



Development and Characterization of a Novel Anti-idiotypic Monoclonal Antibody to Growth Hormone, Which Can Mimic Physiological Functions of Growth Hormone in Primary Porcine Hepatocytes

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ABSTRACT: B-32 is one of a panel of monoclonal anti-idiotypic antibodies to growth hormone (GH) that we developed. To characterize and identify its potential role as a novel growth hormone receptor (GHR) agonist, we determined that B-32 behaved as a typical Ab2 β based on a series of enzyme-linked immunosorbent assay assays. The results of fluorescence-activated cell sorting, indirect immunofluorescence and competitive receptor binding assays demonstrated that B-32 specifically binds to the GHR expressed on target cells. Next, we examined the resulting signal transduction pathways triggered by this antibody in primary porcine hepatocytes. We found that B-32 can activate the GHR and Janus kinase (2)/signal transducers and activators of transcription (JAK2/STAT5) signalling pathways. The phosphorylation kinetics of JAK2/STAT5 induced by either GH or B-32 were analysed in dose-response and time course experiments. In addition, B32 could also stimulate porcine hepatocytes to secrete insulin-like growth factors-1. Our work indicates that a monoclonal anti-idiotypic antibody to GH (B-32) can serve as a GHR agonist or GH mimic and has application potential in domestic animal (pig) production. (**Key Words:** Porcine Growth Hormone, Monoclonal Anti-idiotypic Antibodies (Mab2s), Porcine Hepatocytes, Janus Kinase (2)-Signal Transducers and Activators of Transcription [JAK2-STAT5])

INTRODUCTION

Growth hormone (GH), a 191-amino acid polypeptide, is primarily synthesised and secreted in the anterior pituitary and has pro-proliferative, anti-apoptotic and metabolic effects in various target tissues (Abdel-Meguid et al., 1987). The diverse biological actions of GH are initiated by the binding of GH to its receptor, which belongs to the cytokine receptor superfamily (Møller et al., 2009). After GH binding to growth hormone receptor (GHR), tyrosine kinase janus kinase 2 (JAK2) is activated, which in turn phosphorylates multiple tyrosines within both itself and the

GHR receptor, as well as multiple signalling pathways. These pathways include the signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase, and extracellular regulated kinases. These signalling pathways act together to contribute to the overall actions of GH (Zhu et al., 2001).

Many studies have reported that special anti-idiotypic antibodies to hormones can serve as hormone mimics; this action is based on the network theory of immune regulation (Jerne, 1974). According to this theory, if the antibodies (Ab1), which are generated by immunising animals with a ligand, are directed against the receptor-binding sites on a ligand, then a subpopulation (Ab2 β) of resulting anti-idiotypic antibodies using Ab1 as immunogens can bind and react to the same receptor because the common epitope is shared by the anti-idiotypic antibodies (Ab2 β) and ligand and these Ab2 antibodies represent the internal image of the ligand (Li et al., 2013). This approach has been used to prepare polyclonal or monoclonal anti-idiotypic antibodies

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(Mab2s) against GH (Elbashir et al., 1990; Gardner et al., 1990; Wang et al., 1994; Roberge et al., 1999; Rajput et al., 2003; Li et al., 2013); however, these anti-idiotypic antibodies cannot be used in animal production (such as domestic pigs) because i) all of these studies except for (Roberge et al., 1999) focused on rodent GHR models, which are mainly used for studies at the experimental level; ii) the ability of these anti-idiotypic antibodies to trigger intracellular signalling pathways and activate GHR is unclear, except for in our previous study (Li et al., 2013). iii) The ability of these anti-idiotypic antibodies to stimulate target cells or animal model to secrete insulin-like growth factors-1 (IGF-1) is unknown. Thus, the applications of anti-idiotypic antibodies to GH in animal production (such as domestic pigs) have not yet been reported.

In this work, porcine hepatocytes were used as a cell model to screen and characterise an active anti-idiotypic antibody that has potential application in domestic animal production. A panel of Mab2s against GH were generated, and we found that one antibody, termed B32, could activate intracellular JAK2-STAT5 signalling and stimulate porcine hepatocytes to secrete IGF-1. The current study suggested that a monoclonal anti-idiotypic antibody to GH (B-32) can serve as an effective GHR agonist or GH mimic and has application potential in domestic animal (pig) production.

MATERIALS AND METHODS

Antibodies and reagents

Phospho-JAK2 and total JAK2 were obtained from Cell Signaling Technology (USA). Phospho-STAT5 and total STAT5 were obtained from Santa Cruz (USA). Antiphosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse antibodies and recombinant GH were purchased from Sigma (Sigma-Aldrich Company, St. Louis, MO, USA). ^{125}I -GH was prepared using chloramine T according to published procedures, and the specific activity of GH was 70 to 130 $\mu\text{Ci}/\mu\text{g}$ protein. Lipofectamine Plus and pCDNA3.1 were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). The ImmunoPure Fab Preparation kit, Cell Lysis Buffer, IP kit, Enhanced chemiluminescence (ECL) and the BCA kit were purchased from Pierce (Pierce Biotechnology Inc, Rockford, IL, USA). Porcine GH receptor cDNAs were obtained from Takara Biotechnology Co., Ltd (Dalian, China). The pre-stained molecular weight standards and all tissue reagents were obtained from Gibco (Gibco, Grand Island, NY, USA). The porcine IGF-1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Hua Yi Medical and Biological Laboratories Co., Ltd (Changchun, China). Unless stated otherwise, all other reagents were from

Sigma-Aldrich (USA).

