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Elevated IGF2 prevents leptin induction and terminal adipocyte differentiation in hemangioma stem cells

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ABSTRACT

Infantile hemangioma is a benign vascular tumor that exhibits a unique yet predictable lifecycle of rapid proliferation followed by spontaneous regression. Recent studies have identified that insulin-like growth factor-2 (IGF2), a fetal mitogen, is highly expressed during the proliferative phase of hemangioma growth. Since hemangiomas arise from CD133+ stem cells, high levels of IGF2 may regulate the activity of the stem cells and therefore, hemangioma growth. The aim of this study was to understand the functional significance of elevated IGF2 in hemangiomas. We show that IGF2 localizes to the CD133+ cells in hemangioma specimens. We, therefore, hypothesized that IGF2 may be regulating the plasticity of hemangioma stem cells. To test our hypothesis, we used CD133-selected cells from hemangiomas to knockdown the expression of IGF2. We found that IGF2 is a mitogen for hemangioma stem cells and prevents leptin induction and full terminal differentiation of hemangioma stem cells into adipocytes. We also show that IGF2 does not alter the initial commitment phase. These findings implicate an important role of IGF2 in expanding hemangioma stem cells and preventing terminal adipocyte differentiation.

Introduction

Infantile hemangioma is a unique vascular tumor that recapitulates both the formation as well as the spontaneous disappearance of blood vessels (Kleiman et al., 2012; Mulliken and Glowacki, 1982). Masses of endothelial cells mark the rapid growth phase. In the spontaneous involuting phase, endothelial hyperplasia diminishes and the blood vessels are replaced with a fibrofatty residuum (Kleiman et al., 2012; Mulliken and Glowacki, 1982). We have recently shown that hemangiomas arise from CD133+ stem cells (Khan et al., 2008). Specially, our findings illustrate that CD133+ hemangioma stem cells can be selected, cultured and implanted in a mouse model to produce Glut-1 positive microvessels, the same blood vessels seen in infantile hemangioma (North et al., 2001). These CD133+ hemangioma stem cells (hemSCs) also differentiate into several cellular lineages including adipocytes (Khan et al., 2008; Roach et al., 2012). The mechanisms that regulate self-renewal and proliferation of these hemSCs are still elusive. Recent studies have shown that the self-sustaining nature of embryonic stem cells is established via endogenous regulators such as fibroblast growth factors (FGF) and insulin-like growth factor-2 (IGF2) (Bendall et al., 2007). IGF2 has been reported to be highly expressed during the proliferating phase of hemangiomas (Picard et al., 2008; Ritter et al., 2002, 2003; Yu et al., 2004) implicating a role of IGF2 in hemSC activity.

IGF1 and IGF2 elicit their biological responses by interacting with two structurally dissimilar receptors. Type 1 IGF receptor (IGFR1) is structurally homologous to the insulin receptor, exhibiting 84% amino acid similarity (Hopkins et al., 2010). Despite their structural similarities, the two receptors have varying functions. The insulin receptor is pivotal in the maintenance of glucose homeostasis, while the IGFR1 regulates cellular proliferation, differentiation, migration and protection from apoptosis (Hopkins et al., 2010). IGF1 and at a reduced affinity IGF2, can both bind to IGFR1. When the ligand binds to the α subunit, it induces a cytoplasmic signal cascade resulting in receptor conformational change. This change allows for autophosphorylation of the β subunits and thus in the activation of tyrosine kinase activity of IGFR1 (Kasuga et al., 1982; LeRoith et al., 1995; Massague, 1985; Rubin et al., 1983). Unlike IGFR1 and insulin receptor, the IGF receptor 2 (IGFR2) is structurally distinct and is believed to have no intrinsic catalytic activity. This receptor is known to have a high binding affinity for IGF2 and mannose 6-phosphate (M6P)-containing ligands. At a reduced affinity it can also bind IGF1, but it does not bind insulin (El-Shewy and Luttrell, 2009). Upon binding of IGF2 to IGFR2, the ligand is internalized and degraded. This mechanism regulates the extracellular levels of IGF2 (El-Shewy and Luttrell, 2009). IGF2/M6P receptor functions as a clearance receptor, however its role as a signal transducer remains

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Table 1
Antibody source and concentration for immunostaining.

Antigen	Ab type	Source (catalog #)	Concentration
CD31	Goat polyclonal	Santa Cruz (sc-1505)	1:200
CD31	Mouse monoclonal	DAKO (M0823)	1:50
CD133	Rabbit polyclonal	Abcam (ab19898)	1:100
Glut-1	Rabbit polyclonal	Abcam (ab15309)	1:50
IGF1	Goat polyclonal	R&D Systems (AF-291-NA)	1:100
IGF2	Goat polyclonal	R&D Systems (AF-292-NA)	1:200
IGFR1	Goat polyclonal	R&D Systems (AF-305-NA)	1:200
IGFR2	Goat polyclonal	R&D Systems (AF2447)	1:200

controversial and not well understood. Since the IGF2/M6P receptor is a transmembrane glycoprotein with no catalytic activity, most of the biological outcomes of IGF2 have always been attributed to its interaction with either IGFR1 (Byrd and MacDonald, 2000) or the insulin receptor isoform A (Byrd et al., 2000). However, growing evidence suggests that the interaction between IGF2 and the IGF2/M6P receptor is accountable for several metabolic actions of the small peptide.

While the precise role of IGF2 in hemangioma remains unknown, up-regulated expression of this fetal mitogen in the proliferative phase suggests a potential role in tumor progression. This elevated IGF2 is in the absence of any altered expression of IGF receptors or IGF binding proteins (Ritter et al., 2002, 2003). Thus, we tested the functional significance of high IGF2 in proliferating hemangioma using human hemangioma specimens as well as hemangioma-derived stem cells.

Material and methods

Hemangioma specimens and hemangioma-derived SCs

Paraffin-embedded hemangioma specimens were obtained from the Department of Pathology Archives at the London Health Sciences Centre (LHSC). The proliferating phase of hemangiomas was confirmed by histological analysis. Placenta specimens were provided by Dr. Victor Han (Department of Physiology and Pharmacology, University of Western Ontario). Proliferating hemangioma-derived CD133+ cells were kindly provided by Dr. Joyce Bischoff (Children's Hospital Boston, Boston MA). We have extensively characterized these CD133+ cells by flow cytometry, immunostaining, qRT-PCR, and functional tests (Khan et al., 2008). All studies were conducted following approval by the Research Ethics Board at The University of Western Ontario, London, Ontario, Canada.

Cell culture

CD133-selected cells from proliferating hemangiomas were cultured on fibronectin-coated (FN; 1 μg/cm^2, FC010-10, Millipore, Temecula, CA) plates in Endothelial Basal Media-2 (EBM2; Lonza, Walkersville, MD) supplemented with 20% FBS (Lonza Inc.) and EGM™-2 SingleQuots® without IGF1 (CC-4176, Lonza) and 1× antibiotic antimycotic media (PSF; Life Technologies Inc., Burlington, Ontario). Cord blood endothelial progenitor cells (cbEPCs) were isolated from mononuclear fraction (2C-150A, Lonza Inc.) as shown by us previously (Keats and Khan, 2012; Khan et al., 2006). Bone marrow-mesenchymal progenitor cells (bm-MPCs; Lonza Inc.) were used as controls for the hemSCs. All cells were cultured under identical conditions and all experiments were performed with a minimum of 4 biological replicates and 3 technical replicates unless otherwise mentioned.

