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Original Article

Insulin-like growth factor 1 regulates growth of endometrial carcinoma through PI3k signaling pathway in insulin-resistant type 2 diabetes

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Abstract: Previous studies have shown that insulin-like growth factor 1 (IGF-1) may be responsible for the higher risk for developing endometrial carcinoma (EMC) in insulin-resistant type 2 diabetes mellitus (T2DM) patients. However, the underlying mechanisms are not understood. Here, we compared T2DM patients with or without EMC. We did not find difference in the serum levels of IGF-1, insulin-like growth factor 2 (IGF-2), IGF-1 binding protein 3, as well as the activation of IGF-1 receptor (IGF1R) in endometrial cells between T2DM patients with or without EMC. However, the levels of IGF2R activation and activation of PI3k, an IGF1R downstream factor, were significantly higher in endometrial cells in T2DM patients with EMC. In vitro analyses of activation of IGF1R, IGF2R, PI3k and CCND1 in EMC cells or IGF2R-overexpressing EMC cells by IGF-1 or IGF-2 suggest that increases in IGF2R in endometrial cells in T2DM may increase PI3k/CCND1-dependent cell growth through loss of competitive binding of IGF-2 to IGF1R, as a possible explanation for the higher risk for developing EMC in T2DM.

Keywords: Insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2), insulin-like growth factor 1 receptor (IGF1R), insulin-like growth factor 2 receptor (IGF2R), Cyclin D1 (CCND1), phosphatidylinositol-3 kinase (PI3k), type 2 diabetes mellitus (T2DM), endometrial carcinoma (EMC)

Introduction

Diabetes is a common chronic disease worldwide [1]. Epidemiologic study suggests that people with diabetes are at significantly higher risk for many forms of cancer and greater cancer mortality, predominantly insulin-resistant type 2 diabetes mellitus (T2DM) [2]. In 2010, the American Cancer Society and the American Diabetes Association recommended regular cancer screening for diabetic patients [2].

The incidence and mortality of cancer and diabetes are increasing with age, although the two diseases have trends of attacking young adults. Cancer and diabetes also share many modifiable risk factors, including obesity, diet, physical activity, tobacco smoking and alcohol drinking. Except for the common risk factors, meta-analyses have revealed T2DM to be an independent risk factor for the development of several different types of cancer [3]. Although the mechanisms that underlie the associations between T2DM and cancer risk remain far from understood, the insulin-like growth factors (IGFs) have been proposed to be important factors [4, 5].

Insulin-like growth factor 1 (IGF-1) is a potent growth factor with a role in cancer pathogenesis, which has been linked in epidemiological studies to cancer. The IGF1R signaling pathway initiates with binding of Insulin-like growth factor 1 (IGFI) to its cell-surface receptor IGF1R to activate phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, to stimulate cell growth and proliferation, and to inhibit programmed cell death [6-8]. In obese individuals, total IGF-1 levels has been shown to be normal or even low due to decreased concentrations of IGF binding

proteins, though the free/active IGF-1 levels are generally higher than in the non-obese. IGF-1 signals some of the same pathways as insulin, including PI3K, ERK, AKT, and mTOR, which as described above could increase cancer cell proliferation and impair apoptosis. IGF-1 can also increase normal cell cycling, leading to increased risk of mutation and malignant transformation. Most circulating IGF-1 is produced by the liver, though paracrine secretion of IGF-1 occurs at the growth plate, and perhaps other tissues [9].

As with insulin, inhibition of IGF-1 signaling has anticancer effects. Knockdown of the IGF-1 receptor enhances chemotherapy sensitivity in some cancers [10-12]. IGF-1 receptor antibody has been explored as a treatment, and recently shown to have in vitro efficacy against some cancers [13]. Nevertheless, although previous studies have suggested that serum IGF-1 in insulin-resistant T2D patients is likely associates with higher risk for developing endometrial carcinoma (EMC) [14-19], the underlying mechanisms are not understood. Here, we addressed these questions.

