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A central role for hepatocyte growth factor in adipose tissue angiogenesis

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Bell LN, Cai L, Johnstone BH, Traktuev DO, March KL, Considine RV. A central role for hepatocyte growth factor in adipose tissue angiogenesis. *Am J Physiol Endocrinol Metab* 294: E336–E344, 2008. First published December 11, 2007; doi:10.1152/ajpendo.00272.2007.—Hepatocyte growth factor (HGF) is a potent mitogenic and angiogenic factor produced in human adipose tissue. In this study, we use 3T3-F442A preadipocytes to study the contribution of HGF to angiogenesis in an in vivo fat pad development model. As observed for human adipocytes, HGF is synthesized and secreted by 3T3-F442A preadipocytes and mature adipocytes. HGF knockdown with small-interfering RNA reduced HGF mRNA expression $82.3 \pm 4.2\%$ and protein secretion $82.9 \pm 1.4\%$ from 3T3-F442A preadipocytes. Silencing of HGF resulted in a $70.5 \pm 19.0\%$ reduction in endothelial progenitor cell migration to 3T3-F442A-conditioned medium in vitro. 3T3-F442A preadipocytes injected under the skin of mice form a fat pad containing mature, lipid-filled adipocytes and a functional vasculature. At 72 h postinjection, expression of the endothelial cell genes TIE-1 and platelet endothelial cell adhesion molecule (PECAM)-1 was decreased 94.4 ± 2.2 and $91.5 \pm 2.5\%$, respectively, in 3T3-F442A fat pads with HGF silencing. Knockdown of HGF had no effect on differentiation of 3T3-F442A preadipocytes to mature adipocytes in vitro or in vivo. In developing fat pads under the skin of HGF overexpressing transgenic mice, TIE-1 and PECAM-1 mRNA was increased 16.5- and 21.4-fold, respectively, at 72 h postinjection. The increase in gene expression correlated with immunohistochemical evidence of endothelial cell migration in the developing fat pad. These data suggest that HGF has a central role in regulating angiogenesis in adipose tissue.

obesity; neovascularization; 3T3-F442A; tie-1; platelet endothelial cell adhesion molecule-1

ADIPOSE TISSUE HAS the potential for extensive growth and expansion throughout the lifetime of an individual, a property that is dependent on the plasticity of the vasculature within this tissue. An extensive capillary network is present in adipose tissue that supports adipocyte metabolism (4, 10) and allows this highly vascularized tissue to actively secrete many hormones, cytokines, and angiogenic growth factors (19). Early studies suggest that angiogenesis and adipogenesis are tightly coupled in prenatal development of adipose tissue (9) and that angiogenesis may precede adipogenesis (4). Interestingly, several recent studies have shown that inhibition of angiogenesis is effective in regulating adipose tissue mass, either by preventing weight gain or inducing weight loss in diet-induced and genetic mouse models of obesity (2, 13, 20).

Recent work from our laboratory has focused on the contribution of hepatocyte growth factor (HGF) to angiogenesis in adipose tissue. HGF is a pleiotropic factor with potent angio-

genic and mitogenic effects (15). We have reported that HGF is produced in human adipose tissue (17, 18), that serum HGF is elevated more than threefold in obese individuals (17), and that adipocytes from obese subjects secrete significantly more HGF than adipocytes from lean individuals in vitro (1). Although we have established that HGF is synthesized in adipose tissue, and it is known that angiogenesis is required for expansion of adipose tissue, the role of HGF in adipose tissue angiogenesis has not been investigated.

In this study, we examine the contribution of HGF to adipose tissue angiogenesis using the 3T3-F442A in vivo fat pad formation model originally developed by Green and Kehinde (8). In this model, 3T3-F442A preadipocytes injected under the skin of BALB/c athymic nude mice differentiate into mature, lipid-filled adipocytes and form fully functional fat pads. More recently, this model has been adapted to the study of the mechanisms through which fat pad neovascularization occurs (7, 16). In particular, Neels et al. (16) have demonstrated that angiogenesis in developing 3T3-F442A fat pads can be quantitated by measuring endothelial cell mRNA content, which correlates with histological evidence of vessel formation. In the current study, HGF gene expression was silenced in 3T3-F442A preadipocytes before injection under the skin of nude mice, and the response to loss of HGF function on fat pad angiogenesis and preadipocyte differentiation was assessed. The effect of elevated HGF to enhance fat pad neovascularization and 3T3-F442A differentiation was also tested in transgenic mice that overexpress HGF. We show that silencing of HGF decreases the ability of 3T3-F442A preadipocytes to stimulate endothelial progenitor cell (EPC) migration in vitro and to promote recruitment of endothelial cells for angiogenesis in developing fat pads in vivo. On the other hand, elevated HGF enhances endothelial cell migration into fat pads. Taken together, the findings of these studies highlight a central role for HGF in regulating angiogenesis in developing adipose tissue.