Immunostaining

Immunohistochemistry was carried out on the formalin-fixed/paraffin-embedded tissue specimens using ImmPACT DAB (3,3′-Diaminobenzidine; (SK-4105; Vector laboratories, Burlington, ON)). Tissue slides were first deparaffinized in xylene and rehydrated through varying ethanol concentrations. Antigen retrieval was then performed using the Antigen Retriever™ (Electron Microscopy Sciences, Hatfield, PA) with 10 mM Tris-EDTA buffer (10 mM Trizma-base, 1 mM EDTA, 0.05% Tween-20, pH 9.0). After antigen retrieval, the slides were blocked for 30 min using 5% blocking serum (Vector laboratories). The tissues were then incubated with various primary antibodies at empirically determined optimal concentrations (Table 1) for 1 h at room temperature. Following primary antibody incubation, a horseradish peroxidase (HRP) conjugated secondary antibody was applied to the slides (1:200; Vector Laboratories). The sections were counterstained with hematoxylin (HH616; Sigma-Aldrich, Oakville, ON) and mounted using Clarion™ Mounting Medium (Sigma-Aldrich). For immunofluorescent double-labeling, sections were first incubated with a primary antibody, followed by fluorophore (FITC or Texas red)-conjugated secondary antibody. The slides were then subjected to a second round of staining for the second antigen. The slides were counterstained with DAPI (Vector Laboratories) and mounted using Fluoromount (Sigma-Aldrich). All images were taken using Olympus BX51 fluorescent microscope equipped with Spot Pursuit™ digital camera

Table 2
Primer sequences for real time RT-PCR.

Gene	Sequence (5–3)	Length (nt)	Amplicon (bp)	Melting point	Reference
IGF1	AGGAAGTACATTTGAAGAACGCAAGT	26	104	62.67	1
	CCTGCGGTGGCATGTCA	17		64.18	
IGF2	Qiagen (QT01670802)				N/A
IGFR1	TCGTGGGAGGGTTGGTGAT	19	63	63.73	1
	CCAGCCTGCTGTTATTTCTCTTTC	24		62.63	
IGFR2	ATCCAACTTTCCTCCATCACAAG	23	83	61.58	1
	TGCTGATCGTTGGGCTTCA	19		63.93	
Insulin R	TCTGTAAGTCACACGGCTGC	20	274	60.06	1
	GGGATGCACTTGTTGTTGTG	20		60.01	
c/EBPα	GCAAACTCACCGCTCCAATG	20	247	64	2
	TTAGGTTCCAAGCCCCAAGTC	21		62.55	
FABP4	AACCTTAGATGGGGGTGTCCTG	22	125	63.51	2
	TCGTGGAAGTGACGCCTTTC	20		63.63	
Leptin	GAACCCTGTGCGGATTCTTGTG	22	268	66.13	2
	TTTCTGGAAGGCATACTGGTGAG	23		62.7	
PPARγ2	ATTGACCCAGAAAGCGATTC	20	154	59.13	2
	CAAAGGAGTGGGAGTGGTCT	20		59.15	
18S rRNA	Qiagen (QT00199367)				N/A

Reference 1: Hu Y et al. Mol Hum Reprod. 2008;14(5):281–9.
Reference 2: Taura D et al. FEBS Letters 2009; 583: 1029–1033.

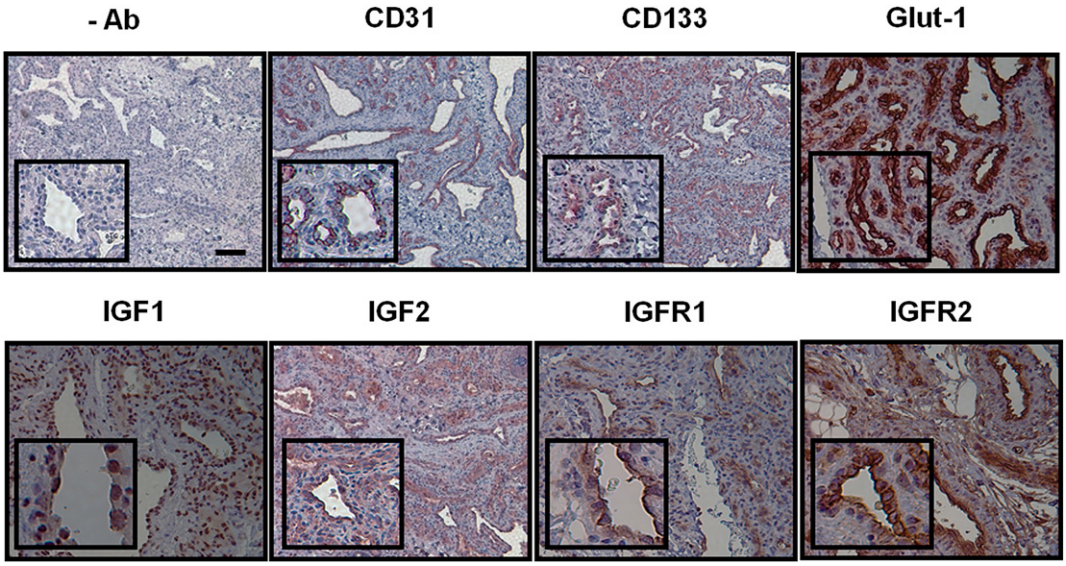

Fig. 1. Insulin-like growth factor-2 localization in hemangioma specimens. Proliferating hemangioma specimens were stained using DAB (brown color) for CD31 (endothelial cell marker), CD133 (stem cell marker), Glut-1 (hemangioma endothelial marker) and for IGF proteins and receptors. Hematoxylin was used for nuclei counterstaining. [Images are presented at a 20× magnification; insets illustrate higher magnification; scale bar represents 200 μm measurement; −Ab represents no antibody control.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Diagnostic Instruments, Inc., Sterling Heights, MI). Images were acquired using Spot Basic Software. At least three different proliferating specimens were used for immunohistochemical localization studies.

RNA isolation and qRT-PCR

Using RNeasy MiniPlus and MicroPlus Kits (QIAGEN, Mississauga, Ontario), total RNA was extracted from cells grown in culture. Purity of the RNA samples was determined by measuring the absorbance at 260:280 nm using Gene Quent™ Spectrophotometer (Pharmacia Biotech, Baie d'Urfe, Quebec). cDNA synthesis was performed with 1 μg of total RNA using iScript cDNA Synthesis Kit (170–8891; Bio-Rad Laboratories, Mississauga, Ontario). Primers used for RT-PCR are listed in Table 2. RT-PCR reactions consisted of 10 μL 2× SYBR® Advantage® qPCR premix (Clontech Laboratories, Inc., Mountain View, CA), 2 μL of both forward and reverse primers (at a 10 μM concentration), 2 μL cDNA, and 6 μL of water. All reactions were performed for 40 cycles using the following temperature profiles: 95 °C for 5 min (initial denaturation); 55 °C for 10 s (annealing); and 72 °C for 12 s (extension). 18S rRNA was used as the housekeeping gene. Data was analyzed by the standard curve method and presented relative to control as shown by us previously (Roach et al., 2012).

Cellular transfection using lentiviral vector plasmid

Cells were plated on FN-coated 12-well plates (growth surface of 3.8 cm^2) at a density of 40,000 cells/cm^2 in complete EBM2 media (described above). The day before transfections, the media was changed to antibiotic/antimycotic-free media. On the day of transfection, the cells were transfected with either control shRNA (sc-108060; Santa Cruz, Santa Cruz, CA) or IGF2 shRNA (sc-39576-SH; Santa Cruz, Santa Cruz, CA) plasmid DNA at a concentration of 0.25 μg/cm^2. Plasmid DNA was administered using lipofectamine (Invitrogen). Cells were incubated for 7 h with the plasmid DNA. After the 7 hour incubation, the cells were supplemented with 1 mL of normal growth media. We then used 1 μg/mL of puromycin for selection. After 2 weeks of puromycin selection, the cells were collected for RNA isolation, and IGF2 knockdown was confirmed using RT-PCR. The knockdown efficiency was confirmed at multiple passages (at least 6 serial passages were tested following transfection). This protocol was used to transfect hemSCs and cbEPCs. We were unable to efficiently knockdown IGF2 in bm-MPCs, therefore; only control bm-MPCs were used in these studies.