Materials and methods

Specimens from patients

A total of 162 T2DM subjects (22 with diagnosis of EMC and 140 without EMC as controls) were included in this study. All the subjects had no accompanying diseases (e.g. Crohn's disease and Parkinson's disease) that may affect the IGF-1 metabolism. All specimens had been histologically and clinically diagnosed at Hebei General Hospital from 2009 to 2015. For the use of these clinical materials for research purposes, prior patient's consents and approval from the institutional research ethics committee were obtained. Receptor and its phosphorylation were measured by Western blot and normalized to total protein or internal control protein. Serum levels of IGF-1, IGF-2 and IGF1BP3 were measured by ELISA.

EMC cell line culture

HEC-1A is a human EMC cell line purchased from American Type Culture Collection (HTB-112, ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and L-glutamine in a humidified chamber with 5% CO_2 at 37°C. The HEC-1A cell line was a subclone of HEC-1B (ATCC HTB-113), isolated in 1968 by H. Kuramoto and associates from a 71-year-old patient with stage IA endometrial adenocarcinoma [20].

Overexpression of IGF2R in HEC-1A cells

The IGF2R construct was cloned using human cDNA as a template. The IGF2R construct or a control scrambled sequence (SCR) were cloned into pcDNA3.1-EGFP to generate the plasmids. Transfection was performed with 2 μg plasmids using the Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfected cells expressing transgenes were selected by flow cytometry based on GFP, which is also included in the plasmid construct.

Western blot

Protein was extracted from the cells by RIPA buffer (Sigma-Aldrich) for Western Blot. The supernatants were collected after centrifugation at 12000×g at 4°C for 20 min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-IGF1R, anti-phosphorylated-IGF1R (pIGF1R), anti-IGF-2R, anti-phosphorylated-IGF2R (pIGF2R), anti-PI3k, anti-phosphorylated-PI3k (p-PI3k) anti-CCND1 and anti-α-tubulin (all purchased from Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software. The phosphorylated protein levels were first normalized to total protein, and then normalized to

