

Original Article

Blockade of IGF/IGF-1R signaling axis with soluble IGF-1R mutants suppresses the cell proliferation and tumor growth of human osteosarcoma

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Abstract: Primary bone tumor, also known as osteosarcoma (OS), is the most common primary malignancy of bone in children and young adults. Current treatment protocols yield a 5-year survival rate of near 70% although approximately 80% of patients have metastatic disease at the time of diagnosis. However, long-term survival rates have remained virtually unchanged for nearly four decades, largely due to our limited understanding of the disease process. One major signaling pathway that has been implicated in human OS tumorigenesis is the insulin-like growth factor (IGF)/insulin-like growth factor-1 receptor (IGF1R) signaling axis. IGF1R is a heterotetrameric $\alpha 2\beta 2$ receptor, in which the α subunits comprise the ligand binding site, whereas the β subunits are transmembrane proteins containing intracellular tyrosine kinase domains. Although numerous strategies have been devised to target IGF/IGF1R axis, most of them have failed in clinical trials due to the lack of specificity and/or limited efficacy. Here, we investigated whether a more effective and specific blockade of IGF1R activity in human OS cells can be accomplished by employing dominant-negative IGF1R (dnIGF1R) mutants. We engineered the recombinant adenoviruses expressing two IGF1R mutants derived from the α (aa 1-524) and β (aa 741-936) subunits, and found that either dnIGF1R α and/or dnIGF1R β effectively inhibited cell migration, colony formation, and cell cycle progression of human OS cells, which could be reversed by exogenous IGF1. Furthermore, dnIGF1R α and/or dnIGF1R β inhibited OS xenograft tumor growth *in vivo*, with the greatest inhibition of tumor growth shown by dnIGF1R α . Mechanistically, the dnIGF1R mutants down-regulated the expression of PI3K/AKT and RAS/RAF/MAPK, BCL2, Cyclin D1 and most EMT regulators, while up-regulating pro-apoptotic genes in human OS cells. Collectively, these findings strongly suggest that the dnIGF1R mutants, especially dnIGF1R α , may be further developed as novel anticancer agents that target IGF signaling axis with high specificity and efficacy.

Keywords: Osteosarcoma, bone tumor, IGF-1R, IGF signaling, dominant-negative mutants, targeted therapy

Introduction

Primary bone tumor, also known as osteogenic sarcoma or osteosarcoma (OS), is the most

common primary malignancy of bone in children and young adults, with a peak incidence in the second decade of life [1-7]. Even though the current treatment protocols, including wide

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margin local resection, pre-operative and/or post-operative chemotherapy, yield a 5-year disease-free survival rate of near 70% [1, 2, 6-9], approximately 80% of patients have either macro- or micro-metastatic disease at the time of diagnosis [7-9]. Furthermore, despite significant improvement in surgical techniques and chemotherapeutic regimens, long-term survival rates have remained virtually unchanged for nearly four decades, largely due to our limited understanding of the disease processes [6-9]. Therefore, there is an unmet critical need to understand the pathogenesis and precise molecular mechanisms leading to the development of OS [1-7].

Like other human tumors, many genetic and epigenetic alterations have been implicated in the development and/or progression of human OS, and these alterations include mutations in oncogenes, tumor suppressor genes, numerous epigenetic modifications, oncometabolic changes and noncoding RNAs [1, 6, 7, 10-27]. One of the major signaling pathways shown to play a critical role in cancer development and progression, including OS tumorigenesis, is insulin like growth factor (IGF) signaling axis [28-32]. In fact, the local expression of IGF-1 is implicated in OS development since the highest levels of IGF-1 usually coincide with the peak incidence of OS; and the increased levels of IGF-1 and IGF-1R have been detected in OS samples [30, 33]. Furthermore, overexpression of IGF-1/IGF-1R signaling contributed to tumor cell survival, metastasis, and resistance to chemotherapeutic drugs; and IGF-1R expression was correlated with a poor prognosis of OS [30, 33, 34].

The IGF signaling axis plays critical roles in many biological processes, including normal growth and development, and cell proliferation and survival, as well as in numerous pathological processes, such as the cancer development and progression, when dysregulated [28-32]. IGFs are members of a ligand family that includes IGF1, IGF2 and insulin. The functions of IGF1, IGF2 and insulin are mediated through interaction with their cell surface receptor tyrosine kinases (RTKs) type 1 IGF receptor, called IGF-1R, or IGF1R, and insulin receptor (INSR) [28, 29, 32]. IGF1R is a heterotetrameric $\alpha 2\beta 2$ receptor, in which the two α subunits are extracellular and together comprise the binding site

for the ligand, whereas the two β subunits are transmembrane proteins that include the intracellular tyrosine kinase domains [32]. IGF1R binds IGF1 with high affinity ($\sim 1-5$ nM) and with significantly lower affinity for IGF2 and insulin (4-5 fold and >100 -fold lower, respectively) [32].

While IGF ligand binding to IGF1R leads to the activation of multiple signaling pathways, two of the best-characterized downstream pathways are Phosphoinositide 3-kinase-Protein kinase B (PI3K-AKT) and RAS-Mitogen-activated protein kinase (MAPK), which are also activated by cell signals, such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), and hepatocyte growth factor (HGF) [28-32]. Since IGF signaling is widely up-regulated in human cancer, targeting IGF1R has long been attractive in the field of cancer research. Numerous strategies including therapeutic antibodies, small molecule inhibitors and shRNA reagents have been devised in the past decades [32]. We previously demonstrated that insulin growth factor binding protein 5 (IGFBP5), a member of the naturally occurring IGF-binding antagonist IGFBP family, can inhibit OS tumorigenesis and metastasis [30, 35-37]. Nonetheless, most of the current IGF1R targeting strategies have been hampered in clinical trials due to the lack of specificity and/or limited efficacy.

In this study, we investigated whether effective and specific blockade of IGF1R activity can block the proliferation and growth of human OS cells. To accomplish this objective, we engineered two dominant-negative IGF1R mutants that express the ligand binding region of the α subunit (i.e., dnIGF1R α) and the extracellular domain of the β subunit (i.e., dnIGF1R β), and investigated whether these soluble decoy receptors could specifically suppress IGF signaling in human OS cells in a dominant-negative inhibition fashion. We found that the exogenous expression of either dnIGF1R α and/or dnIGF1R β effectively inhibited cell migration, colony formation, and cell cycle progression of human OS cells. The inhibitory effect on cell proliferation by the dnIGF1R mutants could be relieved by exogenous IGF1 protein. Furthermore, exogenous expression of dnIGF1R α and/or dnIGF1R β significantly inhibited OS xenograft tumor growth *in vivo*, with the greatest

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inhibition of tumor growth shown by dnIGF1R α . Mechanistically, the dnIGF1R mutants were shown to effectively down-regulate PI3K/AKT and RAS/RAF/MAPK signaling pathways, as well as the expression of BCL2, Cyclin D1 and most EMT regulators, while up-regulating the expression of pro-apoptotic genes in human OS cells. Collectively, these findings strongly suggest that the dominant-negative IGF1R mutants, in particular dnIGF1R α , may be further developed as novel anticancer agents that target IGF signaling pathway with high specificity and efficacy.

