

**Autocrine secretion of insulin-like growth factor-I mediates growth hormone-stimulated DNA synthesis and proliferation in primary cultures of adult rat hepatocytes**

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**Abstract**-----The intracellular signaling pathway of growth hormone (GH)-stimulated DNA synthesis and proliferation was investigated in primary cultures of adult rat hepatocytes. DNA synthesis and cell proliferation were detected in hepatocyte parenchymal cells grown in serum-free, defined medium containing GH (100 ng/ml). GH-stimulated hepatocyte DNA synthesis and proliferation were almost completely blocked by TG101209 ( $10^{-6}$  M), a selective Janus kinase (JAK)2 inhibitor, U-73122 ( $10^{-6}$  M), a selective phospholipase C (PLC) inhibitor, and a monoclonal antibody to insulin-like growth factor-I (IGF-I) receptor (100 ng/ml) or anti-secretion agents such as somatostatin ( $10^{-6}$  M) and BAPTA/AM ( $10^{-7}$  M). In addition, blocking monoclonal antibodies to IGF-I, but not transforming growth factor- $\alpha$ , completely inhibited GH-induced hepatocyte DNA synthesis and proliferation. IGF-I levels in the culture medium increased rapidly versus baseline levels within 5 min in response to GH (100 ng/ml), and the maximum IGF-I level (100 pg/ml) was reached 20 min after GH stimulation. Autocrine secretion of IGF-I into the culture medium was inhibited by a growth-inhibitory dose of TG101209, U-73122, somatostatin, or BAPTA/AM. These data indicate that the proliferative mechanism of action of GH is mediated mainly through a GH receptor/JAK2/PLC-stimulated increase in the autocrine secretion of IGF-I by primary cultured hepatocytes, followed by stimulation of the 95 kDa IGF-I receptor tyrosine kinase signaling pathway.

**Keywords:** growth hormone (GH), hepatocyte proliferation (cultured hepatocytes), insulin-like growth factor (IGF)-I, autocrine secretion

## 1. Introduction

The anterior pituitary produces growth hormone (GH), which modulates critical cellular functions, including cell and tissue growth, differentiation, and carbohydrate and lipid metabolic processes. GH binds to GH receptors and also functions indirectly via insulin-like growth factor I (IGF-I). In the presence of GH, the liver produces IGF-I (Lund et al., 1986). When GH binds to its own receptor, Janus kinases (JAK)<sub>1/2</sub> are activated, downstream pathways including signal transducer and activator of transcription (STAT) phosphorylation are triggered, and nuclear translocation and gene expression are induced. GH signaling pathways are required for normal liver regeneration, but the intracellular signaling mechanisms leading to hepatocyte proliferation have yet to be fully determined (Li et al., 2002; Zerrad-Saadi et al., 2011; Pennisi et al., 2013).

We previously showed that GH receptors induce DNA synthesis and hepatocyte cell division and that stimulation of the GH receptor/JAK2/phospholipase C (possibly PLC- $\alpha$ )/Ca<sup>2+</sup> pathway and the 95 kDa receptor tyrosine kinase (RTK)/phosphatidylinositol 3-kinase (PI3K)/extracellular signal-regulated kinase (ERK)<sub>2</sub>/mammalian target of rapamycin (mTOR) pathway is involved in this process. This 95 kDa RTK was identified as the IGF-I receptor using a monoclonal antibody against the phospho-IGF-I receptor (unpublished observation). The relationship between these two signaling pathways is not well understood. We hypothesized that GH stimulates the GH receptor, followed by JAK2/PLC/Ca<sup>2+</sup>-mediated autocrine secretion of a putative primary mitogen. Subsequently, activation of the p95 kDa IGF-I RTK/PI3K/ERK<sub>2</sub>/mTOR pathway induces DNA synthesis and cell division (Kimura and Ogihara, 1998) in primary cultured hepatocytes.

To test this model, demonstrating that treatment of primary cultured hepatocytes with GH actually leads to increased secretion of a putative primary mitogen is important. If so, determining how autocrine secretion of this mitogen via GH receptor stimulation is regulated is also important. Moreover, investigating how the putative mitogen leads to stimulation of hepatocyte DNA synthesis and cell proliferation is also critical. Here our data show that in primary cultured hepatocytes, GH actually induced autocrine secretion of IGF-I through the JAK2/PLC/Ca<sup>2+</sup> pathway, which then activated IGF-I RTK/PI3K/ERK2/mTOR signaling to induce hepatocyte DNA synthesis and proliferation.

## **2. Materials and Methods**

**2.1. Materials** The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): GH (human recombinant), aphidicolin, dexamethasone, somatostatin, verapamil hydrochloride, and aprotinin. Monoclonal antibodies against IGF-I, monoclonal antibodies against transforming growth factor (TGF)- $\alpha$ , rat IgG, BAPTA/AM [1,2-Bis(2-aminophenoxy)ethane-*N, N, N', N'*-tetraacetic acid tetrakis(acetoxymethyl ester)], and AG538  $\alpha$ -Cyano-(3, 4-dihydroxy)cinnamoyl-(3', 4'-dihydroxyphenyl) ketone were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). TG101209 (N-tert-butyl-3-(5-methyl-2-(4-(4-methylpiperazin-1-yl)phenylamino)pyrimidin-4-ylamino)benzenesulfonamide) was obtained from Med Chem Express (Monmouth Junction, NJ, USA). U-73122 (1-[6-[17 $\beta$ -3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl]-1H-pyrrol-2, 5-dione), U-73343 (1-[6-[17 $\beta$ -3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl]-2, 5-pyrrolidine-dione), GF109203X (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-

yl)maleimide), 2,4-dideoxyadenosine, H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), LY294002 (N-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine), and rapamycin were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). PD98059 (2'-amino-3'-methoxyflavone) was obtained from Calbiochem-Behring (La Jolla, CA, USA). Anti- IGF-I receptor, phospho- IGF-I receptor Tyr<sup>1316</sup>, ERK1/2, and phospho-ERK2 Thr<sup>202</sup>/Tyr<sup>204</sup> monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ, USA). [Methyl-<sup>3</sup>H] thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade.

**2.2. Animals** Male Wistar rats weighing 200-220 g were obtained from Tokyo Experimental Animal Co. (Tokyo, Japan). Adaptation to a light-, humidity-, and temperature-controlled room occurred over a minimum 3-day period prior to beginning the experiments. Rats were fed a standard diet and given tap water *ad libitum*. All animals used in this study were given humane care in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Institutional Animal Care and Use Committee of Josai University (No. JU19032 and JU20035).

**2.3. Isolation and culture of hepatocytes** Rats were given an intraperitoneal injection of 45 mg/kg sodium pentobarbital to induce anesthesia. Hepatocytes were obtained using the two-step *in situ* collagenase perfusion technique to disaggregate the adult rat liver (Seglen, 1976). In brief, the liver was first perfused via the portal vein with calcium-free

Hank's salts-HEPES buffer (pH 7.4) at 37 °C and a flow rate of 30 ml/min for 10 min. The second step was performed with the same buffer containing 0.025% collagenase (Type II) and 0.075% CaCl<sub>2</sub> at a flow rate of 30 ml/min for 10-11 min. Trypan blue exclusion was used to assess viability, which was >97%. Hepatocytes (3.3 × 10<sup>4</sup> cells/cm<sup>2</sup>) were cultured as described (Kimura and Ogihara, 1997).

**2.4. Assessment of DNA synthesis and cell division** DNA synthesis was determined by measuring [<sup>3</sup>H]-thymidine incorporation into acid-precipitated cellular components (Morley and Kingdon, 1972). Protein concentrations were measured using a modified Lowry procedure in which bovine serum albumin (BSA) was the standard (Lee and Paxman, 1972). Because cultured hepatocytes cannot be dispersed quantitatively with EDTA/trypsin treatment, we determined DNA synthetic activity with the cellular protein-based method (Nakamura et al., 1983) instead of the cell number-based method. Nuclei rather than cells were counted to assess proliferation (Kimura and Ogihara, 1997).