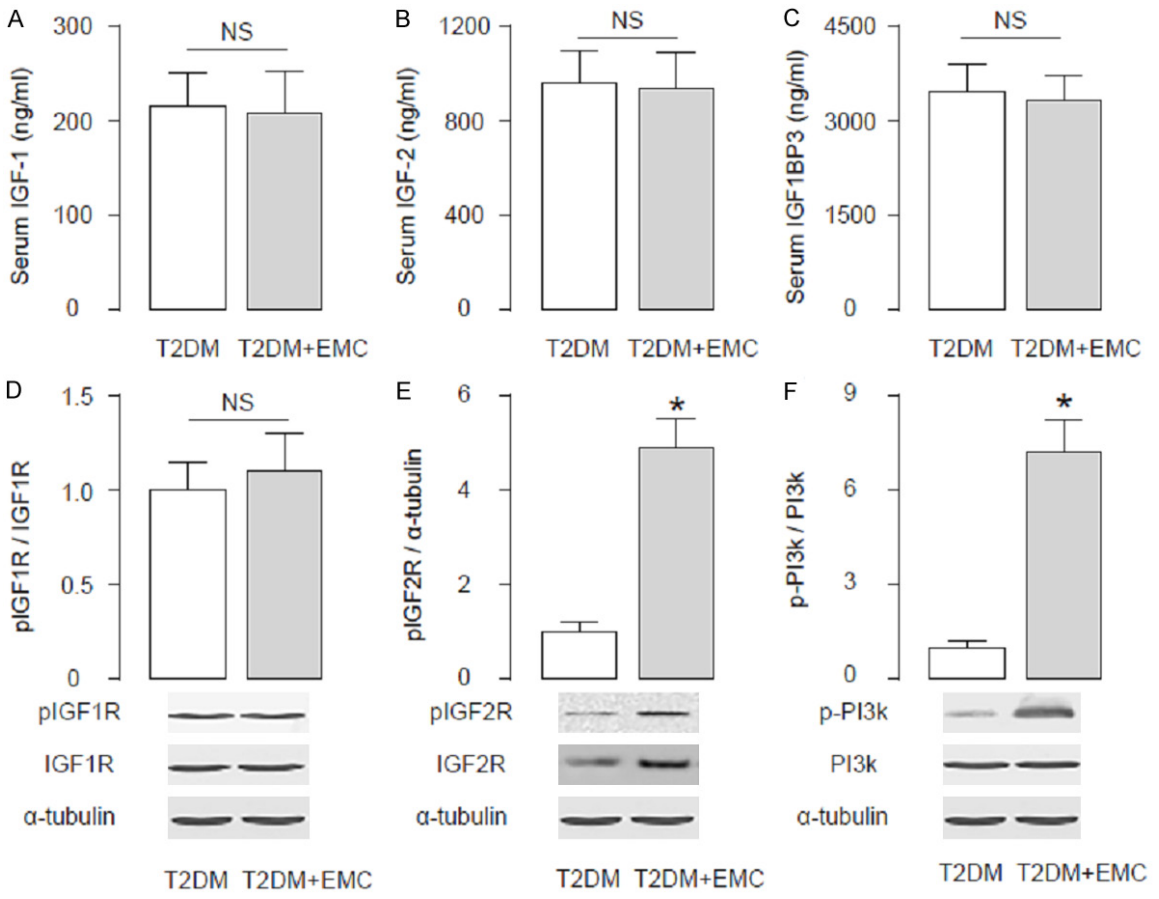

Figure 1. Activation of IGF2R signaling in EM cells hallmarks in T2DM with EMC. A total of 162 T2DM subjects (22 with diagnosis of EMC and 140 without EMC as controls) were included in this study. (A-C) The serum levels of IGF-1 (A), IGF-2 (B), and IGF1BP3 (C) between the T2DM patients with and without EMC. (D, E) The levels of activation of IGF1R (phosphorylated IGF1R (pIGF1R) vs total IGF1R) (D), activation of IGF2R (phosphorylated IGF2R (pIGF2R) vs α-tubulin) (E) and activation of PI3k (phosphorylated PI3k (p-PI3k) vs total PI3k) (F) in EM cells between the T2DM patients with and without EMC by Western blot. *p<0.05. NS: non-significant.

controls. CCND1 were first normalized to α-tubulin, and then normalized to controls.

Cell viability assay

For assay of cell growth, cells were seeded into 24 well-plate at 10000 cells per well and subjected to a Cell Proliferation Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm. Experiments were performed 5 times.

Statistical analysis

All statistical analyses were carried out using GraphPad prism 6.0 (GraphPad Software Inc. La Jolla, CA, USA). All values are depicted as mean ± SD and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups.

Results

Activation of IGF2R signaling in EM cells hallmarks in T2DM with EMC

A total of 162 T2DM subjects (22 with diagnosis of EMC and 140 without EMC as controls) were included in this study. First, we examined