Isolation and culture of porcine hepatocytes

Porcine hepatocytes endogenously express GHR and have been demonstrated to be a valuable system for studying the mechanism of action of GH and signalling pathways induced by GH (Brameld et al., 1995; Brameld et al., 1999; Elsasser et al., 2007; Ramsay et al., 2010). Hepatocytes from pigs (~60 kg body weight) are reportedly responsive to GH (Elsasser et al., 2007; Ramsay et al., 2010). Therefore, porcine hepatocytes isolated from pigs weighing ~60 kg were used in the current study. The pigs (Landrace) were stunned by electric shock and exsanguinated. The livers were immediately excised, and the left lateral lobe was removed, after which the porcine hepatocytes were isolated via the two-step collagenase perfusion technique according to Caperna et al. (2005) and Fernández-Figares et al. (2004); the cell yield from each liver lobe preparation in this study was $1.3 \pm 0.2 \times 10^9$ viable hepatocytes. The Trypan blue dye exclusion assay indicated a viability of approximately 80 to 85% for the isolated hepatocytes. Hepatocytes (5.0×10^6) were seeded into T-100 flasks, pre-coated with pigtail collagen, and cultured as previously described (Caperna et al., 2005). The cells were treated as described as below.

Chinese hamster ovary cell transfection and culture

Porcine GH receptor cDNAs were subcloned into the mammalian expression vector pCDNA3.1 according to the manufacturer's instructions. Chinese hamster ovary (CHO) cells were transfected with pCDNA3.1-rGHR or an empty vector (as a control) using Lipofectamine Plus according to the manufacturer's protocols. Stably transfected cells were selected in G418 (1 mg/mL), which was followed by a selection of single cells for clonal expansion, after which receptor binding assays utilising ^{125}I -GH were performed on individual clones to detect GHR-expressing lines. Each clone was further characterised via Scatchard analysis to determine the receptor number. In this study, one GHR clone (termed CHO-GHR), which expresses high levels of GHR, was selected for subsequent studies. In this study, CHO-GHR cells were used to screen and characterise Mab2s that can bind the GHR.

The CHO-GHR cells were cultured as previously described (Smit et al., 1996).

Preparation of polyclonal anti-growth hormone antibodies

Rabbits were intradermally immunised with 1 mg GH emulsified in Freund's complete adjuvant. The rabbits received 5 similar intradermal booster injections of GH at two-week intervals, and the rabbits were bled weekly after the first booster injection. After a series of immunisations,

all rabbits generated high titres of antibodies against GH, as determined by ELISA. Immunoglobulin G (IgG) fragments were purified from each rabbit by ammonium sulphate precipitation followed by protein-A affinity chromatography. In addition, we also preliminarily evaluated the rabbit anti-GH polyclonal antibodies to determine whether these polyclonal IgG antibodies contain neutralising antibodies that can inhibit GH binding to the GHR expressed on porcine hepatocytes or CHO-GHR cells. In our pre-experiment, we found that the rabbit anti-GH polyclonal antibodies generated by each rabbit could all inhibit GH binding to the GHR expressed on target cells based on a receptor-binding assay. After satisfying this requirement, these polyclonal IgG antibodies were then used as antigens to immunise mice to prepare anti-idiotypic antibodies to GH in the subsequent experiments.

Preparation of monoclonal anti-idiotypic antibodies

Six- to eight-week-old female BALB/c (weighing 15 to 20 g) (Experimental Animal Centre of Jilin University) were intraperitoneally immunised with 0.2 mg of rabbit anti-GH-F(ab')₂ in Freund's complete adjuvant (Rabbit anti-GH F(ab')₂ fragments were prepared using the Pierce kit according to the manufacturer's instructions). For the booster immunisation, the mice received similar booster injections of rabbit anti-GH F(ab')₂ in incomplete Freund's adjuvant at 2-week intervals. Three days after the final injections, the splenocytes were collected and fused with Sp2/0 myeloma cells using 50% PEG1500. After fusion, the cells were distributed in 96-well cell culture plates. Hybridomas were selected in hypoxanthine aminopterin thymidine (HAT) medium. The culture supernatants of hybridomas selected on HAT medium were tested by ELISA to determine the presence of antibodies against the rabbit anti-GH-F(ab')₂. Antibody-secreting hybridisms were cloned at least twice via limiting dilution.

Enzyme-linked immunosorbent assay

To detect the rabbit anti-GH polyclonal antibodies, microtitre plates (96 wells) were coated with 100 µL of 1 µg/mL GH, and incubated overnight at 4°C. After washing three times with phosphate-buffered saline with 0.5% Tween (PBST), the microtitre plates were blocked with 2% bovine serum albumin (BSA) for 1 h in 37°C. Rabbit antisera diluted in 2% BSA were added and incubated for 2 h. After washing, the HRP-conjugated goat anti-rabbit IgG was added and incubated for 1 h in 37°C. The plates were washed again, and the colorimetric reaction was developed for 15 min using the tetramethylbenzidine (TMB) substrate. The reactions were stopped by the addition of 3 M H₂SO₄ into each well (50 µL/well), and the absorbance was measured at 450 nm using an automatic

ELISA plate reader (Multiskan FC, Thermo Fisher Scientific Inc., Cambridge, MA, USA).

