

## Human Recombinant H2 Relaxin Induces AKT and GSK3 $\beta$ Phosphorylation and HTR-8/SVneo Cell Proliferation

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### ABSTRACT

Relaxin is essential for trophoblast development during pregnancy. Evidence shows that relaxin increases trophoblast cell migration capacity. Here, we show the effect of relaxin on protein kinase B (AKT) activation and glycogen synthase kinase 3-beta (GSK3 $\beta$ ) inactivation as well as on the proliferation of HTR-8/SVneo cells, a model of human extravillous trophoblast (EVT).

HTR-8/SVneo cells were treated with different doses of human recombinant (rH2) relaxin in serum-deprived conditions and treated for increasing time with 1 ng/mL of rH2 relaxin. Western blot analysis was performed to detect pAKT, AKT, pGSK3 $\beta$ , GSK3 $\beta$ , and actin expression. Proliferation of HTR-8/SVneo cells was analyzed by MTS assay.

rH2 relaxin treatment increased the ratio of pAKT/AKT, pGSK3 $\beta$ /GSK3 $\beta$ , and proliferation in HTR-8/SVneo cells. Furthermore, AKT and GSK3 $\beta$  activation by rH2 relaxin was inhibited by a phosphoinositide 3-kinase (PI3K) inhibitor.

This study suggests that rH2 relaxin induces AKT and GSK3 $\beta$  phosphorylation as well as proliferation in HTR-8/SVneo cells.

### INTRODUCTION

Human relaxin is a 6kDa, two-chain peptide hormone, which is structurally related to the insulin family. In humans, relaxin family peptides have been identified as relaxin-1, -2, and -3 as well as INSL3, INSL4, and INSL5. Human relaxin-2 is predominantly expressed in humans (1). Relaxin-2 (H2 relaxin) is produced by the corpus luteum in women (2) and the prostate in male (3). Human relaxin is the ligand for four G-protein coupled receptor, relaxin family peptide 1 receptor (RXFP1 receptor) for H2 relaxin, RXFP2 receptor for H1 relaxin and INSL3, RXFP3 for INSL4, and RXFP4 for INSL5 (4). In human cells, H2 relaxin has a higher affinity for RXFP1 than for RXFP2 (5). RXFP1 and RXFP2 couple to the protein G alpha S (G $\alpha$ s) to increase cyclic adenosine monophosphate (cAMP), but only RXFP1 can interact with G $\alpha$ i3 to activate cAMP accumulation via G $\beta$  $\gamma$  phosphoinositide 3-kinase (PI3K)-protein kinase C (PKC- $\zeta$ ) pathway to stimulate adenylyl cyclase 5 (AC5) (1). RXFP1 receptor is distributed in the brain, kidney, heart, lung, and prominently in reproductive tissues (6). Relaxin is involved in various biophysical processes such as anti-fibrotic (7, 8), angiogenesis (9), macrophage infiltration (10), uterus and placenta growth (11), and induced preterm parturition (12).

Relaxin is referred to as a pregnancy-related hormone. However, the role of relaxin in women is still unclear. Based on experiments in rodents and pigs, relaxin plays a major role in promoting maturation, growth, and softening of the uterine cervix (13), growth of the vagina and uterus, and growth and development of the mammary glands and nipples (14, 15). Decidua and placental relaxin has local autocrine and/or paracrine roles that contribute to connective tissue remodeling at the maternal-fetal interface during late pregnancy and parturition. Relaxin blood level varies during the different stages of pregnancy. The highest level (1.18 ng/mL) is observed during the first trimester of pregnancy (6–12 weeks) (16), during which the placenta grows and invades the uterus.

The human placenta consists of trophoblast cells, which exhibit several functions such as fetal protection, nutrition, and gas exchange, as well as hormone production (17). The trophoblast consists of relatively undifferentiated villous trophoblast (vCTB), intermediated trophoblast, terminally differentiated syncytiotrophoblast (CTB), and extravillous trophoblast (EVT) cells that invade the maternal decidua. These differentiated trophoblast cells arise from a putative trophoblastic stem (TS) cell population. It has been proposed that vCTB contains a TS cell population in the villous basement membrane (18). EVT presents two

distinct phenotypes, a proliferative and an invasive phenotype (19). Trophoblast cells proliferate, invade, and differentiate to establish contact with the maternal circulation during the first trimester of pregnancy. Proliferation of trophoblastic cells is regulated by the epidermal growth factor (EGF), leukemia inhibitory factors, vascular endothelial growth factor (VEGF), granulocyte colony-stimulating growth factor (G-CSF), and transforming growth factor (TGF) (20). Thus, we speculate that relaxin increases EVT proliferative capacity.