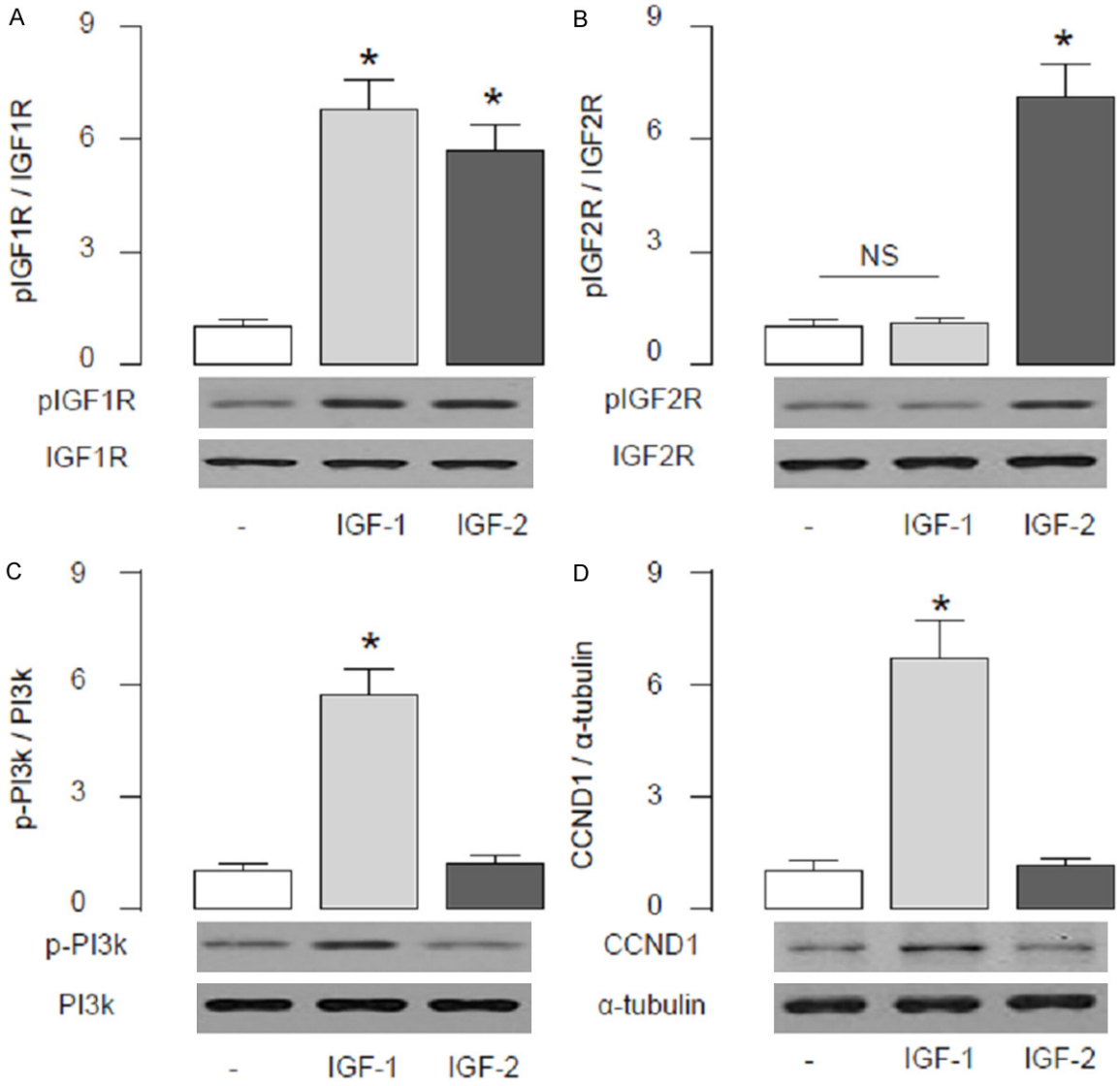

Figure 2. IGF-2 functions as a decoy ligand for IGF1R and does not activate downstream signaling cascades PI3K and CCND1. In order to study the underlying mechanisms, we used a human EMC cell line HEC-1A. Thus, IGF-1 or IGF-2 was given to the cultured HEC-1A cells and the activation of IGF1R, IGF2R and PI3k was examined. (A-D) The activation of IGF1R (phosphorylated IGF1R (pIGF1R) vs total IGF1R) (A), activation of IGF2R (phosphorylated IGF2R (pIGF2R) vs total IGF2R) (B), activation of PI3k (phosphorylated PI3k (p-PI3k) vs total PI3k) (C) and activation of CCND1 (CCND1 vs α-tubulin) (D) by Western blot. *p<0.05. NS: non-significant. N=5.