Growth assay

Growth of hemangioma SCs and cord blood EPCs was assayed by plating cells on FN-coated 96-well plate at a density of 2000 cells/well. The cells were cultured in complete EBM2/20% FBS (minus IGF1) media for 8 days. Media changes were performed every other day and cell counts were determined on days 2, 4, and 8 using Scepter 2.0 Automated Cell Counter (Millipore). The data was confirmed with tetrazolium salt assay. Briefly, cells were incubated with WST-1 (630118, Clontech) for 3 h. Cell viability was then determined by measuring the absorbance at 450 nm using Multiskan® FC Microplate Photometer (Thermo Scientific®, USA). Reference absorbance of 690 nm was measured and subtracted from the 450 nm reading.

Cell proliferation

Cells were plated on fibronectin-coated 96-well plate at a density of 2500 cells/well. The cells were first cultured in complete EBM2/20% FBS media for 24 h to allow cells to attach. Following cell attachment, media was removed and the cells were treated with EBM2/1% FBS media (control), EBM2/1% FBS + 100 ng/mL of recombinant human IGF1 (291-G1; R&D Systems, Burlington, ON), or EBM2/1% FBS + 100 ng/mL of recombinant human IGF2 (292-G2; R&D Systems). Cells were incubated for 48 h and then counted. Some cells were pretreated with 5 μg/mL neutralizing anti-human IGFR1 (AF-305-NA; R&D Systems) and anti-human IGFR2 (AF2447; R&D Systems) antibodies prior to being exposed to IGF2. These concentrations were determined empirically and are also in accordance with previously published studies (Maeng et al., 2009).

Adipogenic differentiation assay

To assay for adipogenic differentiation, we plated hemSCs on 12-well plates (growth surface of 3.8 cm^2) at a density of 40,000 cells/cm^2 in Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (control media) or with StemPro® Adipogenesis Differentiation media (adipogenic media; A10070-01, Life Technologies Inc.). After 7 days, some cells were stained

using Oil Red-O (O0625-25G; Sigma-Aldrich) for adipocyte staining, while other cells were used for RNA isolation and RT-PCR to quantify adipogenic differentiation (primer sequences are presented in Table 2).

Leptin ELISA

To measure leptin production in hemangioma SC following adipogenic differentiation, we collected culture media on day 7 of adipogenic differentiation (time of harvest; 48 hour media). Leptin levels were measured using Human Leptin Quantikine® ELISA (DLP00; R & D Systems) using the manufacturer's protocol. Leptin levels in the media are presented as pg/mL culture media.

Migration assay

In order to test if IGF2 in hemangioma may be involved in the recruitment of circulating EPCs to the tumor, we performed migration assay using cbEPCs. We have previously shown that cbEPCs are molecularly and functionally similar to the hemangioma-derived endothelial cells (Khan et al., 2006). Fibronectin was used to coat 6.5-mm Transwells with 8.0-μm-pore polycarbonate membrane inserts (BD Falcon™ Cell Culture inserts; BD Biosciences, Franklin Lakes, NJ) for 24 h at 37 °C. Control- and IGF2-shRNA transfected cbEPCs were seeded onto the inserts at the density of 25,000 cells/insert. The lower chambers contained either EBM2/1%FBS media (control media), EBM2/1%FBS + 100 ng/mL IGF2 media, or EBM2/1%FBS + 200 ng/mL IGF2 media. The cells were

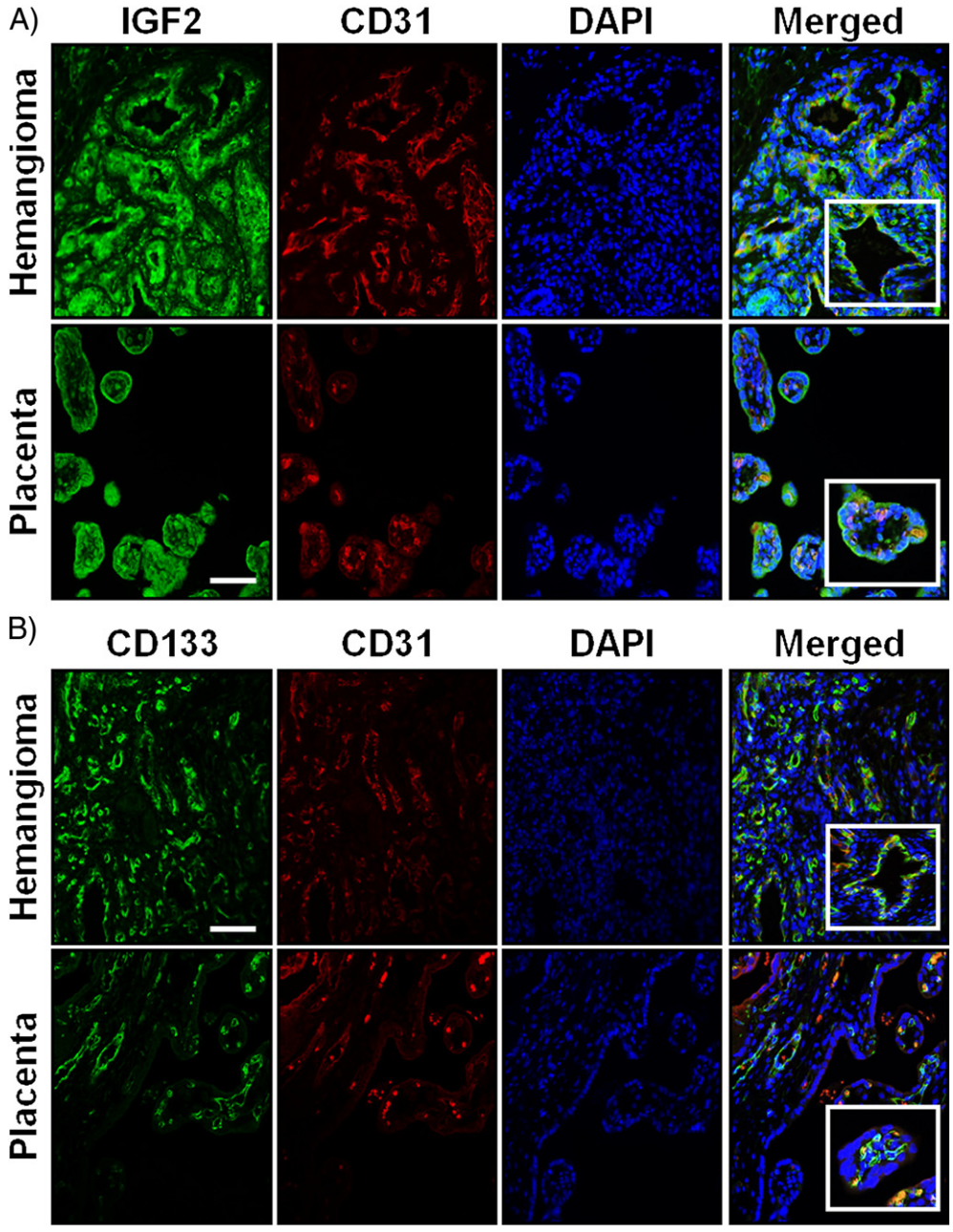

Fig. 2. CD31+/CD133+ cells are the source of IGF2 production in hemangioma. (A) Proliferating hemangioma and early gestation (22–25 weeks) placenta specimens were double-labeled for IGF2 (green), and CD31 (red). DAPI (blue) was used as counterstain. Staining illustrates complete co-localization of IGF2 with CD31 in hemangioma and placenta tissues. (B) Immunofluorescent staining showing CD133 (green) and CD31 (red) positivity in hemangioma and placenta specimens. [Images of slides (both panels) were taken at 20× magnification; insets illustrate high magnification; placenta was used as the positive control; scale bar represents 200 μm measurement.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

allowed to migrate for 24 h at 37 °C. The number of cells migrated after 24 h were measured after fixing the cells in cold methanol, staining with DAPI (Vector Laboratories) and mounting the inserts on slides. Four images were taken at 20× magnification for cell quantification. Each image was computed using Image J software (http://rsbweb.nih.gov/ij/).

