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# Internalization and Nuclear Localization of Porcine Growth Hormone in Porcine Hepatocytes

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**HL conceived and designed the study. PH and YL contributed in experimental work, acquisition of data analyzed and drafting of the manuscript. RL and XZ statistically evaluated the data. PH, RL and HL wrote the article.**

**Key words**
**Porcine growth hormone, Internalization, Subcellular localizations.**

## ABSTRACT

The internalization and subcellular distribution of growth hormone (GH) from humans and rats have been extensively reported. However, to date, there are no descriptions of the cell behaviour of porcine GH in domestic animals. Thus, freshly isolated porcine hepatocytes were used as a somatic cell model to study the internalization and subcellular localization of porcine growth hormone (pGH), and the results showed that pGH could be internalized into porcine hepatocytes in a time-dependent manner. The results also showed that pGH could be translocated into cell nuclei in a time-dependent manner. In addition, the subcellular localization, according to immunoelectron microscopy, showed that pGH was localized in cytoplasmic organelles, *e.g.*, the mitochondria and Golgi apparatus. The present study shows the first exploration of the behaviour of pGH in a somatic cell model of a domestic animal (pig), providing a basis for further studies on the physiological function of pGH based on its subcellular localization.

## INTRODUCTION

Porcine growth hormone (pGH), a polypeptide hormone with 191 amino acids, is synthesized and secreted in the porcine anterior pituitary (Abdel-Meguid *et al*., 1987). pGH acts as an effective growth promoter in swine, increases daily gain, promotes protein deposition and reduces lipid accretion (Etherton and Bauman, 1998). pGH binds to porcine growth hormone receptor (pGHR) in the cell membrane and exhibits biological activity; once combined, Janus kinase 2 (JAK2) is activated, initiating several down-stream intracellular signalling pathways, including signal transducers and activators of transcription 1, 3 and 5 (STATs 1, 3 and 5) as well as extracellular signal-regulated kinase 1/2 (ERK1/2). These signalling molecules transport to the nucleus to regulate gene transcription (Brooks *et al*., 2014; Brooks and Waters, 2010; Waters, 2016). Previous studies have reported that protein molecules exhibit functions based on subcellular protein locations (Bryant and Stow, 2005; Wang and Hung, 2009). In general, the traditional view that GH displays biological functions by binding to cell membrane-GHR has become a paradigm. However, many experimental findings have challenged this paradigm. It has been reported that after GH binds to GHR, GH is rapidly internalized into the cytoplasm (Lobie *et al*., 1994), and previous studies have considered that internalized GH is targeted to the lysosome for degradation. However, except for localization in the lysosome, GH is also localized in other organelles, including the mitochondria and Golgi apparatus (Lobie *et al*., 1994), suggesting that GH may function in subcellular fractions. Indeed, Strous indicated that the GH-GHR complex is internalized into the cytoplasm and subsequently transmits signals (van Kerkhof *et al*., 2000). In addition to cytoplasmic localization, GH has also been shown to be localized in the nucleus. Lobie *et al*. (1994) first studied and reported the phenomenon of the nuclear localization of GH in rodents systematically.

However, currently, the biological functions of nuclear-GH remain unknown (Conway-Campbell *et al*., 2008). Interestingly, GH appears as a dimer with GHR in cell nuclei, namely, the GH-GHR complex, suggesting that nuclear-GH may function in a GHR dimer-dependent manner. Recently, Wasters *et al* reported that nuclear-GHR could regulate cell proliferation and also induce tumour formation (Conway-Campbell *et al*., 2007). Taken together, these studies indicate that the cytoplasmic and nuclear localizations of GH have important biological properties, and this notion should be deeply explored in the future. However, until recently, to our knowledge, there have been no descriptions of the cell behaviour and properties

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of GH in domestic animals, namely, whether pGH can be internalized into the cytoplasm and be translocated to the nucleus. Indeed, these questions remain unclear. Therefore, it is worthwhile to explore the behaviour of pGH in porcine hepatocytes.

To resolve the above-mentioned questions, porcine hepatocytes were used as a cell model to study the behaviour of PGH, it has been demonstrated that porcine hepatocyte is an ideal model to study the interactions between pGH and pGHR (Brameld *et al*., 1995, 1999; Lan *et al*., 2015). The experimental results indicated that pGH internalization is rapid and time-dependent, and internalized pGH is primarily localized in the mitochondria and Golgi apparatus. Furthermore, pGH can be translocated to cell nucleus in a time-dependent manner. In summary, the present study demonstrated the first exploration of the cell behaviour and properties of pGH, and these findings provide a basis for further studies on the physiological function of pGH based on its subcellular localization.

