Insulin-like growth factor 2 promotes the adipogenesis of hemangioma-derived stem cells

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Abstract. Infantile hemangioma (IH), which is the most common tumor in infants, is characterized by rapid proliferation followed by spontaneous regression into fibro-fatty tissue in childhood. However, its specific mechanism has not been clarified. Our previous studies showed that insulin-like growth factor 2 (IGF-2) is increased in the proliferative phase of IH, which is deemed to form from hemangioma-derived stem cells (HemSC). However, it remains unclear whether IGF-2 can promote the adipogenic differentiation of HemSCs and the signaling mechanisms involved require further elucidation. In the present study, CCK-8 assay was used to detect the effect of different concentrations of IGF-2 on the proliferation of HemSCs. Immunohistochemistry was applied to observe the expression of IGF-2 and its receptors in cells. Oil red o-staining of adipogenesis was conducted after cells recevied no treatment or were induced with IGF-2 or IGF-2 plus OSI-906 for 10 days. Cells were cultured in EGM-2/FBS-10% alone or containing IGF-2, IGF-2 plus OSI-906 or IGF-2 plus LY294002 and the protein expression of C/EBPα, C/EBPβ, PPARγ, adiponectin, p-AKT and total AKT was determined using western blot analysis. In another experiment, cells were treated with 25, 50 or 100 μM propranolol, or vehicle. C/EBPα, C/EBPβ, PPARγ and IGF-2 were analyzed using western blot analysis or reverse transcription-quantitative polymerase chain reaction. Results indicated that IGF-2 significantly promoted the cell proliferation and lipid accumulation of HemSCs. The expression of phosphorylated AKT (p-AKT), C/EBPα, C/EBPβ, PPARγ and adiponectin was increased in IGF-2-treated HemSCs culture, whereas these changes were repressed by the inhibition of either the IGF-1 receptor (IGF-1R) or phosphoinositide 3-kinase (PI3K). Our previous research showed that propranolol accelerated adipogenesis in HemSCs and induced the upregulation of IGF-2. The results of the present study indicate that IGF-2 is able to accelerate adipogenesis, and the propranolol-induced promotion of dysregulated adipogenesis may be mediated by the IGF-2 via IGF-1R and PI3K pathways.

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Introduction

Infantile hemangioma (IH), which is a unique vascular tumor that recapitulates both the formation as well as the spontaneous disappearance of blood vessels, is a type of benign tumor with high incidence in infants (1,2). Most IHs finally involute and resolve without intervention during childhood. The lesions that result from the atrophy of IHs can form permanent residual changes, including scarring and fibro-fatty residuum (3). Currently, the leading candidate for the cellular origin of IHs is hemangioma stem cells (HemSC), which have been demonstrated to be highly proliferative in culture and capable of differentiating into multiple cell types. including endothelial cells, adipocytes, and osteocytes. Thus, it is of considerable interest to investigate the mechanism of adipogenesis in HemSCs.

IGF-2 is a small but powerful polypeptide that has been shown to have several biological functions and play an important role in regulating tumor growth (4). As an endogenous regulator, the insulin-like growth factor-2 (IGF-2) could sustain the nature of embryonic stem cells (5). Several studies also have demonstrated that the expression of IGF-2 was markedly upregulated during adipogenesis in cells (6-8). Moreover, recent studies have shown that IGF-2 was highly expressed during the proliferating phase of hemangiomas (9-11), implicating the potential role of IGF-2 in HemSCs activity. IGF-2 binds to three kinds of receptors: type-1 IGF receptor (IGF-1R), type-2 IGF receptor (IGF-2R), and the insulin receptor (IR). The activation of each receptor by the ligand results in different biological effects (4). The insulin receptor

is pivotal in the maintenance of glucose homeostasis, and the IGF-1R regulates cellular proliferation, differentiation, migration, and protection from apoptosis (12). Although IGFR-2R has no intrinsic catalytic activity, it has been postulated that it may serve as a membrane-bound IGF-binding protein that inactivates IGF-2 (13). Most biological outcomes of IGF-2 have been attributed to its interaction with IGF-1R or IR (14). Several other studies have confirmed that the phosphoinositide 3-kinase (PI3K)/AKT branch of the IGF-1R signaling pathway is a significant influence on adipocyte differentiation (15,16). These previous findings raise the following questions: What role does IGF-2 play in the adipogenesis of HemSCs? It is modulated by IGF-1R or PI3K/AKT signaling?

In the present study, we investigated the hypothesis that IGF-2 affects the proliferation and adipogenic differentiation of HemSCs through IGF-related pathways. In recent years, propranolol has been used as a first-line therapy in many cases. Despite the widespread use of this effective therapy, its mechanism of action in IHs is not yet understood. However, previous studies have shown that propranolol could accelerate adipogenesis in HemSCs. Thus, we hypothesized that propranolol-induced promotion of the dysregulated adipogenesis may be mediated by IGF-2 via IGF-1R/PI3K/AKT pathway. The findings of the present study may provide novel insights into the specific mechanism of propranolol's effects on IH.