Statistical analysis

The data were expressed as means ± SEM. Where appropriate, differences were determined analysis of variance (ANOVA) with Bonferroni's correction. P values <0.05 were considered statistically significant.

Results

IGF2 localizes to CD133 + hemangioma endothelium

Our first objective was to identify the target cell type of IGF signaling. Specifically, we wanted to know the main producer of IGF2 and the cell type(s) expressing the receptors for the IGF proteins. Therefore, we screened for the expression of IGF proteins and receptors using immunohistochemistry. We observed that hemangioma specimens were positive for CD31 (endothelial marker), CD133 (stem cell antigen), Glut-1 (glucose transporter; highly expressed on hemangioma endothelium), IGF1, IGF2, IGFR1 and IGFR2 (Fig. 1). CD31 and CD133 were localized to the endothelium. IGF2 immunoreactivity was observed in the same cells that exhibited positivity for CD31, and CD133. There was no IGF2 immunoreactivity in perivascular cells or interstitial cells. IGF1 was found to be mostly nuclear and less restricted as compared to IGF2. The IGF receptors were localized primarily to endothelial cell membranes. To confirm co-localization of IGF2 in CD31 + ECs, we performed double label immunofluorescent staining using proliferative hemangioma specimens. We used early placenta tissue (gestation age of 22–25 weeks) as a positive control since high IGF2 has been previously reported (Forbes and Westwood, 2008). Analysis of the specimens demonstrated complete co-localization of IGF2 with CD31 and CD31 with CD133 in hemangioma and placenta specimens (Fig. 2, A and B). The results show that the ECs comprising the blood vessels of hemangioma are CD133 positive, and are the major target cell type for IGF2 signaling.

Robust expression of IGFs in hemangioma-derived stem cells

Our next objective was to determine the expression of IGF proteins and receptors in hemangioma-derived CD133 + cells. For these experiments, we used bone marrow-mesenchymal progenitor cells (bm-MPCs) as the positive control. bm-MPCs have been shown to express high levels of IGF2 as compared to mature fibroblasts (Brendel et al., 2005). Previous studies have shown almost undetectable mRNA and protein levels of IGF2 in venous and lymphatic malformations, congenital hemangiomas, pyogenic granulomas, and hemangioendotheliomas (Picard et al., 2008; Yu et al., 2004). Therefore, we believe that bm-MPCs represent the most ideal positive control cell type for IGF2 studies in hemangioma. Using qRT-PCR, we confirmed that hemangioma cells in fact express IGF2 transcripts (Fig. 3, A and C). IGF receptors were also found to be expressed in hemangioma cells (Fig. 3, B and C). This in vitro model offers an exciting opportunity to study the functional significance of IGF2 since the hemangioma cells retain expression of IGF2 and corresponding receptors in culture.

IGF2 does not affect the growth potential of hemangioma stem cells

To assess the role of IGF2 in hemangioma stem cells, we first knocked down IGF2 expression using lentiviral shRNA plasmids in hemSC2 (Fig. 3A; cells that produced the highest level of IGF2). Transfected cells were purified using puromycin selection and the efficiency of knockdown was confirmed by real time RT-PCR. We were

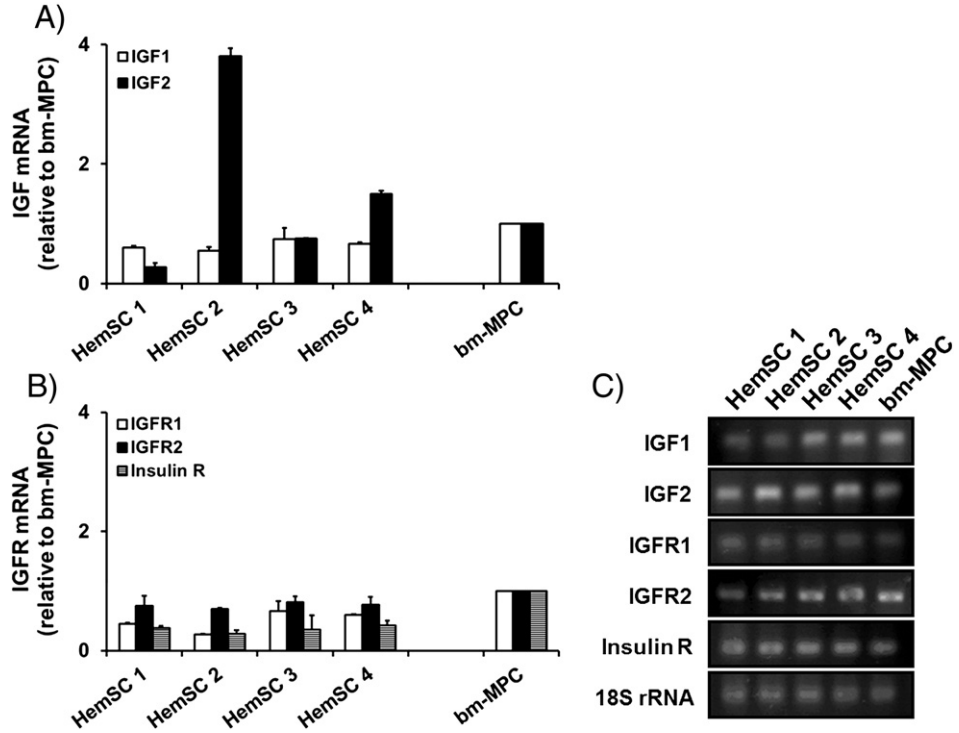

Fig. 3. Real time RT-PCR analysis of IGF/IGFR transcripts in hemangioma SCs. mRNA levels of IGFs (A) and IGF receptors (B) in individual hemangioma CD133 + cell preparations (different patient samples) and bm-MPCs were determined by real time RT-PCR (data normalized to 18S rRNA; relative to bm-MPCs; error bars from technical replicates). The specificity of the amplification was determined by melting curve analysis (data not shown) and gel electrophoresis (C). PCR products at the end of 40 cycles (plateau phase) were run on 2% agarose gel to determine the size of the amplicon.

able to achieve greater than 75% IGF2 suppression in hemSCs (Fig. 4A). To assess the effect of IGF2 on the growth of hemangioma cells, we cultured control shRNA and IGF2-shRNA transfected cells in complete growth media (EBM2/20% FBS). Cells were cultured for a period of 8 days and cellular viability was measured at days 2, 4, and 8. Our results show that the growth potential of the cells was not affected upon IGF2 suppression in the hemSCs (Fig. 4B). Although, a slight increase in the number of viable cells was noted at day 2. The effect was normalized at day 4 and remained undistinguishable from controls through day 8.

IGF2 is a mitogen for hemangioma stem cells

To further study the autocrine role, we assessed whether IGF2 is a mitogen for hemSCs. IGF2 shRNA transfected cells were cultured for 48 h in EBM2 media with 1% serum and recombinant IGF1 or IGF2. Addition of IGF1 and IGF2 increased cell proliferation (Fig. 4C) suggesting that both IGF1 and IGF2 are mitogens for hemSCs. To test whether IGF2 may be causing the cellular changes indirectly through IGF1 (since they share receptors and hemangioma cells express both IGF1 and IGF2), we compared the effect of recombinant IGF1 in both control and IGF2-shRNA transfected cells. Interestingly, we did not find any significant changes in cell proliferation (Fig. 4D) suggesting that the actions of IGF1 and IGF2 are independent of each other. Next, we wanted to see if the effect of IGF2 is mediated by its interaction with the IGF receptors. To test this, we pre-treated cells with IGFR1 and IGFR2 neutralizing antibodies. These neutralizing antibodies have been shown to block the effect of exogenous IGF2 (Maeng et al., 2009). Our results demonstrate that blocking either IGFR1 or IGFR2 inhibits the effect of IGF2, as seen by decreased proliferation of hemSCs (Fig. 4E). IGF2 shRNA transfected cells showed 47.5% inhibition with IGFR1 neutralizing antibody and 55.6% with IGFR2 neutralizing antibody. These studies suggest a role of IGFR1 and IGFR2 in mediating the mitogenic effects of IGF2.

