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ORIGINAL ARTICLE

SiRNA-mediated IGF-1R inhibition sensitizes human colon cancer SW480 cells to radiation

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Abstract

Purpose. Insulin like growth factor receptor 1 (IGF-1R) is well-documented to play a key role in radiation response and tumor radiosensitivity, thus offering an attractive clinic drug target to enhance tumor sensitivity to anti-cancer radiotherapy. *Material and methods.* Human colon carcinoma SW480 cells were transfected with the specific small interference RNA (siRNA) expression vector (pkD-shRNA-IGF-1R-V2) designed to target IGF-1R mRNA. The expression of IGF-1R mRNA and its protein among the transfected and untransfected cells were detected by semi-quantitative RT-PCR and ELISA assay. The changes in cell radiosensitivity were examined by MTT assay. *Results.* Transfection of mammalian expression vector pkD containing IGF-1R siRNA was shown to reduce IGF-1R mRNA levels by up to 95%. ELISA assay detected a similar inhibition of IGF-1R protein levels in cells transfected with IGF-1R siRNA. SW480 cells transfected with the expression vector for siRNA significantly rendered cells more sensitive to radiation and the highest radiation enhancement ratio was 2.02 ± 0.08. *Conclusion.* These data provide the first evidence that specific siRNA fragment (pkD-shRNA-IGF-1R-V2) targeting human IGF-1R mRNA is able to enhance colon cancer radiosensitivity. Also results indicated that, combining IGF-1R siRNA and radiation significantly enhances antitumor efficacy compared with either modality alone.

Colon cancer is the second leading cause of cancer-related deaths in the world [1–4]. Colon cancer is most commonly subtypes of gastrointestinal (GI) cancers and is characterized by a particularly poor prognosis with approximately 90% of patients dying within five years of diagnosis [5–8]. Most colon cancer patients are inoperable at diagnosis and are treated with radiotherapy and/or chemotherapy. Currently, although radiotherapy is an important modality of colon cancer treatments, this therapeutic strategy alone could not improve efficiently the long-term survival rate [9,10]. Therefore, to establish an effective treatment, novel drug compounds combined with radiotherapy are urgently needed [11,12]. Specific molecular targets for enhancing radiation-mediated cell killing are heavily weighted toward the improvement of the cure rate for cancer [12–14]. It is now well established that, in addition to inducing nuclear DNA damage, therapeutic ionizing radiation (IR) can activate specific signalling transduction that can influence the overall cell survival [15,16]. Accumulated evidence suggests a strong correlation between IR-induced gene expression and cellular radiosensitivity [15,17,18]. Using colon cancer cell lines with different radiosensitivities, studies identified several potential candidate genes resistant to IR [17–20]. Of particular interest among the up-regulated genes is IGF-1R, a negative regulator of the p^{53} tumor suppressor, which was shown to be a critical component in response to IR [21]. IGF-1R expression levels were found to be increased in radioresistant colon carcinoma SW480 cells [20–23], indicating that IGF-1R could be a suitable target for re-sensitization of radiation-resistant colon cancer cells.

IGF-1R is an oncogene, and it promotes the growth of tumor cells [23]. Amplification or overexpression of IGF-1R has been found in a variety of

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human carcinomas, including colon cancer patients with poor prognosis [24,25]. Most importantly, IGF-1R expression is found to affect cell response to radiotherapy and chemotherapy [21,26]. Therefore, IGF-1R may serve as a potential target to enhance the tumor radiosensitivity.

siRNA has been extensively studied as a means of attenuating the expression of specific gene transcripts both *in vitro* and *in vivo*. siRNAs are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a process termed RNA interference (RNAi) [27–30]. In this study, we applied vector-mediated delivery of siRNA to knock-down the expression of IGF-1R gene in radioresistant human colon cancer SW480 cells to investigate whether inactivating IGF-1R can enhance the sensitivity to therapeutic dose of IR (0–20 Gy of γ-ray). Our data show that IGF-1R gene expression in SW480 cells is specifically suppressed by IGF-1R siRNA. IGF-1R siRNA transfected cells combined with IR significantly inhibited cell proliferation. Therefore, a combination of IGF-1R siRNA with radiotherapy would be a preferential strategy to enhance the therapeutic efficacy for radioresistant colon cancer cells.