Materials and methods

Preparation of hemangioma specimens. The Ethics Committee of the Second Affiliated Hospital of Anhui Medical University (PJbb2017-004) approved the collection of abscized human hemangiomas. The tissues were used instantly for cell isolation and cultivation in *in vitro* experiments.

Isolation and identification of HemSCs. The proliferating IH tissues resected from the patients were immediately immersed in a growth medium at 4˚C [Dulbecco's modified Eagle's medium, high glucose, 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), and 1% penicillin-streptomycin (PS; Beyotime Institute of Biotechnology, Haimen, China)] and then quickly taken to our laboratory. The fatty and skin tissue were resected, the samples were rinsed three times in phosphate-buffered saline (PBS) and then minced. A 0.2% compound of collagenase (cat. no. 17454; Serva Electrophoresis GmbH, Heidelberg, Germany) was used to digest the samples at 37˚C for 2 h until they were chylous. They were then filtered through a 100-micron cell strainer. From this single cell suspension, cells expressing CD133 were selected using a magnetic beads technology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and then cultured on fibronectin-coated plates (Corning Incorporated, Corning, NY, USA) in EGM-2 media (cat. no. CC-4176; Lonza Group, Ltd., Basel, Switzerland) that was supplemented by 20% FBS and 1% PS.

Proliferation assay. Logarithmic growth phase HemSCs were seeded into 96-well tissue culture plates (Corning Incorporated) at an initial density of $2x10^3$. After serum starvation for 24 h, IGF-2 (cat. no. 100-12-50UG; PeproTech, Inc., Rocky Hill, NJ, USA) at 0, 10, 20, 100, and 200 ng/ml, respectively, was added to EBM-2 containing 5% FBS in the experimental groups. After 72 h in the culture, CCK-8 reagents (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) were added to disparate wells under different treatments. The absorbency was read at 490 nm using a microplate reader (Biotek ELx 800; BioTek Instruments, Inc., Winooski, VT, USA).

To examine the growth kinetics of IGF-2-treated HemSCs, HemSCs were seeded into 96-well plates (Corning Incorporated) at a density of $1.5x10^3$ cells/well. After serum starvation, the cells were treated with 100 ng/ml IGF-2 containing 5% FBS. CCK-8 was added at 0, 1, 3, 5, and 7 days after culture and incubated at 37˚C for 4 h. The results of the OD values at 490 nm were tested by a microplate reader (Biotek Elx 800; BioTek Instruments, Inc.).

Immunohistochemistry. When the cells had increased by 70% in 24 well plates, they were washed in PBS followed by fixing in 4% poly-formaldehyde for 15 min, drying in air for 5 min, and permeabilizing in 0.5% Triton X-100 for 10 min. Endogenous peroxidase activity was inactivated using 3% hydrogen peroxide for 5-10 min at room temperature. The slides were incubated in blocking solution (reagent A) for 15-20 min, and then the blocking solution was out-welled without washing. Between each step, the cells were washed in PBS, the slides were incubated overnight at 4˚C, and the primary antibodies were diluted by PBS to achieve the required concentration. The primary antibodies were replaced by PBS in the negative control group. The secondary antibodies were incubated for 10-15 min at 37˚C. The cells were observed under a bright-field microscope for 3-10 min immediately after the DAB solution (cat. no. K166724B; 50 μl DAB: 1 ml DAB substrate, kept away from light) was added to the slides, which underwent hematoxylin staining for 0.5-1 min and gum seal after drying at room temperature. The cells were photographed using bright-field microscope illumination (Olympus IX71; Olympus Corporation, Tokyo, Japan).

Oil red O-staining. To evaluate the adipogenic differentiation of IGF-2-treated HemSC, $5.0x10^4$ cells were seeded on 6-well plates in EGM-2/10% FBS. When the cells were oversaturated, the original medium was changed to adipogenic differentiation media (cat. no. HUXMF-90031; Cyagen Biosciences, Santa Clara, CA, USA) with IGF-2 (100 ng/ml), IGF-2 (100 ng/ml) plus OSI-906 (cat. no. S1091; 1 μM; Selleck Chemicals, Houston, TX, USA), or no treatment for 10 days. The oil red o-stained cells were observed using an inverted microscope. Photos were taken using the Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan).

Western blot analysis. HemSCs were grown on 10-cm tissue culture plates (Corning Incorporated). The cells were cultured in EGM-2/FBS-10% containing IGF-2 (100 ng/ml), IGF-2 (100 ng/ml) plus OSI-906 (1 μM), IGF-2 (100 ng/ml) plus LY294002 (cat. no. HY-10108; 10 μM; MedChem Express Co., Ltd., Shanghai, China), or no treatment. Protein extracts were subjected to SDS-PAGE on 8-15% polyacrylamide gels. The samples were then transferred to PVDF membranes. The membranes were blocked by 5% non-fat milk for 2 h and then incubated first with primary antibodies overnight at 4˚C and then with secondary antibodies. The following primary antibodies