MATERIALS AND METHODS

Animals. Male BALB/c (6–8 wk old) athymic nude mice were purchased from Charles River Laboratories (Wilmington, MA). Transgenic mice overexpressing HGF under the control of the metallothionein I promoter on the C57Bl/6J background (23) were originally obtained from Dr. Glenn Merlino [National Institutes of Health, (NIH) Bethesda, MD] and maintained as a breeding colony at Indiana University. All animal housing and protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

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Cell culture and injection in vivo. 3T3-F442A preadipocytes were obtained from Dr. Howard Green (Harvard University, Cambridge, MA) and maintained in DMEM + 10% bovine calf serum (BCS). 3T3-F442A preadipocytes were differentiated to mature adipocytes in vitro using DMEM + 10% FBS with 10 μ g/ml insulin (Sigma-Aldrich, St. Louis, MO) for 10 days. To assess the effect of HGF on differentiation in vitro, preadipocytes were differentiated in medium containing 10% FBS, 10 μ g/ml insulin, and 100 ng/ml HGF (R&D Systems, Minneapolis, MN). To initiate fat pad formation in vivo, 1.5×10^7 undifferentiated 3T3-F442A preadipocytes in 0.05 ml DMEM + 10% BCS were injected just under the skin on the back of mice. 3T3-F442A preadipocytes were labeled before injection in mice with Qtracker 800 Qdot nanocrystals (Molecular Probes, Eugene, OR), and injected cells were subsequently identified using the LICOR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) for excision from the mouse. 3T3-F442A-derived and epididymal fat pads (used as a control) were removed at various time points to quantitate endothelial cell and adipocyte gene expression. As previously observed (16), injected 3T3-F442A preadipocytes were retained within a connective tissue pocket formed by the fascia separating the skin and underlying muscle tissue. The presence of the fascia enclosing the injected cells facilitated the dissection and removal of the injected cells. Furthermore, care was taken to remove only injected cells to exclude sample contamination with endogenous mouse tissue. The presence of Qtracker 800-stained cells attached to the mouse skin following excision of the fat pads confirmed that mouse tissue was not included when the 3T3-F442A-derived fat pad was removed.

RNA isolation and real-time RT-PCR. Total RNA was isolated from cells and tissues by guanidinium thiocyanate-phenol-chloroform extraction, and cDNA was synthesized from 0.5 μ g of total RNA using random hexamers (Applied Biosystems, Foster City, CA) in a 100- μ l reaction. mRNA levels of all genes investigated were determined by Real-Time RT-PCR using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Five microliters of cDNA (25 ng of total RNA) were amplified in a reaction volume of 25 μ l, and cycling conditions were one cycle for 2 min at 50°C and one cycle for 3 min at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C. Primer sets (Invitrogen, Carlsbad, CA) shown in Table 1 were used at a concentration of 200 nM. Expression of all genes was normalized to 36B4 (mouse acidic ribosomal phosphoprotein) expression using the ΔC_T method. There was no difference in 36B4 expression in 3T3-F442A preadipocytes and adipocytes following differentiation in vitro or in vivo.

Silencing of HGF gene expression using small-interfering RNA. SMARTpool siGENOME duplex-2 reagent and siCONTROL Non-targeting siRNA no. 1 (scrambled sequence) were purchased from Dharmacon (Lafayette, CO). The siGENOME duplex-2 reagent targeted the sense sequence 5'-GAAACUGCAUCAUUGGUAUU-3' and the antisense sequence 5'-UUACCAUGAUGCAGUUUCUU-3' of the murine HGF mRNA. Small-interfering RNAs (siRNAs) were

reconstituted in $1 \times$ siRNA buffer [20 mM KCl, 6 mM HEPES buffer (pH 7.5), and 0.2 mM MgCl₂]. For transfection, 3T3-F442A preadipocytes were washed and trypsinized from growth flasks, resuspended in Dulbecco's PBS (without Mg²⁺ or Ca²⁺), and electroporated with 400 nM siRNA using a Bio-Rad Gene Pulser (Bio-Rad) set at 0.24 kV and 960 μ F capacitance. Cells were plated and allowed to recover for 72 h before use in any experiments. Approximately 50% of the cells survived electroporation, and there was no difference in survival between cells receiving duplex-2 or scrambled sequence siRNA.