Material and methods

Plasmid preparation

Short hairpin RNA (shRNA) plasmid expressing vectors (PkD-shRNA-IGF-1R-V2 and PkD-shRNA-NegCon-V1 as the negative control) were purchased from Upstate Biotechnology (Wetzlar, Germany). To amplify the vectors, cloning was performed. The PkD plasmid vectors were transformed into the competent *E.Coli* DH5α bacteria. After ampicillin selection, the purified plasmid DNAs (by Miniprep Kit, QIAGEN) from the obtained clones, were tested for identification of the IGF-1R and NegCon shRNAs plasmid vectors by bidigestion of the clones with *PVUII* bidigest enzyme (Fermentas UAB, Lithuania).

Cell culture and plasmid transfection

The human colon cancer SW480 cells (from ATCC) were cultured in DMEM medium (Gibco BRL, Paisley, UK) supplemented with 10% of fetal bovine serum (FBS), 2 mM of L-Glutamine and 1 ml/l Gentamicin 40 mg/ml at 37˚C with 5% CO_2. Transfection of PkD-shRNA-IGF-1R-V2 or PkD-shRNA-NegCon-V1 plasmids was performed with FuGENE HD Transfection Reagent (Roche, Mannheim, Germany) in 24-well plates following manufacturer's instructions. Briefly, the day before transfection, confluent layer of the cells was trypsinated, counted and resuspended. Suspension of 8×10^4 of cells were plated in antibiotic-free DMEM medium into each well of the 24 well plates, so that they could become about 60–80% confluence next day at the time of transfection. The cells were transfected with a mixture of 2 μg PkD-shRNA-IGF-1R-V2 or PkD-shRNA-NegCon-V1 plasmids and 6 μl of FuGENE HD Transfection Reagent (The ratio of DNA plasmid / FuGENE HD Transfection Reagent was 1/3) in 500 μl serum and antibiotic free-DMEM medium per well. FBS (100 μl/well) was added at 6 h post transfection. At 26 h post transfection, the medium was replaced by complete medium and cultured in a humidified 37˚C incubator with 5% CO_2. The cells were harvested at different time points. RT-PCR, enzyme-linked immunosorbent assay (ELISA) or other experiments were then performed.

All of the following experiments were done into four groups: 1. untreated SW480 cells, 2. SW480 cells transfected ones with FuGENE HD Transfection Reagent only, 3. SW480 cells transfected with PkD-shRNA-IGF-1R-V2 and 4. SW480 cells transfected with PkD-shRNA-NegCon-V1. All experiments were repeated at least three times and groups 1, 2 and 4 were applied as controls.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. The first strand of cDNA was obtained using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas UAB, Lithuania). For semi-quantitative analysis of IGF-1R mRNA, human β-actin gene was used as an internal control. DNA primer sequences were designed as follows: for human IGF-1R gene (GenBank accession No. : NM_000875) sense 5′ACAGAGAACCCCAAGACTGAG 3′and antisense 5′TGATGTTGTAG GTGTCTGCGG 3′; and for human β-actin, sense 5′ AGAAAATCTG GCACCACACC3′, and antisense 5′ AGGAAG GAAGGCTGGAAGAG 3′. The PCR cycling conditions for the IGF-1R cDNA included preincubation for 5 min at 95˚C and followed by 30 cycles of 50 s at 95˚C, 50 s at 59˚C, 60 s at 72˚C and a final extension for 7 min at 72˚C. PCR products were identified using electrophoresis on 1.5% agarose gels containing ethidium bromide (Et-Br). Gel images were obtained and the densities of PCR products were quantified using UVI-TEC gel analysis software. All the experiments were repeated on at least three occasions.

Determination of IGF-1R protein expression of cultured cells

To determine IGF-1R protein expression of cultured cells, cells (transfected and control cells) were removed by trypsinization at different times after

transfection, washed in PBS and lysed in RIPA buffer containing protease inhibitors. Protein levels of IGF-1R were measured using an ELISA kit (Millipore, Germany) for human IGF-1R.