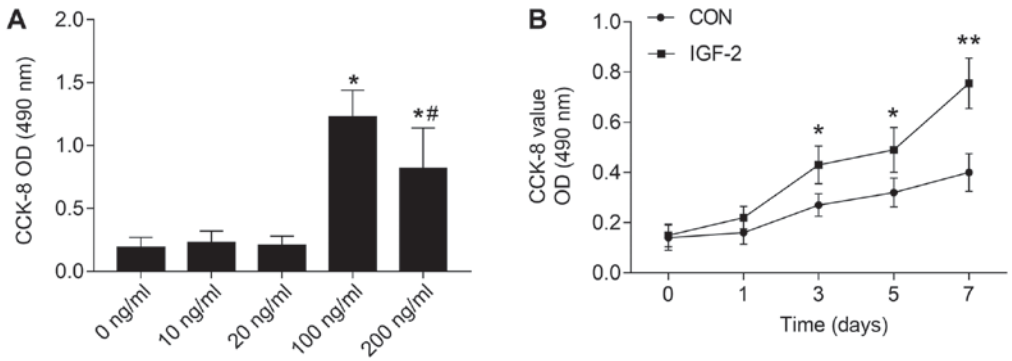

Figure 1. IGF-2 stimulated the proliferation of HemSCs *in vitro*. (A) IGF-2 promoted the proliferation of HemSCs *in vitro*; IGF-2 at 100 and 200 ng/ml significantly promoted the proliferation of HemSCs as compared with the control group, and IGF-2 at 100 ng/ml exhibited the highest proliferation activity among all groups. (B) Growth curves of IGF-2 treated HemSCs. The proliferation of IGF-2-treated HemSCs (days 1-7) was promoted compared with the untreated group. Values are mean ± SD, n=3; *P<0.05 and **P<0.01 vs. Control group; $^{\#}$P<0.05 vs. 100 ng/ml. IGF-2, insulin-like growth factor-2; HemSC, hemangioma stem cell.

Table I. Primers.

Gene	Primers (5'-3')
PPARγ (F)	TCTCCAGCATTCTACTCCACA
PPARγ (R)	CAGGCTCCACTTTGATTGC
C/EBPα (F)	TGGACAAGAACAGCAACGAG
C/EBPα (R)	TTGTCACTGGTCAGCTCCAG
C/EBPβ (F)	TTTCGAAGTTGATGCAATCG
C/EBPβ (R)	CAACAAGCCCGTAGGAACAT
β-actin (F)	CTG GAACGGTGA AGGTGACA
β-actin (R)	AAGGGACTTCCTGTAACAATGCA

F, forward; R, reverse.

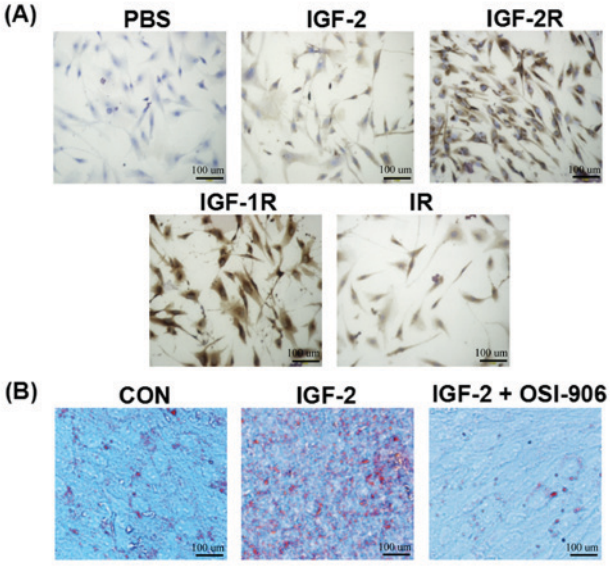

Figure 2. Expressions of IGF-2 and its receptors in HemSCs and the effect of IGF-2 on the adipogenesis of HemSCs. (A) Immunohistochemistry of HemSCs using antibodies specific to IGF-2, IGF-2R, IGF-1R, and IR. (B) IGF-2 induced differentiation of HemSCs into adipocytes. HemSCs that were cultivated for 10 days in adipogenic differentiation media; adipogenic differentiation media with IGF-2 (100 ng/ml); adipogenic differentiation media with IGF-2 (100 ng/ml) plus OSI-906 (1 μM). After Oil Red O-staining, phase-contrast microscopy was used to observe lipid droplets. Scale bar, 100 μm. IGF-2, insulin-like growth factor-2; HemSC, hemangioma stem cell; IGF-1R, IGF-1 receptor; IR, insulin receptor.

were used: Anti-PPARγ (cat. no. bs-0530P), anti-C/EBPα (cat. no. bs-1630R), anti-C/EBPβ (cat. no. bs-1396R), anti-adiponectin (cat. no. 0471R; all from BIOSS, Beijing, China), and anti-pAKT (cat. no. 9271), anti-AKT (cat. no. 9272), anti-IGF-2 (cat. no. 25690), and anti-β-actin (cat. no. 3700; Cell Signaling Technology, Inc., Danvers, MA, USA).