**2.5. Levels of p95 kDa RTK and ERK1/2** We performed western blotting to identify phosphorylated and total p95 RTK. Cells were lysed, and the protein concentration in the supernatant and cytosol was determined with a modified Lowry procedure with BSA as a standard (Lee and Paxman, 1972). Proteins (30 µg/lane) in the supernatant were separated with SDS-PAGE on a 10% polyacrylamide gel. After transfer to a membrane, p95 RTK was detected with monoclonal anti-phospho-RTK antibody according to the manufacturer's instructions, as previously described. This antibody detected a 95-kDa protein, which is the IGF-I receptor β subunit (Garofalo and Rosen, 1989). Blots were

incubated with horseradish peroxidase-conjugated secondary antibodies, bands were visualized with enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) and exposure to Hyperfilm (Kodak, Tokyo), and bands were quantified using densitometry (NIH image, ver. 1.6 for Macintosh). The levels of phosphorylated p95-kDa protein (p-p95 kDa) tyrosine kinase (Tyr1316) were normalized to levels of total p95-kDa protein (Hernández-Sánchez et al., 1995).

We performed western blotting with an anti-phospho-ERK1/2 monoclonal antibody as described above to detect phosphorylated ERK isoforms (pERK1; p-p44 MAPK and pERK2; p-p42 MAPK). pERK levels were normalized to total ERK levels as described (Kimura et al, 2007). Data are shown as arbitrary units as the mean  $\pm$  standard error of the mean (S.E.M.). Autoradiograms represent 3-4 independent experiments using different cell preparations.

## **2.6. Measurement of the concentration of IGF-I secreted into the culture medium**

IGF-I secretion into the culture medium after treatment with GH was determined with an enzyme-linked immunosorbent assay (ELISA) kit for IGF-I. In brief, hepatocytes were cultured in serum-containing Williams' medium E at a cell density of  $3.3 \times 10^4$  cells/cm<sup>2</sup>. After attachment of hepatocytes for 3 h, cells were rinsed three times with 1.0 ml phosphate-buffered saline (PBS) and then preincubated for 5 min in 1.0 ml PBS supplemented with 1.0 mM CaCl<sub>2</sub>, 5.5 mM glucose, and 0.10 U/ml aprotinin (pH 7.4). GH (100 ng/ml) was then added in the presence or absence of test molecules (Grosheva et al., 2016, Naito et al., 2016). Conditioned medium (50  $\mu$ l) was sampled after different incubation times, and IGF-I levels were measured with an ELISA kit (Biosensis, Thebarton, South Australia, Australia). Absorbance at 490 nm was

determined with a micro-plate reader (Tecan Japan, Kanagawa, Japan). A standard curve was created in the linear range (31.25-2000 pmol/ml). The minimum detectable limit for this ELISA was approximately 5.0 pmol/ml.

## **2.7. Immunolocalization of IGF-I**

Isolated hepatocytes were allowed to attach to collagen-coated glass slides and cultured as described in section 2.3. After attachment for 3 h, hepatocytes were washed two times with serum-free Williams' medium E and cultured with or without GH (100 ng/ml) for 0-30 min. Attached hepatocytes were washed two times with PBS (pH 7.4), and then incubated with 3.7% (v/v) neutralized paraformaldehyde at room temperature for 15 min. Fixed hepatocytes were permeabilized with PBS containing 0.1% (v/v) Triton X-100 for 10 min at room temperature. Then, hepatocytes were treated with blocking buffer (PBS containing 1% (w/v) BSA). Hepatocytes were washed two times with PBS, and treated with primary unlabeled antibody buffer containing 0.4 µg/ml anti-mouse IGF-I monoclonal antibody and 0.1% BSA in (w/v) in PBS for 3 h. Then, a fluorescence-tagged secondary antibody (PBS containing 0.2% BSA and 1 mg/ml AlexaFluor<sup>®</sup>488) was used to detect the first antibody. In addition, hepatic nuclei were labeled with 1 ng/ml propidium iodide for 30 min in the dark. Labeling of IGF-I was observed using a fluorescence microscope (Leica, DMI8).

**2.8. Data analysis and statistics** Data are expressed as means ± S.E.M. Group comparisons were made by analysis of variance for unpaired data followed by post-hoc analysis using Dunnett's multiple comparison test. Differences of  $P < 0.05$  were considered to be statistically significant.



### **3. Results**

#### **3.1. Effects of specific inhibitors of the GH receptor/JAK2/PLC pathway, adenylate cyclase/protein kinase A (PKA) pathway, and anti-secretion agents on GH-induced hepatocyte DNA synthesis and proliferation**

At the time that the culture medium of adult rat hepatocytes was changed to serum-free medium or PBS (3 h after plating), GH (100 ng/ml) with or without specific inhibitors of the GH receptor/JAK2/PLC pathway, adenylate cyclase/PKA pathway, or anti-secretion agents such as BAPTA/AM or somatostatin were added to examine the effects of these inhibitors on GH-induced hepatocyte DNA synthesis and proliferation (Fig. 1). GH-induced hepatocyte DNA synthesis and proliferation were almost completely blocked by the addition of TG101209 (a selective JAK inhibitor,  $10^{-6}$  M) (Pardanani et al., 2007) or U-73122 (a selective PLC inhibitor,  $10^{-6}$  M) (Thompson et al., 1991). Moreover, when primary cultured hepatocytes were treated with verapamil (L-type  $\text{Ca}^{2+}$  channel blocker,  $10^{-6}$  M), somatostatin ( $10^{-6}$  M), which inhibits granule secretion (Toro et al., 1988), or BAPTA/AM (a membrane-permeable  $\text{Ca}^{2+}$  chelator,  $10^{-7}$  M) (Ren et al., 2003, Szabo et al., 2008) for 5 h to determine the degree of involvement of  $\text{Ca}^{2+}$  mobilization in GH-induced hepatocyte DNA synthesis and proliferation, significant inhibition was observed following treatment with somatostatin ( $10^{-6}$  M), verapamil ( $10^{-6}$  M), and BAPTA/AM ( $10^{-7}$  M), suggesting involvement of  $\text{Ca}^{2+}$  mobilization and autocrine secretion of primary growth factors. When combined with GH, GF109203X, a specific PKC inhibitor (Toullec et al., 1991), 2, 4-dideoxyadenosine, a specific adenylate cyclase inhibitor (Holgate et al., 1980), and H-89, a PKA inhibitor (Zusick et al., 1994), did not affect GH-induced hepatocyte DNA synthesis and proliferation. In

addition, a monoclonal antibody against IGF-I receptor (100 ng/ml) completely inhibited hepatocyte DNA synthesis and proliferation, but IgG (100 ng/ml) did not. These inhibitors on their own did not affect GH-induced hepatocyte DNA synthesis and proliferation (Fig. 1).

### **3.2. Time course of GH-induced phosphorylation of p95 kDa IGF-I receptor tyrosine kinase: effects of U-73122**

Next, the time course and patterns of GH stimulation of p95 kDa IGF-I RTK phosphorylation, as detected with western blotting, were examined in primary cultured hepatocytes. Phosphorylated IGF-I RTK (p-p95 kDa IGF-I RTK) was significantly induced after treatment for 20 min. The levels peaked (2.0-fold increase compared to the control) at 30 min after addition of GH (100 ng/ml) and then decreased to baseline levels over 60 min (Fig. 2). U-73122 ( $10^{-6}$  M), a selective PLC inhibitor, completely blocked GH-induced p95 kDa IGF-I RTK phosphorylation.