## MATERIALS AND METHODS

*Antibodies and reagents*

pGH proteins were obtained from Sigma (USA). pGH was labeled with FITC according to our previous methods (Li *et al*., 2013). 1A3, a mouse monoclonal antibody against pGH, was generated in our laboratory (Li *et al*., 2013). Anti-growth hormone receptor, goat Anti-mouse IgG H&L (12 nm Gold) was obtained from Abcam (UK). HRP-conjugated goat anti-mouse antibodies were obtained from Cell Signalling Technology (USA). Cell lysis buffer (RIPA kit) and Difco skim milk were obtained from BD Biosciences (USA). Enhanced chemiluminescence (ECL), BCA kits and polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco (USA). The Nuclear/Cytosol Fractionation Kit was obtained from Cell Biolabs (USA). Unless otherwise stated, other reagents were obtained from Sigma (USA).

*Isolation of primary porcine hepatocyte*

Porcine hepatocytes were isolated using a two-step collagenase perfusion technique as previously described with slight modifications (Hong *et al*., 2016; Salaver and Iynedjian, 1982). The cell isolation method was approved by the Animal Ethical Committee of Jilin Agricultural University. Briefly, pigs (Junmu No. 1, ~10 kg body weight, provided by the Experimental Animal Centre, Jilin University) were anaesthetized, the abdominal cavity and chest were opened to expose the inferior vena cava and hepatic portal vein. The porcine livers were successively perfused with the calcium- and magnesium-free Hank's buffer (pH 7.4) and collagenase buffer containing 0.05% (w/v) collagenase at 37°C for 10 min. Subsequently, the minced livers were filtered through a sterile cell strainer nylon mesh. The cells were collected by centrifugation. Cell viability was subsequently determined by trypan blue dye exclusion.

*Confocal laser scanning microscopy*

In the present study, freshly isolated porcine hepatocytes were used. Before the experiment, the porcine hepatocytes were serum starved for 2 h in DMEM medium containing 3% BSA, penicillin and streptomycin. The cells were subsequently washed with phosphate buffered solution (PBS) and incubated with FITC-pGH (100 ng/mL) for different durations. After incubation and three washes, the cell samples were fixed with 4% paraformaldehyde for 10 min at 37°C. The cell nuclei were countered stained with Hoechst 33258. After washing with PBS, the cell samples were visualized by confocal laser scanning microscopy (Olympus FV1000).

*Isolation of subcellular fractions*

After pGH treatment as described as above, nuclear and cytosol proteins of the cells were isolated using a nuclear and cytosol extraction kit according to the manufacturer's instructions. Isolated proteins from cytosol and nuclear fractions were subjected to Western-blot analyses as described below.

*Western-blot analysis*

The Western-blot analyses were performed as previously described (Hong *et al*., 2016; Wang *et al*., 2017). Briefly, the proteins from the cytosol and nucleus were fractionated by SDS-PAGE and subsequently transferred onto PVDF membranes. After blocking with 5% BSA for 1 h, the membranes were incubated with an anti-pGH antibody overnight at 4°C. After washing three times, the membranes were incubated with HRP-conjugated secondary antibodies. The concentration of antibodies used in accordance with the instructions. After the final three washes, the immunoprotein bands were detected using an ECL detection system.

*Immunoelectron microscopy*

Purified freshly isolated porcine hepatocytes were incubated with 100 ng/mL of pGH for 60 min at 37°C and 5% $CO_2$, and subsequently, the cells were centrifuged at 3000 r/min for 10 min. The pellet was washed with 0.1 M phosphate buffer, pH 7.4, at 4°C and fixed in 4%