To detect the Mab2s to GH, microtitre plates were coated with purified anti-GH F(ab')₂ fragments or with F(ab')₂ fragments of control antibodies overnight at 4°C. Next, the wells were washed twice with PBST, and non-specific sites were blocked with 2% BSA per well for 1 h at 37°C. The wells were washed three times with PBST, the culture supernatants from the hybridomas were added, and the wells were incubated at 37°C for 1 h. After washing three times, goat anti-mouse IgG-FC HRP secondary antibody was added and incubated for 1 h at 37°C. After a further incubation for 1 h at 37°C, the colorimetric reaction was developed for 15 min using TMB substrate. The reactions were stopped by the addition of 3 M H₂SO₄ to each well (50 µL/well), and the absorbance was measured at 450 nm using an automatic ELISA plate reader (Multiskan FC, Thermo Fisher Scientific Inc., USA).

Fluorescence-activated cell sorting analysis

Porcine hepatocytes or CHO-GHR cells were detached from the plate and washed twice with phosphate-buffered saline (PBS). The cells were then suspended in PBS containing 1% BSA at a density of 4×10⁵ cells/mL. The cells were incubated with 100 µL in either a negative control medium or in one of the positive hybridoma supernatants for 1 h at 4°C. After incubation, the cells were washed twice and then incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG for 1 h at 4°C in the dark. After incubation, the cells were washed and resuspended in 0.5 mL fluorescence-activated cell sorting (FACS) buffer. A flow cytometric analysis was performed using a FACS Calibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analysed using the Cell Quest software.

Competitive enzyme-linked immunosorbent assay

Competitive ELISAs were performed to determine whether Mab2s screened by flow cytometry can mimic an epitope of GH. Microtitre plates (96 wells) were coated with F(ab')₂ fragments of Mab2s or control antibody for 1 h at 37°C. After three washes with PBST, the plates were blocked with 1% BSA for 1 h at 37°C. After washing three times with PBST, a constant amount of rabbit anti-GH antibodies (Ab1) together with increasing concentrations of the GH were added to the plates and incubated for 1 h at 37°C. The plates were washed again, and goat anti-mouse IgG (Fc fragment-specific)-HRP secondary antibody was added to the wells. After a further incubation for 1 h at 37°C, the plates were washed three times, and the TMB substrate was added to develop the colorimetric reactions for 15 min at 37°C. The reactions were stopped by the addition of 3 M H₂SO₄ into each well (50 µL/well), and the absorbance was

measured at 450 nm using an automatic ELISA plate reader (Multiskan FC, Thermo Fisher Scientific Inc., USA).

A similar competitive ELISA was performed. The microtitre plates were coated with the F(ab')₂ fragments of rabbit anti-GH antibodies, and a constant amount of Mab2s together with increasing concentrations of GH were added to these wells. The subsequent treatments were performed as described as above.

Indirect immunofluorescence assay

Porcine hepatocytes cultured on glass coverslips coated with collagen were washed with PBS and then fixed with 4% paraformaldehyde at 37°C for 20 min. The coverslips were washed with PBS, and the cells then were incubated with 1% BSA for 1 h. After another wash with PBS, the cells were incubated with Mab2s or isotype control monoclonal antibody (negative control) for 30 min at the 37°C. The cells were washed again with PBS and incubated with tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-mouse IgG secondary antibody (1:100 dilutions) for 30 min at the 37°C in the dark. The cells were washed again three times and incubated with 4,6-diamidino-2-phenylindole (DAPI) for 15 min at 37°C (DAPI was used to observe the cell nucleus). The cells were again washed in triplicate with PBS and analysed using confocal laser scanning microscopy (Olympus FV1000, Olympus, Tokyo, Japan).

For the co-localisation analysis, porcine hepatocytes were simultaneously stimulated with FITC-GH and TRITC-B-32 for 30 min at 37°C, and the cells were then treated as described as above.

Competitive receptor-binding assay

The porcine hepatocytes or CHO-GHR cells were incubated overnight in serum-free Dulbecco's Minimal Eagle's Medium (DMEM) containing 1% BSA. After washing with PBS, the cells were incubated with 1 mL DMEM containing 1% BSA, ¹²⁵I-GH (5,000 CPM/well) and various concentrations of unlabelled GH, Mab2s or control antibody for 3 h. The cells were then washed twice, detached from the plate, and then counted in a gamma counter. Non-specific binding was determined in the presence of excess unlabelled GH (3 µg/mL).

Immunoprecipitation and Western immunoblotting

Porcine hepatocytes or CHO-GHR cells were stimulated with GH (20 nM) or B-32 (20 nM) for 20 min at 37°C. The cells were then detached from the tissue plates and washed three times with tris-buffered saline-Tween 20 (TBST). The cells were collected by centrifugation (1,500 rpm for 10 min) and then re-suspended at 1×10⁷ cells/mL in TBST. The cells (1×10⁷ cells/precipitation) were placed on ice and lysed with cell lysis buffer according to the manufacturer's

suggestions. The cell lysates were centrifuged at 12,000 g for 15 min to remove debris. Immunoprecipitation was performed using anti-GHR antibody (10 µg). The IP Kit was used to isolate the immunoglobulin-bound proteins according to the manufacturer's suggestions. The immunoprecipitated samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% BSA at 37°C for 2 h, then washed three times for 10 min each in TBST, and then incubated with the anti-phosphotyrosine monoclonal antibody (clone 4G10, which reacts with total phosphorylated tyrosine). After three washes with TBST, the membranes were incubated for 1 h with a goat anti-mouse-HRP secondary antibody. After washing with TBST, the proteins on the membranes were detected using the ECL detection system.