Cell culture and treatment with propranolol. HemSCs were maintained on fibronectin-coated 10-cm plates in EGM-2/FBS-20%. The medium was changed every two days. HemSCs at passage numbers 5 to 15 were used in all experiments. During the media changes, propranolol hydrochloride (cat. no. P8688; Sigma-Aldrich, St. Louis, MO, USA) was added to the culture media at the indicated concentrations.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cDNA was synthesized by the reverse transcription of 2 μg of total RNA using a PrimeScript™ RT reagent (Takara, Dalian, China). A semi-quantitative real-time polymerase chain reaction was conducted using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the SYBR Premix Ex TaqTM (Takara). The results were normalized to β-actin expression levels, and the samples were analyzed in triplicate. The gene expression levels of C/EBPα, C/EBPβ and PPARγ were then determined (Table I).

Statistical analyses. The results of two independent experiments were expressed as the mean ± SD (n=3 for each experiment). In comparing the two groups, an unpaired Student's t-test was used to assess the differences, and analysis of variance was applied for the analysis of the mean values among multiple groups. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

IGF-2 stimulated cell proliferation in HemSCs. A CCK-8 assay was performed to determine whether IGF-2 influenced the proliferation of HemSCs *in vitro*. The results showed that 10-200 ng/ml of IGF-2 promoted the proliferation of HemSCs. The concentration at 100 ng/ml was the most appropriate among all the groups (Fig. 1A). When 100 ng/ml IGF-2 was used for the cell growth curve, the IGF-2-treated cells promoted increased cell proliferation compared to the non-treatment group from day 1 to day 7 (Fig. 1B).

The expression of IGF-2 and its different receptors in HemSCs. As shown in Fig. 2A, the expression of IGF-2, IGF-2R, IGF-1R, and IR was positive in the HemSCs. In cell localization, the dyeing of IGF-2 was primarily in the cytoplasm. IGF-2R, IGF-1R, and IR were in both the membrane and the cytoplasm.

IGF-2 enhanced lipogenesis during differentiation of HemSCs into adipocytes. In order to determine whether the induction of IGF-2 accelerated the differentiation of adipocytes, HemSCs was exposed to IGF-2 (100 ng/ml), IGF-2 (100 ng/ml) plus OSI-906 (1 μM), or no treatment for 10 days in adipogenic differentiation media (Cyagen Biosciences). Phase-contrast microscopy was used after oil red o-staining. The results indicated that IGF-2 treatment stimulated the accumulation of lipogenesis in the HemSCs, which was shown by the increase in cell numbers and the density of lipid droplets in the HemSCs treated with adipogenic differentiation media containing IGF-2 compared to the adipogenic differentiation media alone or the adipogenic differentiation media containing IGF-2 plus OSI-906 (1 μM) group (Fig. 2B). The results further confirmed the adipogenic effects of IGF-2 on HemSCs. The results also showed that the IGF-1R inhibitor (OSI-906, 1 μM) suppressed the IGF-2 inducing lipid accumulation.

IGF-2 enhanced adipogenic differentiation and AKT protein phosphorylation inhibited by OSI-906 or LY294002. We also aimed to determine whether IGF-2 could regulate the adipogenic differentiation of HemSCs into adipocytes via IGF receptors. The results of the western blot analysis showed that the expressions of disparate transcription factors and adipocyte specific proteins were increased in the IGF-2 treated group compared to the control group. C/EBPα, C/EBPβ, PPARγ, and adiponectin were increased, which indicated that IGF-2 enhanced adipogenesis in HemSCs. Interestingly, the IGF-2-treated medium in the presence of OSI-906 or LY294002 or LY294002 alone could repress the IGF-2-induced adipogenesis and inhibit the protein expressions of C/EBPα, C/EBPβ, PPARγ, and adiponectin (Fig. 3A). In addition, the p-AKT level was attenuated compared with that in the IGF-2 treated cells (Fig. 3B). These findings suggested that the IGF-2 mediated the induction of adipocyte differentiation by upregulating the phosphorylation of AKT via the IGF-1R-PI3K signaling pathway in the HemSCs.

Effects of propranolol on HemSCs. HemSCs were grown in EGM-2/20% FBS at vrious nanomolar concentrations of propranolol (0, 50, and 100 μM). During 24 h of adipogenic differentiation, the gene expression levels of C/EBPα, C/EBPβ (CCAAT/enhancer-binding protein) and PPAR (peroxisome proliferator-activated receptor) were observed. In the 50-μM propranolol solution, the C/EBPα RNA level was no changed compared with the control group, whereas it was increased after intervention in 100 μM propranolol solution compared to the control group. The RNA levels of C/EBPβ and PPARγ were significantly elevated compared with the control group after intervention with 50 and 100 μM of propranolol solution (Fig. 4B). The changes in the levels of protein expression of C/EBPα, C/EBPβ, and PPARγ were similar to those in RT-qPCR in the detection of the adipogenic differentiation index in HemSCs. However, no significant change in C/EBPα was found (Fig. 4A). Interestingly, several concentrations of propranolol-treated HemSCs showed marked increases in IGF-2 compared to the control group (Fig. 4C). Furthermore, IGF-2 may have been involved in this effect, which was shown by its increased levels following the treatment by pronanolol.