Glutaraldehyde for 2 h, followed by centrifugation at 1000 r/min for 5 min. The pellet was washed three times with 0.1 M phosphate buffer, pH 7.4, for 15 min, and the precipitate was fixed in 1% osmium tetroxide for 2 h at 4°C; dehydrated in a graded ethanol series of 50%, 70%, 80%, 90%, 95% and 100% ethanol; and finally embedded in Araldite epoxy resin. Ultrathin sections of 70 nm were obtained, and the slices were washed three times in 0.1 M phosphate buffer, pH 7.4, followed by blocking for 40 min at 37°C with TBST blocking buffer (0.1% Tween 20, 3% bovine serum albumin) and incubating with an anti-pGH antibody at room temperature for 90 min. Subsequently, the sections were washed with TBST buffer as previously described, and following incubation with a gold-labelled secondary antibody, the slices were washed three times with 0.1 M phosphate buffer, pH 7.4, and incubated with 4% uranyl acetate, pH 7, for 10 min. Electron micrographs were obtained using a Hitachi Limited HITACHI H-7650 electron microscope.

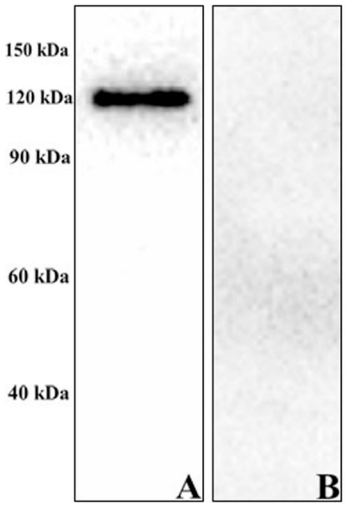


Fig. 1. Western blot analysis to detect the expression of pGHR in porcine hepatocytes. Porcine hepatocytes were collected and lysed using cell lysis buffer, and the cell extracts were analysed by Western blotting using an anti-growth hormone receptor antibody (A) or control antibody (Rabbit IgG) (B). The figures represent at least three independent experiments.

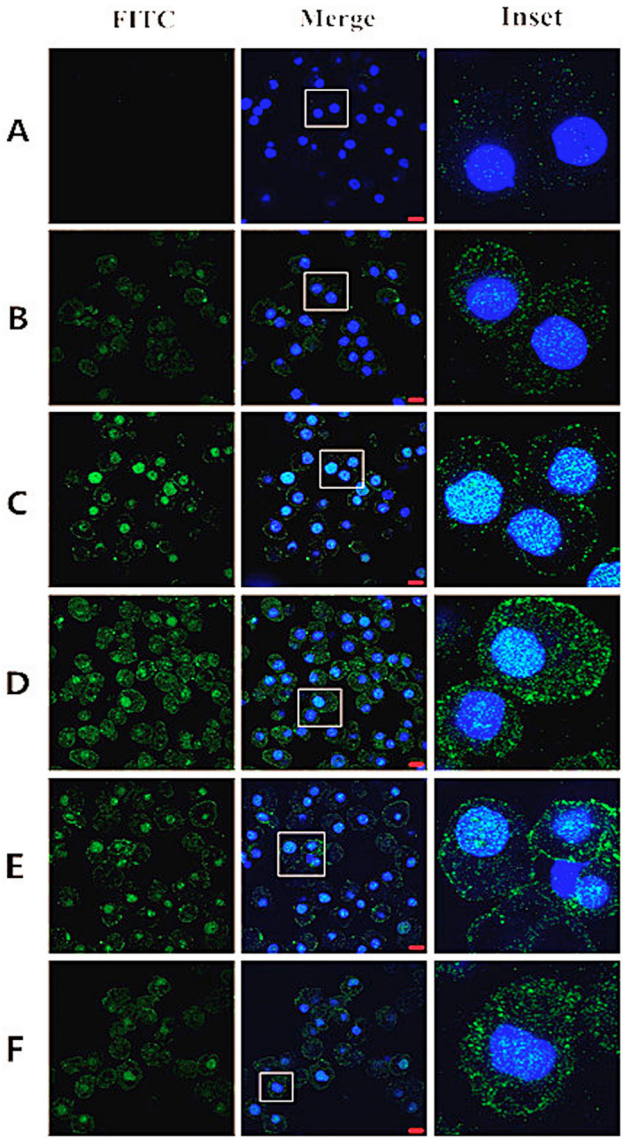


Fig. 2. CLSM analysis to observe the internalization and nuclear localization of pGH in porcine hepatocytes. Porcine hepatocytes were serum starved, washed and incubated with FITC-pGH (100 ng/mL) for 0, 15, 30, 60, 75 and 90 min and subsequently fixed with 4% paraformaldehyde and stained with Hoechst 33258 (nucleus) (A-F). The cell samples were visualized by confocal laser scanning microscopy. Bar = 10 μm. The images represent at least three independent experiments.