Relaxin interacts with RXFP1 to stimulate the PI3K pathway on endothelial cell (21), retina and Sertoli cells in rats (22). In human monocytic cells (THP-1), relaxin stimulates  $G_{\alpha s}$  and PI3K/PKC $\zeta$ , leading to increased cAMP production. PI3K is required for increased VEGF (vascular endothelial growth factor) transcript levels in THP-1 and mouse mesangial Cells (MMC) (23). In lung cells, relaxin, via  $G_{\beta\gamma i3}$ , activates PKA-independent ERK (extracellular signal-regulated kinases) (24). The PI3K pathway regulates many biological processes such as metabolism, cell proliferation, and apoptosis (25). PI3K activation leads to phosphorylation of AKT and its downstream target, GSK3 $\alpha$  and GSK3 $\beta$  (26). Thus, phosphorylated AKT and GSK3 $\beta/\alpha$  can be considered as indicators of the PI3K/AKT pathway activation. This study was carried out to determine the effect of relaxin on the PI3K/AKT/GSK3 $\beta$  pathway and trophoblast proliferation using HTR-8/SVneo cells as a model of human trophoblast cells.

## MATERIAL AND METHODS

### 1. Cell culture and treatment

Human trophoblast-derived HTR-8/SVneo cells were donated by Dr. Benjamin K Tsang, University of Ottawa (Ottawa, Canada). Cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640, Life Technologies, New York, NY, USA) with 10% (v/v) fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), and antibiotics (penicillin 100U/mL, streptomycin 100  $\mu$ g/mL, and fungizone 250  $\mu$ g/mL). Ten thousand cells in 100 $\mu$ L of media were seeded in each well of 96-well plates for MTS assay,  $0.85 \times 10^5$  cells were seeded in each well of a 6-well plate for western blotting. Once at 50% confluence, cells were treated with or without recombinant human relaxin (provided by Dr. Uenomori, BAS Medical Inc., San Mateo, CA, USA) and/or PI3K inhibitor (Ly294002, Cell Signaling Technology, Danvers, MA, USA) in serum free medium for 24 h.

### 2. Protein extraction

Cells were harvested and washed twice with phosphate buffered saline (PBS). Protein lysates were generated by using the M-PER kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, harvested cells were lysed using M-PER with protease inhibitor cocktail and halt phosphatase inhibitor cocktail (Thermo Scientific). Cell extracts were then centrifuged for 15 min at 14000 rpm at 4°C. The supernatant was transferred to fresh tubes and protein levels were assayed promptly using Bradford dye and a microplate reader according to the manufacturer's protocol (Bio Rad Laboratories Inc., Hercules, CA, USA).

### 3. Gel electrophoresis

For gel electrophoresis, proteins (60  $\mu$ g) were solubilized in loading buffer (loading buffer: 2.5  $\mu$ L NuPage LDS sample Buffer (4 $\times$ , Invitrogen), 1  $\mu$ L NuPage Sample reducing Agent (10 $\times$ ) (NP0004, Invitrogen), 6.5  $\mu$ L deionized water), heated (70°C) for 10 min and electrophoresed on NuPage Novex 12% (v/v) BisTris Mini Gels (NP0322BOX) from Life Technologies, California. Proteins were transferred onto immobilon-P polyvinylidene difluoride (PVDF) membranes (0.45 $\mu$ m) using the wet transfer method. After protein transfer, membranes were blocked with 30 mL of 5% (w/v) skim milk in Tris-Buffered Saline with Tween 20 (TBS-T) for 1 h at room temperature. After blocking, blots were incubated at 4°C overnight with 1:1000 diluted rabbit polyclonal antibody against AKT (60 kDa, catalog number 9272), pAKT (S473, 60 kDa, catalog number 9271S), pGSK (S9, 45 kDa, catalog number 9336S), mouse monoclonal antibody against GSK3 $\beta$  (45 kDa, catalog number 9832S), or Actin (42 kDa, catalog number 4958) in immunoreaction enhancer solution for primary antibody (Toyobo Co, LTD, Osaka, Japan). All primary antibodies were purchased from Cell Signaling Technology. The membranes were then washed with TBS-T and incubated for 1 h at room temperature with 1:5000 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T with 5% milk for AKT, pAKT, and pGSK3 $\beta$ . For actin and GSK3 $\beta$ , the membrane was incubated with 1:1000 diluted HRP conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) in immunoreaction enhancer solution (Toyobo Co, LTD). The membranes were then washed with TBS-T 3 times. The bands were detected by using image reader Fujifilm LAS-3000mini (Tokyo, Japan) after adding 1 mL of enhanced chemiluminescent (ECL) Western blotting detection Reagent (GE Healthcare, Buckinghamshire, UK) and the intensity of the bands was quantified by using ImageJ software (NIH, Bethesda, MD, USA).

