

Original Article

Insulin-like growth factor-1 promotes cell cycle progression *via* upregulation of cyclin D1 expression through the phosphatidylinositol 3-kinase/nuclear factor- κ B signaling pathway in FRTL thyroid cells

Meng REN^{1,#,§}, Xia ZHONG^{1,#}, Chun-yan MA¹, Ying SUN², Qing-bo GUAN¹, Bin CUI¹, Jun GUO¹, Hai WANG¹, Ling GAO^{1,*}, Jia-jun ZHAO^{1,*}

¹Shandong Provincial Hospital, Shandong University, Jinan 250021, China; ²Department of Endocrinology, Yantai Yuhuangding Hospital, Yantai 264000, China

Aim: Insulin-like growth factor-1 (IGF-1) is an important hypertrophic and cell cycle progression factor for a number of cell types. It has been proven that IGF-1 is involved in the regulation of thyroid proliferation and cell cycle progression; however, the exact mechanism of this regulation has not been fully elucidated. In the present study, we investigated the effect of IGF-1 on the expression of cyclin D1, an important cell cycle regulatory protein, and a signaling pathway involved in IGF-1's effect on cyclinD1 expression in FRTL thyroid cells.

Methods: FRTL thyroid cells were treated with IGF-1 or vector control for 24 h. As appropriate to individual experiments, a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, and/or a nuclear factor- κ B (NF- κ B) inhibitor, BAY11-7082, were added 1 h prior to IGF-1 treatment. Western blotting was used to detect cyclin D1 protein expression. Immunofluorescence was performed to analyze the expression of I κ B α , an NF- κ B inhibitory protein. Cell cycle analysis was performed by fluorescence activated cell sorting (FACS).

Results: IGF-1 increased the cyclin D1 expression in thyroid cells. This increase was blocked by pretreatment with LY294002 or BAY11-7082. Further studies showed that IGF-1 specifically induced NF- κ B activity. Treatment with IGF-1 could accelerate cell cycle progression from G₀/G₁ to S phase, whereas this progression was inhibited by the presence of LY294002 or BAY11-7082.

Conclusion: In summary, the results of the present study show that in FRTL cells, IGF-1 promotes cell cycle progression via an upregulation of cyclin D1 expression, at least partially through the PI3K/NF- κ B signaling pathway.

Keywords: insulin-like growth factor-1; phosphatidylinositol 3-kinase; nuclear factor- κ B; cyclin D1

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Introduction

During the G₁ phase of the eukaryotic cell division cycle, signals transduced from activated growth factor receptors regulate progression through the cell cycle^[1,2]. These signals can cause cells to either enter the G₁ phase of the cell cycle and divide or exit from the cell cycle. Cyclin D1

is a G₁ phase cell cycle protein. Accumulation of this kind of protein by stimulation of extracellular signals promotes cell proliferation. It has been reported that in thyroid papillary carcinomas the expression of cyclin D1 is elevated; this elevation is thought to be a contributory factor in thyroid tumorigenesis^[3]. However, information is limited regarding the relationship between cyclin D1 and growth factors as well as the relevant signaling pathway in normal thyroid cells.

Insulin-like growth factor-1 (IGF-1) has been widely proven to regulate cell proliferation and an array of other cell functions. Furthermore, IGF-1 is believed to be an important hypertrophic and cell cycle progression factor for a number of cell types^[4,5]. However, the specific signaling

These authors contributed equally to this work.

§ Now in Department of Endocrinology, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510120, China.

* Correspondence to Dr Jia-jun ZHAO and Dr Ling GAO.

E-mail jj_zhao61@yahoo.com.cn; gaoling@medmail.com.cn

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pathways involved in the effect of IGF-1 on cyclin D1 expression and cell cycle progression in thyroid cells have been less well characterized.

