced TCF-20 and Nrf2-related genes. Similar to antioxidants, the induction

of TCF-20 by excessive GH upregulated antioxidant-related genes and promoted lung metastasis in acromegaly mice (Figure 7A~E). In contrast, GHR and TCF-20 inhibition attenuated GH-induced breast cancer development and metastasis (Figure 7A~E). In previous studies, NRF2 activation stimulated lung metastasis by inducing Ho-1, leading to heme degradation and ROS reduction [32,33]. In addition, supplementing the diet of mice harboring tumors with either a pharmacological or dietary (vitamin E) antioxidant promoted metastasis by increasing intracellular Bach1 [31]. We speculate that like other antioxidants, including N-acetylcysteine and vitamin E, GH also acted as an antioxidant and promoted tumor aggressiveness and metastasis by reducing oxidative stress in breast cancer.

This study had certain limitations. First, the investigation was focused on GH but not IGF-1. GH induces the production of IGF-1 in the liver. Excess IGF-1 production may affect tumor development and metastasis. However, to elucidate the specific impact of GH on tumor aggressiveness and metastasis, we injected a GH antagonist in the peritumoral region subcutaneously, not systemically, and observed a significant reduction in tumor volume. Second, we did not examine the clinical impact of GH. As acromegaly is a rare disease, no breast tumor tissue was obtained from patients with acromegaly at our institution. While the clinical impact of GH has been previously confirmed in a meta-analysis of several clinical studies, further validation experiments are necessary.

V. CONCLUSION

In conclusion, our results suggest that excess GH in acromegaly possess a strong potential to promotes tumor development and metastasis. This is the first paper to study tumor development and metastasis in an acromegaly mouse model. We identified a molecular mechanism by which GH-induced TCF-20 and Nrf2 related genes promote tumor aggression and metastasis. These findings can aid in the development of improved strategies for managing cancer in acromegaly.

REFERENCES

1. Raben MS. Growth Hormone. New England Journal of Medicine 1962;266:31-5.
2. Greenwood FC, Landon J. Growth Hormone Secretion in Response to Stress in Man. Nature 1966;210:540-1.
3. Martin JB. Neural Regulation of Growth Hormone Secretion. New England Journal of Medicine 1973;288:1384-93.
4. Katznelson L, Laws ER, Jr, Melmed S, Molitch ME, Murad MH, Utz A, et al. Acromegaly: An Endocrine Society Clinical Practice Guideline. The Journal of Clinical Endocrinology & Metabolism 2014;99:3933-51.
5. Melmed S, Colao A, Barkan A, Molitch M, Grossman AB, Kleinberg D, et al. Guidelines for Acromegaly Management: An Update. The Journal of Clinical Endocrinology & Metabolism 2009;94:1509-17.
6. Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, et al. The prevalence of pituitary adenomas. Cancer 2004;101:613-9.
7. Melmed S. Pathogenesis of pituitary tumors. Nature Reviews Endocrinology 2011;7:257-66.
8. Colao A, Grasso LFS, Giustina A, Melmed S, Chanson P, Pereira AM, et al. Acromegaly. Nature Reviews Disease Primers 2019;5:20.
9. Boguszewski CL, Boguszewski MCdS. Growth Hormone's Links to Cancer. Endocrine Reviews 2018;40:558-74.
10. Gillam MP, Ku CR, Lee YJ, Kim J, Kim SH, Lee SJ, et al. Somatotroph-Specific Aip-Deficient Mice Display Pretumorigenic Alterations in Cell-Cycle Signaling. Journal of the Endocrine Society 2017;1:78-95.
11. Boguszewski CL, Ayuk J. MANAGEMENT OF ENDOCRINE DISEASE: Acromegaly and cancer: an old debate revisited. European Journal of Endocrinology 2016;175:R147-R56.
12. Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, Wei M, Madia F, Cheng C-W, et al. Growth Hormone Receptor Deficiency Is Associated with a Major Reduction in Pro-Aging Signaling, Cancer, and Diabetes in Humans. Science Translational Medicine 2011;3:70ra13-70ra13.