### 4. MTS assay

The proliferation of cultured HTR-8/SV neo cells was assessed by MTS assay. Cell Titer 96 Aqueous One Solution Cell Proliferation kit (Promega Corporation, Madison, WI, USA), which contains 3-(4,5-dimethyl thiazole-2-yl)-5-(3-carboxymethyloxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt (MTS) and phenazine

## RELAXIN UP-REGULATES PAKT, PGSK3 $\beta$ , PROLIFERATION ON HTR8

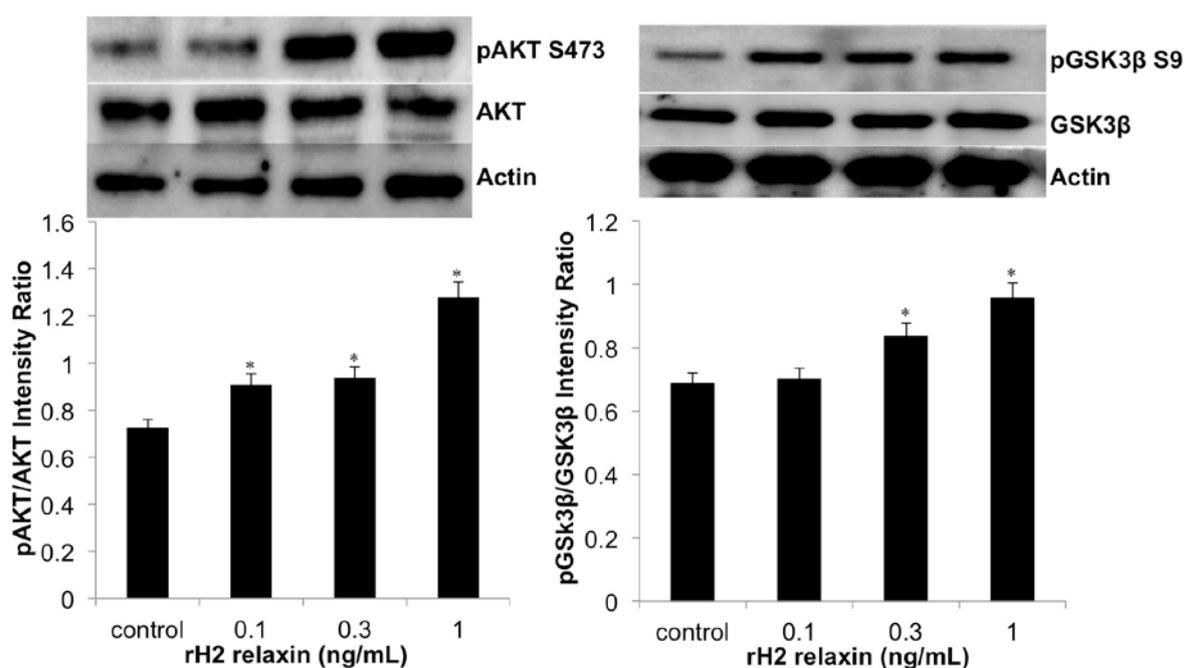
methosulfate, was used for this assay. The assay is based on cellular reduction of tetrazolium into formazan production. The amount of formazan production is directly proportional to the number of live cells. The assay was performed according to the manufacturer's instructions. After 24 h of incubation, 20  $\mu$ L of MTS reagent was added into 100  $\mu$ L of medium in each well of 96-well plates and the cells were incubated in 5% CO<sub>2</sub>, humidified atmosphere at 37°C for another 2 h. The absorbance of soluble formazan was measured at 492 nm using a microplate reader (MTP-120 Corona Electric, Osaka, Japan). The experiment was repeated five times in quadruplicates.

### 5. Statistical analysis

Results are presented as mean  $\pm$  S.D. Comparisons between control and treatment groups were performed by using the one way ANOVA. A p-value <0.05 was considered statistically significant.