Discussion

Traditionally, IH has been regarded as a benign tumor of the microvasculature. The present findings showed that the cellular origin of IHs was HemSCs, which has the subject of extensive research in the biology of IH (1). In the present study, the spontaneous disappearance of IHs provided a new angle to explore the mechanisms controlling the phases of the proliferation and the involution of hemangiomas. Several previous studies attempted to confirm the molecules that stimulated the adipogenic differentiation of HemSCs to aid the development of drugs for the treatment of IH. However, whether IGF-2 promotes or inhibits the adipogenic differentiation of HemSCs *in vitro* remains unclear. In this study, we observed that IGF-2 significantly improved the proliferation of HemSCs in a dose-dependent manner. *In vitro*, the highly enhanced differentiation of HemSCs was derived from the proliferating IH tissues. Furthermore, the IGF-2-enhanced differentiation appeared to be mediated by IGF-1R through the activation of the PI3K-AKT signaling pathway in HemSCs.

IGF-2 is key in regulating tumor growth and in several biological functions (4). IGF-2 stimulated both the proliferation and the adipogenesis of cell populations derived from adult rat bone (6). We presupposed that IGF-2 might influence the proliferation and differentiation of HemSCs. Our research showed that IGF-2 significantly facilitated the proliferation of HemSCs in a dose-dependent manner, and it increased the lipid accumulation of HemSCs *in vitro*. The inhibition of IGF-1R by OSI-906 blocked the IGF-2-induced adipogenic differentiation of HemSCs. Adipogenesis is closely regulated by transcription factors including members of the PPARs and CCAAT/enhancer-binding protein (C/EBP) families (17). C/EBPs have diverse roles in the regulation of pre-adipocyte differentiation. Furthermore, in pre-adipocytes, C/EBPβ accelerates the induction rate of C/EBPα, which is an integral part of the genetic cascade that causes adipogenesis (18). C/EBPα is another transcription factor that is induced during adipocyte differentiation, which cooperates with PPARγ to stimulate the adipocyte process and, adiponectin promotes preadipocyte differentiation via the PPARγ pathway (19). The results of the present research demonstrated that IGF-2 played