Western-Blotting analysis

Prior to the experimental treatments, the growth media of hepatocytes were replaced with serum-free media for 6 h, and the serum-free media was then removed from the T-100 flasks; porcine hepatocytes were challenged with GH, Mab2s or control antibody at the indicated concentrations and for the indicated times, and all stimulations were carried out at 37°C. The cells were solubilised in lysis buffer. The protein concentrations were determined and 30 µg of total protein was loaded on the 4% to 10% SDS-PAGE gels. The proteins were transferred to the PVDF membranes, which were probed with the phospho-JAK2 and phospho-STAT5 antibodies following the manufacturer's instructions. The membranes were then incubated with HRP-conjugated secondary antibody, and the proteins detected by using an ECL plus kit. The blots were stripped via incubation in stripping solution for 30 min at 55°C, blocked and re-probed for JAK2 and STAT5.

Medium insulin-like growth factors-1 analysis

The freshly isolated hepatocytes were seed into 100 mm flasks. The hepatocyte medium was exchanged for basal medium 10 h after plating. The hepatocytes were exposed to 20 nM GH (positive control), 20 nM B32 or 20 nM control antibody (negative control) for 6, 12, and 24 h. After incubation for the indicated time (6 to 24 h), the medium was collected and stored at -80°C prior to being assayed for IGF-1. The IGF-1 levels in the medium obtained from each study condition were determined using a commercially available porcine-specific ELISA kit according to the manufacturer's instructions.

Statistical analysis

The data are presented as the mean values±standard error of triplicate samples from six independent hepatocyte

culture preparations. “n” is the number of animals used in each experiment. The results were analysed with a one-way analysis of variance using the Statistical Analysis System (SAS) software (SAS version 9.0; Institute Inc., Cary, NC, USA) followed by the Student-Newman-Keuls test. A p-value of <0.05 was considered statistically significant.

RESULTS

Generation of monoclonal anti-idiotypic antibody against anti-growth hormone antibodies (Mab2)

After a series of immunisations, the splenocytes from the immunised mice were fused with mouse myeloma cells (Sp2/0-Ag14). The supernatants from all of the resulting hybridomas were screened by ELISA, and 52 positive antibody-secreting clones were identified (data not shown). We then further screened the potential Mab2s that can bind to GHR on porcine hepatocytes or CHO-GHR cells. Five clones exhibited strong positive signals. Of these clones, one Mab (referred to as B-32) was selected for further characterisation because of its unique biological properties (Figure 1), which are described below. Hybridoma cells were intraperitoneally injected into liquid paraffin-primed BALB/c mice to induce ascetic fluids, which were purified by ammonium sulphate precipitation followed by protein-A affinity chromatography. Isotype analysis identified the Mab B-32 to be IgG1.

Characterisation of monoclonal anti-idiotypic antibodies

Competitive assays were performed to assess whether the anti-idiotypic monoclonal antibody (B-32) possesses an

internal image nature (Ab2 β). The results indicated that GH could inhibit the interaction between the anti-GH antibodies and B-32. Moreover, GH could also compete with B-32 for binding to anti-GH antibodies in a dose-dependent manner, suggesting that B-32 and GH share a common epitope (Figure 2).

Determination of B-32 binding to growth hormone receptor

Indirect immunofluorescence and competitive receptor-binding assays were carried out to determine whether B-32 specifically binds to GHR. First, the cells were observed using confocal laser scanning microscopy. As shown in Figure 3A, B-32 (a) but not control antibody (b) bound to the hepatocytes surface, suggesting that B-32 is likely to bind GHR on hepatocytes. In addition, although both GH and B-32 showed similar staining patterns in the cytoplasm of hepatocytes, B-32 failed to localise in the nucleus, whereas GH was subjected to rapid nuclear translocation (Figure 3B). To further confirm whether B-32 can specifically bind to GHR on porcine hepatocytes or CHO-GHR cells, competitive receptor-binding assays were performed. As shown in Figure 4, unlabelled GH inhibited the binding of 125 I-GH to cells, and B-32 also displaced 125 I-GH in a dose-dependent manner; the isotype-matched control antibody did not exert an effect. These data demonstrate that B-32 specifically binds to GHR.

Activation of porcine growth hormone receptor by B-32

The above-mentioned experiments demonstrated that B-32 specifically binds to GHR in porcine hepatocytes or

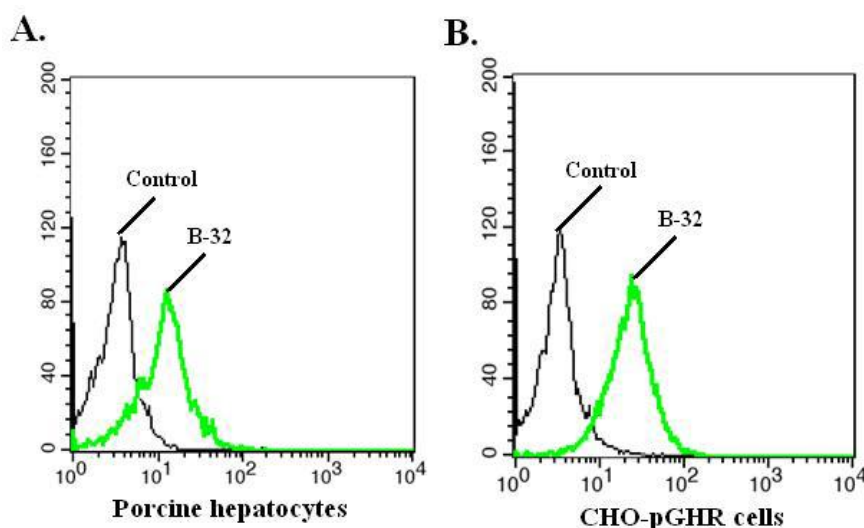


Figure 1. Screen for potential anti-idiotypic monoclonal antibodies based on their ability to bind growth hormone receptor (GHR) on the porcine hepatocytes or Chinese hamster ovary (CHO)-GHR by fluorescence-activated cell sorting (FACS). Porcine hepatocytes or CHO-GHR cells were collected, centrifuged to eliminate cell debris and incubated with negative control media (black line) or B-32 supernatants (green line) for 1 h at 4°C. The cells were then pelleted, washed and incubated with fluorescein isothiocyanate-conjugated secondary antibody for 1 h in the dark at 4°C. The cells then were resuspended in FACS buffer and analysed by flow cytometry. The figures represent at least three independent experiments.