### **3.3. Effect of inhibition of the GH receptor/JAK2/PLC or adenylate cyclase/PKA pathways on GH-induced phosphorylation of ERK1/2**

Primary cultured hepatocytes were treated with U-73122 ( $10^{-6}$  M), verapamil ( $10^{-6}$  M), somatostatin ( $10^{-7}$  M), or BAPTA/AM ( $10^{-7}$  M) for 45 min to determine the contribution of  $Ca^{2+}$  mobilization in the GH-induced phosphorylation of ERK1/2. Treatment with U-73122 ( $10^{-6}$  M), somatostatin ( $10^{-7}$  M) or verapamil ( $10^{-6}$  M) (Figure 3, upper) significantly blocked GH-induced ERK2 phosphorylation, but not ERK1 phosphorylation, suggesting a role for  $Ca^{2+}$  mobilization. On the other hand, GH (100 ng/ml)-induced phosphorylation of ERK2 was not blocked by the PKC inhibitor,

GF109203X, the adenylate cyclase inhibitor, dideoxyadenosine, or the PKA inhibitor, H-89. Thus, neither PKC nor PKA appears to be involved in the GH receptor/JAK2/PLC signaling pathway. When used in the absence of GH, these compounds did not affect ERK1/2 phosphorylation during 60 min of culture (data not shown).

#### **3.4. Effects of various concentrations of monoclonal antibodies against TGF- $\alpha$ or IGF-I on GH-induced DNA synthesis and proliferation in hepatocytes**

To investigate the relationship between the GH receptor/JAK2/PLC pathway and the IGF-I RTK/PI3K/ERK2/mTOR pathway, we tested the idea that GH receptor activation induces secretion of IGF-I and/or TGF- $\alpha$  via the GH receptor/JAK2/PLC/Ca<sup>2+</sup> pathway, followed by activation of the IGF-I RTK/PI3K/ERK2/mTOR pathway and subsequent hepatocyte division. We tested the effects of a monoclonal antibody against IGF-I (12.5 to 75 ng/ml) or TGF- $\alpha$  (12.5 to 75 ng/ml) on hepatocyte proliferation induced by 100 ng/ml GH. The anti-IGF-I monoclonal antibody (62.5 ng/ml) almost completely inhibited DNA synthesis and cell division induced by GH in hepatocytes (Figs. 4A and 4B). The IC<sub>50</sub> values were approximately 29 ng/ml for DNA synthesis and 25 ng/ml for division after 5 h. No effects were seen with the anti-TGF- $\alpha$  monoclonal antibody (1-100 ng/ml). Thus, secretion of endogenous IGF-I, but not TGF- $\alpha$ , plays a role in GH-induced DNA synthesis and cell division. In the absence of GH, the monoclonal antibodies did not significantly change DNA synthesis or cell division after treatment for 5 h (data not shown).

#### **3.5. Time- and dose-dependent effects of GH-induced secretion of IGF-I into the**

### **culture medium**

To further test our hypothesis, we next determined whether GH induces secretion of IGF-I from primary hepatocytes. We added GH (100 ng/ml) for 1-40 min and then performed ELISA for IGF-I to assess the time course of IGF-I secretion into PBS (Fig. 5A). Significant increases in IGF-I were detected 5 min after addition of GH. The IGF-I concentration (100 pg/ml) peaked 20 min after addition of GH, and levels remained significantly increased for up to 40 min. IGF-I secretion induced by GH was almost completely blocked by the addition of a monoclonal antibody to GH receptor (80 ng/ml) (Fig. 5A). Moreover, GH induced IGF-I in a dose-dependent manner during 20 min of culture. Significant stimulation of IGF-I secretion by GH was seen at 30 ng/ml GH, and reached a plateau at 100 ng/ml GH.

### **3.6. Effects of specific inhibitors of the GH receptor/JAK2/PLC/Ca<sup>2+</sup> pathway and the IGF-I signaling pathway on GH-induced IGF-I secretion into the culture medium**

We additionally assessed the signaling pathway using the JAK inhibitor, TG101209, the PLC inhibitor, U-73122, the membrane-permeable Ca<sup>2+</sup> chelator, BAPTA/AM, somatostatin, the PKC inhibitor, GF109203X, and the L-type Ca<sup>2+</sup> channel blocker, verapamil.

The anti-GH receptor monoclonal antibody (80 ng/ml), TG101209 (10<sup>-6</sup> M), and U-73122 (10<sup>-6</sup> M) inhibited the 100 ng/ml GH-induced increase in IGF-I (Fig. 6). U-73343 (10<sup>-6</sup> M), which is an inactive analog of U-73122, did not change GH-induced IGF-I concentrations (data not shown). Thus, the GH receptor/JAK2/PLC pathway is critical for GH-induced stimulation of IGF-I secretion by cultured hepatocytes.

Somatostatin inhibits secretion of some gastrointestinal and pancreatic hormones by blocking  $\text{Ca}^{2+}$  mobilization (Toro et al., 1988). Somatostatin ( $10^{-7}$  M, 20 min) blocked GH-induced IGF-I secretion by hepatocytes. Verapamil ( $10^{-6}$  M) and BAPTA/AM ( $10^{-7}$  M) showed similar effects, suggesting that intracellular  $\text{Ca}^{2+}$  is important for IGF-I secretion. In contrast, GF109203X ( $10^{-6}$  M), dideoxyadenosine ( $10^{-6}$  M), and H-89 ( $10^{-6}$  M) did not affect GH-induced IGF-I levels, suggesting that PKC and adenylate cyclase/PKA are not involved in GH-induced IGF-I secretion (Fig. 6).

We next assessed blockers of growth-related signal transducers, including AG538 (IGF-I receptor kinase inhibitor;  $10^{-6}$  M) (Blum et al., 2000), LY294002 (PI3K inhibitor;  $3 \times 10^{-7}$  M) (Vlahos et al., 1994), PD98059 (ERK inhibitor;  $10^{-6}$  M) (Alessi et al., 1995), and rapamycin (mTOR inhibitor; 10 ng/ml) (Chung et al., 1992). In the presence of GH (100 ng/ml), growth-inhibitory doses of these compounds did not significantly change the increase in IGF-I secretion stimulated by GH (Fig. 6), indicating that RTK, PI3K, ERK, and mTOR are involved in hepatocyte mitogenesis but not IGF-I secretion. Moreover, a monoclonal antibody against IGF-I receptor (100 ng/ml) as well as IgG (100 ng/ml) did not affect GH-stimulated IGF-I secretion. When used alone, the signal transducer inhibitors did not affect basal concentrations of IGF-I in the medium (Fig. 6).

To confirm the above results, we examined immunolocalization of IGF-I using an immunofluorescence technique. As shown in Fig. 7 (panel A), IGF-I (green signals) was detected in the cultured hepatocytes (at time 0). When hepatocytes were cultured with GH (100 ng/ml) for 5 min, IGF-I was significantly lost from cultured hepatocytes, and nuclei (brown signals) were visible (panel B). IGF-I was almost completely depleted after 20 min of culture (panel C). In contrast, when combining GH (100 ng/ml) with U-73122

( $10^{-6}$  M), IGF-I secretion into the culture medium was almost completely inhibited (panel D).

#### 4. Discussion

We have shown previously that GH-induced DNA synthesis and proliferation are mediated by GH receptors in primary cultures of adult rat hepatocytes. Moreover, GH receptor-mediated hepatocyte mitogenesis involves activation of both the GH receptor/JAK2/PLC (possibly PLC-r)/ $\text{Ca}^{2+}$  pathway and the p95 kDa IGF-I RTK/PI3K/ERK2/mTOR pathway. GH-mediated JAK2 phosphorylation appears to act upstream of p95 kDa IGF-I RTK phosphorylation (unpublished data). However, the relationship between these signaling pathways in hepatocyte mitogenesis was unknown.