Effect of IGF2 on the differentiation potential of hemangioma stem cells

We subjected hemSCs to adipogenic induction in order to study whether endogenous IGF2 may be regulating hemSC plasticity. After 7 days of adipogenic induction, we measured the expression of both early and late adipogenesis-specific genes. Specifically, we measured peroxisome proliferator activated receptorγ2 (PPARγ2) and c/EBPα as specific markers of early adipogenic commitment (Fig. 5A). We also measured fatty acid binding protein-4 (FABP4), a cytoplasmic protein involved in fatty acid uptake, transport, and metabolism. For assessment of terminal differentiation, we measured the levels of leptin[16–18]. Oil Red O staining was performed for qualitative assessment of differentiation (Fig. 5B). The expression level of PPARγ2, c/EBPα, and FABP4 was significantly elevated when the cells were treated with adipogenic media (Fig. 5C). Real time RT-PCR analysis revealed that the absence of endogenous IGF2 (through shRNA) does not drastically alter the expression of these early adipogenesis genes. Although slight

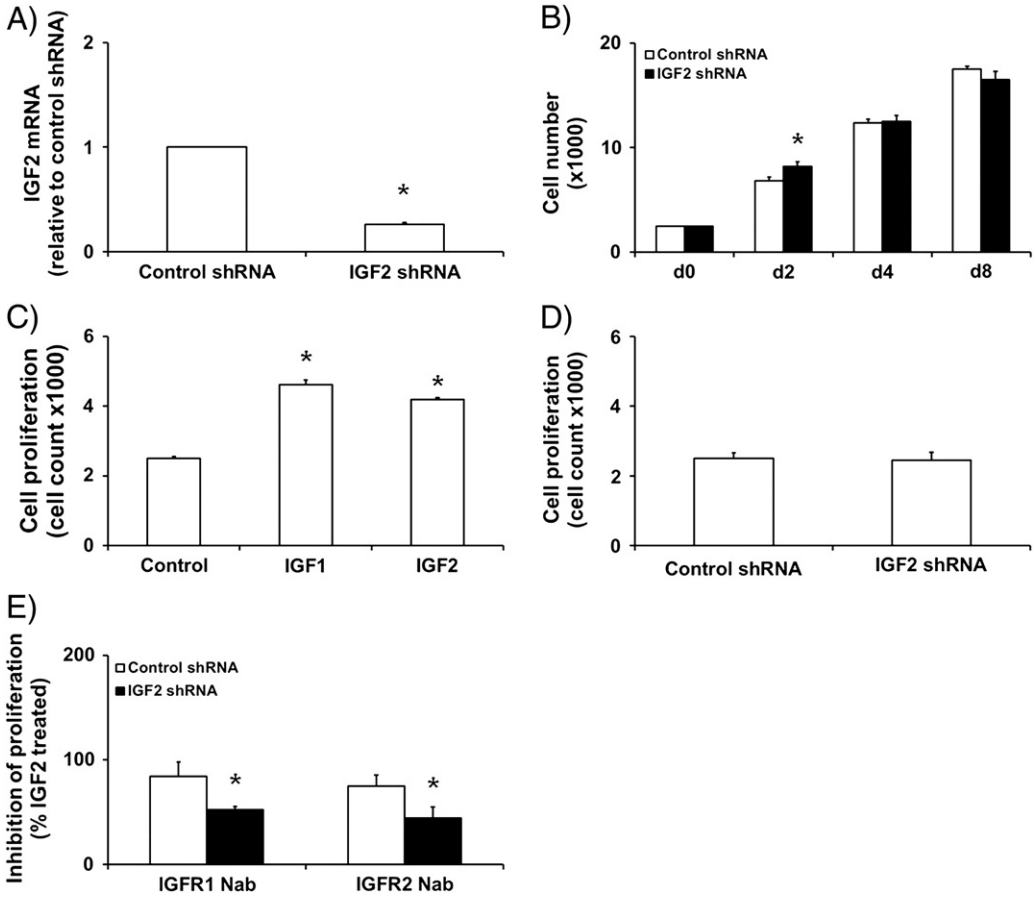

Fig. 4. IGF2 causes hemSC proliferation. CD133+ hemSC2 were transfected using lentiviral shRNA plasmid DNA targeting IGF2. Knockdown efficiency was confirmed using real time RT-PCR after 2 weeks of puromycin selection (A) [*$p<0.05$ compared to control shRNA]. (B) Cellular growth of hemSCs with or without IGF2 suppression in complete growth media [*$p<0.05$ compared to control shRNA]. (C) Cell proliferation in IGF2-shRNA transfected cells cultured in either EBM2/1% FBS (control) media, EBM2/1% FBS + 100 ng/mL IGF1 media, or EBM2/1% FBS + 100 ng/mL IGF2 media for 48 h [*$p<0.05$ compared to control media]. (D) Effect of exogenous IGF1 (100 ng/mL) on control- and IGF2-shRNA transfected cells. (E) Cells transfected with IGF2 shRNA plasmids exhibited a decrease in IGF2-induced cellular proliferation when pre-treated with neutralizing 5 μg/mL IGFR1 or IGFR2 antibodies [*$p<0.05$ compared to IGF2 treated cells].