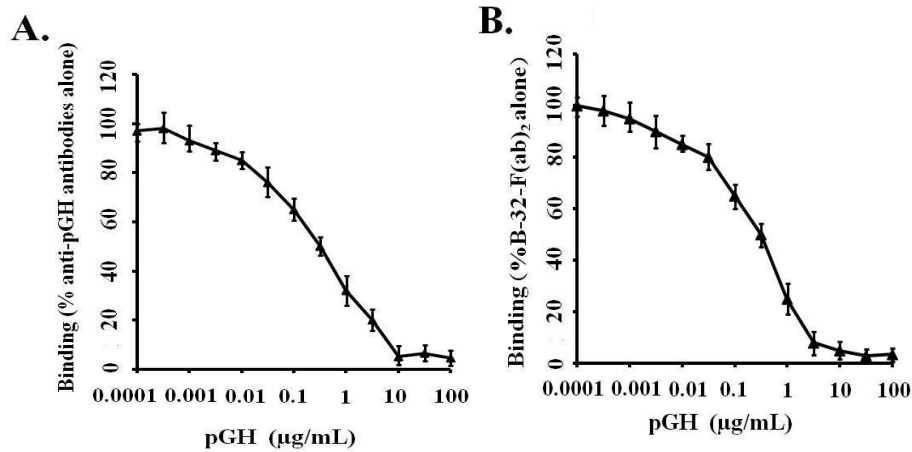


Figure 2. Interactions between anti- growth hormone (GH) antibodies, B-32 and GH. (A) GH inhibits the binding of anti-GH antibodies to B-32. Microtitre plates were coated with F(ab')₂ fragments of B-32, and a constant amount of rabbit anti-GH antibodies together with increasing concentrations of GH were added. (B) Microtitre plates were coated rabbit anti-GH-F(ab')₂, and a constant amounts of F(ab')₂ fragments of B-32 together with increasing concentrations of GH were added. After incubation and washing, goat anti-mouse IgG (Fc fragment-specific)-horseradish peroxidase econdary antibody was added to the wells. The colorimetric reactions were developed using tetramethylbenzidine, and the absorbance was measured using an enzyme-linked immunosorbent assay reader. The data shown were measured in triplicate and represent two independent experiments.

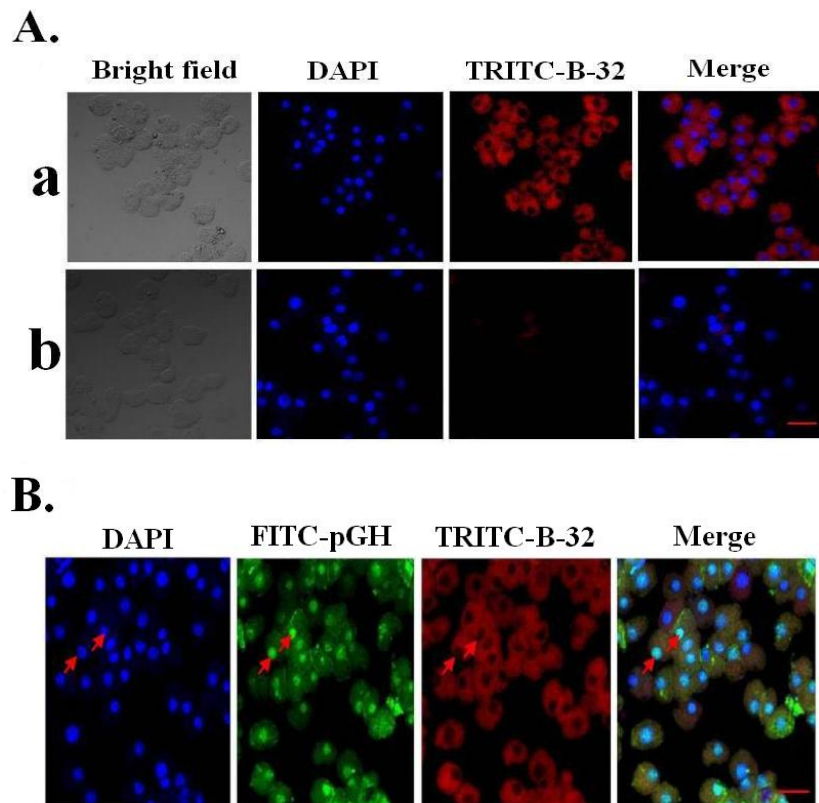


Figure 3. (A) Binding of B-32 to porcine hepatocytes. The hepatocytes were fixed with 4% paraformaldehyde, blocked with 1% bovine serum albumin, washed, and incubated with B-32 (a) and control antibody (b). The cells were then incubated with TRITC-labelled goat anti-mouse IgG secondary antibody. After washing, the hepatocytes were fixed and analysed by confocal microscopy. (B) Co-localisation analysis of growth hormone (GH) and B-32 in porcine hepatocytes. The hepatocytes were simultaneously treated with TRITC-B-32 and fluorescein isothiocyanate (FITC)-GH for 30 min at 37°C. After washing, the hepatocytes were fixed and analysed by confocal microscopy. The confocal images were generated using an Olympus FV1000 confocal laser scanning microscope. Bar = 10 nm. The confocal images shown represent at least three independent experiments.