Thus, we tested the hypothesis that stimulation of autocrine secretion of a putative mitogen occurs via the GH receptor/JAK2/PLC/ $\text{Ca}^{2+}$  pathway in primary cultured hepatocytes, and that DNA synthesis and cell proliferation mediated by the putative mitogen occur by stimulation of the downstream RTK (p95 kDa)/PI3K/ERK2/mTOR signaling pathway (Kimura and Ogihara, 1998).

In our current study, we again observed that GH-mediated hepatocyte DNA synthesis and cell division were nearly completely inhibited by the JAK2 inhibitor, TG101209 ( $10^{-6}$  M), and the PLC inhibitor, U-73122 ( $10^{-6}$  M). In addition, a monoclonal antibody against IGF-I receptor completely inhibited GH-stimulated hepatocyte DNA synthesis and proliferation (Figs. 1A and 1B). Treatment with JAK2/PLC inhibitors produced no significant changes in the hepatocyte nucleus number or cellular protein levels. In addition, U-73343 ( $10^{-6}$  M), an inactive analog of U-73122, did not affect GH-induced DNA synthesis and proliferation (Fig. 1) or GH-induced IGF-I secretion (data not shown).

Moreover, after 4 h of treatment with TG101209 ( $10^{-6}$  M) or U-73122 ( $10^{-6}$  M), DNA ladders characteristic of apoptotic cells were not observed upon analysis of DNA on agarose gels (data not shown). In primary cultured hepatocytes, GH-induced DNA synthesis and cell division were significantly inhibited by somatostatin ( $10^{-7}$  M; inhibits granule secretion), BAPTA/AM ( $10^{-7}$  M; a membrane-permeable  $\text{Ca}^{2+}$  chelator), and verapamil (L-type  $\text{Ca}^{2+}$  channel blocker;  $10^{-6}$  M) after 5 h of treatment (Fig. 1), suggesting the importance of  $\text{Ca}^{2+}$  mobilization in autocrine secretion of primary growth factor(s). Possible primary mitogens were IGF-I or TGF- $\alpha$ , because hepatocytes express both IGF-I and TGF- $\alpha$  mRNA, and these cytokines are produced and secreted by hepatocytes (Fausto et al., 2006; Michalopoulos and DeFrances, 1997).

Western blotting showed that a phosphorylated IGF-I RTK band (p-p95 kDa IGF-I RTK) was significantly upregulated after treatment with GH (100 ng/ml) for 20 min, with a peak (about a 2.0-fold increase compared with the control) at 30 min (Fig. 2). U-73122 ( $10^{-6}$  M) completely blocked GH-induced 95 kDa IGF-I RTK phosphorylation, suggesting involvement of  $\text{Ca}^{2+}$  mobilization. Furthermore, significant inhibition of GH-induced ERK2 phosphorylation was observed following treatment with U-73122 ( $10^{-6}$  M), somatostatin ( $10^{-7}$  M), or verapamil ( $10^{-6}$  M) (Fig. 3), suggesting involvement of  $\text{Ca}^{2+}$  mobilization in ERK2 phosphorylation.

GH-induced hepatocyte DNA synthesis and cell division were completely inhibited with an anti-IGF-I monoclonal antibody, suggesting autocrine secretion (Fig. 4). IGF-I is stored in parenchymal hepatocytes, and this cytokine is secreted into the culture medium after GH treatment. We previously showed a similar autocrine mechanism for DNA synthesis and cell division that is mediated by a prostacyclin IP receptor agonist or prostaglandin EP<sub>1</sub> following autocrine production of TGF- $\alpha$ , which subsequently

induces hepatocyte mitogenesis via the epidermal growth factor/TGF- $\alpha$  receptor/PI3K/ERK2/mTOR pathway (Kimura et al., 2001; Kimura et al., 2009). In our current study, however, the blocking monoclonal antibody against TGF- $\alpha$  did not affect DNA synthesis or cell division (Fig. 4), indicating that TGF- $\alpha$  is not involved in GH receptor/JAK2/PLC/Ca<sup>2+</sup> signaling. Here we found that IGF-I did play a principal role in GH-stimulated hepatocyte DNA synthesis and proliferation (Fig. 4). Accordingly, our previous study showed that IGF-I directly induced hepatocyte proliferation via RTK/PI3K/ERK/mTOR signaling (i.e., IGF-I receptor pathway) (Kimura and Ogihara, 1998). Moreover, Desbois-Mouthon et al. showed that hepatocyte proliferation during liver regeneration *in vivo* is impaired in mice with liver-specific IGF-I receptor knockout (Desbois-Mouthon et al., 2006).

GH is the primary regulator of IGF-I synthesis and is secreted by hepatocytes. GH also binds to the cell surface GH receptor, causing activation of the JAK2 tyrosine kinase and triggering downstream pathways including STAT phosphorylation (Li et al., 2002). However, this mechanism is not involved in hepatocyte DNA synthesis and proliferation, because SH-4-54 (10<sup>-6</sup> M), a specific inhibitor of STAT, did not significantly affect hepatocyte DNA synthesis and proliferation (unpublished data).

We next assessed how rapid IGF-I secretion is regulated after GH stimulation of hepatocytes. We showed significant GH-induced time- and dose-dependent autocrine secretion of IGF-I (Figs. 5A and 5B). Stimulation of IGF-I secretion by GH was completely blocked by a monoclonal antibody to GH receptor and the JAK inhibitor, TG101209 (Fig. 6). In addition, rapid secretion of IGF-I stimulated by GH was blocked by the PLC inhibitor, U-73122 (Thompson et al., 1991) (Fig. 6), but not by the inactive analog, U-73343 (data not shown). Thus, GH-induced hepatocyte DNA synthesis and



cell division mainly involve functional GH receptors on hepatocytes, stimulation of the JAK2/PLC/Ca<sup>2+</sup> pathway, and induction of IGF-I secretion (Figs. 1, and 4). On the other hand, PLC/PKC and adenylate cyclase/PKA signaling are unlikely to play a role in these processes, because GF109203X (a PKC inhibitor; 10<sup>-6</sup> M), dideoxyadenosine (an adenylate cyclase inhibitor; 10<sup>-6</sup> M), and H-89 (a direct PKA inhibitor; 10<sup>-6</sup> M) failed to block secretion of IGF-I in the presence of GH (Fig. 6).

Blockers of growth-related signal transducers including AG538 (an IGF-I RTK inhibitor), LY294002 (a PI3K inhibitor), PD98059 (an ERK inhibitor), and rapamycin (an mTOR inhibitor), did not significantly change the GH-induced IGF-I levels in the medium (Fig. 6). These drugs may inhibit growth-related signals downstream of the IGF-I receptor pathway without influencing IGF-I secretion induced by GH receptor/JAK2/PLC/Ca<sup>2+</sup> signaling (Fig. 6). Furthermore, the results of immunofluorescence analysis support the notion that GH-induced autocrine secretion of IGF-I is mediated by PLC activation (Fig. 7A-7D).

In light of these results, we conclude that GH stimulation induces GH receptor/JAK2/PLC activation, which increases membrane phosphatidylinositol turnover and intracellular Ca<sup>2+</sup> levels, resulting in autocrine IGF-I secretion. IGF-I then directly stimulates DNA synthesis and cell proliferation via p95 kDa IGF-I RTK/PI3K/ERK2/mTOR signaling in primary cultured hepatocytes. By exploiting this mechanism, GH-induced hepatocyte DNA synthesis and cell proliferation may induce liver regeneration following partial hepatectomy or liver damage *in vivo*. Fig. 8 shows a schematic of the proposed GH-stimulated intracellular signaling pathways for upregulating hepatocyte DNA synthesis and cell proliferation.