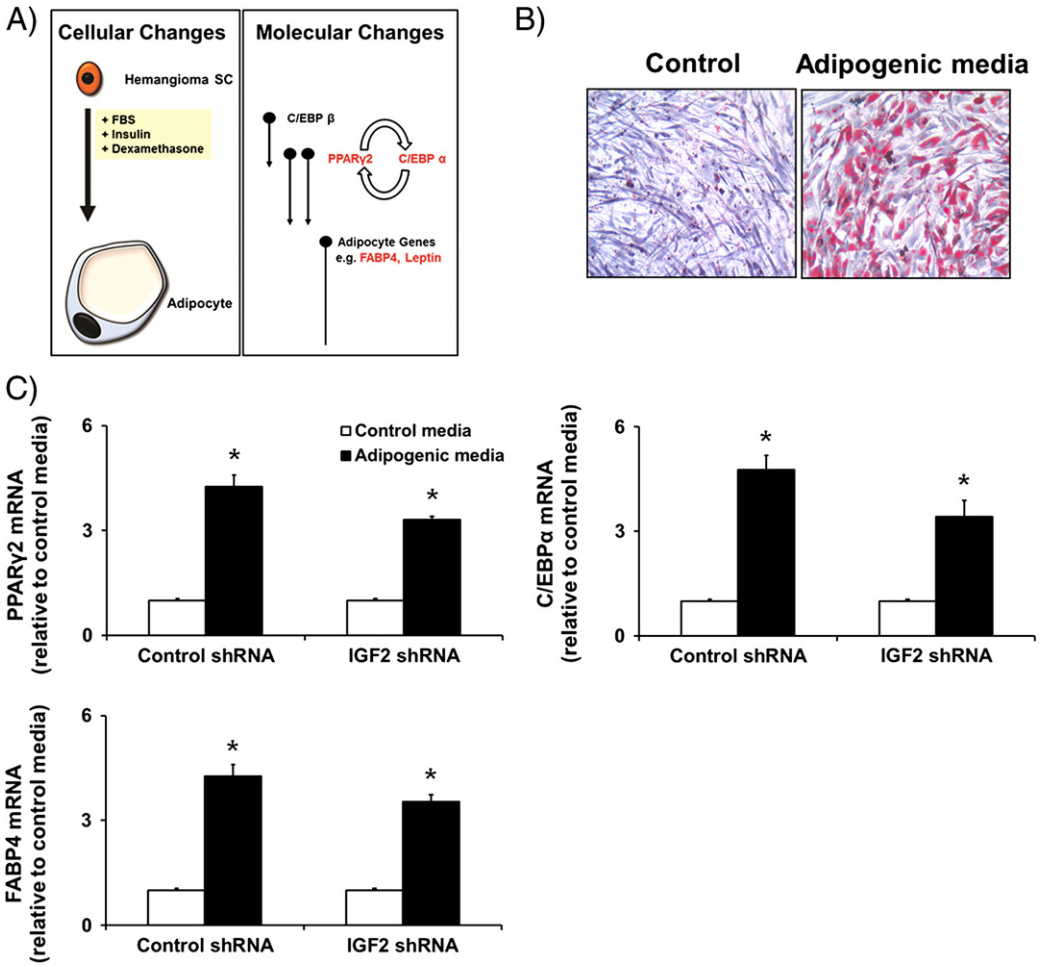

Fig. 5. Effect of IGF2 on the differentiation potential of hemSCs. Panel (A) shows a schematic of cellular and molecular changes underlying hemSC to adipocyte differentiation and the key transcription factors involved [red font indicates the markers measured in our study]. (B) Representative Oil Red O staining of undifferentiated hemSCs (control) and hemSCs undergoing adipogenic differentiation at a 20× magnification [image taken at day 7]. (C) Real time RT-PCR analysis revealed that in the presence (control shRNA) or absence of endogenous IGF2 (IGF2 shRNA), transcript expression of PPARγ2, c/EBPα, and FABP4 were all significantly elevated when the cells were cultured in adipogenic media [*$p<0.05$ compared to respective control media; hemSC2]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reductions were noted in the IGF2-shRNA transfected cells, these differences were not statistically significantly. These findings are consistent with positive Oil Red O staining suggesting that fatty acid update and lipid accumulation are not different in IGF2 shRNA transfected cells.

IGF2 regulates leptin expression in hemangioma stem cells

Leptin is one of the key hormones produced by mature adipocytes and serves as a marker of terminal adipocyte differentiation. We wanted to know whether IGF2 would regulate terminal differentiation of hemSCs into adipocytes. Our results show that endogenous IGF2 prevents leptin induction in hemSCs (Fig. 6A). This induction was restored when the cells were transfected with IGF2-shRNA. In comparison, control bm-MPCs exhibited leptin induction following 7 day treatment in identical differentiation media (Fig. 6B). Since bm-MPCs also express IGF2, we tested whether the level of IGF2 production correlates with the ability to induce leptin. We used another hemSC preparation (hemSC4; Fig. 3A) and performed similar experiments following IGF2 shRNA transfection. We were able to reduce IGF2 expression in hemSC4 to almost undetectable levels (Fig. 6C). These IGF2 shRNA transfected cells showed higher leptin mRNA expression even in non-adipogenic (basal) conditions (Fig. 6D). Induction in the adipocyte differentiation media further increased leptin expression in these IGF2-shRNA transfected cells (Fig. 6E). This was accompanied by over 20-fold induction in PPARγ2, C/EBPα, and FABP4 (as compared to approximately 4-fold induction seen in hemSC2; Fig. 5). Next, we determined whether the cells are producing and releasing leptin protein. Using leptin ELISA, we show that IGF2 shRNA transfected cells have higher leptin protein in the culture media as compared to control shRNA cells with further increase following adipocyte differentiation (Fig. 6F). We wanted to confirm that the endogenous production of IGF2 is regulating leptin expression in hemangioma cells; hence, we performed the adipogenesis assay on control shRNA transfected cells with neutralizing IGFR antibody treatments. We cultured the cells in adipogenic media with or without 5 μg/mL IGFR1 or IGFR2 neutralizing antibodies for 7 days (media changed every other day). Our results show that IGFR1 neutralization does not alter the expression of adipogenesis-specific genes including leptin. However, when we treated the cells with IGFR2 neutralizing antibody for 7 days, we noted induction of leptin mRNA indicating that endogenous IGF2 may regulate leptin expression through IGFR2 signaling (Fig. 6G).

IGF2 promotes migration of endothelial cells

We also studied whether IGF2 may have a role in recruiting circulating endothelial progenitor cells. Elevated levels of endothelial progenitor cells have been reported in proliferating hemangioma specimens (Kleinman et al., 2003) coinciding with elevated IGF2 expression levels (Ritter et al., 2002). Furthermore, the possibility exists that high IGF2 may also enhance proliferation of EPCs. For these