The transcription factor nuclear factor- κ B (NF- κ B) is a key regulator of gene transcription that is involved in the control of cellular proliferation and apoptosis^[6-9]. In most cell types, NF- κ B is maintained in an inactive form in the cytoplasm by an association with its inhibitory protein, I κ B α . A wide variety of extracellular signals may lead to the phosphorylation and degradation of I κ B α protein; degradation of I κ B α frees NF- κ B to enter the nucleus and activate transcription of target genes^[10]. In some cell types other than thyroid cells, the effects of NF- κ B on cyclin D1 expression and cell cycle progression have been reported; these results indicate that NF- κ B acts by increasing the abundance of cyclin D1^[11,12]. In addition, some growth factors, such as IGF-1, induce expression of NF- κ B activity in various cell types; this induction corresponds with a proliferative response^[13,14]. These findings prompted us to investigate whether IGF-1 influences cyclin D1 expression in thyroid cells by affecting the activity of NF- κ B.

The aim of the current study was to examine the signaling pathway related to IGF-1-mediated cyclin D1 expression in thyroid cells and its relationship to cell cycle control. Our results demonstrate that IGF-1 promotes cell cycle progression and upregulates cyclin D1 expression by stimulating the PI3K/NF- κ B pathway in thyroid cells.

Materials and methods

Cell culture and treatments FRTL thyroid cells (ATCC catalog no. CRTL-1486) were grown in Coon's modified F12 media supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and a mixture of six hormones: bovine TSH (1 mU/mL), insulin (10 μ g/mL), hydrocortisol (0.4 ng/mL), transferrin (5 μ g/mL), somatostatin (10 ng/mL), and glycyl-L-histidyl-L-lysine acetate (10 ng/mL) (hereafter termed "6H"; Sigma, St Louis, MO, USA). Fresh 6H medium was added every two or three days, and cells were passaged every 7–10 days. When grown to 90% confluence in 6H medium, the cells were then washed and starved in medium containing 0.2% fetal bovine serum in the absence of both TSH and insulin (4H medium) and were cultured for an additional two days before being used. In individual experiments, starved cells were washed twice with PBS, then IGF-1 was added as noted for 24 h. In some experiments, the PI3K inhibitor LY294002 (10 μ mol/L) or the NF- κ B inhibitor BAY11-7082 (50 μ mol/L; Sigma, St Louis, MO, USA), was added for 1 h prior to IGF-1 treat-

ment. Cells that were maintained in 4H without any treatment were used as a control.

Western blotting After being washed with phosphate-buffered saline (PBS), FRTL cells were collected in a lysis buffer containing 1 \times PBS, 1% nonylphenylpolyethylenglycol P-40 (NP-40), 0.1% SDS, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 1 mmol/L sodium orthovanadate and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were then centrifuged at 12 000 r/min for 10 min at 4 °C. The resulting supernatants (whole-cell lysates) were collected and either frozen at -80°C or used immediately. Cytoplasmic proteins were collected with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, USA). Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, USA). Samples (60 μ g) were heated for 30 min at 60°C, then analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk for 1 h and then incubated with the specific primary antibody overnight at 4°C, washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. After that, immune complexes were detected using the enhanced chemiluminescence method, and the same membrane was re-blocked with anti- β -actin monoclonal antibody. The immunoreactive bands were quantified using the AlphaImager 2200. Values were corrected by the absorbance of the internal control (β -actin).

Immunofluorescence The location and expression of I κ B (NF- κ B inhibitory protein) were examined using immunofluorescence. FRTL-5 cells were seeded on polyornithine-coated glass coverslips and treated as described above. The antibody used was rabbit anti-I κ B (Santa Cruz, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). The resultant immunofluorescence was viewed under a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). All images were acquired using the same intensity and photodetector gain to allow quantitative comparisons of the relative levels of immunoreactivity between sections.

Cell cycle analysis by fluorescence activated cell sorting (FACS) Cell cycle analysis was performed using FACS. Briefly, subconfluent thyroid cells were rendered quiescent by incubation in serum-free media for 48 h and then stimulated with or without inhibitors for 1 h, followed by treatment with IGF-1 (50 ng/mL) for 24 h. Cells were harvested by trypsinization, washed with PBS and stained with propidium iodide (PI) using the Coulter DNA PREPTM reagents kit (Beckman Coulter, USA). Samples were analyzed by FACS

on a Coulter Elite ESP (Beckman Coulter, USA) using standard filter sets. Results were expressed graphically and were quantified as the fraction of cells in G₀/G₁, S, or G₂/M phase using Coulter cytologic software.