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## 5. Figure legends

Fig. 1. Effects of specific inhibitors of the GH receptor/JAK2/PLC pathway and adenylate cyclase/PKA pathway, and anti-secretion agents on GH-induced hepatocyte DNA synthesis and proliferation. Hepatocytes at a cell density of  $5.0 \times 10^4$  cells/cm<sup>2</sup> were plated and allowed to attach for 3 h (time 0) as described in Section 2. The medium was then removed, and serum-free Williams' medium E was added. Hepatocytes were cultured in the presence of GH (100 ng/ml) with or without various test molecules. DNA synthesis (A) and cell division (B) were measured 5 h after GH was added. The inhibitors and their concentrations were: TG101209 ( $10^{-6}$  M), U-73122 ( $10^{-6}$  M), U-73343 ( $10^{-6}$  M), GF109203X ( $10^{-6}$  M), verapamil ( $10^{-6}$  M), BAPTA/AM ( $10^{-7}$  M), dideoxyadenosine ( $10^{-6}$  M), H-89 ( $10^{-6}$  M), somatostatin ( $10^{-7}$  M), monoclonal antibody against IGF-I receptor (100 ng/ml), and IgG (100 ng/ml). Data are shown as the means  $\pm$  S.E.M. of three independent experiments. \*P < 0.05, \*\*P < 0.01 vs. control.

Fig. 2. Time course associated with GH-induced phosphorylation of p95 kDa IGF-I receptor tyrosine kinase (RTK): effects of U-73122.

Hepatocytes at a cell density of  $5.0 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1. After a medium change, liver extracts from cultured hepatocytes treated with GH and/or test agents for 45 min were prepared.

Western blot image, p95 kDa IGF-I RTK phosphorylation (A); Effect of U-73122 ( $10^{-6}$  M) on GH (100 ng/ml)-induced phosphorylation of p95 kDa IGF-I RTK (expressed as the ratio of p-p95 kDa IGF-I RTK/95 kDa IGF-I RTK) (B). Results are expressed as a percentage of the respective control value (mean  $\pm$  S.E.M. of three separate

experiments). \*P < 0.05, \*\*P < 0.01 compared with control. The arrow points to the time of GH addition (panel B).

Fig. 3. Effects of anti-secretion agents on GH-induced phosphorylation of ERK1/2.

Hepatocytes at a cell density of  $5.0 \times 10^4$  cells/cm<sup>2</sup> were plated and cultured for 3 h as described in the legend to Fig. 1 and cultured for an additional 45 min with or without anti-secretion agents. Liver extracts from cultured hepatocytes treated with saline or GH (100 ng/ml) with or without test agents were prepared as described in the Materials and Methods. Tissue extracts were immunoprecipitated with an IGF-I antibody and immunoblotted with an anti-PY antibody. Western blot image (A); effects of anti-secretion agents on ERK1/2 phosphorylation (B). The concentrations of the various inhibitors of signal transducers were: U-73122 ( $10^{-6}$  M), U-73343 ( $10^{-6}$  M), GF109203X ( $10^{-6}$  M), verapamil ( $10^{-6}$  M), BAPTA/AM ( $10^{-7}$  M), dideoxyadenosine ( $10^{-6}$  M), H-89 ( $10^{-6}$  M), and somatostatin ( $10^{-7}$  M). Results are expressed as a percentage of the respective control value (mean  $\pm$  S.E.M. of three separate experiments). \*\*P < 0.01 compared with control.

Fig. 4. Effects of various concentrations of a monoclonal antibody against TGF- $\alpha$  or IGF-I on DNA synthesis and proliferation. Hepatocytes at  $5.0 \times 10^4$  cells/cm<sup>2</sup> were grown for 3 h, and then the medium was removed, and serum-free Williams' medium E was added. Cells were then grown in the presence of various concentrations of monoclonal antibodies against TGF- $\alpha$  (12.5-75 ng/ml) or IGF-I (12.5-75 ng/ml) in the presence of GH (100 ng/ml) for 5 h. Hepatocyte DNA synthesis (A) and cell division (B) were measured. Data are the means  $\pm$  S.E.M. of three independent experiments. \*P



< 0.05, \*\*P < 0.01 compared with the respective control.

Fig. 5. Time course of the induced secretion of IGF-I into the culture medium by GH: effects of a monoclonal antibody (mAb) to GH receptor (A); dose-dependent increase in IGF-I secretion induced by GH (B). Hepatocytes were isolated and cultured and then incubated for various lengths of time (0-40 min) in the presence of GH (100 ng/ml) with or without a mAb to GH receptor (80 ng/ml) (A), and various doses of GH (1 to 1000 ng/ml) for 20 min (B). IGF-I concentrations in 150  $\mu$ l culture medium were assessed with ELISA at various times. The results are expressed as the means  $\pm$  S.E.M. of three separate experiments. \*P < 0.05, \*\*P < 0.01 compared with the respective control. The arrow points to the time of GH addition (panel A).

Fig. 6. Effects of blocking the GH receptor/JAK2/PLC/Ca<sup>2+</sup> pathway and IGF-I signaling pathway on IGF-I secretion induced by GH.

Hepatocytes were grown in the presence of GH (100 ng/ml) for 20 min with or without different inhibitors. Then, ELISA was used to measure the IGF-I concentrations in 100  $\mu$ l culture medium. The concentrations of the various inhibitors of signal transducers were: monoclonal antibody against GH receptor (80 ng/ml), TG101209 ( $10^{-6}$  M), U-73122 ( $10^{-6}$  M), GF109203X ( $10^{-6}$  M), BAPTA/AM ( $10^{-7}$  M), somatostatin ( $10^{-6}$  M), AG1478 ( $10^{-6}$  M), LY294002 ( $3 \times 10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml), monoclonal antibody against IGF-I receptor (100 ng/ml), and IgG (100 ng/ml). The results are expressed as the means  $\pm$  S.E.M. of three separate experiments. \*\*P < 0.01 compared with the respective control.

Fig. 7. Representative fluorescent images of control and GH-treated hepatocytes with or without U-73122. Hepatocytes were cultured and immunolabeled as described in the Materials and Methods section. A, control (at 0 min); B, treatment with 100 ng/ml GH (at 5 min); C, treatment with 100 ng/ml GH (at 20 min); D, treatment with 100 ng/ml GH and  $10^{-6}$  M U-73122 (at 20 min). Scale bar: 100  $\mu$ m.

Fig. 8. Schematic of the GH receptor-mediated intracellular signal transduction pathways that play a role in DNA synthesis and cell division in primary cultured hepatocytes. GH: growth hormone, GH receptor: growth hormone receptor, JAK2: Janus kinase 2, RTK: receptor tyrosine kinase, PLC: phospholipase C, PIP<sub>2</sub>: phosphatidylinositol 4, 5-bisphosphate, DG: diacylglycerol, IP<sub>3</sub>: inositol 1,4,5-trisphosphate, PI3K: phosphoinositide 3-kinase, ERK2: extracellular signal-regulated kinase 2, mTOR: mammalian target of rapamycin, IGF-I: insulin-like growth factor-I