Immunohistochemistry for endothelial cells. Developing fat pads were removed from the mice attached to the underside of the skin. Fat pads were preserved in 4% formaldehyde, followed by paraffin embedding, and then cut into thin sections (5 μ m). Deparaffinized sections were treated with citrate retrieval buffer (90°C, 20 min) and incubated with 2% H₂O₂ to block endogenous peroxide for 10 min. Sections were incubated with rabbit anti-CD31 IgGs (Lab Vision, Fremont, CA) for 1 h followed by incubation with anti-Rabbit IgGs for 30 min (Vector Labs, Burlingame, CA). Antigen-antibody complexes were developed by incubating sections with VECTASTAIN ABC Reagent (horseradish peroxidase) and detected by accumulation of diaminobenzidine substrate (Sigma-Aldrich). The sections were counterstained with hematoxylin to visualize cell nuclei.

In vitro EPC Transwell migration assays. 3T3-F442A preadipocytes with and without HGF silencing were cultured in DMEM + 1% BCS for 72 h, and conditioned culture medium was collected and stored at -80°C. For the migration assays, highly proliferative late-outgrowth EPC clones were isolated from human umbilical cord blood as previously described (11). Migration of EPCs to 3T3-F442A preadipocyte conditioned medium with and without HGF silencing was assessed as previously described with minor modifications (11). Briefly, bottom surfaces of Transwell insert membranes, with a pore diameter of 8 μ m, were coated with rat collagen type I to enhance adhesion. EPCs (3×10^4) were added to each insert, and inserts were placed in a 24-well plate with the lower wells containing undiluted nonconditioned control, conditioned siCONTROL, or conditioned siHGF medium (3 inserts/well per treatment). After allowing cells to migrate for 4 h, the lower aspect of the inserts was stained with Diff Quick (Dade Behring, Deerfield, IL) to visualize the cells that had migrated through the membrane. Cells retained on the top surface of the insert membranes were eliminated using a cotton-tipped applicator. Three separate fields across the bottom of the insert, covering ~80% of the available surface, were photographed using a $\times 40$ objective. The number of migratory cells in each field was quantitated using Image J (NIH). The number of migratory cells was averaged across the three fields and then across the three wells to derive the number of migratory cells per treatment in a given experiment. Migration in the absence of medium conditioning (undiluted control medium) was determined in each experiment and subtracted from the conditioned medium values. On average, 525 ± 42 cells migrated across the membrane in the absence of medium conditioning for the six experiments performed.

Table 1. Primer sequences used for real-time RT-PCR assays

mRNA	Forward (5'-3')	Reverse (5'-3')
HGF	TGTTCTTGGTGTGTCATTGTTCCCT	AATATGCTACTCGAAGAAAAATAACAGG
LpL	TTCTCCTCCTACTCCTCCTC	TGTCCTCAGTCGTGTCTTCA
TIE-1	GCCCTTTTAGCCTTGGTGTG	CTTACCCGATCCTGACTGG
PECAM-1	CTGGGAGGTCGTCCATGT	CACAGGACTCTCGCAATCC
PPAR γ	CCAGAGCATGGTGCCTTCGCT	CAGCAACCATTGGGTCAGCTC
C/EBP α	GAACAGCAACGAGTACCGGGTA	GCCATGGCCTTGACCAAGGAG
36B4	AAGCGGCTCCTGGCATTGCTCT	CCGCGGGGCGACGAGTGGT

HGF, hepatocyte growth factor; LpL, lipoprotein lipase; PECAM, platelet endothelial cell adhesion molecule; PPAR, peroxisome proliferator-activated receptor.

HGF and vascular endothelial growth factor secretion assays. HGF release in the culture medium was measured using a mouse-specific HGF enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Tokyo, Japan). Vascular endothelial growth factor (VEGF) release in the culture medium was also measured by ELISA (R & D Systems). Secretion was normalized to either total DNA content of cells quantitated by Hoechst 33258 fluorometry (3), or total protein concentration of cells determined with the D_C Bio-Rad Protein Assay (Bio-Rad Laboratories).