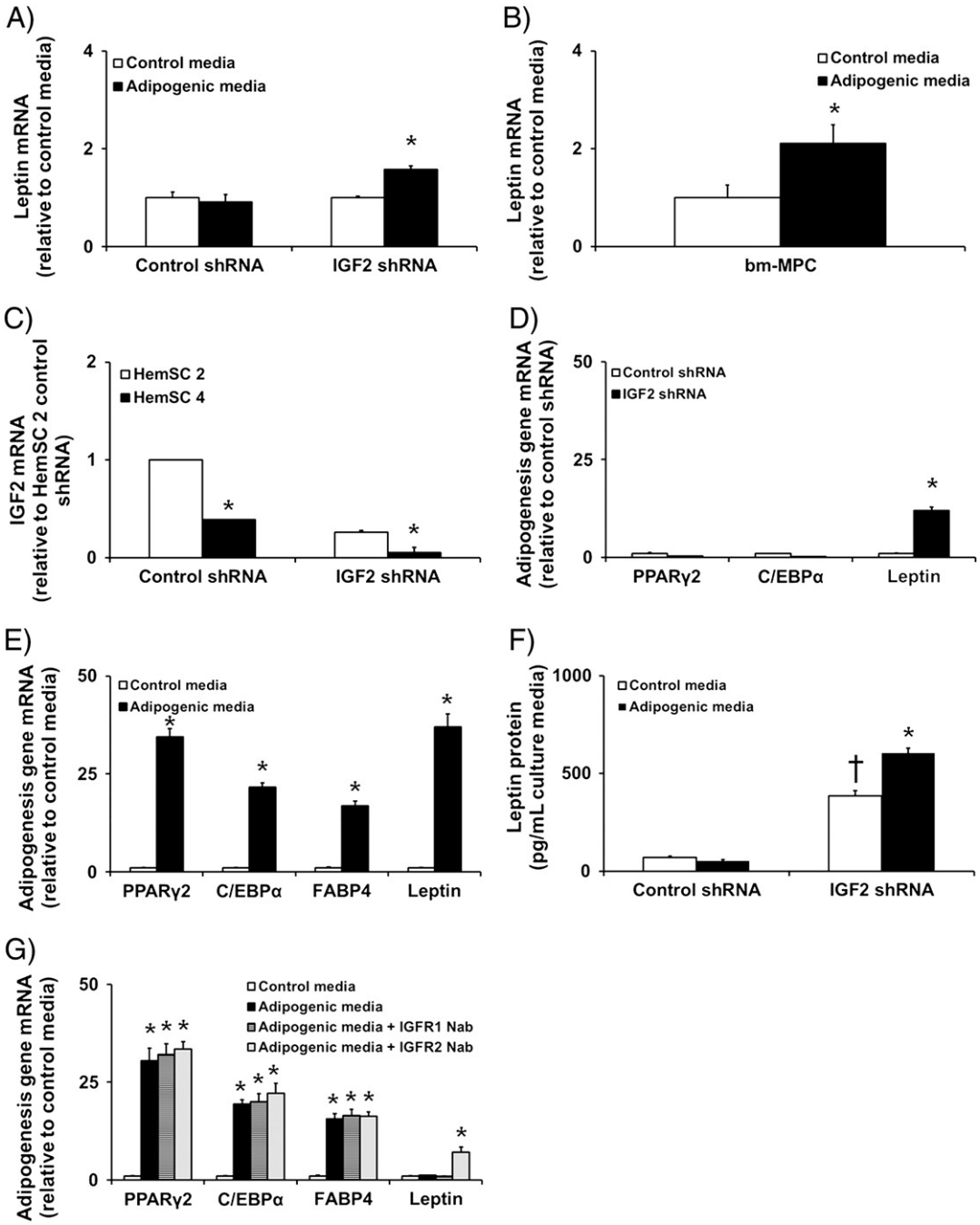

Fig. 6. IGF2 regulates leptin expression in hemangioma stem cells. Real time RT-PCR analysis of control and IGF2 shRNA transfected cells showed leptin expression (A) only in the IGF2 shRNA cells following adipogenic differentiation [*p<0.05 compared to the control media]. (B) Treatment of bm-MPCs to adipogenic media for 7 days induced leptin expression [*p<0.05 compared to the control media]. (C) shRNA knockdown of IGF2 in hemangioma SC 2 and 4 [*p<0.05 compared to the respective control shRNA]. (D) Expression of adipogenic genes in hemSC4 under basal conditions (EBM2/20% FBS growth media) [*p<0.05 compared to control shRNA]. (E) Induction of adipogenesis-specific genes in hemSC4 (IGF2-shRNA transfected) following 7-day treatment with differentiation media [*p<0.05 compared to control media]. (F) Leptin protein levels in culture media following adipocyte differentiation measured with ELISA [*p<0.05 compared to control media; †p<0.05 compared to control shRNA cells in control media; hemSC4]. (G) Effect of neutralizing IGFR1 and IGFR2 on adipogenic differentiation of hemSC4 control shRNA cells [*p<0.05 compared to control media].

studies, we also knocked down endogenous IGF2 in cbEPCs to avoid confounding variables and a possible saturation effect. Similar to hemSCs, we were able to achieve a significant suppression of IGF2 in cbEPCs (Fig. 7A). Suppressing IGF2 in cbEPCs significantly reduced the growth potential of the cells in complete endothelial media (Fig. 7B). This is in contrast to the CD133+ hemSCs. We next assayed for potential mitogenic role of IGF2 in cbEPCs. Unlike hemSCs, IGF1 and IGF2 failed to increase cell proliferation (Fig. 7C).

The ability of IGF2 to promote cellular migration was assessed by measuring the capability of cbEPCs to migrate through 8 μm pores in the presence of exogenous IGF2. Inserts seeded with cbEPCs were incubated in either control media (EBM2/1% FBS) or media supplemented with IGF2 (100 ng/mL or 200 ng/mL). After 24 h, the inserts were stained with DAPI. EPC migration increased in the presence of 200 ng/mL of IGF2 as compared to control (Fig. 7D). Although these results are statistically significant, the increase was modest. We

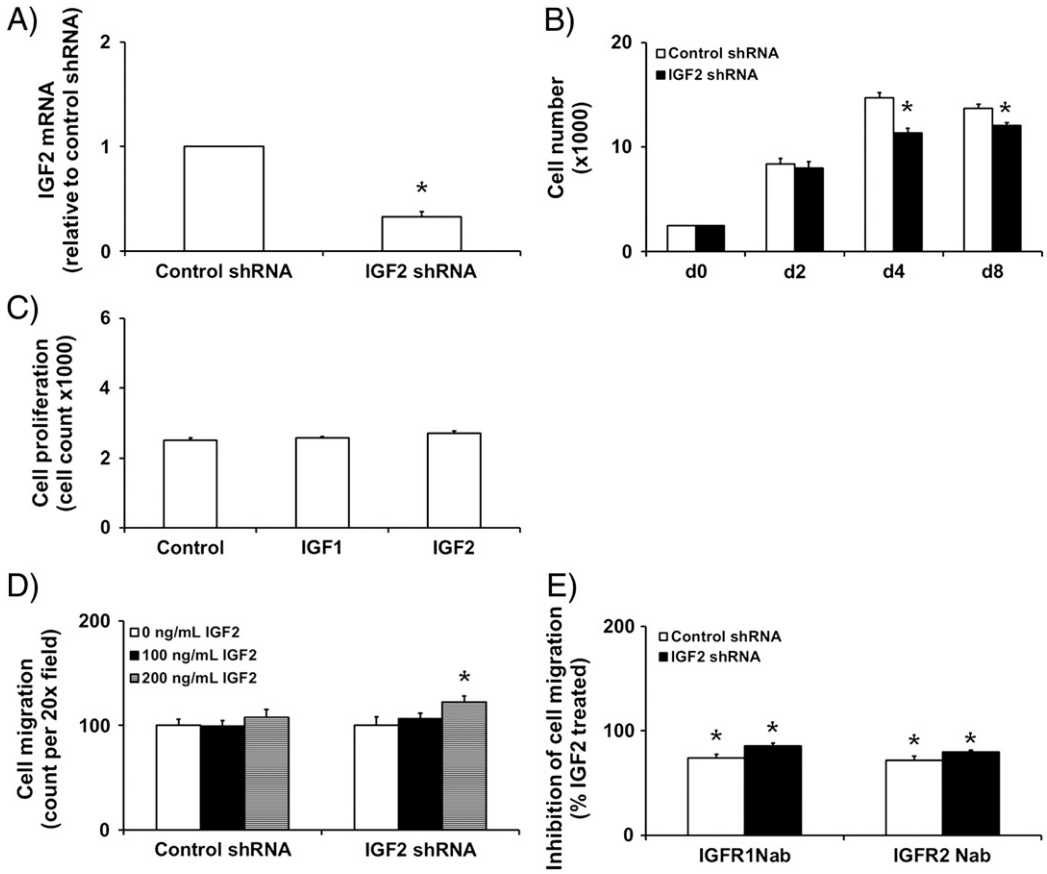

Fig. 7. IGF2 promotes migration of endothelial cells. cbEPCs were transfected using lentiviral shRNA plasmid DNA targeting IGF2. Knockdown efficiency was confirmed using real time RT-PCR after 2 weeks of puromycin selection (A) [n = 4; *p<0.05 compared to control shRNA]. (B) Cellular growth of cEPCs with or without IGF2 suppression in complete growth media at days 2, 4, and 8 [n = 4; *p<0.05 compared to control shRNA]. (C) Cell proliferation in IGF2-shRNA transfected cells cultured in either EBM2/1% FBS (control) media, EBM2/1% FBS + 100 ng/mL IGF1 media, or EBM2/1% FBS + 100 ng/mL IGF2 media for 48 h. (D) The migration of cbEPCs was measured using a transwell assay with either 100 ng/mL or 200 ng/mL of IGF2 as chemoattractant [n = 4; *p<0.05 compared to respective control media]. (E) cbEPCs were pre-treated for 30 min with neutralizing antibodies targeting IGFR1 and IGFR2 and seeded on 8.0 μm pore inserts. Treated cells were incubated in control media or media supplemented with 200 ng/mL IGF2. The analysis demonstrated a significant decrease in the migratory ability of cbEPCs when the cells were pre-treated with neutralizing receptor antibodies. [n = 4; *p<0.05 compared to IGF2 treated cells].

pre-treated cbEPCs with IGFR1 and IGFR2 neutralizing antibodies and measured EPC migration again. We observed that inhibition of the IGF receptors decreased the migratory ability of EPCs by approximately 15–20% (Fig. 7E).