Data analysis All experiments were repeated at least three times independently. Values are given as means±SD. Data were analyzed using SPSS 10.0 software. Statistical significance was assessed by ANOVA and unpaired Student's *t* test. A *P*<0.05 was considered to be statistically significant.

Results

The effect of IGF-1 on cyclin D1 expression and the involvement of the PI3K pathway To determine the effect of IGF-1 on cyclin D1 expression, FRTL cells were incubated with IGF-1, and the cyclin D1 protein expression was measured by Western blotting. These results demonstrated that the level of cyclin D1 protein was increased significantly following IGF-1 treatment (*P*<0.05), indicating that in thyroid cells IGF-1 could up-regulate the cyclin D1 expression. Next, the pathway mediating the IGF-1-induced increase in the cyclin D1 level was examined by using a selective inhibitor of the PI3K pathway, LY294002. These results showed that LY294002 decreased the IGF-1-induced increase in the cyclin D1 protein expression by 40% (Figure 1).

The inhibitory effect of IGF-1 on IκBα proteins It is known that the activation of NF-κB is regulated, in part, through a cellular process involving degradation of its inhibitory protein IκBα, which allows NF-κB to translocate to the nucleus and trigger transcription. To study the effects of IGF-1 on NF-κB, immunofluorescence was performed on thyroid cells with a specific antibody to IκBα. As shown in Figure 2, intense staining for IκB in the cytoplasm was shown in the untreated cells, while treatment of FRTL cells with 50 ng/mL IGF-1 resulted in a decrease in IκBα expression as compared with the untreated cells (*P*<0.05). Meanwhile, LY294002 pretreatment diminished the degradation of IκBα that was induced by IGF-1 (*P*<0.05). Furthermore, our previous study showed that IGF-1 pretreatment increased NF-κB DNA binding activity, which could be blocked by LY294002 (data not shown). Together, these results suggest that IGF-1 promotes the activation of NF-κB via PI3K.

Effect of NF-κB inhibitor BAY11-7082 on cyclin D1 expression stimulated by IGF-1 An essential role for NF-κB in the regulation of cyclin D1 expression has been reported in several cell models. The NF-κB inhibitor BAY11-7082 can prevent IκBα phosphorylation and proteasome-mediated IκBα degradation, resulting in the inactivation of NF-κB. Therefore, in the current study, BAY11-7082 was

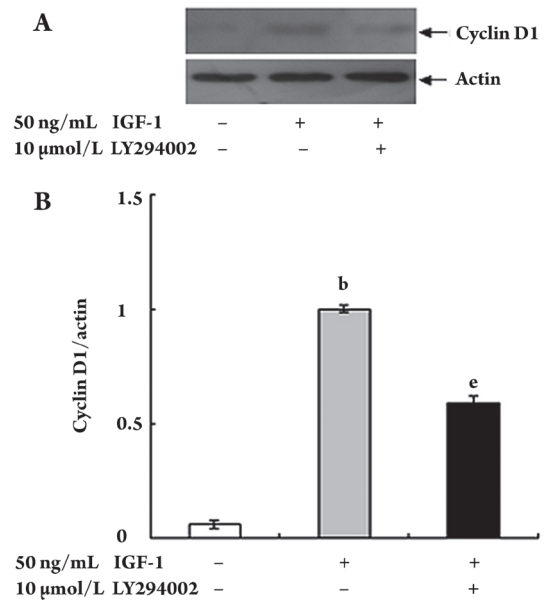


Figure 1. Expression of cyclin D1 in FRTL cells. In the presence or absence of LY294002 (10 μmol/L), starved cells were pretreated with IGF-1 for 24 h. Equal amounts of total protein were loaded and a Western blot was performed to measure cyclin D1 protein expression. (A) A representative Western blot result is shown. (B) Expression of cyclin D1 was quantified by densitometric analysis and expressed as mean±SD for three separate experiments. ^b*P*<0.05 vs the control group, ^e*P*<0.05 vs the IGF-1-treated group.