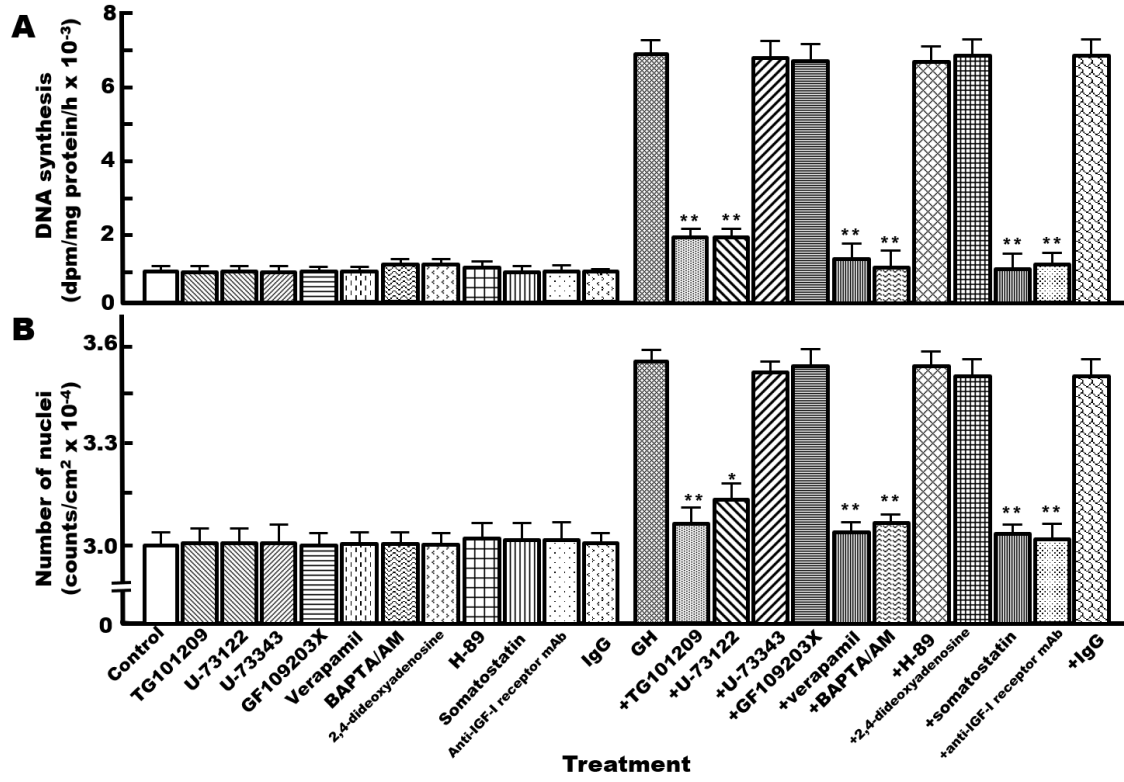


Fig.1 Kurihara, K. et al.

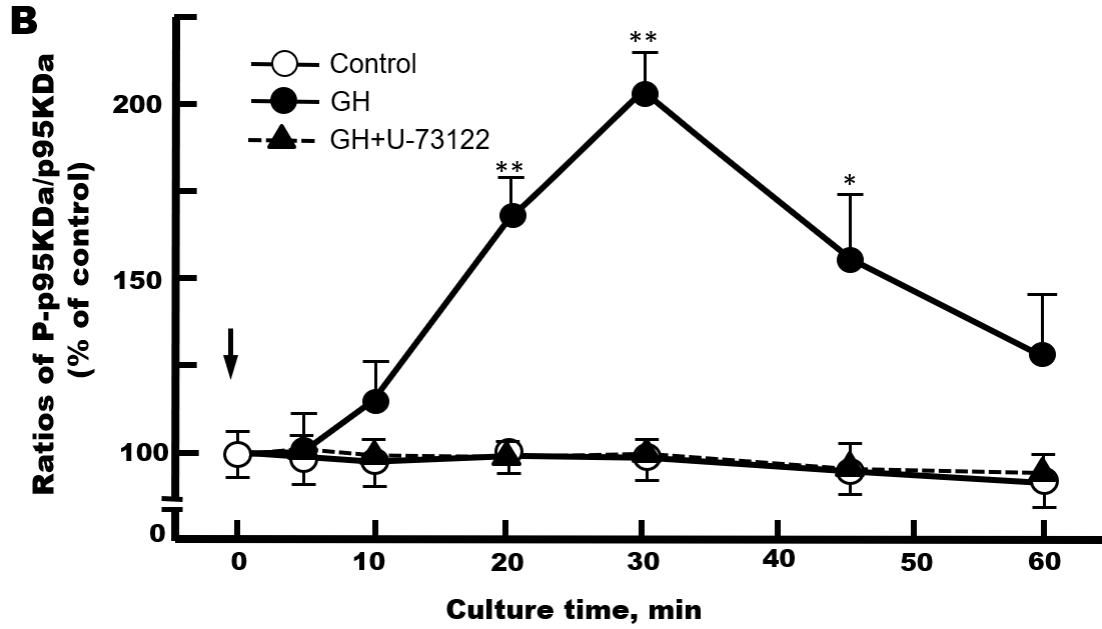
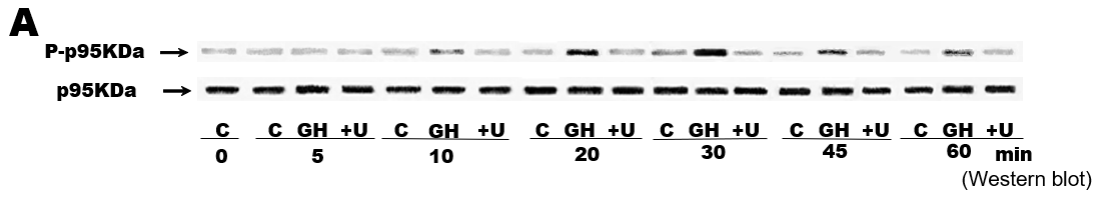


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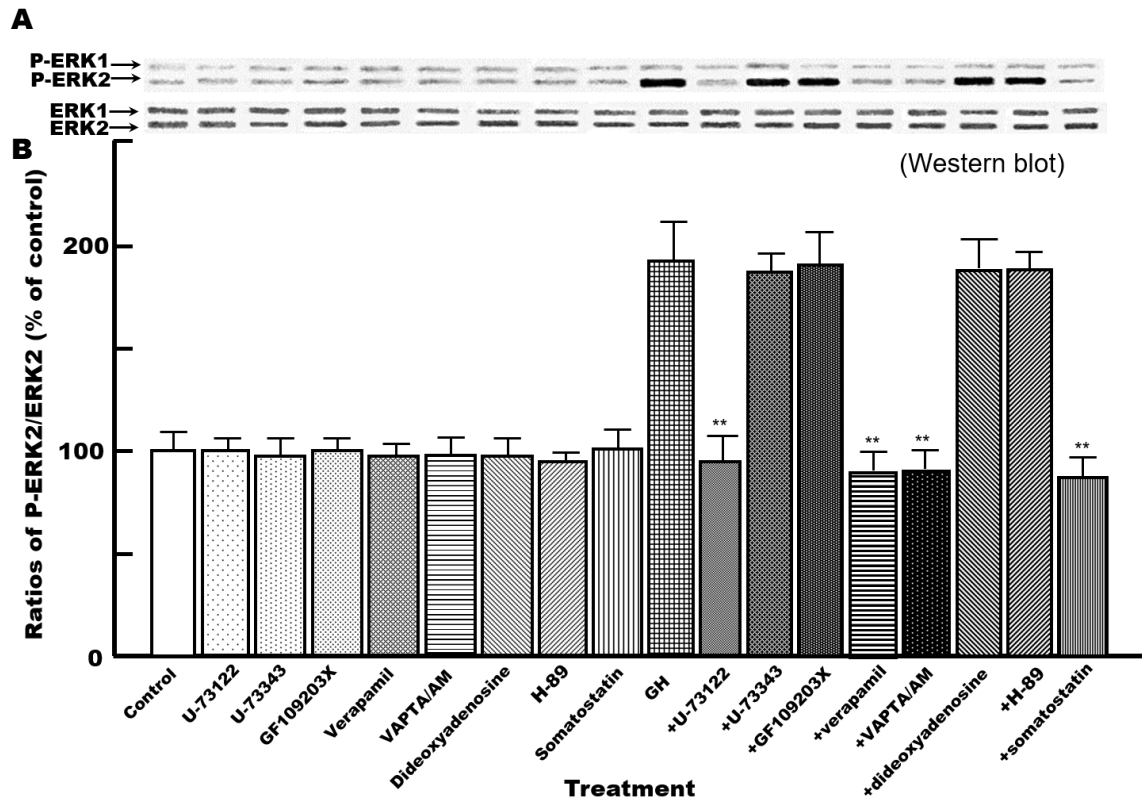