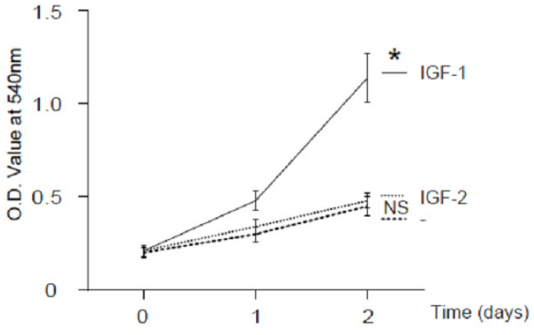

Figure 3. IGF-1, but not IGF-2, increases EMC cell growth. Next, we examined the effects of IGF-1 or IGF-2 on EMC cell growth in an MTT assay. We found that IGF-1, but not IGF-2, increased EMC cell growth. -: negative control, no growth factors. *p<0.05 (IGF-1 vs IGF-2 or -). NS: non-significant (IGF-2 vs -). N=5.

the serum levels of IGF-1, IGF-2, and IGF-1 binding protein 3 (IGF1BP3) between the T2DM patients with and without EMC. We did not detect difference in serum IGF-1 (**Figure 1A**),

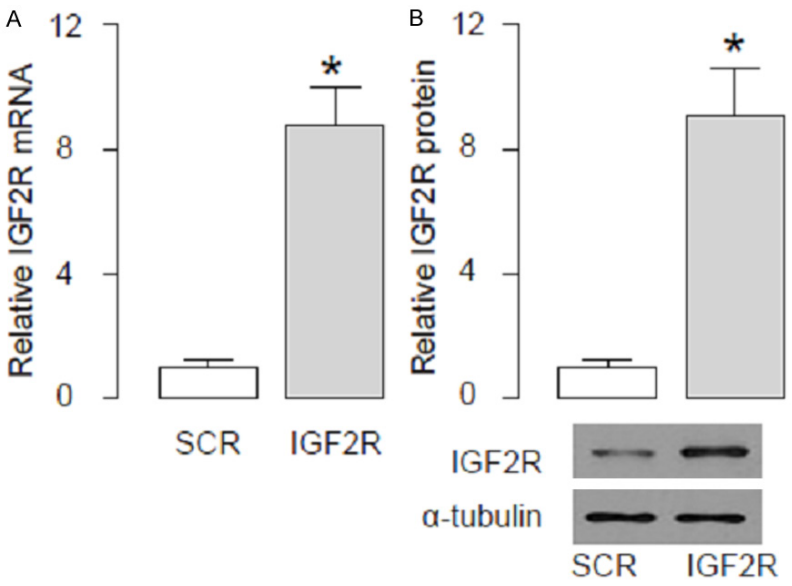

Figure 4. Generation of IGF2R-overexpressing EMC cells. We generated IGF2R-overexpressing HEC-1A (IGF2R) and control SCR cells. (A, B) The overexpression of IGF2R was confirmed at mRNA level by RT-qPCR (A), and at protein level by Western blot (B). *p<0.05. N=5.

serum IGF-2 (**Figure 1B**) and serum IGF1BP3 (**Figure 1C**) between two groups. Next, we examined the levels of IGF1R phosphorylation in endometrial (EM) cells, and found that there was no difference between T2D patients with or without EMC (**Figure 1D**). However, the levels of phosphorylated IGF2R (**Figure 1E**) as well phosphorylation of PI3k (**Figure 1F**), an IGF1R downstream factor, were significantly higher in endometrial cells in T2DM patients with EMC. These data suggest presence of a complex regulation of PI3k signaling by IGF1R and IGF2R signaling in EM cells in T2DM.