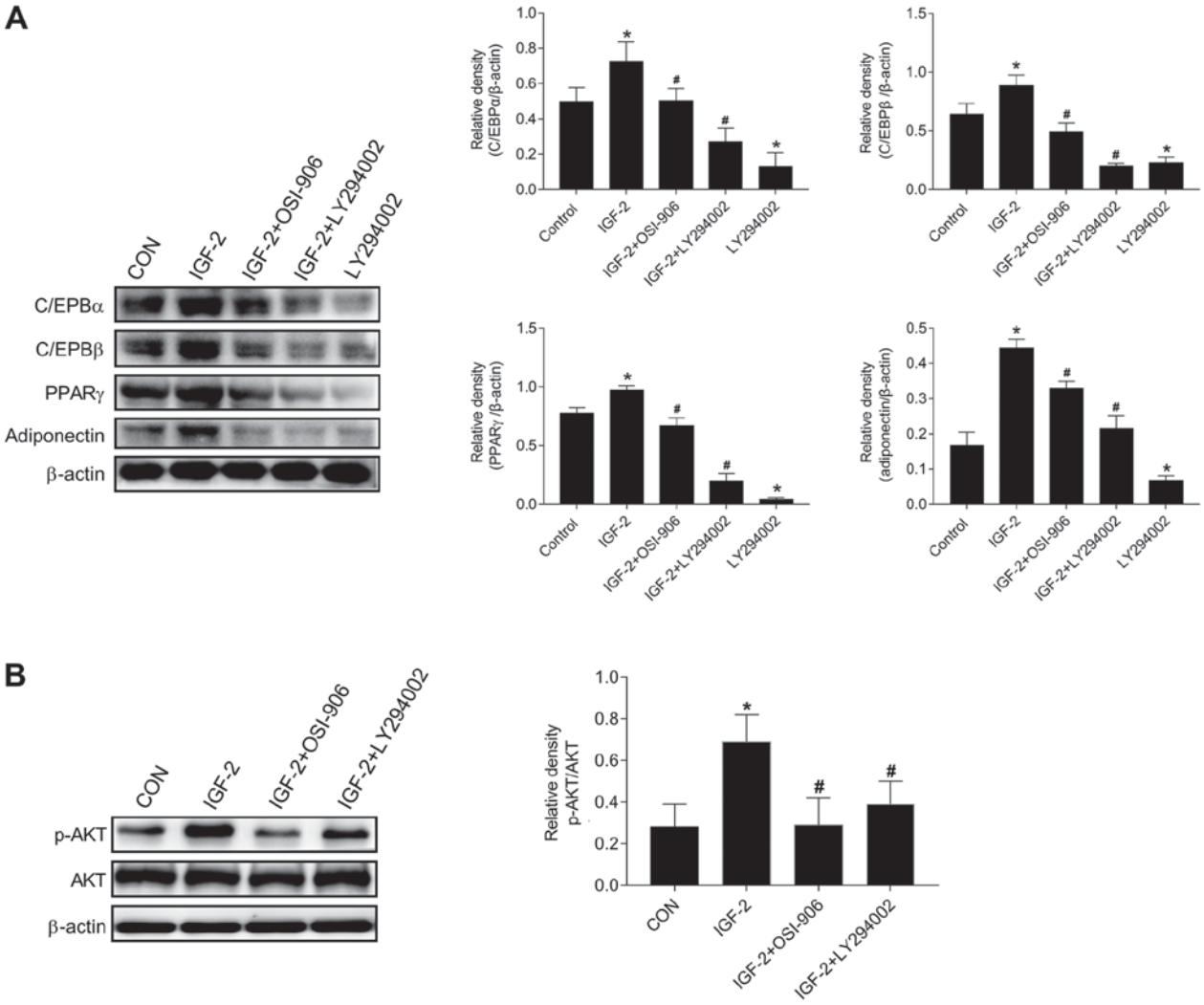

Figure 3. Effects of IGF-2 on the expression of adipogenic markers in HemSCs. The C/EBPα, C/EBPβ, PPAR, and adiponectin protein levels were analyzed by western blot analysis. (A) The results showing protein levels in untreated and treated HemSCs for C/EBPα, C/EBPβ, PPARγ, and adiponectin in IGF-2 (100 ng/ml), IGF-2 (100 ng/ml) with OSI-906 (1 μM), IGF-2 (100 ng/ml) with LY294002 (10 μM), and LY294002 (10 μM). β-actin was used as a loading control. (B) Western blot analysis showed p-AKT and total AKT as well as β-actin protein bands in confluent HemSCs cultures exposed to the indicated treatments for 1 h. Groups: No treatment; IGF-2 (100 ng/ml); IGF-2 (100 ng/ml) plus OSI-906 (1 μM); IGF-2 (100 ng/ml) plus LY294002 (10 μM). Quantification of the p-AKT protein levels showed an obvious increase in the IGF-2 group ($P<0.05$). No changes were detected in the total AKT protein levels among the groups ($P>0.05$). Values are mean ± SD. n=3, $^{*}P<0.05$ vs. Control group. $^{\#}P<0.05$ vs. IGF-2. IGF-2, insulin-like growth factor-2; HemSC, hemangioma stem cell; p-AKT, phosphorylated AKT.

an important role not only in the early-stage of adipogenesis but also in the later stage of lipid accumulation, which was indicated by the upregulated C/EBPα, C/EBPβ, PPARγ, and adiponectin protein levels of the IGF-2-HemSCs, which was confirmed by the results of oil red o-staining. IGF-2R functions as a type of decoy receptor via the binding and inactivation of IGF-2, which appeared to increase proliferation and stimulate differentiation in granulosa cells via the IGF-1R (20,21). Several studies confirmed that the PI3K/AKT branch of the IGF-1R signaling pathway influences adipocyte differentiation (15,16). In 3T3-L1 pre-adipocytes, the inhibition of PI3K by LY294002 restrained adipocyte differentiation (22). In this research, we found that treatment with the IGF-1R inhibitor-OSI-906 and PI3K inhibitor-LY294002 in combination with IGF-2 reduced the expression of C/EBPα, C/EBPβ, PPARγ, and adiponectin with compared with treatment with IGF-2 alone. The same changes were also observed in the LY294002 single-treated group compared to the group without IGF-2. Furthermore, the phosphorylation of AKT was promoted by the treatment with IGF-2, whereas it was inhibited by the treatment with OSI-906 or LY294002. These findings suggest that the effects of IGF-2 on adipocyte differentiation in HemSCs are through the IGF-1R/PI3K/AKT pathway.

Propranolol has proved to be efficacious in problematic IHs. Previous studies have found that propranolol changed HemSCs proliferation and differentiation into adipocytes (23). In basal media, we demonstrated that treatment with propranolol caused an obvious increase in transcripts in multiple pro-adipogenic genes in the absence of adipogenic stimulation. The RNA levels of PPARγ and C/EBPβ were significantly increased compared with the control group after intervention with 50 and 100 μM of propranolol solution. In contrast, C/EBPα was not changed by 50 μM of propranolol. These results were in accordance with Wong, Li, and England (23-25). The protein expression level of