## RESULTS

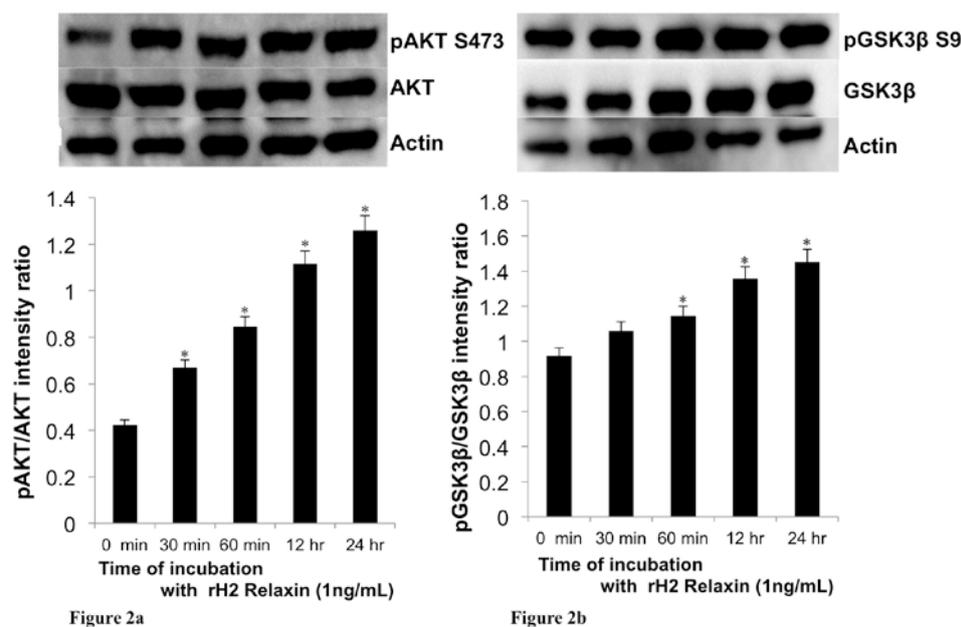
### Effect of increasing concentrations of rH2 relaxin on AKT and GSK3 $\beta$ phosphorylation in HTR-8/SVneo cells



**Figure 1:** Effect of increasing rH2 relaxin concentrations (0.1, 0.3, and 1 ng/mL) on AKT (1a) and GSK3 $\beta$  (1b) activation in HTR-8/SV neo cells treated for 24 h. Western blot followed by densitometric analysis of the bands was performed. Actin was used as a protein loading control. Values represent the mean  $\pm$  SD of at least three independent experiments. \*: p < 0.05 (compared to untreated control; 0 ng/mL)

HTR-8/SVneo cells were treated with increasing concentrations of relaxin (0.1, 0.3, and 1 ng/mL) for 24 h after serum starvation. Cells were lysed and western blot analysis was performed to detect AKT, pAKT S473, GSK3 $\beta$ , pGSK3 $\beta$  S9, and actin expression. The AKT antibody used can detect total AKT1, AKT2, and AKT3. An antibody against pAKT S473 was used to detect AKT phosphorylation on serine 473. The bands were detected by image analyzer and quantified by using imageJ software. Compared to untreated control cells, treatment with increasing concentrations of rH2 relaxin increased the ratio of pAKT/AKT and pGSK3 $\beta$ /GSK3 $\beta$  (Figure 1). The effect of 1 ng/mL of relaxin on both AKT and GSK3 $\beta$  phosphorylation was significant (p < 0.05) compared to the untreated control cells.

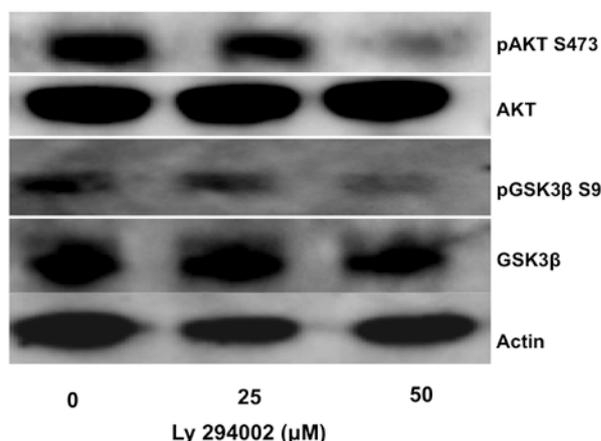
**Time-dependent effect of rH2 relaxin on AKT and GSK3 phosphorylation in cultured HTR-8/SVneo cells**