Statistical analyses. All data are expressed as means \pm SE. Statistical comparisons of all in vitro experiments were done by paired *t*-test. Comparison of gene expression in fat pads with and without HGF silencing and from HGF-overexpressing mice and wild-type littermates was done by unpaired *t*-test. A *P* value of <0.05 was considered significant. All statistics were done using GraphPad Prism software.

RESULTS

3T3-F442A preadipocytes express HGF mRNA and secrete HGF protein. To determine if 3T3-F442A adipocytes secrete HGF as we previously observed for human adipocytes, HGF mRNA and protein secretion were quantitated. Both 3T3-F442A preadipocytes and mature adipocytes express HGF, and differentiation does not alter mRNA levels (78.4 ± 10.8 vs. 62.1 ± 6.1 relative units; $n = 3$). A significant increase in lipoprotein lipase (LpL) mRNA after 10 days of differentiation in vitro confirmed that the cells had matured to adipocytes (10.3 ± 2.0 vs. $1,866.0 \pm 132.0$ relative units; $P = 0.005$; $n = 3$). Interestingly, despite similar levels of HGF mRNA expression in undifferentiated and fully differentiated cells, secretion of HGF protein was significantly greater from preadipocytes compared with mature cells (Fig. 1). Adipose tissue secretes a number of angiogenic growth factors in addition to HGF, including VEGF. As shown in Fig. 1, 3T3-F442A preadipocytes secreted 15.5 \pm 0.2-fold more HGF than VEGF in the culture medium over a 48-h period. Differentiation increased VEGF secretion 4.4 \pm 1.1-fold over that of preadipocytes, and 3T3-F442A adipocytes secreted more VEGF than HGF ($P = 0.02$). These data demonstrate that 3T3-F442A preadipocytes and adipocytes synthesize and secrete HGF.

Expression and secretion of HGF is reduced by siRNA in 3T3-F442A preadipocytes. To examine the role of HGF in fat pad neovascularization and adipocyte differentiation in vivo,

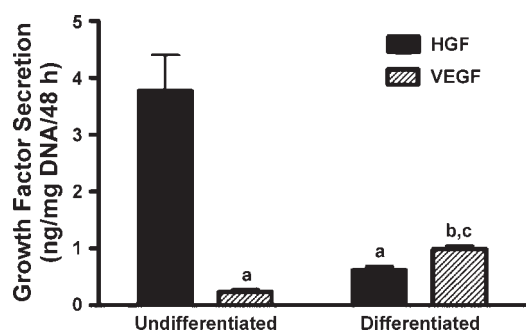


Fig. 1. 3T3-F442A cells secrete hepatocyte growth factor (HGF) protein. Undifferentiated 3T3-F442A preadipocytes and mature adipocytes differentiated in vitro were cultured for 48 h, and HGF or vascular endothelial growth factor (VEGF) secretion in the medium was quantitated by specific enzyme-linked immunosorbent assay (ELISA). Values represent the means \pm SE for 3 separate experiments. ^a $P < 0.05$ relative to HGF secretion from undifferentiated cells. ^b $P = 0.02$ relative to VEGF secretion from undifferentiated cells. ^c $P = 0.02$ relative to HGF secretion from differentiated cells.

siRNA constructs were used to silence HGF expression in 3T3-F442A preadipocytes. Electroporation with 400 nM siRNA resulted in a significant $82.3 \pm 4.2\%$ decrease in HGF mRNA levels (Fig. 2A), which translated to a significant $82.9 \pm 1.4\%$ decrease in HGF secretion from 3T3-F442A preadipocytes compared with control cells that had been electroporated with a scrambled sequence (Fig. 2B). The effect of HGF knockdown on 3T3-F442A differentiation to mature adipocytes was tested in vitro. Following 10 days of differentiation, expression of the adipocyte-specific genes CCAAT/enhancer binding protein- α (C/EBP α), peroxisome proliferator-activated receptor (PPAR)- γ , and LpL was significantly increased in differentiated adipocytes compared with that in undifferentiated cells, indicating successful differentiation to mature adipocytes. Silencing of HGF had no effect on differentiation of preadipocytes to mature adipocytes in vitro (Fig. 3).