Ionizing Radiation (IR)

Briefly, the cells seeded at 0.4×10^4cells/well in a 96-well microtiter plate and transfected with vector constructed after culturing for 24 h. At 48 h post transfection, cells were treated with different doses of ^{60}Co (Varian; the radiation doses were 0, 1, 5, 10, and 20 Gy, respectively; the dose efficiency was 1.6 Gy/min). After 24, 48 and 72 h of irradiation, survival fractions of cells were obtained using MTT assay. All samples were made in triplicate.

Statistical analysis

All statistical analyses were performed by using SPSS13.0. Comparisons among all cell groups were performed with the one and two ways (for IR) analysis of variance *(ANOVA)* test. If statistical significance was found, the Tukey *post hoc* test was used. Values of $p < 0.05$ were considered to be statistically significant.

Results

IGF-1R-specific siRNA can efficiently block expression of IGF-1R at mRNA level in human colon cancer SW480 cells

The transiently knock-down efficiencies of PkD-shRNA-IGF-1R-V2 in SW480 cells was first evaluated using semi-quantitative RT-PCR. Results are displayed in Figure 1. Twenty-four hours after transfection of cells with pkD-shRNA-IGF-1R-V2, the relative levels of IGF-1R RNA were decreased by 65% ± 2 and, which were further reduced significantly ($p < 0.001$) at 48 h (93 ± 2%) and followed by a slight increase at 72 h (89 ± 1%). However, the three other cell-groups had no effect on the expression of IGF-1R mRNA ($p > 0.05$) (Figure 1). The expression of B-actin mRNA was not affected in any of the groups.

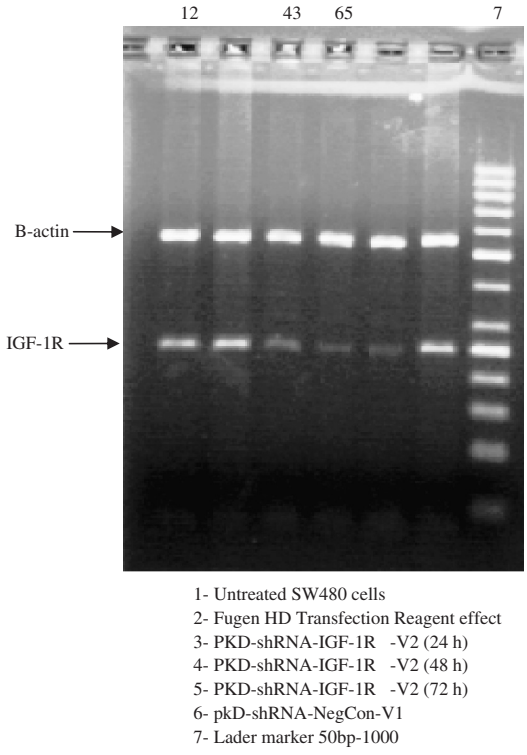

Figure 1. Down-regulation of IGF-1R mRNA in colon cancer cells following transient transfection with IGF-1R specific siRNA. Total RNA was extracted at the indicated times after transfection and IGF-1R mRNA was quantitated by semi-quantitative RT-PCR. Shown are the relative mRNA levels of IGF-1R in reference to β-actin expression. Data are expressed as the mean ± standard deviation.

Effect of pkD-shRNA-IGF-1R-V2 plasmid on expression of IGF-1R Protein in experimental and control cell groups

To further establish the silencing effects of the pkD-shRNA-IGF-1R-V2 on IGF-1R expression, cells were transfected with pkD-shRNA-IGF-1R-V2 or pkd-shRNA-NegCon-V1. The transfected and blank controls cells were collected at 24, 48, 72 and 96 h, and expression of IGF-1R was quantified by means of sandwich ELISA. The greatest decrease in expression of IGF-1R was seen at the 48-hour time point; there was a 91.2 ± 2.8% decrease in the expression of IGF-1R. We also evaluated the effects and durability of our transient transfection with the pkD-shRNA-IGF-1R-V2 during the course of four days by collecting cells from our transfected cells and control cells and again assessing levels of IGF-1R on ELISA. We again found a 91.2 ± 2.8% knockdown at 48 h, but an 81.2 ± 1.8% knockdown of IGF-1R was still evident four days after transfection in those cells treated with the pkD-shRNA-IGF-1R-V2. However, three control cell groups had no effect on the expression of IGF-1R protein ($p > 0.05$) (Figure 2).