IGF-2 functions as a decoy ligand for IGF1R and does not activate downstream signaling cascades PI3K and CCND1

In order to study the underlying mechanisms, we used a human EMC cell line HEC-1A. Thus, IGF-1 or IGF-2 was given to the cultured HEC-1A cells and the activation of IGF1R, IGF2R and PI3k was examined. We found that IGF1R was significantly and seemingly activated by either IGF-1 or IGF-2 (**Figure 2A**). However, IGF2R was only significantly activated by IGF-2, but not by IGF-1 (**Figure 2B**). Interestingly, PI3k (**Figure 2C**) and Cyclin D1 (CCND1) (**Figure 2D**) were only activated by IGF-1, but not by IGF-2. These data suggest that IGF-2 may function as a decoy ligand for IGF1R and it may cause alternative phosphorylation of the IGF1R which does not activate downstream signaling cascades PI3k and CCND1. Thus, presence of IGF-2 may compete with IGF-1 for functional binding to IGF1R. Meanwhile, presence of IGF2R may reduce free IGF-2 and thus increase the effects of IGF-1.

IGF-1, but not IGF-2, increases EMC cell growth

Next, we examined the effects of IGF-1 or IGF-2 on EMC cell growth in an MTT assay. We found that IGF-1, but not IGF-2, increased EMC cell growth (**Figure 3**). These data support our hypothesis, in which IGF-2 may function as a decoy ligand for IGF1R and it may cause alternative phosphorylation of the IGF1R which does not activate downstream signaling cascades PI3k and CCND1 to increase cell growth.

Generation of IGF2R-overexpressing EMC cells

Since our clinic data showed T2DM with EMC had a higher level of IGF2R in EM cells than T2DM without EMC, and since our in vitro data showed that IGF-2 might be a decoy ligand for IGF1R, we hypothesize that high IGF2R levels in EM cells may attenuate the competing effects of IGF-2 on IGF-1-mediated activation of IGF1R signaling on cell growth, and thus increase EM proliferation and increase the risk of developing EMC. To prove it, we generated IGF2R-overexpressing HEC-1A and control SCR cells. The overexpression of IGF2R was confirmed at mRNA level by RT-qPCR (**Figure 4A**), and at protein level by Western blot (**Figure 4B**).

IGF2R-overexpressing EMC cells increase the sensitivity of IGF1R to IGF-1

Then we treated these cells with combined IGF-1 and IGF-2. We found that the activation of IGF1R by IGF-1 and IGF-2 was not significantly