used to determine the role of NF-κB in the IGF-1-stimulated cyclin D1 expression pathway in thyroid cells. The effect of IGF-1 on cyclin D1 protein levels was significantly blunted by BAY11-7082 (*P*<0.05). Furthermore, in FRTL cells cultured in the presence of IGF-1 with LY294002 and BAY11-7082 together, a more significant reduction of cyclin D1 protein level was observed as compared to cells cultured in the presence of IGF-1 with LY294002 or BAY11-7082 alone (Figure 3). Thus, activation of the NF-κB pathway appears to be required for IGF-1 mediated upregulation of cyclin D1 expression.

The involvement of PI3K and the NF-κB pathway on cell cycle progression stimulated by IGF-1 The ability of IGF-1 to cause entry into the cell cycle was confirmed by FACS analysis of cell distribution in each phase of the cell cycle. When FRTL cells were rendered quiescent by a 48-h incubation in starvation media, 79.5%±4.3% of cells were in G₀/G₁, 17.7%±3.0% were in G₂/M, and almost no cells were in S phase. When the quiescent FRTL cells were incubated for 24 h with 50 ng/mL IGF-1, the proportion of cells in G₀/G₁ phase was decreased to 73.9%±2.3%, the proportion in S phase was increased to 6.2%±1.2% (*P*<0.05), and the

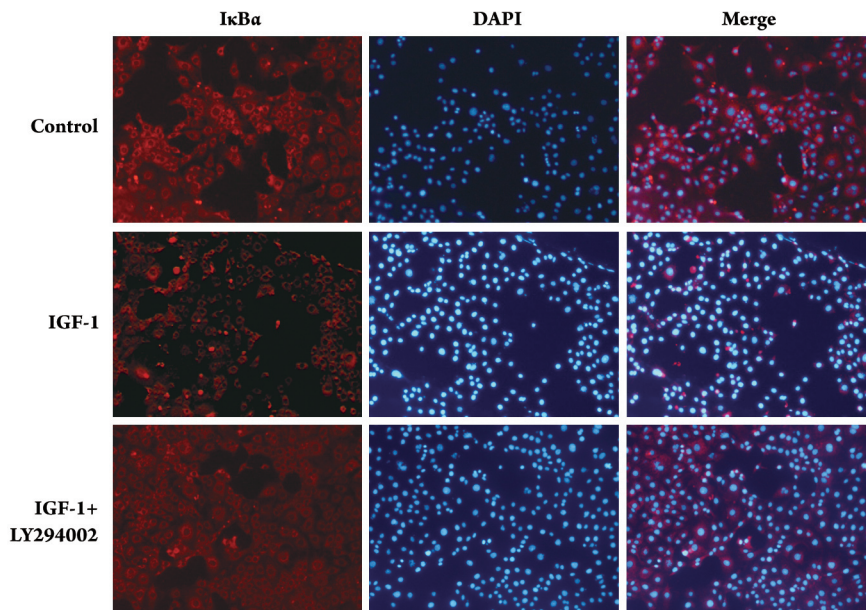


Figure 2. The immunofluorescence of I κ B α in FRTL-5 cells. Following 48 h of starvation and pretreatment for 1 h with the PI3K inhibitor LY294002, subconfluent FRTL cells were treated with 50 ng/mL IGF-1 in the absence or presence of inhibitor for 24 h. The staining of I κ B α was detected by immunofluorescence with anti-I κ B α antibody (TRITC-conjugated). The blue in the images depicts the DAPI-stained nuclei. The merged images were obtained after superposition of the red (I κ B α) and blue (DAPI) channels. The images are representative of three separate experiments. Magnification: $\times 100$.