**Figure 2:** Time-dependent effect of rH2 relaxin (1 ng/mL) on AKT (a) and GSK3β (b) phosphorylation in HTR-8/SVneo cells was performed. Actin was used as an internal loading control. Values represent the mean ± SD of at least three independent experiments. \*: p < 0.05 (compared to time 0 min)

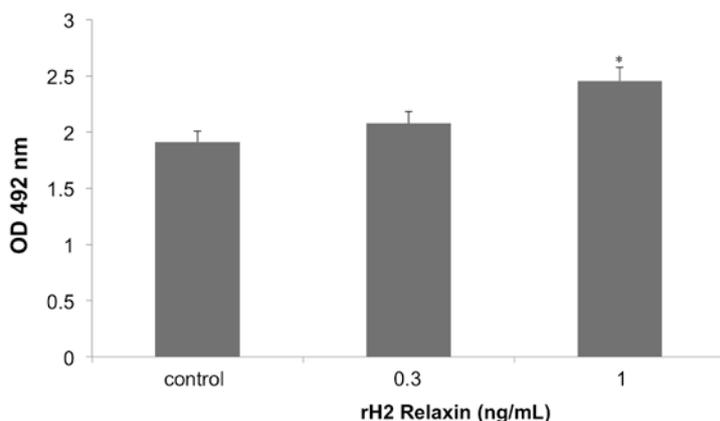
HTR-8/SVneo cells were treated with 1 ng/mL of rH2 relaxin 30 min, 60 min, 12 h, and 24 h. Cells were lysed for western blot analysis of AKT, pAKT, GSK3β, pGSK3β, and actin expression. The ratio of pAKT/AKT and pGSK3β/GSK3β were significantly increased (\*: p < 0.05) with 1 ng/mL of rH2 relaxin compared with untreated cells (0 min).

**Effect of a PI3K inhibitor (Ly294002) on relaxin-induced AKT and GSK3β phosphorylation**



**Figure 3:** PI3K inhibitor, Ly294002, downregulates AKT and GSK3β phosphorylation induced by rH2 relaxin in HTR-8/SVneo cells. To evaluate the effect of relaxin on the PI3K pathway, the PI3K inhibitor, Ly294002, was used to block PI3K. Cells were pretreated with Ly294002 for 30 min and, then, stimulated with rH2 relaxin (1 ng/mL) for 24 h.

To elucidate the role of the PI3K pathway in the effect of relaxin, HTR-8/SVneo cells were incubated with Ly294002, a specific inhibitor of PI3K. Ly294002 is a stable synthetic compound that acts as a competitive pan-PI3K inhibitor. Ly294002 inhibited relaxin-induced AKT and GSK3β activation. As shown in Figure 3, relaxin-induced AKT activation was partially blocked with 25μM of Ly294002. A bigger effect was observed with 50 μM Ly294002. Ly294002 inhibited AKT phosphorylation, leading to dephosphorylation of its downstream target GSK3β.

**Effect of increasing concentrations of rH2 relaxin on proliferation of HTR-8/SVneo cell**

**Figure 4:** rH2 relaxin effect on proliferation of HTR-8/SVneo cells. Proliferation was determined by MTS assay in presence or absence of different concentrations of rH2 relaxin for 24 h. Values represent the mean  $\pm$  SD of more than five independent experiments. \*:  $p < 0.05$  (compared to untreated control; 0 ng/mL)

Relaxin-induced proliferation of HTR-8/SVneo cells was evaluated by MTS assay. After incubation with increasing concentrations of rH2 relaxin for 24 h, MTS reagent was added for 2 h. As shown in Figure 4, rH2 relaxin increased the number of viable HTR-8/SVneo cells. Significant increase ( $p < 0.05$ ) in the number of HTR-8/SVneo cells was observed with 1 ng/mL of rH2 relaxin for 24 h compared to untreated cells (0 ng/mL).

## DISCUSSION

The relaxin receptor is found in the female reproductive system, including the ovary, uterine endometrium, and placenta. Thus, we assumed that relaxin has some physiological role on the reproductive system. Placental tissue contains a heterogeneous population of cells, including villous trophoblast, syncytiotrophoblast, and extravillous trophoblast cells (18). Controlled proliferation and self-renewal of trophoblast cells play an important role in the successful establishment of pregnancy during the implantation period. This is facilitated by several cytokines and growth factors present in the uterine microenvironment (20). During pregnancy and parturition, relaxin acts as a systemic pregnancy hormone secreted by the corpus luteum and an autocrine/paracrine hormone at the maternal-fetal interface formed by the decidua, placenta, and fetal membranes (27).