Materials and methods

Cell culture

Human osteosarcoma cell line 143B and HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 293pTP and RAPA cells were HEK293 derivative lines that were previously characterized [38, 39]. All cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO₂ as described [40-44]. Human IGF-1 was purchased from PEPRO TECH (Cat# G502522, Cranbury, NJ). Unless indicated otherwise, other chemicals/reagents were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).

Construction and generation of recombinant adenoviruses expressing the dominant-negative IGF1R mutants, dnIGF-1R α and dnIGF-1R β , or GFP

Recombinant adenoviruses were generated by using the AdEasy technology as described [45-48]. Briefly, for the construction of the dnIGF-1R α , the coding region for amino acids 1-524 of the human IGF1R (NM_000875.3) was PCR amplified and cloned into the adenoviral shuttle vector, pAdTrace-CMV as described [40, 49-51], yielding pAdTrace-dnIGF1R α . For the construction of the dnIGF-1R β , the coding region for amino acids 741-936 of the human IGF1R was PCR amplified and cloned into the pDisplay vector (Invitrogen, Carlsbad, CA) and maintained in-frame with the Igk signal peptide. The fragment containing the coding sequences of Igk signal peptide-IGF1R AA 741-936 was then cloned into the pAdTrace-CMV

vector, yielding pAdTrace-dnIGF1R β . The transgenes are driven by the CMV promoter as independent expression cassettes. All PCR amplified sequences and cloning junctions were verified by DNA sequencing. Detailed information about vector construction is available upon request.

Both pAdTrace-dnIGF1R α and pAdTrace-dnIGF1R β were used for homologous recombination with the pAdEasy1 backbone vector in BJ5183 bacterial cells, generating the recombinant adenoviral plasmids pAdR-dnIGF1R α and pAdR-dnIGF1R β , which were subsequently used to generate adenoviruses in 293pTP and/or RAPA cells as reported [46, 52-56]. The resultant adenoviruses were designated as AdR-dnIGF1R α and AdR-dnIGF1R β , both of which also express red fluorescent protein (RFP) as a tracking marker of infection efficiency. An adenovirus that expresses GFP only was used as a mock virus control [57-60]. Polybrene (final concentration at 4 μ g/mL) was added to all adenovirus infections to enhance adenoviral transduction efficiency [61-63].

RNA isolation and touchdown real-time quantitative PCR (TqPCR) analysis

RNA was isolated with the TRIzol Reagent (Invitrogen) by following the manufacturer's instructions and subjected to reverse transcription reactions using random hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The resultant cDNA products were diluted and used as qPCR templates. PCR primers were designed by using the Primer3 Plus program [64]. The quantitative PCR analysis was carried out using our previously optimized TqPCR protocol [44, 65, 66]. Briefly, the SYBR Green qPCR reactions (Bimake, Houston, TX) were set up according to manufacturer's instructions. The cycling program was modified by incorporating 4 cycles of touchdown steps prior to the regular cycling program as described [67-72]. *GAPDH* was used as a reference gene. All sample values were normalized to *GAPDH* expression by using the 2^{- $\Delta\Delta$ CT} method. The qPCR primer sequences are shown in the [Supplementary Table 1](#).

Wound healing/cell migration assay

Subconfluent 143B cells were seeded in 6-well cell culture plates, infected with the indicated adenoviruses, and cultured to confluence (usu-

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ally within 24 h). Wound injuries were introduced by scratching the monolayer cells with 10 μ L pipette tips, and floater cells were removed by washing. The wound areas were monitored under bright field and photographed at 0, 12, 24, and 36 hours post wounding as described [73-76]. Each assay condition was set up in triplicate.

Colony formation assay

Approximately 3,000 of 143B cells infected with different adenoviruses were seeded into 6-well culture plates in triplicate, and cultured for 8 days. The cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The stained colonies were recorded photographically and quantitatively determined as described [77-79].

WST-1 cell proliferation analysis

Exponentially growing cells were first infected with adenoviruses for 16 h, replated into 96-well plate at 30% confluence in triplicate, and treated with or without human IGF-1 at the predetermined optimal concentration 30 ng/mL. At 0 h, 24 h, 48 h, 72 h after IGF1 treatment, WST-1 substrate working mix was added to the wells and incubated for 1 h, and then subjected to absorbance readings at 450 nm with the use of a microplate reader as described [54, 73, 79-84].

Cell cycle/flow cytometry analysis

Exponentially growing 143B cells were infected with adenoviruses for 16 h, replated into 60 mm cell culture dishes in triplicate, and treated with or without human IGF-1 (30 ng/mL) for 24 hours. The cells were fixed with 70% ethanol, washed with PBS and stained with the PI and RNase stain solution (BD Biosciences Pharmingen, Cat # 550825). The stained cells were then subjected to FACS using the BD™ LSR II Flow Cytometer. The acquired flow cytometry data were analyzed with the ModFit Lt software as described [57, 66, 69, 79].

Subcutaneous tumor cell implantation, xenograft tumor formation, and Xenogen bioluminescence imaging

The animal use and care in this study was approved by the Institutional Animal Care and

Use Committee, and all animal experimental procedures were carried out in accordance with the approved guidelines. Subcutaneous cancer cell implantation procedure was performed as described [35, 36, 85-89]. Briefly, 143B cells were first retrovirally tagged with firefly luciferase, yielding 143B-FLuc. Subconfluent 143B-FLuc cells were infected with different adenoviruses. After 24 h post infection, the cells were collected, resuspended in sterile PBS, and injected subcutaneously into the flanks of athymic nude mice (Envigo/Harlan Research Laboratories, n=5/group, female, 6-8 week old, 2×10^6 cells per injection site). The animals were maintained *ad lib* in the biosafety barrier facility. Tumor volumes were measured with a digital clipper at days 7, 10, 13 and 16. The mice were also subjected to Xenogen IVIS 200 imaging at days 7, 10, 13 and 16. The pseudoimages were obtained by superimposing the emitted light over the grayscale photographs of the animals. Quantitative analysis was done with Xenogen's Living Image V2.50.1 software as described [73, 79, 85]. At 16 days after implantation, the mice were euthanized, the tumors were retrieved for volume and weight measure, and also histological evaluation.

Hematoxylin and Eosin (H&E) and immunohistochemistry staining

The retrieved tumor masses were fixed with 10% buffered formalin and embedded in paraffin. Serial sections at 5 μ m of the embedded specimens were carried out, and subjected to H & E staining as described [49, 56, 90-94]. The immunohistochemical staining was carried out as described [37, 88, 95-97]. Briefly, slides were deparaffinized and rehydrated, followed by antigen retrieval treatment through boiling the slides for 5 min in 0.01 M citrate buffer. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 15 min. Non-specific binding was blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories) for 60 min at room temperature. The slides were incubated at 4°C with primary antibody against PNCA (sc-56, 1:300 dilution, Santa Cruz Biotechnology) overnight, and then with donkey anti-mouse IgG secondary antibody conjugated with HRP (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. The staining was visualized with 5% 3, 3'-diaminobenzidine tetrachloride (DAB), and counter-

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stained with light green. The minus anti-PNCA and mouse IgG staining groups were used negative controls. The staining results were recorded under a bright field microscope.