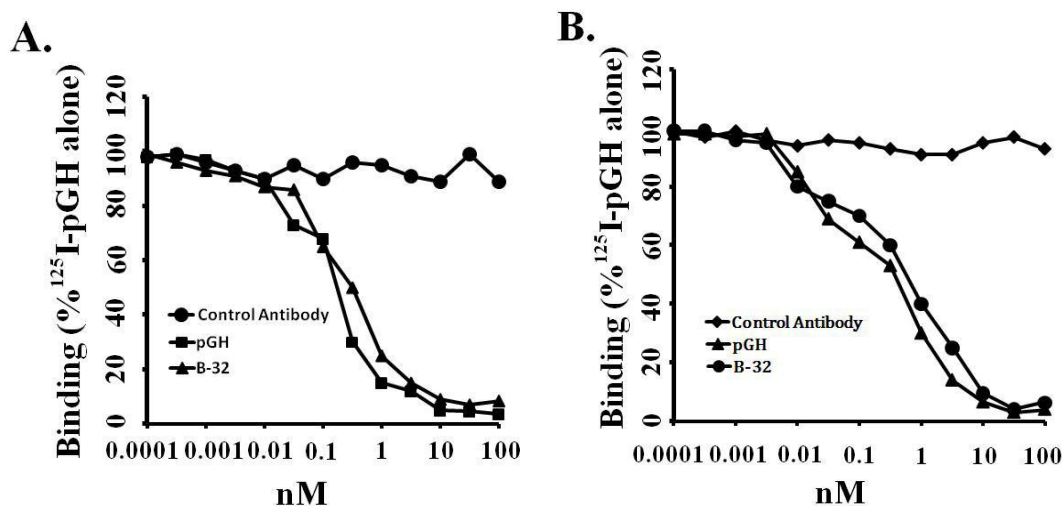


Figure 4. Displacement of ^{125}I -growth hormone (GH) from porcine hepatocytes and CHO-GHR638 cells by unlabelled GH, B-32 or control antibody. Porcine hepatocytes (A) and CHO-GHR cells (B) were incubated with the indicated amounts of ^{125}I -GH and increasing concentrations of unlabelled GH, B-32 or control antibody for 2 h at 4°C. The cells were then washed and detached from the plate and counted in a gamma counter. The data shown represent two independent experiments.

CHO-GHR cells, but its ability to activate GHR was unclear. Therefore, we first tested the ability of the anti-idiotypic monoclonal antibody B-32 to activate GHR in porcine hepatocytes or CHO-GHR cells. Serum-starved cells were treated with 20 nM GH or B-32 for 20 min. GHR was immunoprecipitated from porcine hepatocytes or CHO-GHR and detected using 4G10. As shown in Figure 5, GH and B-32, but not the control antibody, activated GHR in porcine hepatocytes and CHO-GHR cells.

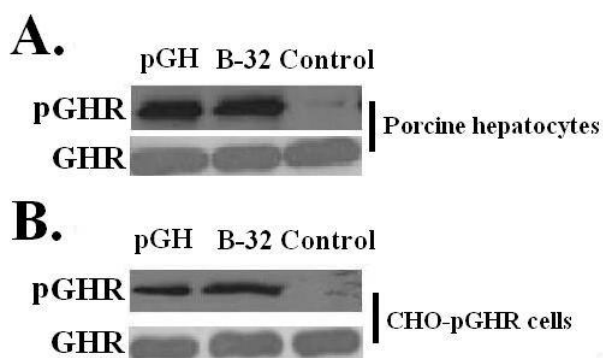


Figure 5. Activation of growth hormone receptor (GHR) by B-32. Serum-starved hepatocytes or CHO-GHR cells were stimulated with GH, B-32 or control antibody (20 nM) for 20 min before detergent extraction, and the porcine hepatocytes or CHO-GHR cells were then collected, washed, centrifuged and lysed with cell lysis buffer. The detergent lysates of the cells were immunoprecipitated using anti-GHR antibody or control antibody. Equal amounts of protein from each treatment were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes and probed with 4G10. The figures represent at least three independent experiments.

Activation of JAK2-STAT5 signalling by B-32

The JAK2-STAT5 pathway has been shown to be central to GH-induced changes in metabolic function, body growth and IGF-I gene transcription (Herington et al., 2012). Therefore, the tyrosine phosphorylation of JAK2/STAT5 was analysed by Western-blotting. Dose-response and time-course experiments were performed in porcine hepatocytes, and representative results are shown in Figure 6A and B, respectively. The dose-response profiles for JAK2 and STAT5 activation by B-32 were similar to those observed for GH. For both ligands, JAK2 and STAT5 activation was easily detected after 5 min of stimulation with concentrations as low as 3 nM, and the activity was maximised at 10 to 40 nM (Figure 6A). The time courses of JAK2 and STAT5 activation by GH or B-32 were also similar in porcine hepatocytes (Figure 6B). For each ligand, JAK2 and STAT5 tyrosine phosphorylation was observed as early as 5 min, maximised at 15 to 45 min, and subsequently began to decline. In addition, the dose-response and time-course experiments were not carried out in CHO-GHR638 cells because we aimed screen for and characterise an anti-idiotypic monoclonal antibody that has application potential in domestic animal (pig) production. Therefore, porcine hepatocytes (somatic cells) may be the best choice, whereas transfected CHO cells (referred to as the overexpression cell model) are not suitable and were only used to screen and characterise the anti-idiotypic antibody to GH in the present study.