## RESULTS

*pGHR expression on porcine hepatocytes*

We first evaluated pGHR expression on porcine hepatocytes from 1.5 kg of Junmu No. 1 pigs, and as shown in Figure 1, pGHR expression was detected as a ~120 KDa band using the pGHR-specific antibody, and the control antibody showed no effect.

*Internalization and nuclear localization of pGH*

To observe the internalization of pGH in porcine hepatocytes, cells were treated with 100 ng/mL FITC-pGH for 0, 15, 30, 60, 75 and 90 min. The results showed that pGH internalization was time depended in porcine hepatocytes. At 0 min after FITC-pGH treatment, as

shown in Figure 2A, no or little fluorescence signalling was observed. At 15 min after FITC-pGH treatment, little FITC-pGH (green signal) signalling was observed in the cytoplasm. In addition, small amounts of pGH were transferred to cell nuclei (Fig. 2B). The internalization and nuclear localization of pGH increases with increasing time, and the nuclear fluorescence signals peaked at 30 min (Fig. 2C) and cytoplasmic fluorescence signals peaked at 60 min (Fig. 2D). The cytoplasmic and nuclear fluorescence signals declined slightly at 60-90 min (Fig. 2D-F).

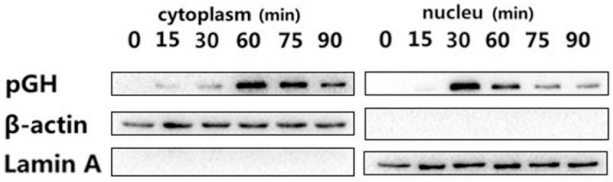


Fig. 3. Western blot analysis to detect the internalization of pGH in porcine hepatocytes. Porcine hepatocytes were incubated with FITC-pGH (100 ng/mL) for different durations, and the cytoplasmic and nuclear extracts were subjected and analyzed by Western blotting using 1A3 (the mouse monoclonal antibody to pGH was generated in our laboratory). Anti-β-actin and anti-lamin A antibodies were used as markers for the cytoplasmic and nuclear extracts, respectively. The experiment was performed at least three times.

*Analysis of pGH internalization in porcine hepatocytes by Western-blot analysis*

Freshly isolated porcine hepatocytes were incubated with 100 ng/mL of pGH for 0, 15, 30, 60, 75 and 90 min, and subsequently, the cytoplasm and nucleus were isolated and protein was extracted. The expression of pGH was detected using Western-blot analysis. The results showed the same trend as the CLSM observations, and pGH was internalized into the cytoplasm in a time-depended manner (Fig. 3).

*Subcellular localization of pGH*

Based on the above results, pGH internalization peaked at 60 min; thus, in the following experiment, porcine hepatocytes were stimulated with pGH (100ng/mL) for 60min, followed by an anti-pGH antibody; a 12nm Gold-conjugated second antibody was used to observe pGH subcellular localization by immunoelectron microscopy. As indicated in Figure 4, pGH was localized to the cell membrane, mitochondria and cell nucleus.

## DISCUSSION

pGH plays important roles in the growth and development of pigs (Chung *et al.*, 1985; Vize and Wells, 1987). The conventional view suggests that after pGH interacts with pGHR on the cell membrane, JAK2 and pGHR are subsequently tyrosine-phosphorylated and down-stream signalling is subsequently activated. However, it has been reported that GH not only displays physiological roles at the cell membrane but also exhibits physiological functions in the cytoplasm and cell nuclei (van Kerkhof *et al.*, 2000; Conway-Campbell *et al.*, 2007, 2008). However, until recently, there have been no studies describing the cell behaviour of porcine GH. In the present study, to our knowledge, we present the first exploration of the biological properties of pGH internalization and subcellular localization.