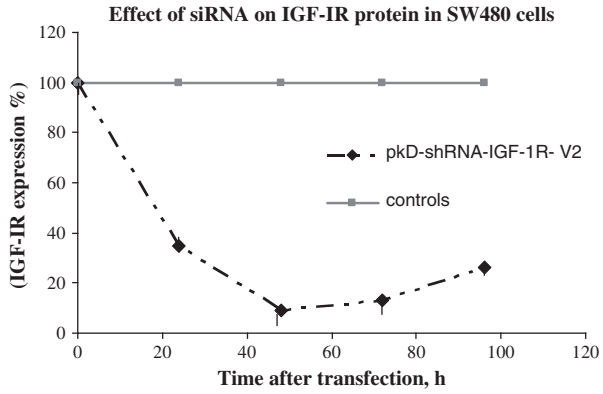

Figure 2. Expression of IGF-1R protein in SW480 cells. Transfection of pkD-shRNA-IGF-1R-V2 results in greatest knockdown at 48 hours, but still provides an 81.2 ± 1.8% knockdown after four days in comparison with midi ones of control cells (untreated SW480 cells, SW480 cells transfected ones with FuGENE HD Transfection Reagent only and cells transfected with PkD-shRNA-NegCon-V1). Data are expressed as the mean ± standard deviation.

Effect of IGF-1R specific siRNA on radiosensitivity of SW480 cells

We next investigated whether the IGF-1R inhibition leads to an increased antitumor effect of irradiation on SW480 cell lines. A series of MTT cell viability assays were conducted to determine the growth-inhibitory effect of three different treatments: anti-IGF-1R shRNA alone, fractionated doses of γ-radiation (1, 5, 10 and 20 Gy) and a combination of the two. The growth inhibition profiles over the 24, 48 and 72 h after radiation treatment with the unionized cell controls for ionizing radiation are depicted in Figure 3A–D.

IGF-1R siRNA by itself exerted growth inhibitory effects on cell survival in SW480 cells (Figure 3A). Irradiation as a monotherapy caused a cytotoxic effect ranging between 2–13% after 24 h, 5–23% after 48 h and 15–63% after 72 h radiation treatment in SW480 cells (Figure 3B–D). Combined therapy with IGF-1R siRNA and irradiation induced a significant decrease of cell growth in SW480 cells ($p < 0.01$). After 24 and 48 h of radiation treatment, cell growth was reduced by about 6–35% and 15–70% in SW480 cells transfected with IGF-1R siRNA (Figure 3B,C). Following increasing incubation time of radiation treatment, this percentage increased progressively up to 72 h, when about 95% of cell growth inhibition was observed in 20 Gy doses of γ-radiation (Figure 3D).

Growth inhibition was time and dose dependent, which significantly decreased the viable cell number 72 h after treatment compared to controls. In addition, the γ-IR dose that induced a decrease of cell viability from 100 to 50% (ID_{50}) was calculated to be 4.3 ± 0.2 Gy in the SW480 cells transfected with pkD-shRNA-IGF-1R-V2, while ID_{50} of control cells, after 72 h radiation were about 8.34 ± 0.58. There were no obvious differences in survival fractions among control cell groups ($p > 0.05$; Figure 3A–D). In the SW480 cells transfected with pkD-shRNA-IGF-1R-V2, survival fraction was significantly reduced ($p < 0.01$); resulting in a calculated radiation-induced cytotoxicity enhancement factor of 2.02 ± 0.08 for the SW480 cells (Figure 3D).