proportion in G₂/M phase was increased to 19.9% \pm 1.9%. However, in the presence of inhibitors of LY294002 or BAY11-7082, IGF-1-mediated cell cycle progression was blocked. Compared with the proportion in IGF-1-only treated cells, the proportion of cells in S phase was decreased to 3.4% \pm 1.1% and 2.9% \pm 1.7%, respectively (both $P < 0.05$). Furthermore, the proportion of cells in G₀/G₁ was increased to 79.4% \pm 3.2% and 78.3% \pm 3.0%, respectively. Furthermore, in FRTL cells cultured in the presence of IGF-1 together with LY294002 and BAY11-7082, a reduction of cells in S phase (2.5% \pm 1.3%) was observed, as compared to cells cultured in the presence of IGF-1 with LY294002 or BAY11-7082 alone. These results show that the PI3K and NF- κ B pathways are involved in IGF-1-induced cell cycle progression from G₀/G₁ to S phase (Figure 4).

Discussion

The main finding of the present study is that stimulation of IGF-1 promotes cell cycle progression and upregulates cyclin D1 expression in cultured FRTL cells. Further research shows, for the first time, that pleiotropic transcription factor NF- κ B is implicated in the signaling pathway of IGF-1-mediated upregulation of cyclin D1 protein in normal thyroid cells.

IGF-1 plays important roles in promoting survival and proliferation of a variety of cell types. IGF-1 stimulates quiescent cells to pass through G₁, S, and G₂/M phases to complete the cell cycle by regulating cyclins. Cyclin D1,

which belongs to the D group of cyclins, is expressed in all mammalian cells except lymphocytes and myeloid cells^[20]. In some cell types, cyclin D1 is rate-limiting for cell cycle progression and its abundance varies with the cell cycle^[15,16]. Here we found that in quiescent FRTL cells cyclin D1 was barely detectable, whereas pretreatment with IGF-1 caused a distinct increase in cyclin D1 expression. Furthermore, our current study showed that, under quiescent conditions, most thyroid cells remained in G₀, and pretreatment with IGF-1 for 24 h increased the number of cells in S phase to a small extent, with a decrease in the number of cells in G₀/G₁. This indicates that IGF-1 promotes cell entry into S phase in thyroid cells. Our results were not consistent with those of a study of glomerular mesangial cells, which showed that IGF-1 increased cyclin D1 levels to promote G₀/G₁ progression but did not induce G₁ to S phase transition^[17]. This discrepancy may be a result of the different cell types used in the two studies.

The biological actions of IGF-1 are mediated by activation of the IGF-1 receptor (IGF-1R). Signals that transferred from the interactions between IGF-1 and the IGF-1R are required for cell cycle progression^[18-20]. IGF-1 stimulates two major signal transduction pathways: the PI3K pathway and the MAPK pathway. Our experiments demonstrated that blocking the PI3K pathway with the PI3K inhibitor LY294002 prevented the progression from G₁ to S phase in response to IGF-1. Moreover, LY294002 also diminished the expression of cyclin D1 stimulated by IGF-1. This was supported by a previous study suggesting that the effect of