Migration of EPCs to medium conditioned by preadipocytes is attenuated with HGF silencing. To assess the functional significance of HGF knockdown in 3T3-F442A preadipocytes with respect to the induction of angiogenesis, the in vitro migratory response of EPCs to medium conditioned by 3T3-F442A preadipocytes was examined. EPC migration was increased significantly by $155.5 \pm 3.8\%$ when cells were exposed to medium conditioned by 3T3-F442A preadipocytes compared with migration stimulated by control medium not conditioned by preadipocytes. As shown in Fig. 4, EPC migration to conditioned medium from 3T3-F442A preadipocytes with HGF knockdown was decreased significantly by $70.5 \pm 19.0\%$. These data demonstrate that inhibition of HGF secretion from 3T3-F442A preadipocytes results in a significant reduction in the ability of the preadipocytes to recruit cells required for vascular formation.

3T3-F442A cells develop into vascularized fat pads in vivo. Injection of 3T3-F442A preadipocytes under the skin of BALB/c nude mice results in formation of a fat pad retained within a pocket formed by the connective tissue fascia separating the skin and underlying muscle tissue (16), permitting clean retrieval of the developing fat pad from the mouse. Labeling of preadipocytes with Q-tracker 800 Qdot nanocrystals before injection confirmed that the injected cells were retained within this connective tissue pocket (data not shown).

Developing fat pads were removed at several time points following injection of 3T3-F442A preadipocytes, and expression of endothelial cell genes and markers of adipocyte differentiation were examined. As shown in Table 2, expression of the endothelial cell markers TIE-1 and platelet endothelial cell adhesion molecule (PECAM)-1 was not detectable in undifferentiated 3T3-F442A preadipocytes cultured in vitro. TIE-1 and PECAM-1 expression was measurable within the developing fat pad as soon as 24 h after injection of 3T3-F442A preadipocytes, and expression increased to levels comparable to that in epididymal fat by day 6 following injection. LpL mRNA was low in developing fat pads at 24 h following injection and increased over the course of the experiment, indicating that 3T3-F442A cells were differentiating to mature adipocytes in vivo (Table 2). These data demonstrate that injected 3T3-F442A cells form fat pads in vivo and that endothelial cell migration and adipocyte differentiation can be monitored by quantitation of cell-specific mRNA.

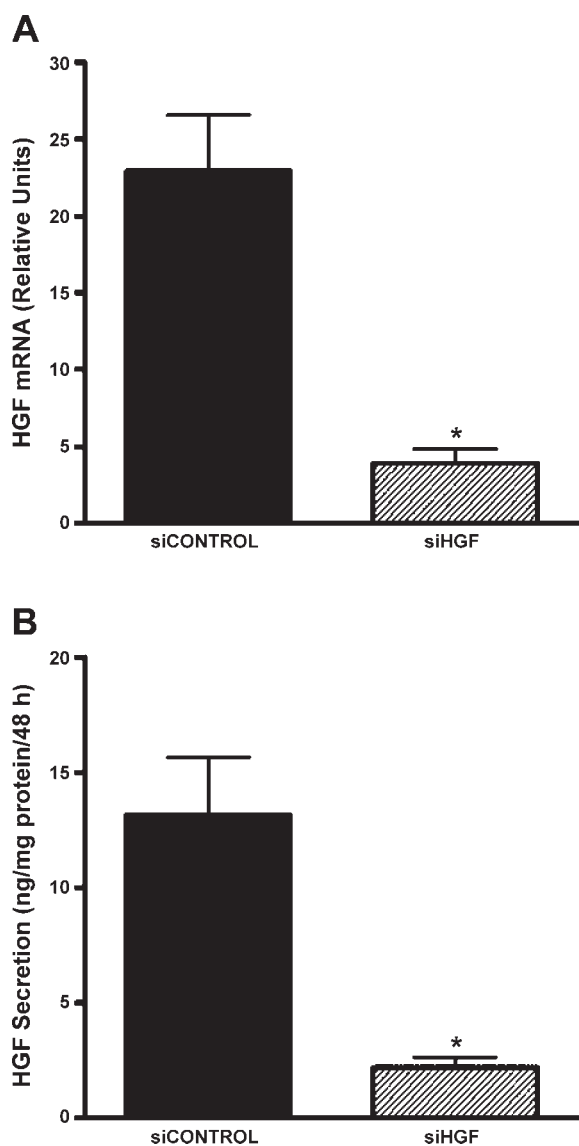


Fig. 2. Silencing of HGF gene expression and protein secretion in 3T3-F442A preadipocytes. *A*: preadipocytes were electroporated with small-interfering (si) RNA targeting HGF mRNA (siHGF) or scrambled siRNA sequence (siCONTROL), and HGF message levels were quantitated after recovery for 72 h (* $P = 0.005$; $n = 5$ experiments). *B*: 3T3-F442A preadipocytes were cultured for an additional 48 h after the recovery period and secretion of HGF protein measured by ELISA (* $P = 0.006$; $n = 5$).