Statistical analysis

All quantitative assays were performed in triplicate and/or repeated in three independent batches. Data were expressed as mean \pm standard deviation. Statistical significances were determined by one-way analysis of variance and the Student t-test. Statistical analysis was performed using the SPSS software version 19.0. A *p*-value less than 0.05 was considered statistically significant.

Results

The soluble forms of IGF-1R decoy receptor function as dominant-negative inhibitors of IGF-1R activity by suppressing cell migration and colony formation of human osteosarcoma (OS) cells

We sought to construct soluble forms of the α and β subunits of the human IGF-1R receptor. As shown in **Figure 1A**, human IGF-1R is a tetramer consisting of two α subunits and two β subunits, in which the α subunits are located in the extracellular space and mainly bind to IGF ligands, whereas the β subunits contain a small extracellular domain, a transmembrane span and a large cytoplasmic domain with tyrosine kinase activity (**Figure 1Aa**). To engineer soluble dominant-negative mutants that may effectively block IGF-1R activity in human OS cells, we constructed two mutants that express the large ligand-binding portion of the α subunits (aa 1-524; aka, dnIGF1R α) and the extracellular portion of the β subunits that interact with the α subunits (aa 741-936; aka, dnIGF1R β), respectively (**Figure 1Ab, 1Ac**).

In order to effectively express these mutants in OS cells, we generated recombinant adenoviruses that express dnIGF1R α and dnIGF1R β , designated as AdR-dnIGF1R α and AdR-dnIGF1R β , respectively. These adenoviruses were shown to effectively infect human OS line 143B cells (**Figure 1B**). Co-infection of AdR-dnIGF1R α and AdR-dnIGF1R β (aka, AdR-dnIGF1R α + β) was utilized to achieve simultaneous inhibition of both α and β subunits. High levels of the adenovirus-mediated expression of the mutants,

either individually or in co-infection, were confirmed by qPCR analysis (**Figure 1Ca, 1Cb**).

When the human OS cells were infected with AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β , the cell wounding assay revealed that the adenovirus-mediated expression of dnIGF1R α , dnIGF1R β , or dnIGF1R α + β significantly inhibited the wound closure at 24 h and 36 h after monolayer cell injury, compared with the GFP control (**Figure 2A**). Colony formation analysis further revealed that dnIGF1R α , dnIGF1R β , or dnIGF1R α + β effectively suppressed the numbers of colonies formed in human OS cells, compared with the GFP control (**Figure 2Ba**). Quantitatively, the expression of dnIGF1R α and dnIGF1R β decreased the numbers of colonies formed to 58.2% and 61.6% of the GFP control numbers, while the colony numbers decreased to 25.8% of the GFP control (**Figure 2Bb**). These *in vitro* results indicate that expression of dnIGF1R α , and/or dnIGF1R β effectively suppressed wounding monolayer cell healing and colony formation in human OS 143B cells.

The dnIGF1R mutants competitively inhibit IGF1-induced cell proliferative activity in human OS cells

Mechanistically, we next tested whether these IGF1R mutants would suppress the IGF signaling activity in dominant-negative inhibition fashion by assessing whether the dnIGF1R mutant-mediated inhibition of cell proliferation could be overcome by exogenous IGF ligands. Using the human IGF1 recombinant protein, we demonstrated that the human OS line 143B cells were highly responsive to IGF1 stimulation in low FBS growth condition in a dose-dependent fashion (in the 0 to 50 ng/mL range) (data not shown). When the OS line 143B cells were infected with AdR-dnIGF1R α , AdR-dnIGF1R β , AdR-dnIGF1R α + β , or AdGFP, we found that exogenous expression of dnIGF1R α , dnIGF1R β , or dnIGF1R α + β significantly inhibited cell proliferation, which could be effectively reversed by the addition of IGF1 (at 30 ng/mL) at as early as 24 h post stimulation, and becoming more pronounced at 72 h (**Figure 3A**). In fact, the addition of exogenous IGF1 in the cells expressing dnIGF1R α , dnIGF1R β , or dnIGF1R α + β effectively restored the cell proliferative activity close to the level of the GFP control's (**Figure 3B**). These results strongly suggest that the

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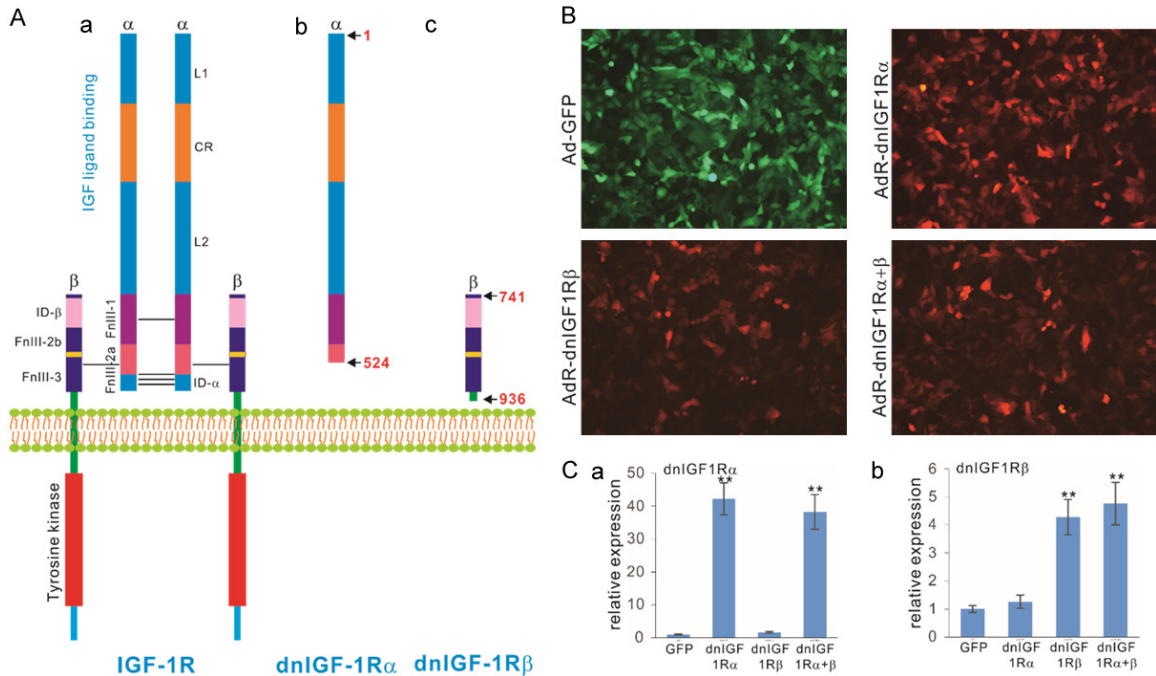