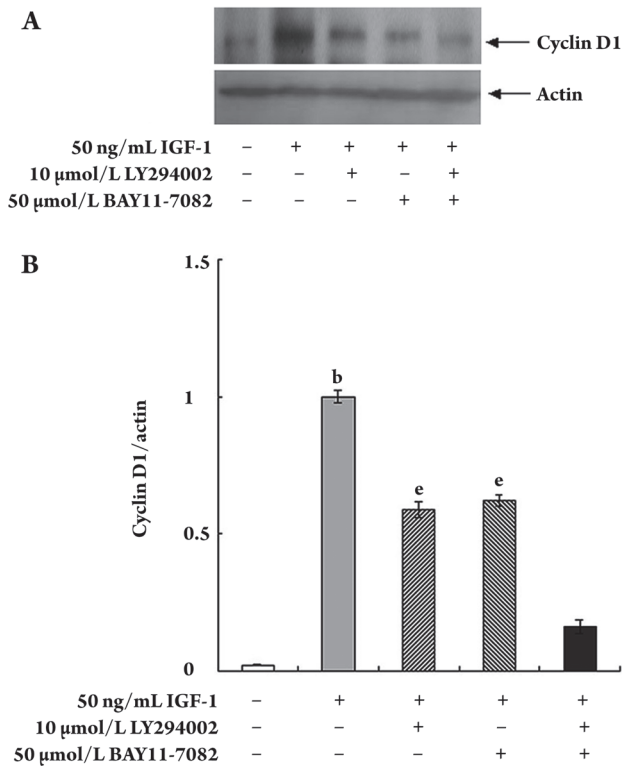


Figure 3. Effect of LY294002 and BAY11-7082 on protein levels of cyclin D1. Starved cells were treated with IGF-1 (50 ng/mL) for 24 h. LY294002 (10 μmol/L) and/or BAY11-7082 (50 μmol/L) were added 1 h prior to IGF-1 treatment. Whole-cell lysates were probed for cyclin D1 and β-actin by Western blotting. Equal amounts of protein were loaded to allow for direct comparisons of cyclin D1 levels in different groups. β-actin was used as a loading control. (A) A representative Western blot is shown. (B) A histogram plot represents the densitometric analysis corresponding to the means±SD of three independent experiments. ^b*P*<0.05 vs the control group, ^e*P*<0.05 vs the IGF-1 group.

IGF-1 on cyclin D1 expression required PI3K activation^[21]. Taken together, our results extend the previous observations in showing that PI3K is involved in the regulation by IGF-1 in cell cycle progression as well as in cyclin D1 expression. Furthermore, IGF-1 also activates the MAPK pathway through activation of Ras and a cascade of successive protein phosphorylation reactions, which also play important roles in proliferative thyroid diseases. However, whether the Ras-MAPK signaling pathway is involved in IGF-1-induced cell cycle progression needs further investigation^[22].

The transcriptional factor NF-κB has been implicated in the regulation of cellular proliferation. Enhanced NF-κB activity, which is apparent during the G₀/G₁ transition in fibroblasts, is induced by mitogenic stimuli in G₀ arrested 3T3 fibroblasts^[23,24]. It has recently been reported that acti-

vation of NF-κB is involved in the IGF-1-mediated neuronal survival against oxidative stress and insulin anti-apoptotic activity^[13,14]. Based on these reports, and because IGF-1 is an essential factor for the proliferation of FRTL cells, we speculate that NF-κB may be involved in the effect of IGF-1 on FRTL cells. To explore whether NF-κB is a downstream target of IGF-1 when inducing cyclin D1 expression, NF-κB cytoplasmic inhibitory protein, IκBα, was measured after stimulation with IGF-1. As compared with unstimulated cells, an obvious decrease in IκBα expression was found in the cells treated with IGF-1. Together with our previous findings that IGF-1 increased NF-κB DNA binding activity in FRTL cells, we speculate that the induction of NF-κB-driven gene expression by IGF-1 is one mechanism for promoting proliferation and cell cycle progression in thyroid cells.

Some previous experiments indicate that NF-κB acts by increasing the abundance of cyclin D1 protein, and subsequently affects G₁-to-S phase transition. Methods for inhibiting NF-κB activity are commonly used to study the role of NF-κB factors in controlling the cell cycle and cyclin D1 expression. In the present study, the influence of the NF-κB signaling pathway on cyclin D1 expression was observed by inhibiting NF-κB activation with a selective inhibitor, BAY11-7082. Our results showed that BAY11-7082 significantly reduced IGF-1-stimulated cyclin D1 expression, consistent with those of a study in human parathyroid tumors that showed that blockade of the NF-κB pathway caused an obvious decrease in cyclin D1 expression^[25]. All together, our results suggest that in normal thyroid cells, the activation of NF-κB mediates the cyclin D1 expression induced by IGF-1.

Since the current study has demonstrated that PI3K and NF-κB are both implicated in the signaling pathway of cyclin D1 expression mediated by IGF-1, we further explored whether both processes were mediated by a common pathway. To date, the role of PI3K in regulating NF-κB transactivation remains controversial. Some studies have shown that the inhibition of PI3K increased DNA-binding activity of NF-κB in monocyte-derived dendritic cells^[26], whereas others indicated that PI3K activation could promote the activation of NF-κB^[27]. In our present experiment, upon investigating the effect of LY294002 on IκBα expression induced by IGF-1, it turned out that the PI3K inhibitor blocked the IGF-1-induced decrease in NF-κB inhibitory protein expression, indicating that NF-κB is a downstream target involved in the IGF-1/PI3K pathway in this normal thyroid line.