Secretion of insulin-like growth factors-1 in cultured porcine hepatocytes under B32 stimulation

The above experiments demonstrated that B32 could activate the JAK-STAT5 signalling pathway, which is

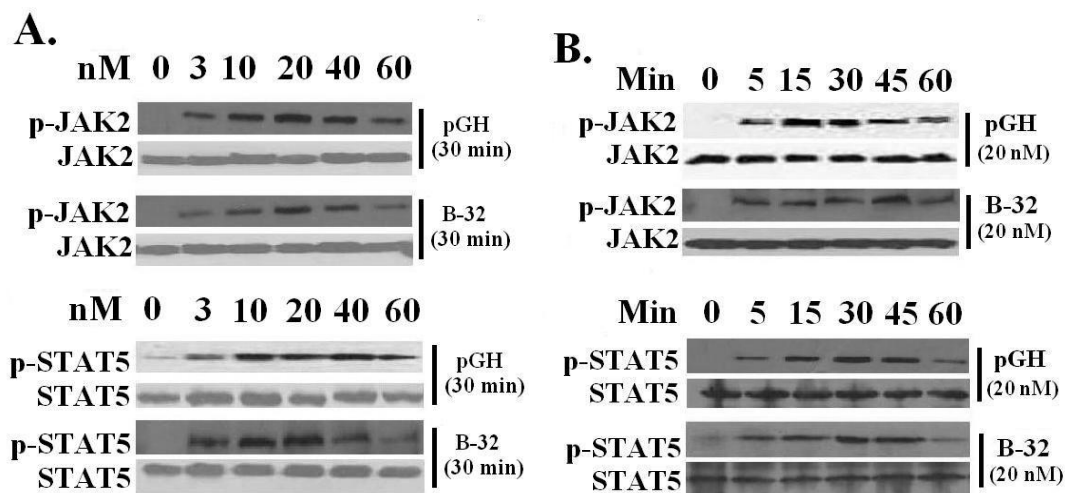


Figure 6. (A) Dose-response of Janus kinase (2) (JAK2) and signal transducers and activators of transcription (STAT5) phosphorylation induced and growth hormone (GH) or B-32. Serum-starved porcine hepatocytes were stimulated with GH or B-32 (0 to 60 nM) for 20 min at 37°C before detergent extraction. Equal amounts of protein from each treatment were resolved by SDS-PAGE, transferred to PVDF membranes and probed with antibodies recognising p-JAK2 and p-STAT5. (B) Time-course of JAK2 and STAT5 phosphorylation by GH and B-32. Serum-starved porcine hepatocytes were stimulated with 20 nM GH or B-32 (0 to 60 min) at 37°C before detergent extraction. Equal amounts of protein from each treatment were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes and probed with antibodies recognising p-JAK2 and p-STAT. In each experiment, protein samples resulting from treatment with each ligand were analysed on separate gels, and immunoblotting conditions was optimised for each to compare time courses rather than relative signal intensities. The figures represent at least three independent experiments.

responsible for IGF-1 protein expression by the liver. Therefore, we analysed the IGF-1 content in the medium in response to B32 treatment to determine whether B32 could prompt porcine hepatocytes to secrete IGF-1 protein. The hepatocytes were treated with GH, B-32 or control antibody for different time intervals. The results show that stimulation with B32 or GH for different incubation times significantly increased the secretion of IGF-1, while treatment with the control antibody or basal medium alone showed no effect (Figure 7). These results indicated that B32 can stimulate the hepatocytes to secrete IGF-1 protein.

DISCUSSION

The earliest reports indicating that anti-idiotypic antibodies to hormones can mimic hormone function described insulin (Sege and Peterson, 1978). Since then, a number of studies have reported that anti-idiotypic antibodies may mimic hormones. The anti-idiotypic antibody approach also has been successfully used in the preparation of polyclonal or monoclonal antibodies to GH (Elbashir et al., 1990; Gardner et al., 1990; Wang et al., 1994; Roberge et al., 1999; Li et al., 2013). Parallel findings have been described, in which antibodies raised against GHR also have biological effects similar to that of GH (Carlsson et al., 1994; Wang et al., 1994; Wang et al., 1996; Mellado et al., 1997; Rowlinson et al., 1998; Wan et al., 2003; Wu et al., 2010). In the present work, we prepared

and characterised a monoclonal anti-idiotypic antibody named B-32. To our knowledge, this report is the first to describe a monoclonal anti-idiotypic antibody (B-32) that could activate GHR on porcine hepatocytes and has application potential in animal (pig) production.