Figure 1. Construction of dominant-negative IGF-1R (dnIGF1R) mutants as decoy receptors to specifically target IGF-1R signaling activity. **A.** Schematic depictions of the hetero-tetrameric structure and domains of the wild type IGF-1R and two dnIGF1R mutants, dnIGF-1R α and dnIGF-1R β . L1, ligand binding domain 1; L2, ligand binding domain 2; CR, cysteine rich domain; ID-a, insert domain a; ID-b, insert domain b; FnIII-1, fibronectin type III domain 1; FnIII-2a, fibronectin type III domain 2a; FnIII-2b, fibronectin type III domain 2b; FnIII-3, fibronectin type III domain 3. The schematic diagrams are not drawn to scale. **B.** The dnIGF1R mutant expressing adenoviral vectors effectively transduce human osteosarcoma (OS) cells. Subconfluent OS line 143B cells were infected with Ad-GFP control virus, AdR-IGF1R α , or AdR-IGF1R β , or co-infected with AdR-IGF1R α and AdR-IGF1R β (AdR-dnIGF1R α + β). Fluorescent signals were recorded at 48 h post infection. Representative images are shown. **C.** Recombinant adenovirus-mediated expression of the dnIGF1R mutants in human OS cells. Subconfluent OS line 143B cells were infected with Ad-GFP control virus, AdR-IGF1R α , or AdR-IGF1R β , or co-infected with AdR-dnIGF1R α + β . Total RNA was isolated at 48 h post infection and subjected to reverse transcription and qPCR analyses with the primers for the coding regions of the dnIGF1R mutants. *GAPDH* was used as a reference gene. “***”, $P < 0.01$ compared with that of the GFP group’s.

dnIGF1R α and dnIGF1R β may function as dominant-negative mutants to suppress the IGF1R signaling activity in human OS cells.

The dnIGF1R mutants suppress OS cell cycle progression by increasing G0/G1 phase and reducing S phase, and effectively inhibit tumor growth in the xenograft OS tumor model

We infected the subconfluent 143B cells with AdR-dnIGF1R α , AdR-dnIGF1R β , AdR-dnIGF1R α + β , or AdGFP, in the presence or absence of human IGF1 stimulation (30 ng/mL) for 48 h and conducted flow cytometry analysis. We found that exogenous expression of dnIGF1R α , dnIGF1R β , or dnIGF1R α + β significantly inhibited cell cycle progression by increasing numbers of cells in G0/G1 phase, while decreasing the numbers of cells in S phase (**Figure 4A**). As expected, the addition of IGF1 (at 30 ng/mL)

effectively reversed the inhibition of cell cycle progression caused by the expression of dnIGF1R α , dnIGF1R β , or dnIGF1R α + β in OS line 143B cells (**Figure 4B**). However, it seems that these mutants had little impact on G2/M phase in the OS cells expressing dnIGF1R α , dnIGF1R β , or dnIGF1R α + β mutant.

We further investigated the effect of these mutants on tumor growth *in vivo*. We infected the subconfluent Firefly luciferase-tagged 143B cells with AdR-dnIGF1R α , AdR-dnIGF1R β , AdR-dnIGF1R α + β , or AdGFP, and the infected cells were injected subcutaneously into the flanks of athymic nude mice. Whole body bioluminescence imaging analysis indicated that the tumor growth was significantly inhibited in the groups injected with OS tumor cells infected with AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β , compared with that of the AdGFP

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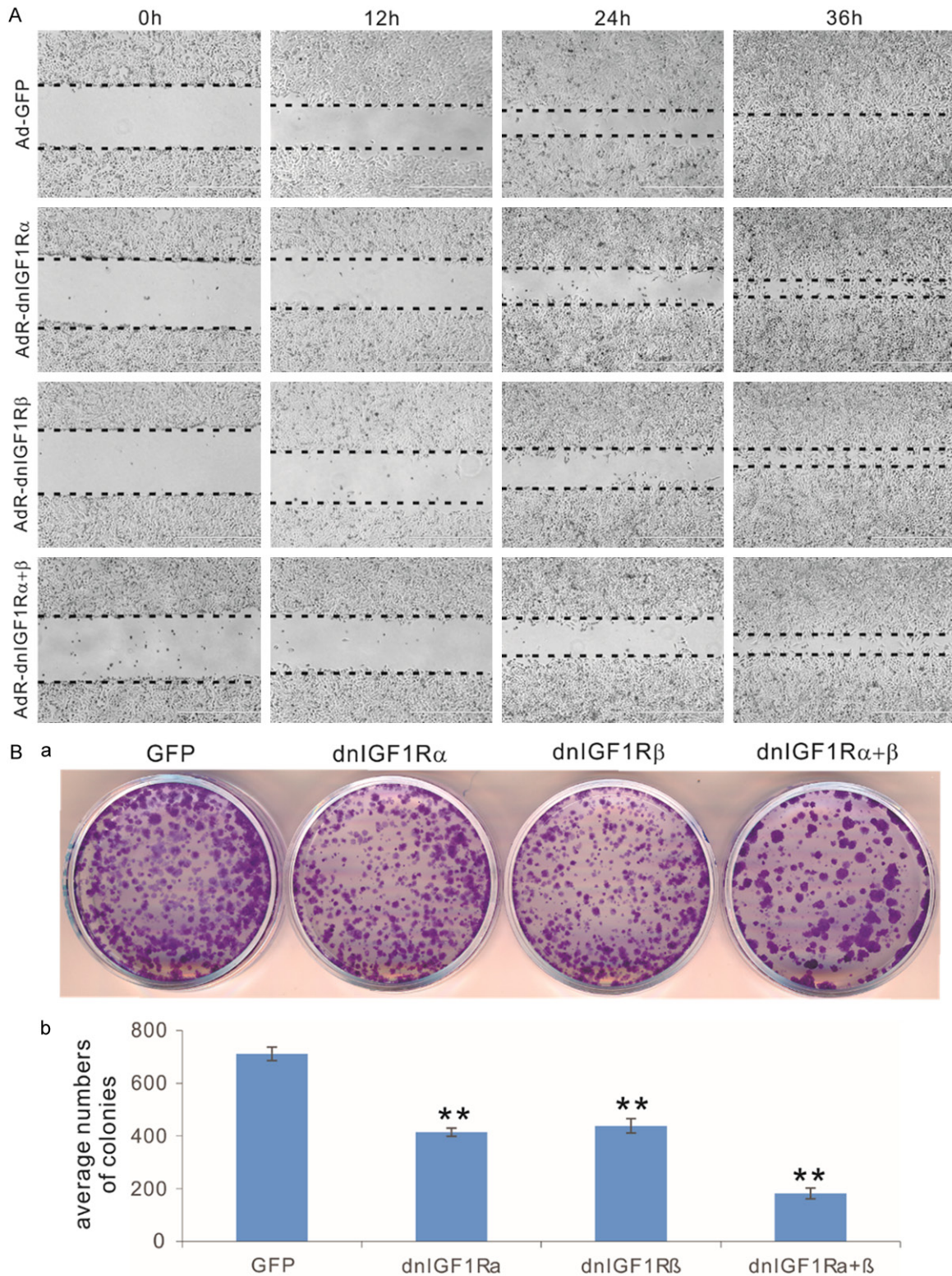


Figure 2. The dominant-negative IGF-1R (dnIGF1R) mutants effectively inhibit cell migration and colony formation of human OS cells. (A) The dnIGF1R mutants inhibit cell wound healing in OS cells. Subconfluent 143B cells were infected with the indicated adenoviral vectors for 16 h and replated at near confluence. The well attached cells were injured with fine pipette tips, and the injured gaps were recorded and monitored for 36 h. Images at approximately same locations were taken under a bright field microscope. The wounding front lines are indicated with the dotted lines. (B) The dnIGF1R mutants inhibit colony formation from human OS cells. Subconfluent 143B cells were

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infected with the indicated adenoviral vectors for 16 h. The same numbers of infected OS cells were reseeded in 12-well cell culture plates in triplicate, and cultured for 10 days with medium changes every 2-3 days. At 10 days post replating, the cells were fixed and stained with crystal violet. Representative images are shown (a), and the established colonies were further quantitatively determined (b). “***”, $P < 0.01$ compared with that of the GFP group’s.