Discussion

Clinically, many cell colon carcinomas are inoperable, and little tumor controlling efficacy is achieved by radiotherapy or chemotherapy, or a combination of both therapies due to tumor resistance. Therefore, developing new therapies to improve the overall outcome of colon cancer patients is in urgent need [9,10,13,31,32]. Although potential targets for therapeutic intervention in therapy-resistant colon cancers have been extensively studied in the last decade, no efficient molecular target has been identified. Our present results demonstrate for the first time that inhibition of IGF-1R with a specific human siRNA sequence (IGF-1R siRNA) is able to re-sensitize radiation-resistant colon cancer SW480 cells. Oncogenes provide a potential target for cancer gene therapy [33,34]. Overexpression of the oncogene IGF-1R is a common event associated with the pathogenesis of most human cancers, including breast cancer, lung and colon cancer [23,25]. Activation of IGF-1R increases the expression of genes that promote cell survival and block apoptosis [23]. Transduction of IGF-1R into NSCLC cells can promote rapid cell proliferation [35]. Interestingly, tumor regression was observed by the inactivation of IGF-1R [36]. Overexpression of IGF-1R in various tumors is believed to inhibit p^{53}, therefore favoring uncontrolled cell proliferation. Studies reported that IGF-1R expression levels were increased in colon cancer cells in response to IR [18,20]. Therefore, specific down-regulation of IGF-1R may be a potential target to enhance radiosensitization. Other groups have applied antisense to attenuate IGF-1R expression resulting in the inhibition of proliferation of cancer cells *in vitro* [37]. Although antisense oligonucleotides are efficient to block IGF-1R, RNA interference is a powerful technique for the specific inhibition of expression of individual genes at the posttranscriptional level. siRNA has a number of advantages over antisense oligonucleotide strategy, in part from the greater resistance of siRNA to nuclease degradation. RNAi and antisense RNA have been compared and RNAi seemed to be quantitatively more efficient and durable in both cell culture and nude mice [28,29,38]. Therefore, the feasibility of using siRNA in molecular cancer therapy and as a mediator of chemosensitization or radiosensitization has been shown by several groups [39,40].