13. Perry JK, Wu Z-S, Mertani HC, Zhu T, Lobie PE. Tumour-Derived Human Growth Hormone As a Therapeutic Target in Oncology. Trends in Endocrinology & Metabolism 2017;28:587-96.
14. Zhang B, Shu X-O, Delahanty RJ, Zeng C, Michailidou K, Bolla MK, et al. Height and Breast Cancer Risk: Evidence From Prospective Studies and Mendelian Randomization. JNCI: Journal of the National Cancer Institute 2015;107.
15. Subramani R, Nandy SB, Pedroza DA, Lakshmanaswamy R. Role of Growth Hormone in Breast Cancer. Endocrinology 2017;158:1543-55.
16. Raccurt M, Lobie P, Moudilou E, Garcia-Caballero T, Frappart L, Morel G, et al. High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland. Journal of Endocrinology 2002;175:307-18.
17. Mukhina S, Mertani HC, Guo K, Lee K-O, Gluckman PD, Lobie PE. Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone. Proceedings of the National Academy of Sciences of the United States of America 2004;101:15166-71.
18. Kaulsay KK, Mertani HC, Törnell J, Morel G, Lee K-O, Lobie PE. Autocrine Stimulation of Human Mammary Carcinoma Cell Proliferation by Human Growth Hormone. Experimental Cell Research 1999;250:35-50.
19. Luque RM, Amargo G, Ishii S, Lobe C, Franks R, Kiyokawa H, et al. Reporter Expression, Induced by a Growth Hormone Promoter-Driven Cre Recombinase (rGHp-Cre) Transgene, Questions the Developmental Relationship between Somatotropes and Lactotropes in the Adult Mouse Pituitary Gland. Endocrinology 2007;148:1946-53.
20. Tavera-Mendoza LE, Brown M. A less invasive method for orthotopic injection of breast cancer cells into the mouse mammary gland. Laboratory Animals 2017;51:85-8.
21. Kocatürk B, Versteeg HH. Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth. Journal of visualized experiments : JoVE 2015:51967.
22. Lee MW, Kim HJ, Yoo KH, Kim DS, Yang JM, Kim HR, et al. Establishment of a bioluminescent imaging-based in vivo

leukemia model by intra-bone marrow injection. Int J Oncol 2012;41:2047-56.
23. Peng Y, Zhang HW, Cao WH, Mao Y, Cheng RC. Exploration of the Potential Biomarkers of Papillary Thyroid Cancer (PTC) Based on RT(2) Profiler PCR Arrays and Bioinformatics Analysis. Cancer Manag Res 2020;12:9235-46.
24. Messeguer X, Escudero R, Farré D, Núñez O, Martı´nez J, Albà MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics 2002;18:333-4.
25. Vallejo AN, Pogulis RJ, Pease LR. PCR Mutagenesis by Overlap Extension and Gene SOE. CSH Protoc 2008;2008:pdb.prot4861.
26. Chesnokova V, Zonis S, Zhou C, Recouvreux MV, Ben-Shlomo A, Araki T, et al. Growth hormone is permissive for neoplastic colon growth. Proceedings of the National Academy of Sciences 2016;113:E3250-E9.
27. Gebert CA, Park S-H, Waxman DJ. Regulation of Signal Transducer and Activator of Transcription (STAT) 5b Activation by the Temporal Pattern of Growth Hormone Stimulation. Molecular Endocrinology 1997;11:400-14.
28. Arumugam A, Subramani R, Nandy SB, Terreros D, Dwivedi AK, Saltzstein E, et al. Silencing growth hormone receptor inhibits estrogen receptor negative breast cancer through ATP-binding cassette sub-family G member 2. Exp Mol Med 2019;51:1-13.
29. Brittain AL, Basu R, Qian Y, Kopchick JJ. Growth Hormone and the Epithelial-to-Mesenchymal Transition. The Journal of Clinical Endocrinology & Metabolism 2017;102:3662-73.
30. Darvekar SR, Elvenes J, Brenne HB, Johansen T, Sjøttem E. SPBP is a sulforaphane induced transcriptional coactivator of NRF2 regulating expression of the autophagy receptor p62/SQSTM1. PLoS One 2014;9:e85262.
31. Wiel C, Le Gal K, Ibrahim MX, Jahangir CA, Kashif M, Yao H, et al. BACH1 Stabilization by Antioxidants Stimulates Lung Cancer Metastasis. Cell 2019;178:330-45.e22.
32. Wang H, Liu X, Long M, Huang Y, Zhang L, Zhang R, et al. NRF2 activation by antioxidant antidiabetic agents accelerates tumor metastasis. Sci Transl Med 2016;8:334ra51.