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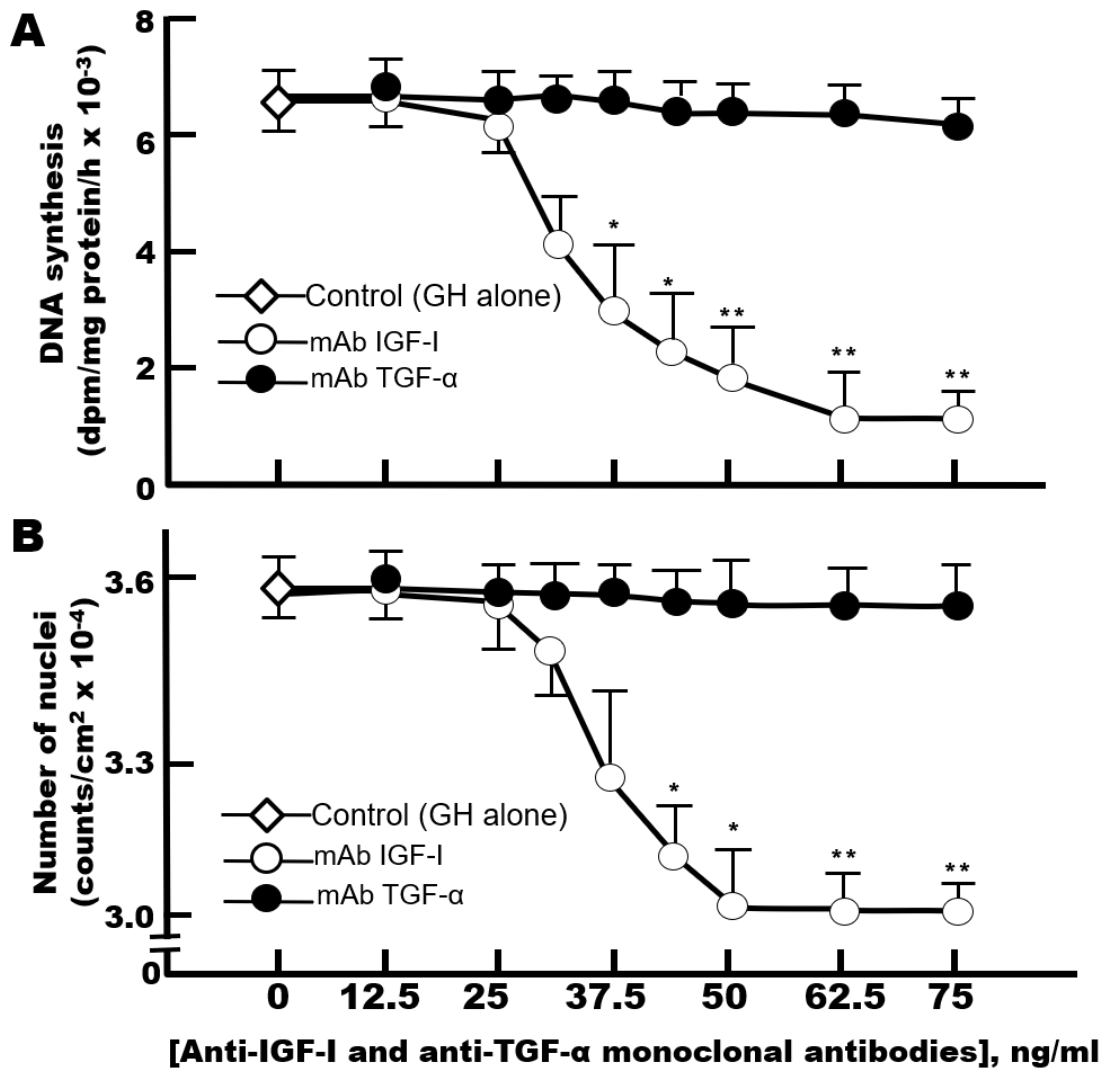


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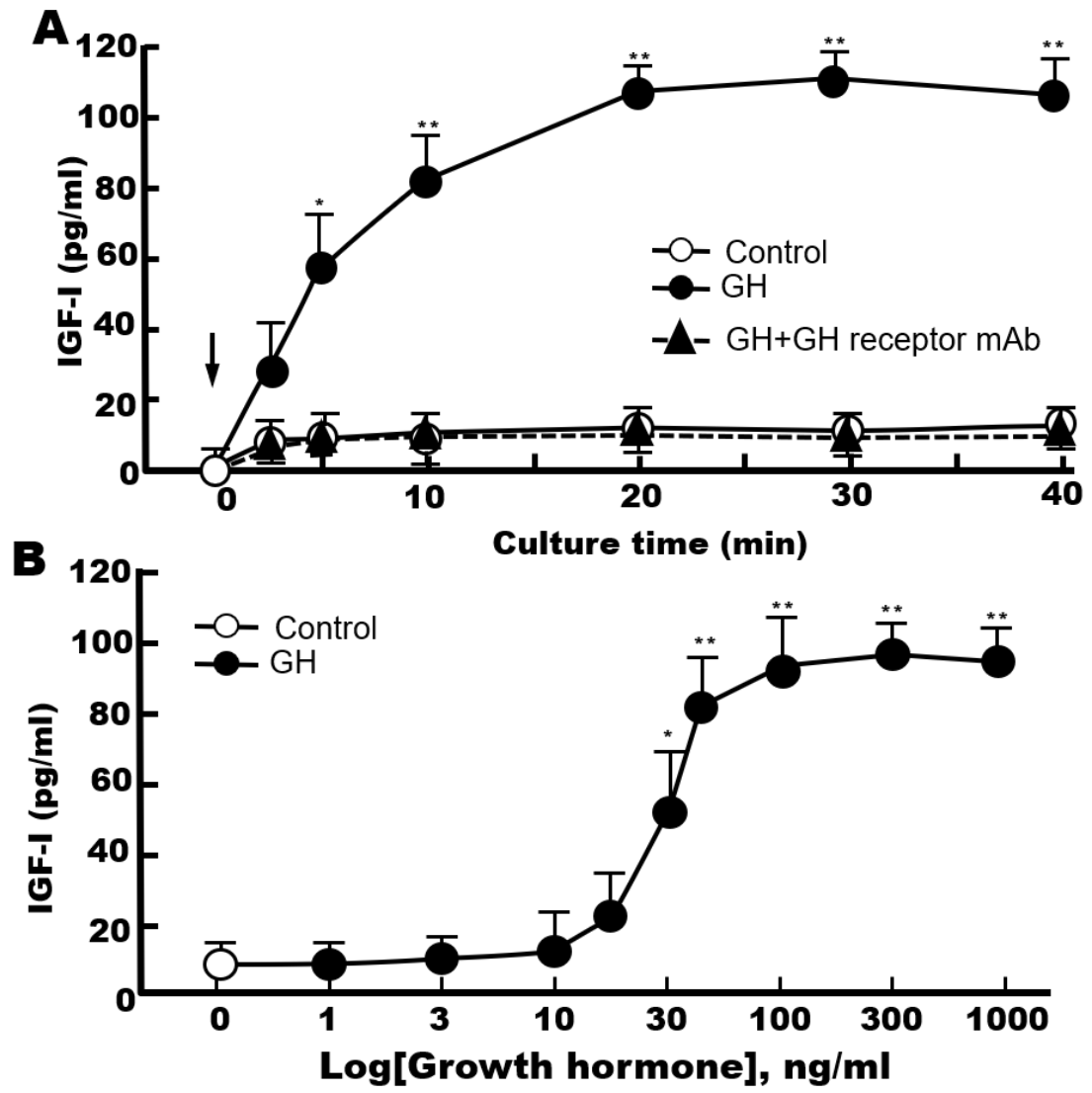