In conclusion, this study shows that IGF-1 stimulates FRTL thyroid cells to progress through the G₁ phase of the

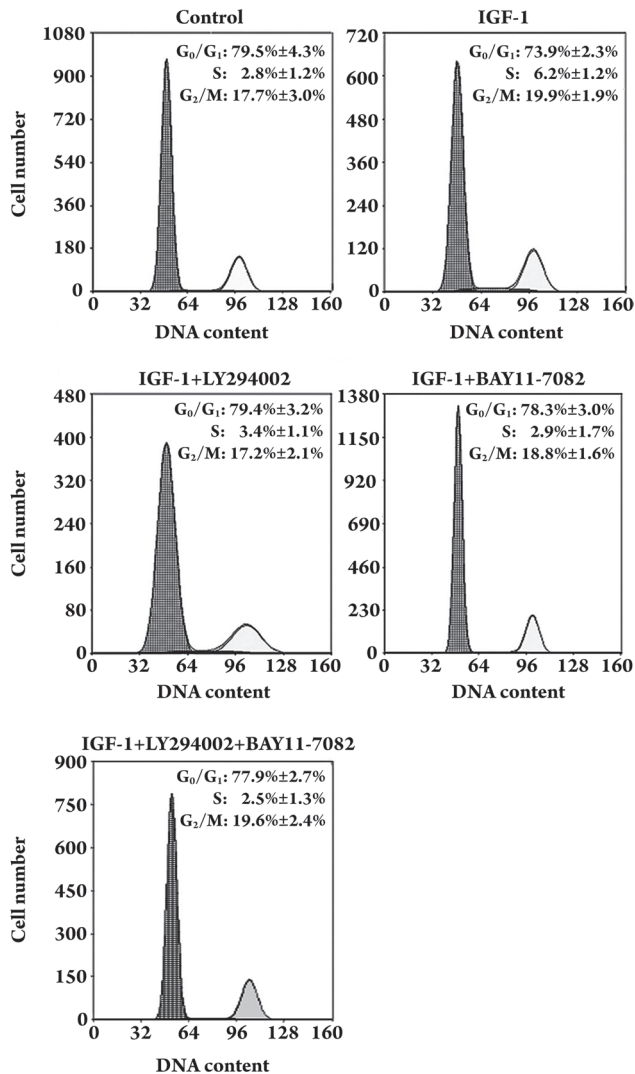


Figure 4. Fluorescence-activated cell sorting (FACS) analysis of cell cycle distribution. Following 48 h of starvation and pretreatment for 1 h with 10 $\mu\text{mol/L}$ LY294002 or 50 $\mu\text{mol/L}$ BAY11-7082, subconfluent FRTL cells were treated with 50 ng/mL IGF-1 in the absence or presence of inhibitor for 24 h. The cells were harvested by trypsinization and subjected to FACS analysis as described in Materials and Methods. Results for quiescent and treated cells were expressed as the percentage of total cells in G₀/G₁, S, or G₂/M phase of the cell cycle. Values represent the means \pm SD of three separate experiments.

cell cycle and exerts its effects on the G₁ phase by upregulating cyclin D1 expression. Our findings also provide novel evidence that the IGF-1/PI3K/NF- κ B pathway is involved in inducing the expression of cyclin D1 in normal thyroid cells. This involvement may explain how IGF-1 plays an important role in the growth of thyroid cells. However, the precise underlying mechanisms and the potential involvement of other signaling pathways still need further investigation.

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Author contribution

Jia-jun ZHAO and Ling GAO designed research; Meng REN and Xia ZHONG performed research; Chun-yan MA and Ying SUN contributed new analytical tools and reagents; Qing-bo GUAN, Bin CUI, Jun GUO, and Hai WANG analyzed data; Meng REN, Xia ZHONG, and Ling GAO wrote the paper.

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