33. Lignitto L, LeBoeuf SE, Homer H, Jiang S, Askenazi M, Karakousi TR, et al. Nrf2 Activation Promotes Lung Cancer Metastasis by Inhibiting the Degradation of Bach1. Cell 2019;178:316-29.e18.
34. Evans A, Jamieson SMF, Liu D-X, Wilson WR, Perry JK. Growth hormone receptor antagonism suppresses tumour regrowth after radiotherapy in an endometrial cancer xenograft model. Cancer Letters 2016;379:117-23.
35. Sayin Volkan I, Ibrahim Mohamed X, Larsson E, Nilsson Jonas A, Lindahl P, Bergo Martin O. Antioxidants Accelerate Lung Cancer Progression in Mice. Science Translational Medicine 2014;6:221ra15-ra15.
36. Gong Y, Zhang K, Xiong D, Wei J, Tan H, Qin S. Growth hormone alleviates oxidative stress and improves the IVF outcomes of poor ovarian responders: a randomized controlled trial. Reproductive Biology and Endocrinology 2020;18:91.

ABSTRACT (IN KOREAN)

말단비대증에서 과도하게 분비되는 성장호르몬에 의한 유방암 공격성과 전이 촉진 기전 연구

<지도교수 이은직>

연세대학교 대학원 의과학과

오 주 헌

말단비대증은 성장호르몬(GH)을 과도하게 분비하는 뇌하수체 종양에 의해 발생하는 질환으로 항상 높은 혈중 GH 수치를 보이는 것을 특징으로 한다. 기존의 역학 연구에서는 말단비대증 환자에서 갑상선암, 유방암을 비롯한 종양의 발생과 전이가 증가하는 것으로 알려져 있다. 반대로 성장호르몬수용체(GHR)에 돌연변이가 있는 라론 증후군 환자에 대한 추적조사 결과에 따르면 GHR에 돌연변이가 있는 사람은 암에 걸리지 않는 것으로 알려졌다. 그러나 현재까지 종양 발달 및 전이에서 GH의 정확한 기전에 대해서는 알려진 바가 거의 없다.

이 논문에서, 우리는 말단 비대증에서 과도한 혈청 GH가 유방암 공격성과 전이를 촉진한다는 것을 보여주었다. 과도한 성장 호르몬은 유방암 종양의 진행과 전이를 촉진한다. 반대로, 성장 호르몬 길항제는 유방 종양 진행을 예방한다. 과도한 성장 호르몬은 항산화제 역할을 하여 종양 전이에 관여하는 항산화 유전자를 증가시킨다. 특히 성장호르몬은 TCF-20 유전자 발현을 증가시킨다. TCF-20은 Nrf2 및 Nrf2 관련 유전자의 전사를 활성화하고 종양

전이 표현형을 증가시킨다. TCF-20 및 성장 호르몬 수용체의 발현을 억제하면 과도한 성장호르몬이 유발하는 종양 진행 및 전이를 상쇄시킨다. 우리는 과도한 GH가 항산화제 역할을 하고 산화 스트레스를 감소시켜 조기 종양 진행 및 전이를 자극한다고 결론지었다. 과도한 GH는 항산화 관련 유전자, 특히 TCF-20, 의존적 종양 진행 및 전이를 자극한다.

핵심되는 말 : 성장호르몬, 인슐린유사성장인자-1, 유방암, 말단비대증, 암 전이

PUBLICATION LIST

1. Kim J, Oh JH, Harlem H, Culler MD, Ku CR, Lee EJ. Therapeutic Effect of a Novel Chimeric Molecule Targeting Both Somatostatin and Dopamine Receptors on Growth Hormone-Secreting Pituitary Adenomas. Endocrinol Metab (Seoul) 2020;35:177-87.
2. Kwon IG, Kang CW, Park JP, Oh JH, Wang EK, Kim TY, et al. Serum glucose excretion after Roux-en-Y gastric bypass: a potential target for diabetes treatment. Gut 2021;70:1847-56.
3. Kang CW, Han YE, Kim J, Oh JH, Cho YH, Lee EJ. 4-Hydroxybenzaldehyde accelerates acute wound healing through activation of focal adhesion signalling in keratinocytes. Sci Rep 2017;7:14192.
4. Han YE, Kang CW, Oh JH, Park SH, Ku CR, Cho YH, et al. Olfactory Receptor OR51E1 Mediates GLP-1 Secretion in Human and Rodent Enteroendocrine L Cells. J Endocr Soc 2018;2:1251-8.
5. Lee YJ, Kang CW, Oh JH, Kim J, Park JP, Moon JH, et al. Downregulation of miR-216a-5p and miR-652-3p is associated with growth and invasion by targeting JAK2 and PRRX1 in GH-producing pituitary tumours. J Mol Endocrinol 2021;68:51-62.