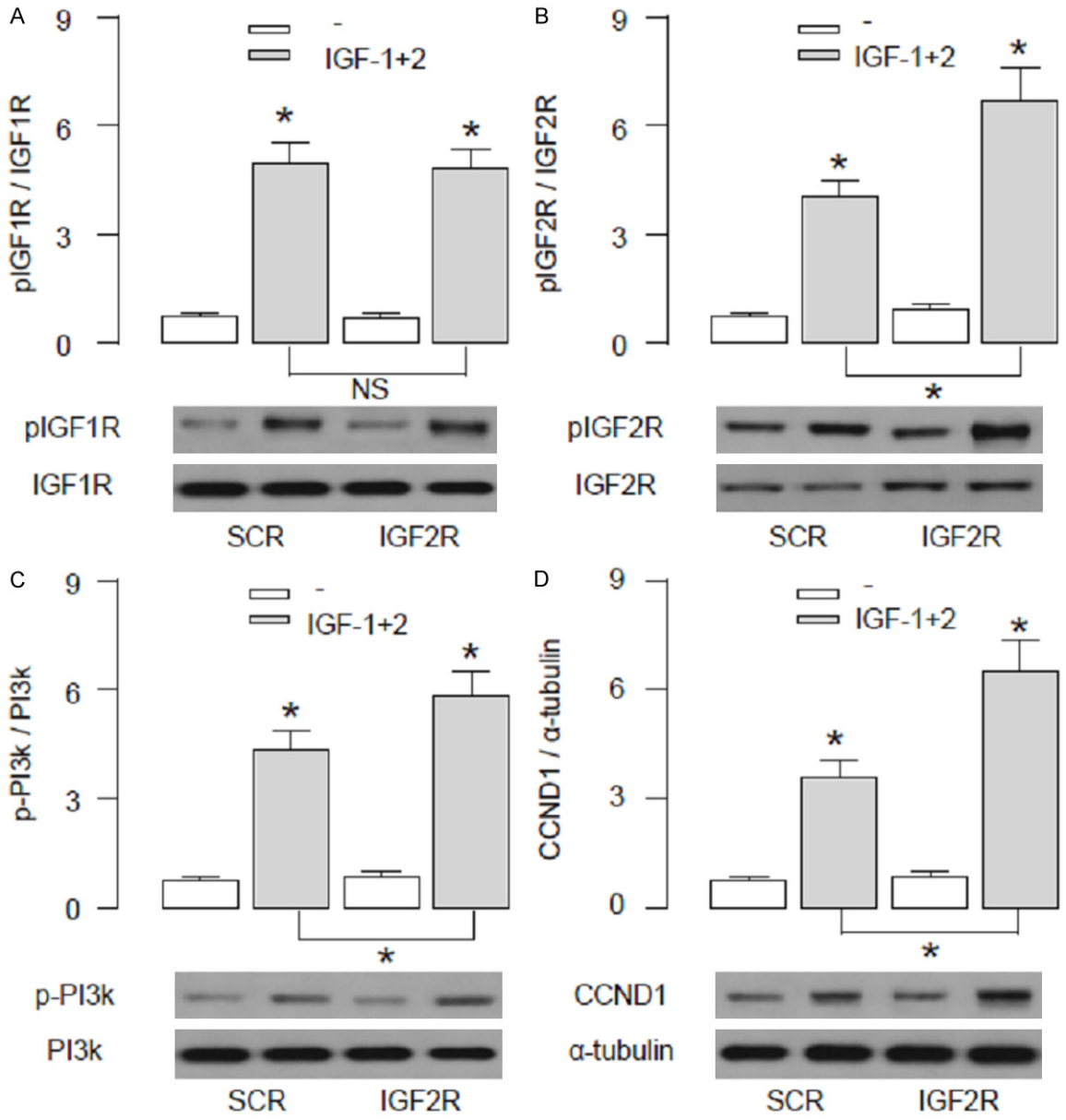

Figure 5. IGF2R-overexpressing EMC cells increase the sensitivity of IGF1R to IGF-1. Then we treated these cells with combined IGF-1 and IGF-2 (IGF-1+2) or no growth factors as a negative control (-). (A-D) The activation of IGF1R (A), IGF2R (B), PI3k (C) and CCND1 (D) by Western blot. *p<0.05. NS: non-significant. N=5.

different between control SCR and IGF2R-overexpressing HEC-1A cells (**Figure 5A**). Overexpression of IGF2R further increased the activation of IGF2R by IGF-1 and IGF-2 (**Figure 5B**). Moreover, the activation of PI3k (**Figure 5C**) and CCND1 (**Figure 5D**) were both further increased in IGF2R-overexpressing HEC-1A cells upon IGF-1 and IGF-2 stimulation. These data suggest that high IGF2R may increase the sensitivity of IGF1R to IGF-1 to increase cell growth.

High IGF2R increases EMC cell growth

Next, we examined the effects of overexpression of IGF2R on EMC cell growth in an MTT assay. We found that overexpression of IGF2R further increased EMC cell growth upon IGF-1 and IGF-2 stimulation (**Figure 6**). These data support our hypothesis, and demonstrate that high IGF2R in T2DM may increase the sensitivity of IGF1R to IGF-1 to increase EM cell growth (**Figure 7**).

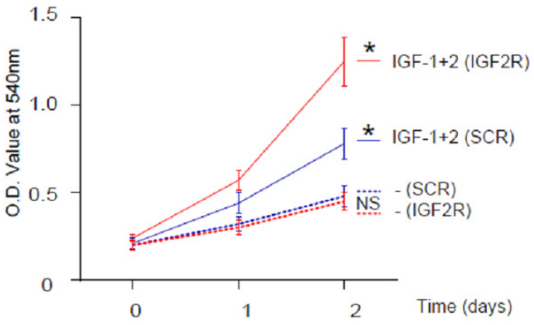

Figure 6. High IGF2R increases EMC cell growth. Next, we examined the effects of overexpression of IGF2R on EMC cell growth in an MTT assay. We found that overexpression of IGF2R further increased EMC cell growth upon IGF-1 and IGF-2 stimulation. *p<0.05. NS: non-significant. N=5.