The GH is an asymmetric molecule. Its interaction with GHR is mediated by two non-equivalent binding sites (site 1 and site 2) (Brooks et al., 2010). A GH-induced GHR conformational change is generally accepted to be required for its activation, although the nature of this conformational change remains unclear. In contrast to GH, the antibody agonist (IgG) is a symmetric molecule that contains two binding sites with the same binding affinity. The activating antibody, Mab263 (a well-known GHR agonist), can reportedly induce conformational changes similar to that induced by GH (Wan et al., 2003). In addition to the above general features, anti-idiotypic antibodies to GH (such as B-32) can also mimic the GHR binding site on GH. The GH has two specific receptor binding sites (site 1, site 2), but the site mimicked by B-32 remains unclear. The high- and low-affinity receptor binding sites of GH are reportedly allosterically coupled (Walsh et al., 2004); GH first binds to one receptor molecule via site 1 (high affinity), which allosterically affects site 2. Subsequently, GH binds another GHR via site 2, suggesting that the natural structures of site 2 but not site 1 is changed. In addition, rabbits were immunised with natural GH in the present study, which contains the natural and unchanged site 2. Therefore, we

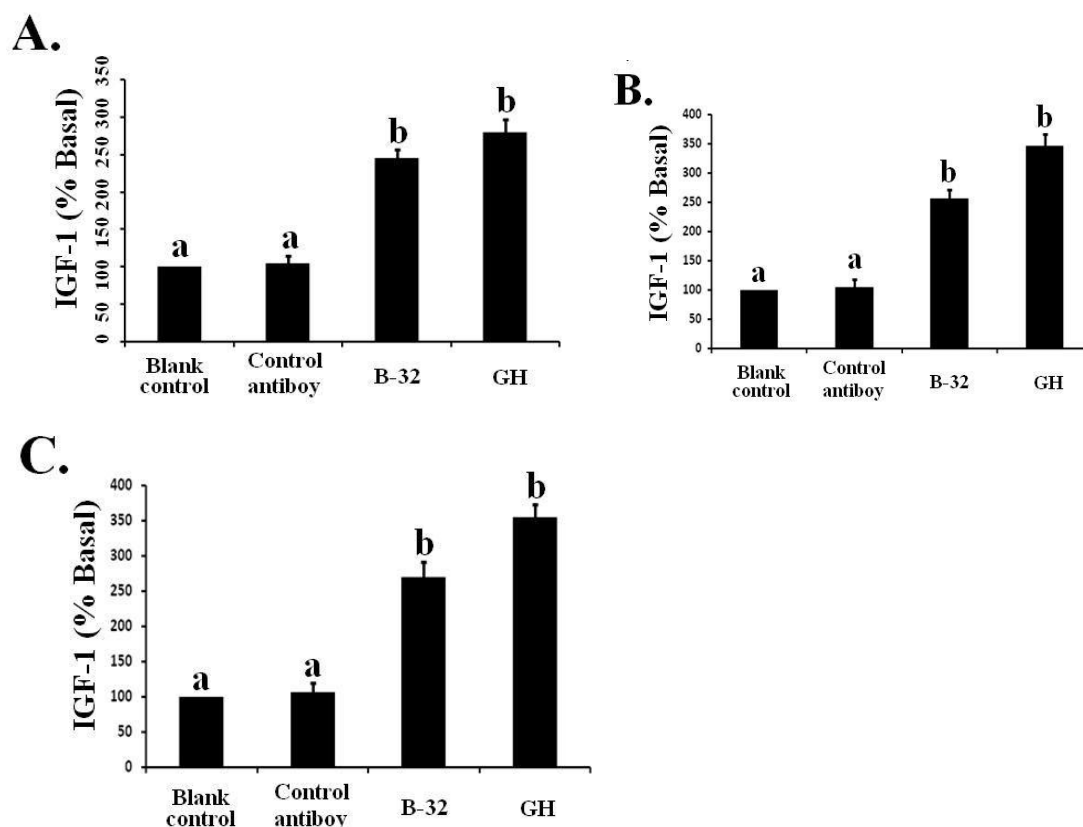


Figure 7. The insulin-like growth factors-1 (IGF-1) levels in the media following incubation with basal medium (blank control), control antibody (negative control), B-32 or growth hormone (GH) (positive control) for 6 h (A), 12 h (B), or 24 h (C). The porcine hepatocytes were treated as described in the materials and methods. After stimulation with the indicated ligands, the medium was collected, and the IGF-I content was measured using a commercially available porcine-specific enzyme-linked immunosorbent assay kit according to manufacturer's instruction. The data are expressed as per cent of IGF-I in the medium of control cultures (basal medium). Bars that do not share the same superscript letter are significantly different ($p < 0.05$).

speculated that B-32 mimics site 1 of GH.

In the current study, we found that the cytoplasm internalised both GH and B-32, which showed similar staining patterns. In addition, B-32 failed to localise in the nucleus, whereas GH was subjected to rapid nuclear translocation (Figure 3B). The nuclear translocation of GH is mediated by GHR, and the nuclear translocation of the GH-GHR complex is mediated by the importin α/β system (Conway-Campbell et al., 2007). In addition to events that occur at the plasma membrane, the GH-GHR complex also can exhibit physiological actions based on its nuclear localisation, which is currently a hot topic. However, the cause of the inability of B-32 to translocate into the nucleus remains unclear and deserves further study.

Jak2 is a key mediator in GH-induced signalling (Milward et al., 2004), and many studies have established STAT5 as a key intracellular mediator of GH action, especially in hepatocytes (Gebert et al., 1999; Chia et al., 2006). Therefore, we mainly studied JAK2/STAT5 phosphorylation induced by B-32 in this study, and the patterns of JAK2-STAT5 activation by B-32 were similar to those observed for GH (Figure 6). In addition, B-32 could

also stimulate porcine hepatocytes to secrete IGF-1 (Figure 7).

Overall, we prepared and characterized an anti-idiotypic monoclonal antibody to GH, B-32, which was found to behave as a typical Ab 2β and could activate GHR and the JAK2-STAT5 signalling pathway. Furthermore, B-32 could induce hepatocytes to secrete IGF-1. These results indicate that B-32 possesses application potential in domestic animal (pig) production, but this application requires further study.

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