Discussion

The salient findings of our study include: 1) IGF2 is a mitogen for hemSCs but not endothelial cells suggesting that IGF2 is a key player in expansion of stem cells only and, 2) endogenous IGF2 may inhibit terminal adipocyte differentiation in hemSCs. The findings of the study are summarized in Fig. 8.

Previous identification of hemangioma-initiating cell has brought us closer to understanding the peculiar mechanisms behind hemangiomagenesis. However, there still remains a gap in our understanding of those mechanisms controlling the proliferating and the involuting phases of hemangioma. Our study has been able to verify an important role of IGF2 in enhancing hemangioma stem cell proliferation. Yet, we have found that IGF2 does not seem to play a role in the early commitment phase of adipogenesis. We have been able to demonstrate that the presence or absence of endogenous IGF2 does not affect the expression of early adipocyte specific transcription factors PPARγ2 and C/EBPα. However, knocking down endogenous IGF2 significantly increased leptin levels in hemSCs. Leptin is a late marker of adipocyte differentiation and is elevated only upon full functional differentiation (Cristancho and Lazar, 2011; Gregoire et al., 1998; Hwang et al., 1997; Rentsch and Chiesi, 1996). Furthermore, leptin has been shown to be increased in bm-MPCs following 7 days of treatment with differentiating media (Donzelli et al., 2011). Our results also show that leptin is induced in the bm-MPCs when cultured under identical conditions as hemSCs. When we further probed leptin induction, we found that the level of IGF2 is important in the ability of hemangioma stem cells to induce leptin. These findings may have significant clinical utility in determining which and how fast the hemangiomas may regress.

One of the most important processes that enable hemangioma to progress and develop is vasculogenesis (SCs to EC differentiation) and angiogenesis (recruitment of ECs). Recruitment of EPCs for neovascularization under pathological conditions, such as, ischemia and tumor progression has become more generally accepted (Gao et al., 2008; Nolan et al., 2007). A study conducted by Maeng et al. (2009) explored the role of IGF2 in the 'early EPCs' (hematopoietic cells). The study demonstrated that IGF2 increased early EPC chemotaxis through the IGF2/M6P receptor-dependent signaling pathway but not IGFR1 pathway. In our study, we have looked at whether the same IGF axis plays a chemotaxis role in vasculogenic EPCs (Khan et al., 2006; Melero-Martin et al., 2007) (also known as late outgrowth EPCs). We know that the late EPCs express all markers of mature endothelial cells and lack markers of hematopoietic cells (Khan et al., 2006; Melero-Martin et al., 2007). These EPCs also create blood vessels in animal models (Melero-Martin et al., 2007). Using these EPCs, we show that IGF2 promotes cell migration

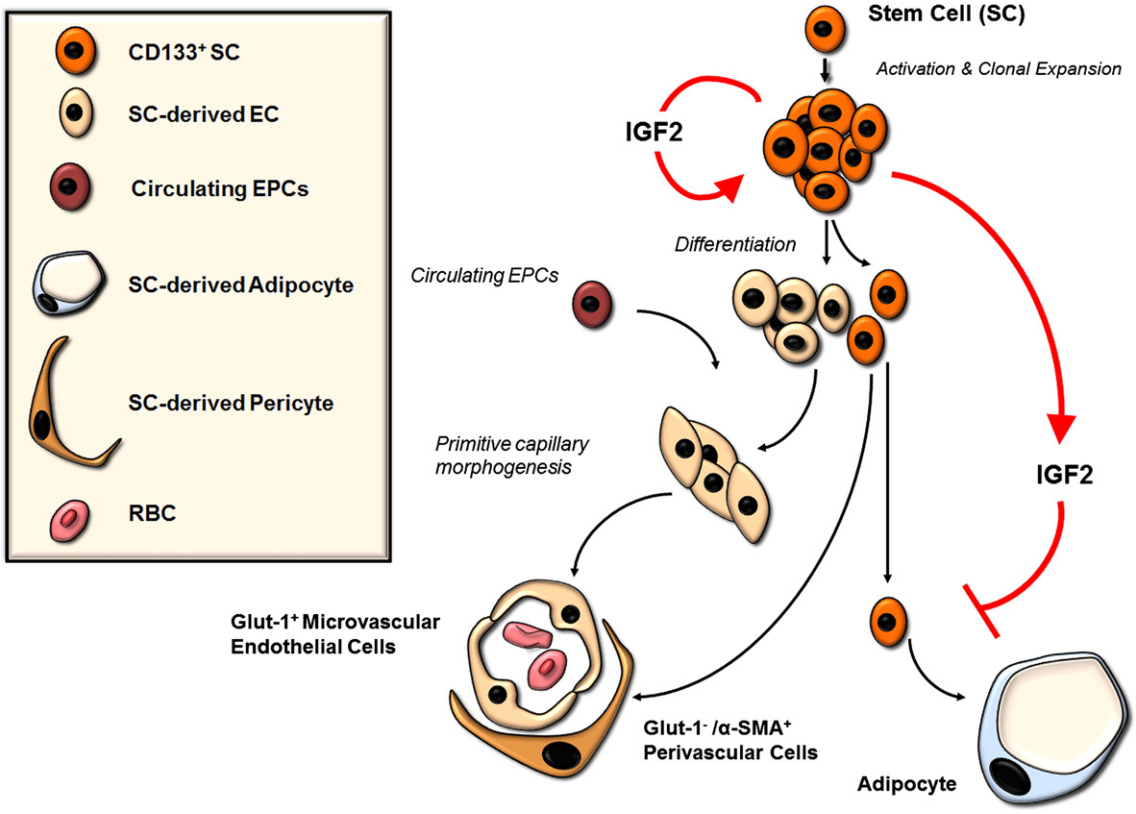

Fig. 8. Schematic diagram illustrating the hypothesis and acquired findings. We hypothesize that hemangiomas arise from atypical activation of CD133 + stem cells. These CD133 + cells give rise to Glut-1 + endothelial cells (during proliferation) and adipocytes (during involution). Recent studies have also shown that CD133 + cells may give rise to pericytes in the hemangiomas. Red lines illustrate the findings of the present study [α-SMA = alpha smooth muscle actin; EPC = endothelial progenitor cell; Glut-1 = glucose transporter-1; IGF2 = insulin-like growth factor-2; RBC = red blood cell; SC = stem cell]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

through IGFR1 and IGFR2. It should be noted that this enhanced migration was achieved with 200 ng/mL IGF2 that we believe is quite high. Whether elevated IGF2 promotes EPC recruitment in hemangiomas remains to be determined. However, taking our study and the one by Maeng and colleagues together, we can speculate that the main chemotactic role of IGF2 may be on the hematopoietic cells that have been shown to be high in hemangiomas.

To better understand the receptor utilization by IGF2, we conducted the studies using neutralizing antibodies against IGFR1 and IGF2/M6P receptor. Interestingly, blocking the IGFR1 and IGF2/M6P receptor (IGFR2) inhibited IGF2-mediated proliferation of hemSCs and also the migratory ability of the cbEPCs. These findings bring an exciting possibility of IGF2 interacting with both IGF receptors to elicit its biological response. Generally, IGF2/M6P receptor is known to have no intrinsic catalytic activity and is well known for its function as a clearance receptor. However, there is increasing evidence for the role of IGF2 in angiogenesis in different systems (Baker et al., 1993; Kim et al., 1998; Lee et al., 2000). Evidence suggests that the interaction between IGF2 and the IGF2/M6P receptor is accountable for several metabolic activities (El-Shewy and Luttrell, 2009). Our results do support this notion. However, to better understand the pathways which may be activated downstream of the IGF receptors, future studies would need to focus on targeting key downstream signaling pathways including PI3K, PKB/AKT, TOR system, and MAPK system (that are known to be implicated in IGF receptor signaling) (Pavelic et al., 2007).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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