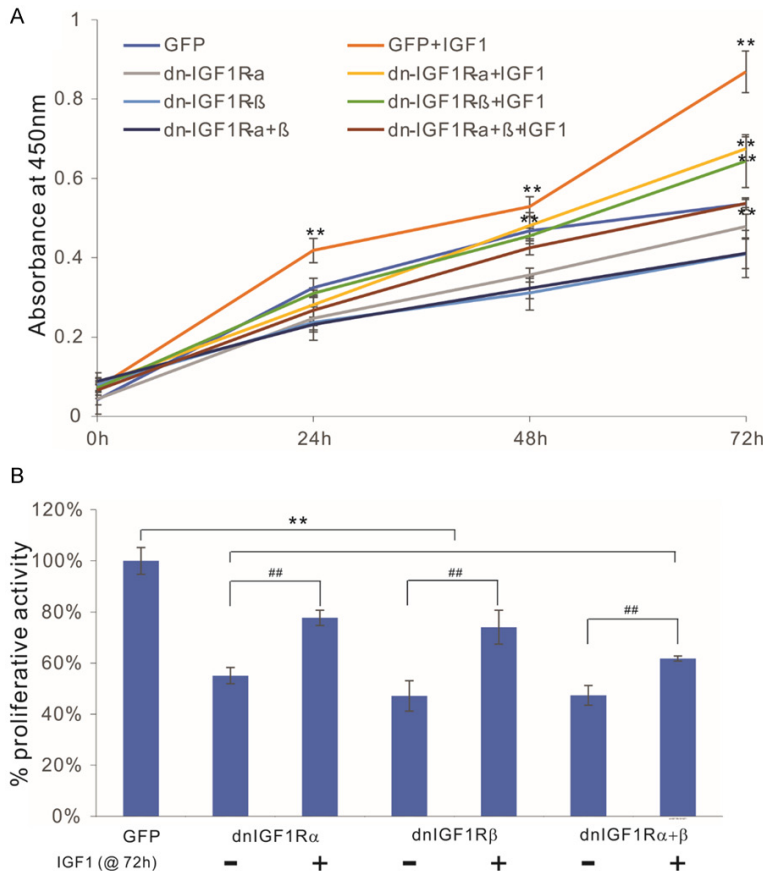


Figure 3. The dominant-negative IGF-1R (dnIGF1R) mutants compete with IGF1-induced cell proliferative activity in human OS cells. Exponentially growing 143B cells were infected with the indicated adenoviral vectors for 16 h and replated into 96-well cell culture plates, with or without IGF1 stimulation (30 ng/mL). At the indicated time points, the cells were subjected to WST-1 assays (A), and the WST-1 assay results at 72 h were further quantitatively analyzed (B). “***”, $P < 0.01$ compared with that of the GFP group’s; “##”, $P < 0.01$ compared with that of the -IGF1 group’s.

group (Figure 5A). Interestingly, the AdR-dnIGF1R α group exhibited the greatest reduction of tumor growth. These results were further confirmed by the quantitative analysis of the bioluminescence imaging data during the course of tumor growth (Figure 5B). The tumor volume changes were in general correlated well with the bioluminescence imaging results and the AdR-dnIGF1R α group showed the greatest reduction of tumor volume growth (Figure 5C).

At the endpoint of day 16, the xenograft tumor masses were retrieved from the athymic nude

mice, and the tumor sizes in the AdR-dnIGF1R α , AdR-dnIGF1R β , and AdR-dnIGF1R α + β groups were smaller than that of the AdGFP group, while the AdR-dnIGF1R α group had the smallest tumor size (Figure 5Da), which was further confirmed by the quantitative measurement of average tumor weight (Figure 5Db). H&E histologic evaluation indicates that the tumor masses retrieved from the AdR-dnIGF1R α , AdR-dnIGF1R β , and AdR-dnIGF1R α + β groups exhibited significantly lower proliferating cells, compared with the AdGFP group (Figure 5Ea). Immunohistochemical staining analysis revealed that PNA-positive cells significantly decreased in the tumor masses retrieved from the AdR-dnIGF1R α , AdR-dnIGF1R β , and AdR-dnIGF1R α + β groups, compared with that in the Ad-GFP group (Figure 5Eb). Collectively, the above *in vivo* results strongly demonstrate that the dnIGF1R mutants could effectively inhibit tumor growth and reduce tumor cell proliferative activity in the xenograft model of human osteosarcoma.

The dnIGF1R mutants inhibit the expression of major downstream mediators of the IGF-1R signaling pathway in OS cells

Lastly, we investigated the impact of the dnIGF1R mutants on the major signaling mediators of the IGF-1R pathway. IGF1R is activated by IGF1 and IGF2, which triggers the auto-phosphorylation of IGF1R and subsequently activates the downstream PI3K/AKT and RAS/RAF/MAPK signaling pathways, promoting proliferation, growth and survival [28, 29, 31]. When the subconfluent 143B cells were infect-

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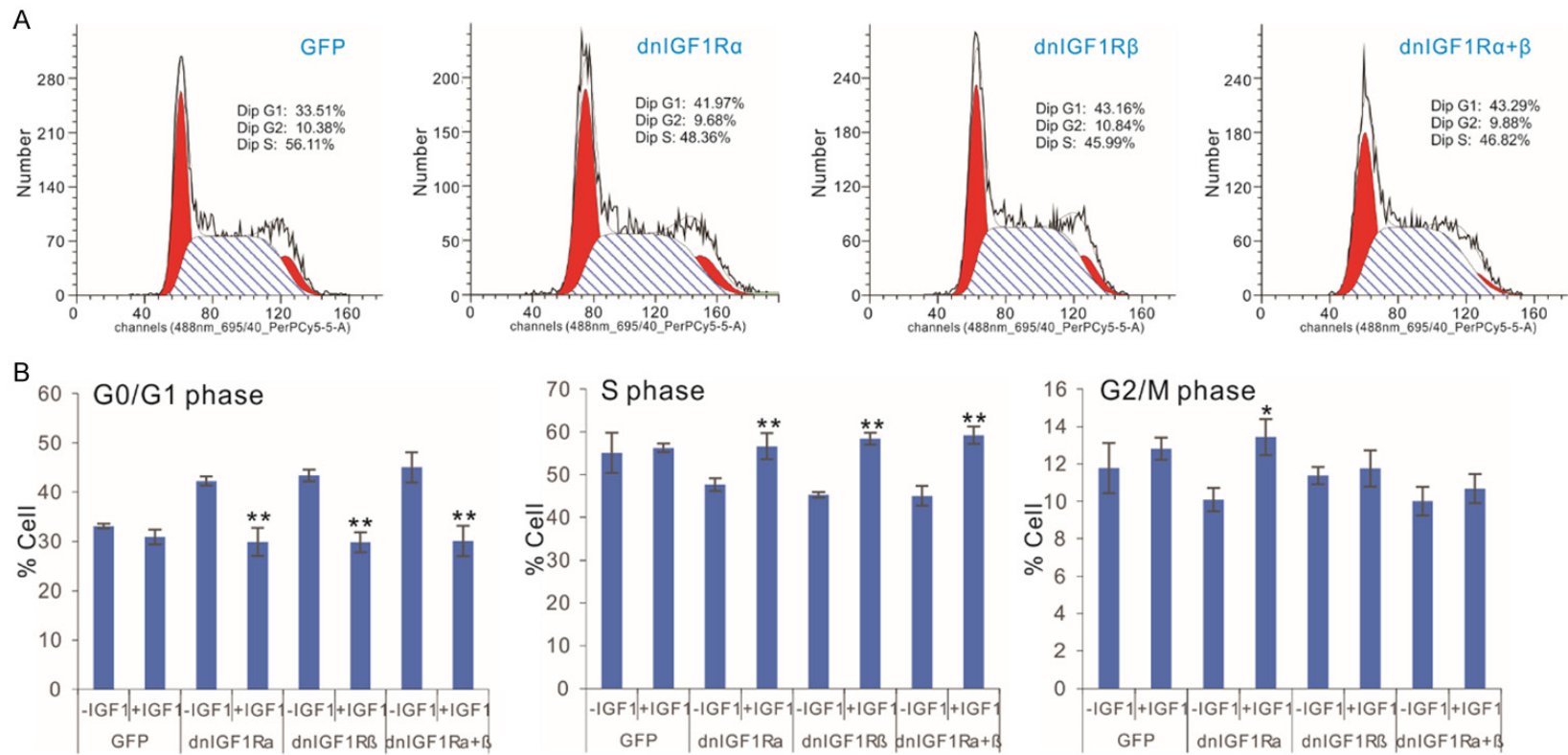