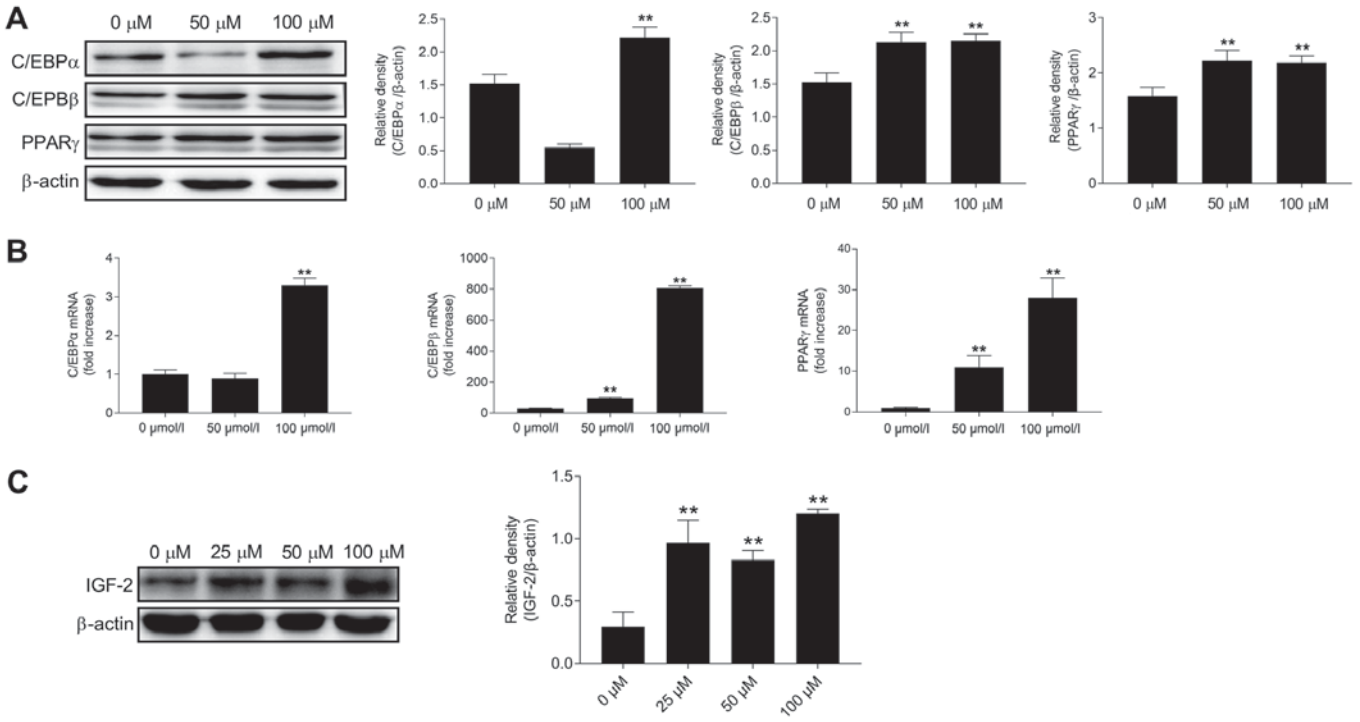

Figure 4. Analysis of the adipogenic gene and protein expression of propranolol-treated HemSCs. (A) Western blot analysis results of protein levels in propranolol-treated HemSCs for C/EBPα, C/EBPβ, and PPAR in 0, 50, and 100 μM. (B) Quantitative RT-PCR was performed to analyze the expression of C/EBPα, C/EBPβ, and PPAR. (C) Western blot analysis showed IGF-2 and β-actin protein bands in propranolol-treated HemSCs. Values are mean ± SD. n=3; **P<0.01 vs. Control group. HemSC, hemangioma stem cell; IGF-2, insulin-like growth factor-2.

C/EBPα, C/EBPβ and PPARγ were obviously enhanced in 100 μM of propranolol, but C/EBPα was not significantly altered in 50 μM group. The changes in the protein expression level of PPARγ, C/EBPα and C/EBPβ were similar to those of RT-qPCR in detection of adipogenic differentiation index in HemSCs. We speculated that the pro-adipogenic gene expression was perturbed during early adipogenesis (23,25). Elevated expressions of gene and protein levels of PPARγ were seen in later stages of adipogenesis during terminal differentiation (18). The gene level was increased in the propranolol treatment group, which explained the increase in lipogenesis observed in propranolol-treated HemSCs. This phenomenon was consistent with the results of Wong, A. (23).

The present study has the following limitations. The population of patients in this study was small. Although we found that propranolol enhanced adipogenesis in HemSCs, we did not explore this effect in an animal study to confirm that propranolol regulates IGF-2, thereby affecting HemSCs in adipogenesis.