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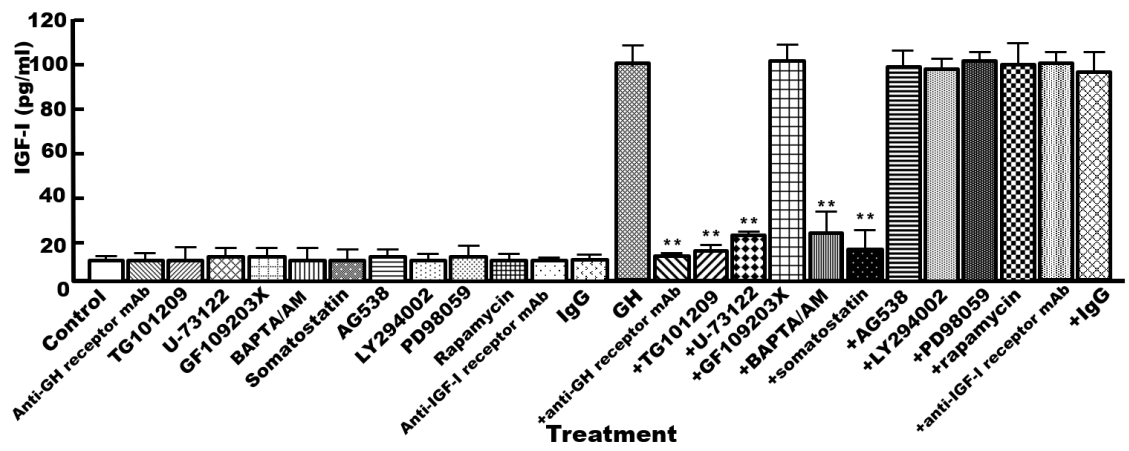


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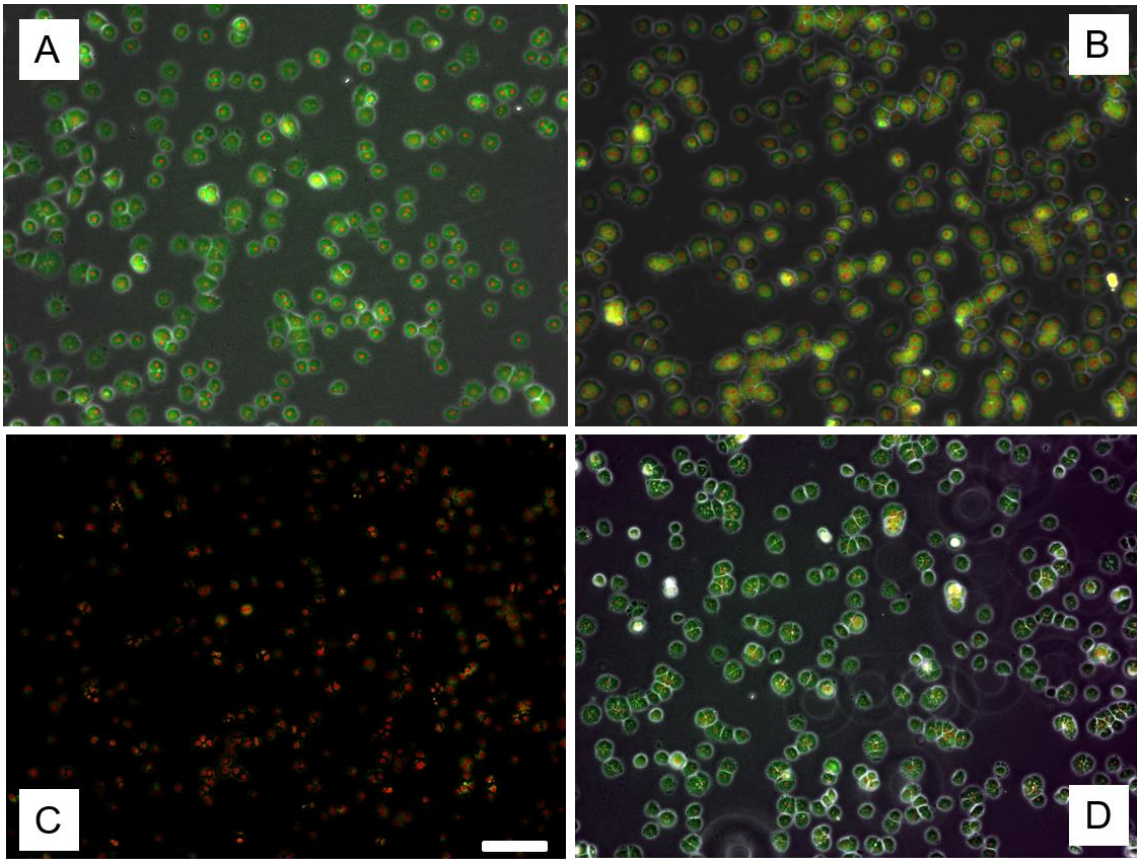


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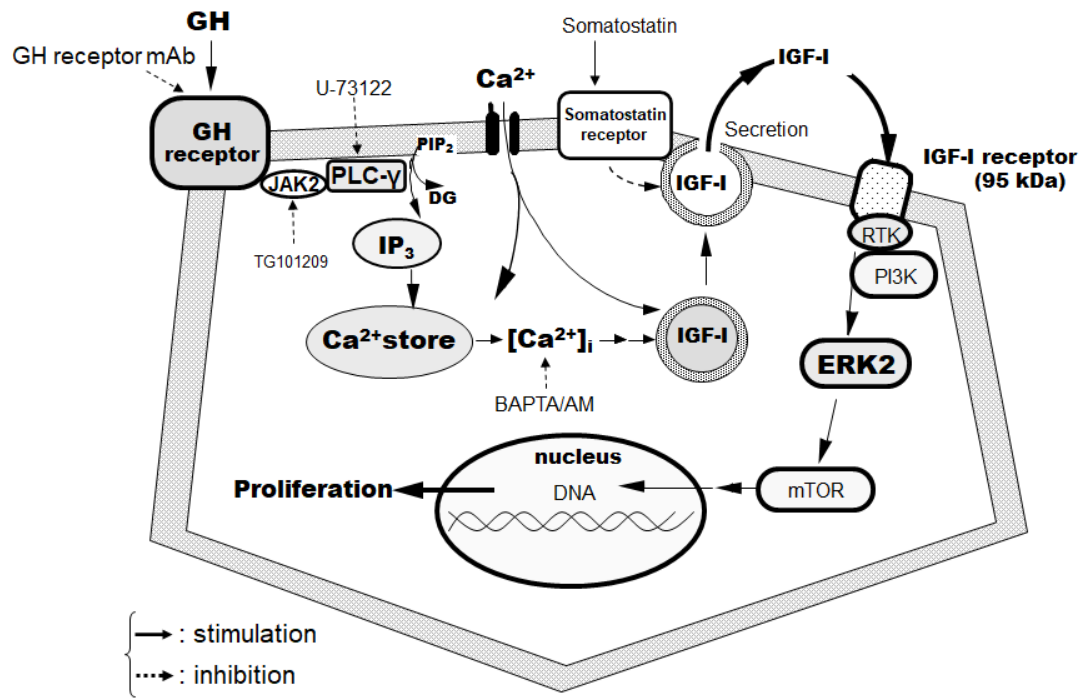


Fig.8 Kurihara, K. et al.