Figure 4. The dominant-negative IGF-1R (dnIGF1R) mutants inhibit OS cell cycle progression by increasing G0/G1 phase and reducing S phase. A. Subconfluent 143B cells were infected with the indicated adenoviral vectors for 48 h and subjected to FACS analysis. The assays were done in triplicate, and representative results are shown. B. Subconfluent 143B cells were infected with the indicated adenoviral vectors and cultured with or without IGF1 (30 ng/mL). At 48 h post infection, the cells were collected and subjected to FACS analysis. “***”, P<0.01 compared with that of the -IGF1 group’s.

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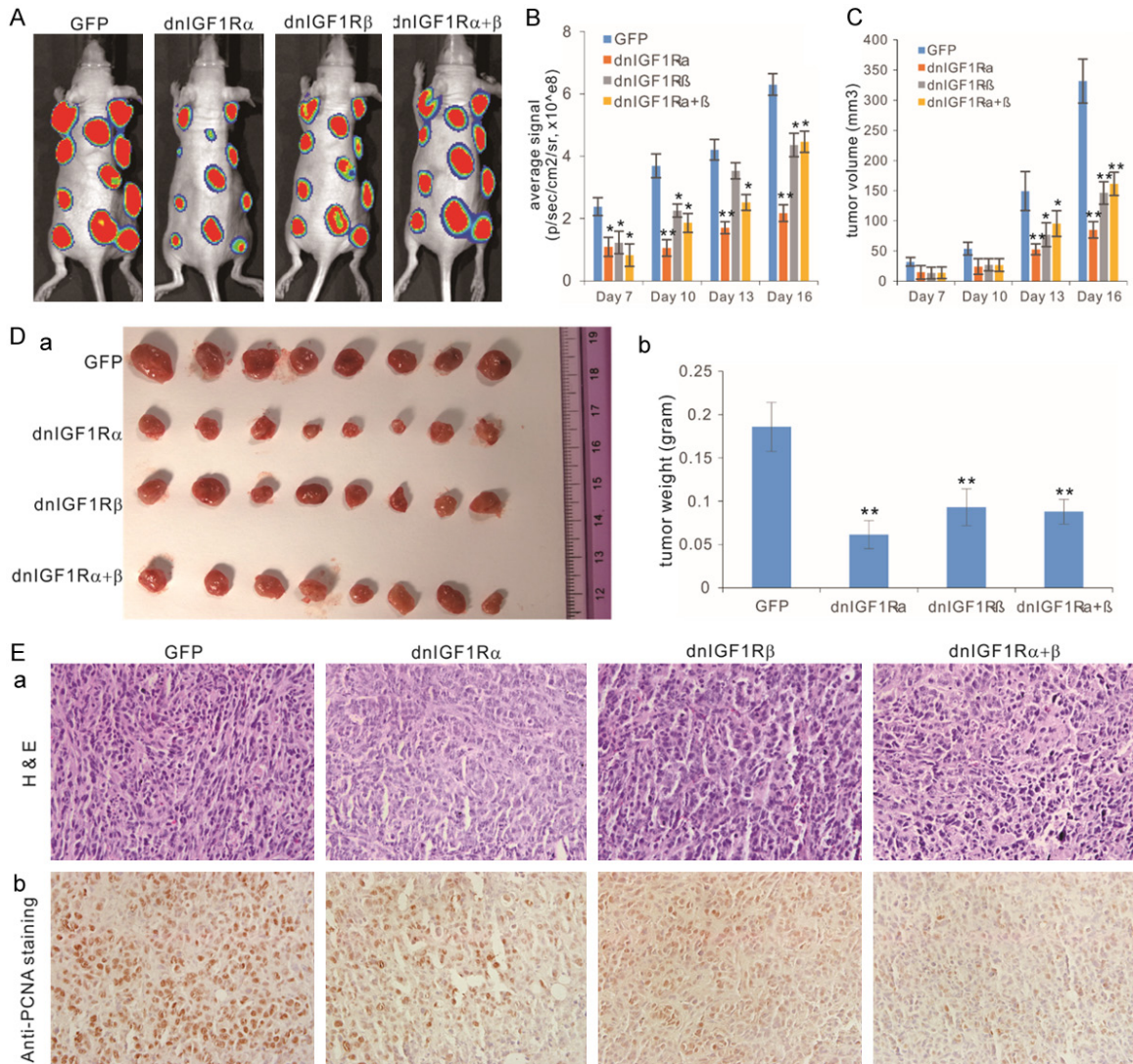


Figure 5. The dominant-negative IGF-1R (dnIGF1R) mutants suppress tumor growth in the xenograft tumor model of human OS. (A) Subconfluent Firefly luciferase-tagged 143B cells were infected with the indicated adenoviral vectors for 24 h, and collected for subcutaneous injection into the flanks of athymic nude mice. The mice were subjected to Xenogen imaging at days 7, 10, 13 and 16. The representative results for day 16 are shown. (B) Quantitative analysis of the average signal from the xenograft imaging. “*”, $P < 0.05$, “***”, $P < 0.01$, compared with that of the GFP group’s. (C) Tumor volumes monitored with clipper measurement. “*”, $P < 0.05$, “***”, $P < 0.01$, compared with that of the GFP group’s. (D) Gross images of the retrieved tumor masses. Representative images of the retrieved tumor masses (a) and average tumor weight of the retrieved masses (b). (E) Histologic and immunohistochemical staining. The retrieved tumor samples were paraffin-embedded and subjected to H & E staining (a) and immunohistochemical staining using a PCNA antibody (b). Minus primary antibody and control IgG were used as negative controls (data not shown). Representative images are shown.

ed with AdR-dnIGF1R α , AdR-dnIGF1R β , AdR-dnIGF1R α + β , or AdGFP for 48 h, qPCR analysis revealed that the expression of PI3KCA, AKT1, BRAF, KRAS, NRAS, ERK1, and MEK1 was significantly inhibited in the OS cells infected with AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β , although the decreased expression of MTOR was less pronounced, compared with that infected with AdGFP (Figure 6A). These

results indicated the dnIGF1R mutants can effectively suppress important downstream mediators of the IGF-1R pathway, including PI3K/AKT and RAS/RAF/MAPK signaling pathways.