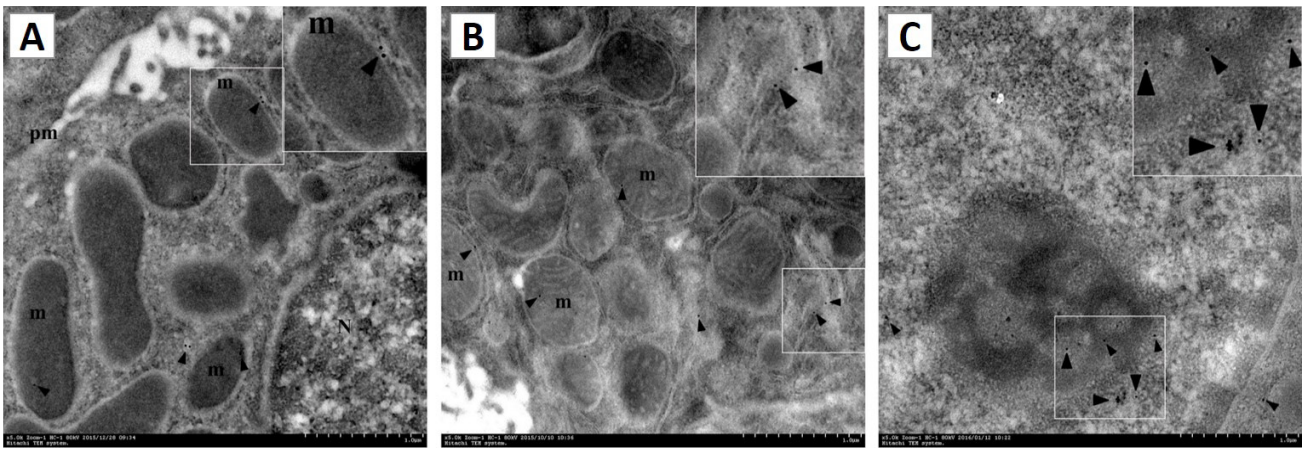


Fig. 4. Subcellular localization of pGH. Immunoelectron microscopy samples were prepared as described in the "Materials and methods" section. Gold grains were localized to the cell membrane (pm), mitochondria (m) and cell nucleus (N) (A-C). Bar = 1μm. The micrograph represents at least three independent experiments.

Indeed, internalized GH from the cell membrane may possess important biological activities. van Kerkhof *et al*. (2000) reported that internalized GH transmits signals. However, whether internalized GH has other physiological activities remains unclear. In the present study, we observed that pGH was also internalized into the cytoplasm in a time-dependent manner (Fig. 2). After porcine hepatocytes were stimulated with pGH for 15 min, FITC-pGH was observed in the cytoplasm using confocal laser scanning microscope (CLSM) (Fig. 2B), and the internalization peaked at 60 min, while the cytoplasmic fluorescence signals declined slightly at 60-90 min (Fig. 2D-2F). However, how GH is transported from the cell membrane into the cytoplasm remains unknown, multiple channels may be involved. Indeed, Lobie *et al*. (1999) reported that caveolin might participate in membrane-GH/GHR internalization. In addition, Strous *et al*. showed that clathrin is involved in GH/GHR internalization from the cell membrane (Strous and Govers, 1999). Furthermore, Strous *et al*. (1996) also reported that the ubiquitin coupling system also plays an important role in GH internalization. These findings suggest that the internalization of GH is a complex physiological procedure, and additional studies are needed in the future.

After GH internalization, except for cytosol localization, GH is also localized in some organelles, including the Golgi apparatus, lysosome, endoplasmic reticulum, and mitochondria (Lobie *et al*., 1994). In the present study, immunoelectron microscopy revealed that pGH was also localized to organelles, particularly mitochondria (Fig. 4), and interestingly, pGH was localized to the membrane of mitochondria, suggesting that pGH localized in the mitochondria membrane may exhibit direct physiological effects on mitochondria. This notion is only a speculation, and additional studies are needed in the future.

Except for cytoplasmic localization, CLSM and immunoelectron microscopy also revealed that pGH is localized in the nucleus of porcine hepatocytes and that nuclear localization occurred a time-dependent manner (Fig. 2). The nuclear fluorescence signals peaked at 30 min and declined slightly at 60-90 min (Fig. 2). In addition, the nuclear localization of GH in other species has previously been described. Many studies have indicated that the nuclear localization of GHR has important biological activities.

Taken together, the present study represents the first analysis of the internalization of pGH in porcine hepatocytes, showing that the internalization of pGH is time dependent. In addition, pGH could translocate to cell nuclei in a time-dependent manner. These findings suggest that pGH localizes to the cytoplasm or nucleus and has potential functions and roles that deserve further study in the future.

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*Statement of conflict of interest*

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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