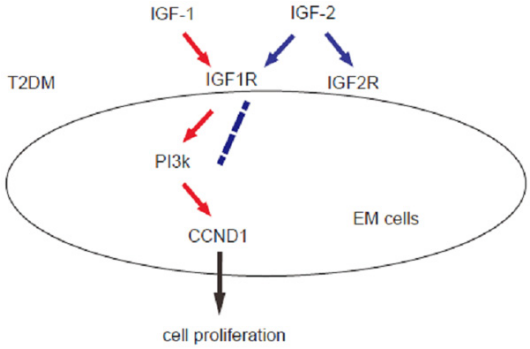

Figure 7. Schematic of the model. High IGF2R in T2DM may increase the sensitivity of IGF1R to IGF-1 to increase EM cell growth.

Discussion

Epidemiological data suggest that hyperinsulinemia in women is associated closely with endometrial carcinoma risk. Recent evidence points insulin and IGF-I to the risk factors for this malignancy. Both insulin and IGFs are demonstrated mitogens for EMC cells.

Insulin receptor and IGFIR are structurally related tyrosine kinase receptors with more than 50% homology. Both receptors are covalent dimers consisting of two extracellular α-subunits containing ligand binding sites and two transmembrane β-subunits containing tyrosine kinase domains. In spite of structural similarities, the two receptors have distinct biological effects: IGFIR signaling has primarily mitogenic effects, whereas insulin receptor activation has mostly metabolic effects. Although there is some interaction between insulin receptor and IGFIR when activated by insulin and IGFs, insulin exerts its effect mostly through IR, and both IGFs bind to IGFIR with high affinity and bind to IR with much lower affinity (<1%). Given the cross-talk between signaling of the IGF1R, insulin receptor, and other growth receptors, it is proposed that IGF-1 signaling could represent a mechanism of resistance to therapies targeting signaling pathways. However, our current understanding of the system in the molecular manner is rather preliminary.

In the current study, we compared T2D patients with or without EMC. We found that the serum levels of IGF-1, IGF-2, IGF1BP3, as well as the levels of activation of IGF1R in EM cells were not different between T2D patients with or without EMC. However, the levels of IGF2R activation and phosphorylation of PI3k, an IGF1R downstream factor, were significantly different between groups. Based on previous studies on other cell types, we know that IGF-2 binds to both IGF1R and IGF2R, while IGF-1 only binds to IGF1R. We confirmed these findings in EMC cells. Interestingly, we found that activation of IGF1R by IGF-2 did not further activate PI3k signaling-mediated activation of cell cycle associated proteins, e.g. CCND1. Thus, these data suggest that IGF-2 may function as a decoy ligand for IGF1R and it competes with IGF-1 for IGF1R binding, while its binding to IGF1R may cause alternative phosphorylation of the IGF1R which does not further activate downstream signaling cascades PI3k and CCND1. Of note, these may be due to phosphorylation of different amino acid residue on IGF1R by IGF-1 and IGF-2. Thus, presence of IGF-2 may compete with IGF-1 for functional binding to IGF1R. Meanwhile, presence of IGF2R may reduce the IGF-2 binding to IGF1R and thus increase the sensitivity of IGF1R to IGF-1 stimulation. These hypotheses were confirmed in our in vitro analyses of a human EMC cell line.

Our study provides a novel model to explain the high risk for developing EMC in T2DM patients. These data demonstrate that not only direct modulation of IGF-1, but also indirect modulation of IGF-2 and IGF2R may help to reduce the susceptibility of the T2DM women to EMC.

Disclosure of conflict of interest

None.

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