We further examined the effect of the dnIGF1R mutants on apoptosis and cell cycle regulators. The qPCR results revealed that the expression

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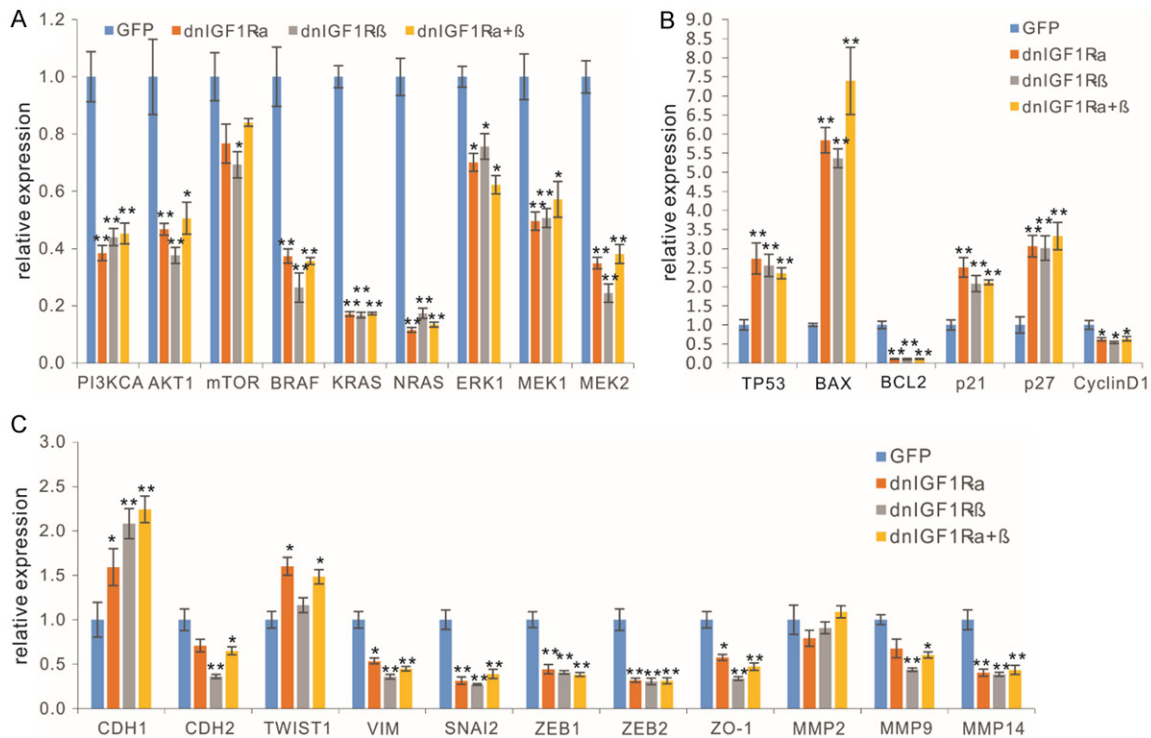


Figure 6. The dominant-negative IGF-1R (dnIGF1R) mutants effectively inhibit the downstream mediators of the IGF-1R signaling pathway in OS cells. Subconfluent 143B cells were infected with the indicated adenoviral vectors for 48 h. Total RNA was isolated and subjected to qPCR analyses of the IGF-1R signal downstream mediators (A), the apoptosis and cell cycle related genes (B), and EMT related genes (C). “*”, $P < 0.05$, “**”, $P < 0.01$, compared with that of the GFP group’s.

of pro-apoptotic regulators and/or cell cycle inhibitors TP53, BAX, p21 and p27 was significantly up-regulated in the OS cells infected with AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β , while anti-apoptotic factor BCL2 and cell cycle promoter Cyclin D1 were down-regulated in the OS cells infected with AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β (**Figure 6B**). We also analyzed the effect of the dnIGF1R mutants on epithelial-mesenchymal transition (EMT) regulators and found that AdR-dnIGF1R α , AdR-dnIGF1R β , or AdR-dnIGF1R α + β inhibited the expression of EMT regulators including CDH2, VIM, SNAI2, ZEB1, ZEB2, ZO-1, MMP9 and MMP14, while up-regulating CDH1, although MMP2 expression was not affected and TWIST1 was slightly upregulated by AdR-dnIGF1R α , or AdR-dnIGF1R α + β , compared with that of the GFP group (**Figure 6C**). Collectively, these results demonstrate that the dnIGF1R mutants can effectively up-regulate the expression of pro-apoptotic regulators while suppressing the expression of anti-apoptotic BCL2, Cyclin D1, and most of the EMT regulators in human OS cells.

Discussion

Since aberrant activation of the IGF/IGF-1R axis has been associated with many pathogenic processes including cancer, this signal axis has long been targeted for therapeutic intervention [32]. IGF1R was indeed the first tyrosine kinase receptor to be targeted by a monoclonal antibody in preclinical studies, which was shown to inhibit breast cancer cell proliferation *in vitro* and xenograft tumor growth in nude mice [98]. Several anti-IGF-1R monoclonal antibodies that bind to the IGF1R α subunit and neutralize IGF binding have been evaluated in clinical trials in cancer patients [32]. However, some of these antibodies bind IGF1R: INSR-A/B hybrid receptors, causing dose-limiting hyperglycemia in patients treated with IGF-1R antibody, or other cases causing endocrine response to IGF-1R blockade and hepatic secretion of growth hormone and IGFbPs that can impair glucose tolerance [32].

Small molecule tyrosine kinase inhibitors (TKIs) of IGF1R represent another major strategy to

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inhibit IGF1R kinase activity [32, 99]. While numerous experimental IGF1R TKIs, such as OSI-906, have shown strong anti-proliferative effects in preclinical studies [99], very few have undergone clinical evaluation and results have been disappointing [32]. A major obstacle is to overcome high levels of sequence homology (~85%) between IGF-1R and INSR-A/B kinase domains, which contain 100% identity in the ATP-binding cleft [100]. Therefore, ATP-competitive IGF1R TKIs also inhibit INSR [101], leading to impairment of metabolic insulin signaling and hyperinsulinemia and dose-limiting hyperglycemia disappointing [32]. Non-ATP competitive IGF1R inhibitors, such as AXL1717 and INSM-18, may overcome the above adverse effects and inhibit IGF-1R without INSR blockade [32, 102]. Thus, some of this class of inhibitors have been moved to various stages of clinical trials.