Based on the results of this study, we conclude that IGF-2 could promote proliferation and adipogenic differentiation in HemSCs. Furthermore, IGF-2 could enhance the adipogenesis of HemSCs through the IGF-1R and PI3K pathways. Propranolol and IGF-2 could play an important role during adipogenic differentiation and may have clinical implications for the treatment of IH.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KZ, FW and JH were involved in drafting the manuscript and data analysis. KZ assisted with acquisition of data. KZ and YL performed and analyzed western blot analyses, RT-qPCR and IHC. JX, HL, DC and XH provided excellent technical assistance. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the second Hospital of Anhui Medical University. Written informed consents were signed by the patients and/or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Kilcline C and Frieden IJ: Infantile hemangiomas: How common are they? A systematic review of the medical literature. Pediatr Dermatol 25: 168-173, 2008.
2. Kleiman A, Keats EC, Chan NG and Khan ZA: Evolution of hemangioma endothelium. Exp Mol Pathol 93: 264-272, 2012.
3. Cheng CE and Friedlander SF: Infantile hemangiomas, complications and treatments. Semin Cutan Med Surg 35: 108-116, 2016.
4. Halje M, Nordin M, Bergman D and Engström W: Review: The effect of insulin-like growth factor II in the regulation of tumour cell growth in vitro and tumourigenesis in vivo. In Vivo 26: 519-526, 2012.
5. Bendall SC, Stewart MH, Menendez P, George D, Vijayaragavan K, Werbowetski-Ogilvie T, Ramos-Mejia V, Rouleau A, Yang J, Bossé M, *et al*: IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature 448: 1015-1021, 2007.
6. Jia D and Heersche JN: Insulin-like growth factor-1 and -2 stimulate osteoprogenitor proliferation and differentiation and adipocyte formation in cell populations derived from adult rat bone. Bone 27: 785-794, 2000.
7. Cha MH, Kim IC, Lee BH and Yoon Y: Baicalein inhibits adipocyte differentiation by enhancing COX-2 expression. J Med Food 9: 145-153, 2006.
8. Bellows CG, Jia D, Jia Y, Hassanloo A and Heersche JN: Different effects of insulin and insulin-like growth factors I and II on osteoprogenitors and adipocyte progenitors in fetal rat bone cell populations. Calcif Tissue Int 79: 57-65, 2006.
9. Picard A, Boscolo E, Khan ZA, Bartch TC, Mulliken JB, Vazquez MP and Bischoff J: IGF-2 and FLT-1/VEGF-R1 mRNA levels reveal distinctions and similarities between congenital and common infantile hemangioma. Pediatr Res 63: 263-267, 2008.
10. Ritter MR, Dorrell MI, Edmonds J, Friedlander SF and Friedlander M: Insulin-like growth factor 2 and potential regulators of hemangioma growth and involution identified by large-scale expression analysis. Proc Natl Acad Sci USA 99: 7455-7460, 2002.
11. Yu Y, Wylie-Sears J, Boscolo E, Mulliken JB and Bischoff J: Genomic imprinting of IGF2 is maintained in infantile hemangioma despite its high level of expression. Mol Med 10: 117-123, 2004.
12. Hopkins A, Crowe PJ and Yang JL: Effect of type 1 insulin-like growth factor receptor targeted therapy on chemotherapy in human cancer and the mechanisms involved. J Cancer Res Clin Oncol 136: 639-650, 2010.
13. Spicer LJ, Voge JL and Allen DT: Insulin-like growth factor-II stimulates steroidogenesis in cultured bovine thecal cells. Mol Cell Endocrinol 227: 1-7, 2004.
14. Byrd JC and MacDonald RG: Mechanisms for high affinity mannose 6-phosphate ligand binding to the insulin-like growth factor II/mannose 6-phosphate receptor. J Biol Chem 275: 18638-18646, 2000.
15. Kim SP, Ha JM, Yun SJ, Kim EK, Chung SW, Hong KW, Kim CD and Bae SS: Transcriptional activation of peroxisome proliferator-activated receptor-gamma requires activation of both protein kinase A and Akt during adipocyte differentiation. Biochem Biophys Res Commun 399: 55-59, 2010.
16. Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG and Hay N: Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev 17: 1352-1365, 2003.
17. Madsen MS, Siersbaek R, Boergesen M, Nielsen R and Mandrup S: Peroxisome proliferator-activated receptor γ and C/EBPα synergistically activate key metabolic adipocyte genes by assisted loading. Mol Cell Biol 34: 939-954, 2014.
18. Musri MM and Parrizas M: Epigenetic regulation of adipogenesis. Curr Opin Clin Nutr Metab Care 15: 342-349, 2012.
19. Choi SK, Park S, Jang S, Cho HH, Lee S, You S, Kim SH and Moon HS: Cascade regulation of PPARγ(2) and C/EBPα signaling pathways by celastrol impairs adipocyte differentiation and stimulates lipolysis in 3T3-L1 adipocytes. Metabolism 65: 646-654, 2016.
20. Adashi EY, Resnick CE and Rosenfeld RG: Insulin-like growth factor-I (IGF-I) and IGF-II hormonal action in cultured rat granulosa cells: Mediation via type I but not type II IGF receptors. Endocrinology 126: 216-222, 1990.
21. Spicer LJ and Aad PY: Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: Role of follicle-stimulating hormone and IGF2 receptor. Biol Reprod 77: 18-27, 2007.
22. Xu J and Liao K: Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. J Biol Chem 279: 35914-35922, 2004.
23. Wong A, Hardy KL, Kitajewski AM, Shawber CJ, Kitajewski JK and Wu JK: Propranolol accelerates adipogenesis in hemangioma stem cells and causes apoptosis of hemangioma endothelial cells. Plast Reconstr Surg 130: 1012-1021, 2012.
24. Li HX, Xiao L, Wang C, Gao JL and Zhai YG: Review: Epigenetic regulation of adipocyte differentiation and adipogenesis. J Zhejiang Univ Sci B 11: 784-791, 2010.
25. England RW, Hardy KL, Kitajewski AM, Wong A, Kitajewski JK, Shawber CJ and Wu JK: Propranolol promotes accelerated and dysregulated adipogenesis in hemangioma stem cells. Ann Plast Surg 73 (Suppl 1): S119-S124, 2014.