The present study used HTR-8/SVneo cells, immortalized first-trimester human trophoblast cells by transfection with a gene encoding the simian virus large T antigen, as a model for EVT cells. HTR-8/SVneo cells express RXFP1 and RXFP2 receptors (19) and RXFP1 has the ability to bind relaxin, leading to G protein alpha S ( $G\alpha_s$ ) and the PI3K/PKC $\zeta$   $\delta$  pathway stimulation and increased cAMP production (28). Thus, we assumed that the effect of relaxin involved the PI3K/AKT pathway in HTR-8/SVneo cells. In human trophoblast cells, PI3K/AKT signaling is activated by EGF as an antiapoptotic factor (29). Other studies reported that PI3K/AKT signaling positively affected human trophoblast cell migration (30), and that PI3K/AKT signaling is activated by insulin growth factor II (IGF-II) in HTR-8/SVneo cells. PI3K/AKT signaling is required for human chorionic gonadotropin (hCG) and EGF-dependent expression of metalloproteinases, MMP-2 and MMP-9, in trophoblast cells (31). Gang (32) reported that PI3K is involved in the activation of AKT induced by growth factors. In the present study, relaxin induced AKT phosphorylation on serine 473. As shown in Figure 1, relaxin increased the pAKT/AKT ratio in a dose- and time-dependent manner in HTR-8/SVneo cells. These results are consistent with those of Vinall (33). They demonstrated that relaxin activates both the PI3K/AKT pathway and the Gs/cAMP/PKA in a prostate cancer cell line. PI3K pathway activation leads to phosphorylation of AKT and its downstream target, GSK3 $\beta$ / $\alpha$  (23). GSK3 $\beta$  is a critical central target in many cell signaling pathways and also an important regulator of several transcription factors, which can affect the expression of numerous genes and cell viability (34).

The increase of pGSK3 $\beta$ /GSK3 $\beta$  induced by relaxin might be an indicator of a survival signaling pathway in HTR-8/SVneo cells. In fact, Lodhi (35) reported that Bcl-2, an anti-apoptotic marker, is increased in HTR-8/SVneo cells after treatment with increasing dose of rH2 relaxin for 24 h. The phosphorylation of AKT and GSK3 $\beta$  is considered as an indicator of the PI3K/AKT pathway activity. In the present study, relaxin phosphorylated AKT and GSK3 $\beta$  via activation of PI3K in HTR-8/SVneo cells. In LNCaP cells, relaxin induced

the PI3K/AKT/GSK3 $\beta$ / $\beta$ -catenin pathway to activate the androgen receptor (36). In primary EVT, wingless (Wnt)-3A induced the phosphorylation of AKT and GSK3 $\beta$  (37). This is also observed in cytotrophoblastic SGHPL-5 cells (38). GSK3 $\beta$  is a component of the Wnt signaling pathway, which regulates the stability of  $\beta$ -catenin and pGSK3 $\beta$  is known to stabilize  $\beta$ -catenin (39). In a mouse mammary tumorigenesis model, pGSK3 $\beta$  is accompanied by an increase in  $\beta$ -catenin and cyclin D1, a transcription factor. This results from a deregulation of the Wnt/ $\beta$ -catenin pathway. On the other hand, pGSK3 $\beta$  (S9) stimulates the oncogenic transcription factor, activator of protein 1 (AP-1), through the MEK1/ERK pathway induced by EGF (40). In the present study, relaxin induced the PI3K/AKT/GSK3 $\beta$  pathway in HTR-8/SVneo cells. Further investigation is necessary to reveal the transcription factors or other proteins involved in the relaxin induction of the PI3K/AKT/GSK3 $\beta$  pathway in HTR-8/SVneo cells. Additionally, the present study showed that relaxin is involved in the proliferation process. As shown in Figure 4, relaxin induced an increase in viable HTR-8/SVneo cells after incubation for 24 h. The signaling pathway involved in proliferation is suspected to include the activation of the PI3K/AKT pathway. As reported in Sertoli cells, relaxin binds to RXFP1 and induces the coupling of the receptor to the Gi protein. Released  $\beta\gamma$  subunits activate a non-receptor tyrosine kinase from the Src family of kinases and the PI3K/AKT pathway, which can possibly stimulate cell proliferation. Src and PI3K activate the Ras/Raf/MEK1/2/ERK1/2 pathway, which also contributes to cell proliferation (20). Further study is needed to reveal the proliferation signaling on HTR-8/SV neo cells.

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