Interestingly, IGF binding proteins (IGFBPs) are a family of naturally occurring IGF inhibitors and have been identified as a focus for drug development. Recombinant human IGFBP3 (rhIGFBP3) was shown to have anticancer activity *in vitro* and *in vivo* [103]. We previously demonstrated IGFBP5 is a critical inhibitor of OS tumorigenesis and metastasis [36]. IGFBP5 expression was also decreased in metastatic OS lesions when compared to primary tumors in patient samples [35]. Furthermore, we found that IGFBP5 acted as a potent tumor suppressor, and over-expression of IGFBP5 inhibited OS cell proliferation, migration and invasion *in vitro*; while inducing apoptosis while siRNA knockdown of IGFBP5 promoted OS growth and lung metastasis *in vivo* [36, 37]. While it is not clear whether IGFBPs have comparable or more potent inhibitory effect than that of dnIGF1R mutant's, it is conceivable that a combination of IGFBPs and dnIGF1R mutants may act synergistically in blocking IGF signaling in cancer cells. An alternative therapeutic strategy is to specifically target the IGF ligands with neutralizing antibodies, such as Dusigitumab (MEDI-573, Medimmune) and xentuzumab (BI 836845), both of which entered clinical trials [104, 105].

Previously, dnIGF1Rs expressing IGF1R residues 1~486, 1~482, 1~950, and 1~976, were constructed and shown to induce apoptosis and inhibit xenograft tumor growth in breast

cancer, lung cancer and gastric cancer [106-108]. Furthermore, an IGF-TRAP was generated by fusing the IGF1R extracellular domain (aa 1-933) to the Fc region of human IgG₁, which was shown to bind IGF ligands and suppress growth of breast cancer xenografts and colon and lung cancer liver metastases *in vivo* [109]. However, the Fc-fusion protein tends to form high molecular weight complexes due to the propensity of cysteine to form disulfides between Fc fragments, and there are currently no IGF-TRAPS in clinical use. In this study, unlike the previous reports we engineered two distinct dominant-negative IGF1R mutants that were derived from the α (aa 1-524) and β (aa 741-936) subunits, respectively, and demonstrated the dnIGF1R mutants could effectively suppress OS cell proliferation and tumor growth *in vitro* and *in vivo*. Our reported work was the first to demonstrate that the 196-aa fragment containing the extracellular domain of the IGF1R β subunit is sufficient and effective to block IGF1R signaling activity. It is conceivable our dnIGF1R mutants, in particular dnIGF1R β , provide more specific interactions with IGF ligands and thus minimize potential interference with insulin functions.

In summary, in order to overcome the lack of specificity and/or limited efficacy of the current IGF1R targeting strategies, we engineered two dominant-negative IGF1R mutants dnIGF1R α and dnIGF1R β , and demonstrated that these soluble decoy receptors could specifically suppress IGF signaling in human OS cells in a dominant-negative inhibition fashion. Specifically, we found that the exogenous expression of either dnIGF1R α and/or dnIGF1R β effectively inhibited cell migration, colony formation, and cell cycle progression of human OS cells, which could be relieved by exogenous IGF1 protein. Furthermore, exogenous expression of dnIGF1R α and/or dnIGF1R β , especially dnIGF1R α alone, significantly inhibited OS xenograft tumor growth *in vivo*. Mechanistically, the dnIGF1R mutants were shown effectively down-regulate PI3K/AKT and RAS/RAF/MAPK signaling pathways, as well as the expression of BCL2, Cyclin D1 and most EMT regulators, while up-regulate the expression of pro-apoptotic genes in human OS cells. Therefore, these findings strongly suggest that these dominant-negative IGF1R mutants, in particular dnIGF1R α , may be further developed as novel anticancer agents

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in the clinical arena that target IGF signaling pathway with high specificity and efficacy.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. List of Oligonucleotides Used in the Study

Gene	Oligo Sequence	Use
human dnIGF1R- α mutant	tccGGATCCaccatgggcATGAAGTCTGGCTCCGGAGGAGGGTC aagaagcttTTAGGTGAAGCTGATGAGATCCCTGTAG	cloning
Human dnIGF1R- β mutant	cgcggggcccgGATGTCATGCAAGTGGCCAACACCACCATG gtcgtcgacTTAGATGATCAGATGGATGAAGTTTTTCATATC	
dnIGF1R- α CDS	CTGACAACGACACGGCCT CTCTCGGCGCTGAGGATG	TqPCR
dnIGF1R- β CDS	AGCCGAAGCAGGAACACC TCAGCCTCGTGGTTGCAG	
PI3KCA	CCCCTCCATCAACTTCTTCA CGGTTGCCTACTGGTTCAAT	
AKT1	AGAAGCAGGAGGAGGAGGAG TCTCCTCACCAGGATCACC	
MTOR	CCAGGCGGAGAACTTGCA TGCGCAGGAAAGGCATGA	
Cyclin D1	TGTTTGCAAGCAGGACTTTG TGGCACAAAGGATTCTTAA	
p27	AGATGTCAAACGTGCGAGTG TCTCTGCAGTGCTTCTCAA	
p21	CACAGGCCCCCTTGAGTG GGGAGGGATGGGGTGGAT	
KRAS	TGTGGTAGTTGGAGCTGGTG TGACCTGCTGTGTCGAGAAT	
NRAS	GACTCGTGGTTCGGAGGC ACCAAGGAGCGGCACTTC	
BRAF	TAAGATGGCGGCGCTGAG CTCCGGAATGGCAGGGTC	
MEK1/MAP2K1	TGCAGTTGGCTCTGCTC AGGAGGCCAAAAGCGAC	
MEK1/MAP2K2	CGCTCCTACATGGCTCCG TCCAGCTCTTTGGCGTCG	
ERK1	CCAGACCATGATCACACAGG CTGAAAAGATGGCCTGTTA	
GAPDH	CAACGAATTTGGCTACAGCA AGGGGAGATTCAGTGTGGTG	
TP53	GGCCCACTTCACCGTACTAA GTGGTTTCAAGCCAGATGT	
BAX	GCCCCACTAACTGTTGCATT AGACTGCAGTGAGCCAAGGT	
BCL2	GGATGCCTTTGTGGAAGTGT AGCCTGCAGCTTTGTTTCAT	
CDH1	AATGAAGCCCCATCTTTG CAGCCAGTTGGCAGTGTCT	
CDH2	GTGCATGAAGGACAGCCTCT GCCACTTGCCACTTTTCCTG	
TWIST1	GAAAGGAAAGGCATCACTATGG AGACACCGGATCTATTTGCATT	
VIM	TGCTCAATGTTAAGATGGCCCT	

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SNAI2	TTCAAGGCATCGTGATGCTGA CATCTTTGGGGCGAGTGAGT GGCCAGCCCAGAAAAAGTTG
ZEB1	CTTGAGCAGCCTAGCCAACT GGAGTGGAGGAGGCTGAGTA
ZEB2	CTTTTGCCCAACTGCTGACC ATCTGTCCCTGGCTTGTGTG
ZO-1	GAACGAGGCATCATCCCTAA GAGCGGACAAATCCTCTCTG
MMP2	CTTCCGTCTGTCCCAGGAT CTGAGCGATGCCATCAAAT
MMP9	CCCGGACCAAGGATACAGT GCCATTACGTCGTCCTTA
MMP14	CCTGCTGGACAAGGTCTGA GCCAGGGGACAGAGAGAAG