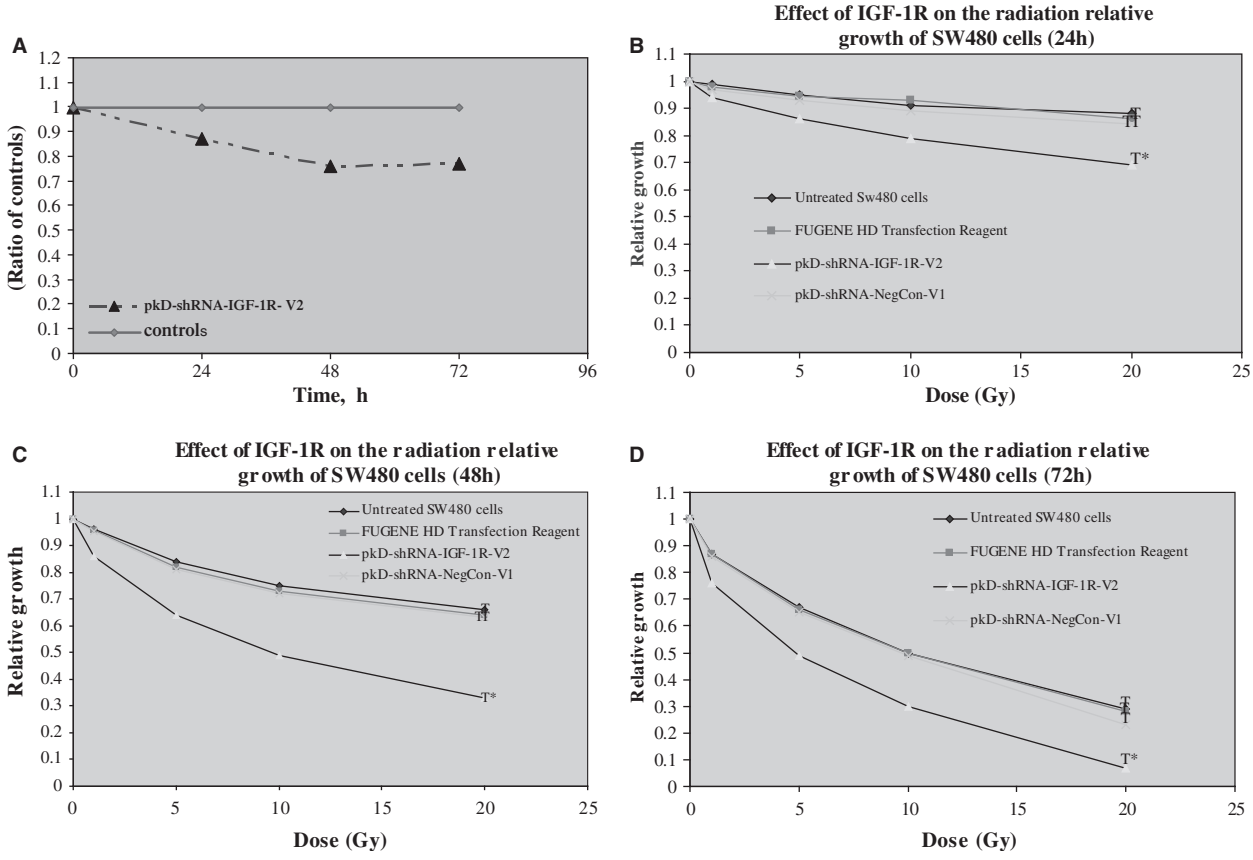

Figure 3. Effect of combined treatment (IGF-1R siRNA and irradiation) on viability of SW480 cells. Cell viability of all four groups of SW480 cells including untreated SW480 cells, FUGENE HD Transfection Reagent, pkD-shRNA-NegCon-V1 as cell controls for siRNA and pkD-shRNA-IGF-1R. Also this entire four cell groups used as cell controls for ionizing radiation (curve A) for irradiation of the same treatment cells in 24 h (B), 48 h (C) and 72 h (D). SW480 cells were seeded in 96-well culture plates (4 000 cells/well) and treated with IGF-1R siRNA, irradiated with 1, 5, 10 and 20 Gy or irradiation combined with IGF-1R siRNA treatment. Results are expressed as relative growth ratios of control cells. Each bar represents the relative cell growth inhibition (mean ± standard deviations) from three independent experiments.*$p < 0.01$, compared to untransfected and transfected cells with pkD-shRNA-IGF-1R-V2 before irradiation.

In the present study, the functional consequences of siRNA-mediated suppression of IGF-1R, using expression PkD vectors for IGF-1R siRNA was established in radioresistant colon cancer cells. Our results demonstrated that inhibition of IGF-1R by siRNA enhanced response to radiation, which was associated with significant reduced levels of cell proliferation (Figure 3) for dual treatment of siRNA and IR. As a result, there was enhanced radiosensitivity in SW480 cells. Although synthetic siRNAs can achieve effective and very rapid knockdown of target genes in mammalian cells, their effects are transient. To circumvent this problem, transfection of siRNA plasmid that stably integrates and expresses interfering double-strand RNA hairpins may be effective at knocking down target genes continually. In this study, IGF-1R siRNA was transfected transiently in SW480 cells and because of the loss of long-term gene silencing, the clonogenicity assay was not performed. In conclusion, we have tested IGF-1R siRNA and found significant inhibition efficiency as determined by both mRNA and protein levels (Figures 1 and 2). Accumulating evidence suggest that different regions of mRNA show a varied efficacy of gene inhibition by siRNA-mediated targeting. Therefore, it appears that the region of siRNA is an efficient target for IGF-1R.

Radiotherapy is widely used in malignant intestinal tumor therapy, but some colon cancers are not sensitive to it. In our study, cell growth was inhibited with the decrease in IGF-1R expression caused by shRNA. PkD-shRNA-IGF-1R-V2 with ionizing radiation treatment significantly inhibited cancer cell growth, compared with IGF-IR siRNA alone in SW480 cells. Furthermore, PkD-shRNA-IGF-IR-V2 with ionizing radiation treatment significantly inhibited cancer cell growth, compared with ionizing radiation treatment alone. Therefore, it is suggested that the silencing of the IGF-IR gene by RNAi and 5-FU has a synergistic effect. Overall, the data presented here provide evidence suggesting that IGF-1R siRNA could be a promising and powerful method for sensitization of radioresistant cancer cells. The study results lay a solid foundation for

gene therapy combined with radiotherapy. New therapeutic modalities in the treatment of human colon cancer are suggested by this study.

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There are no conflicts of interest to be declared.

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