Loss of HGF activity in developing fat pads results in impaired neovascularization in vivo. The effect on endothelial cell recruitment of silencing HGF mRNA expression in 3T3-F442A preadipocytes before injection under the skin of nude mice was assessed at 72 h postinjection. As shown in Fig. 5, HGF mRNA remained reduced significantly by $73.9 \pm 12.0\%$ at the end of 72 h of *in vivo* fat pad development. Decreased endothelial cell recruitment was evidenced by a significant $94.4 \pm 2.2\%$ reduction in TIE-1 mRNA and a $91.5 \pm 2.5\%$ decrease in PECAM-1 mRNA in the developing fat pad (Fig. 5).

We performed immunohistochemical staining for PECAM-1 (CD31) in fat pads removed 72 h after injection to assess the location and organization of endothelial cells in developing fat

pads. As shown in Fig. 6, *A* and *B*, endothelial cells are not detectable in the injected cell mass at 72 h postinjection. However, staining for PECAM-1 along the edge of the muscle layer adjacent to the fat pad developing from siCONTROL preadipocytes (Fig. 6*A*) is greater than that next to siHGF-

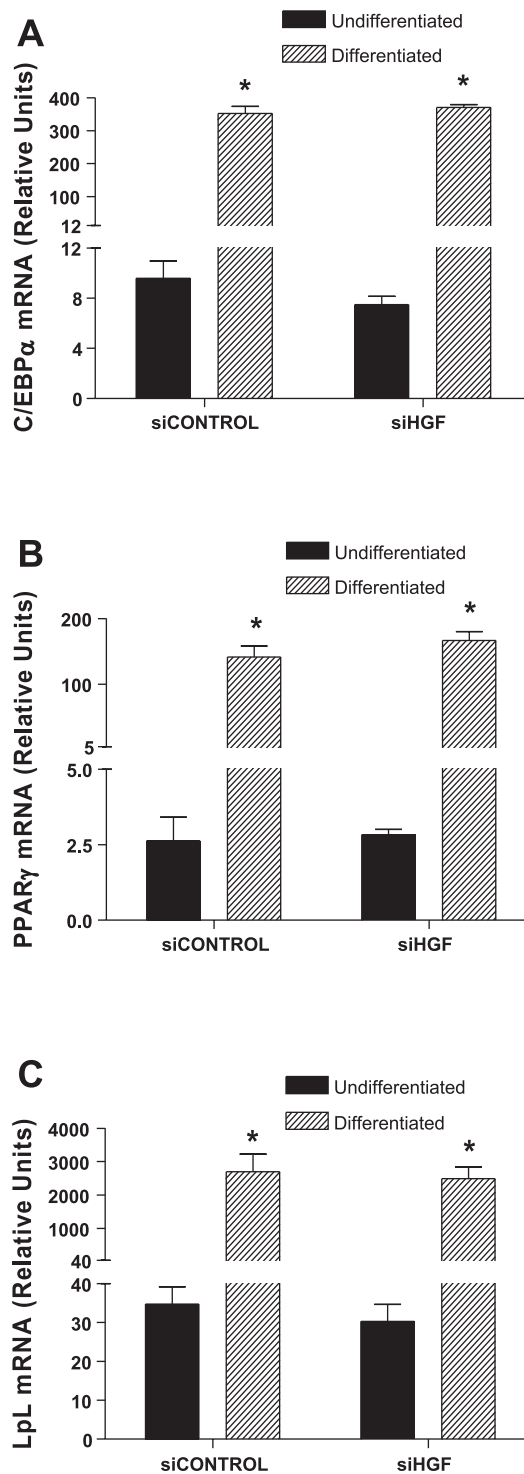


Fig. 3. HGF silencing has no effect on differentiation of 3T3-F442A preadipocytes to mature adipocytes *in vitro*. Preadipocytes with (siHGF) and without (siCONTROL) HGF silencing were differentiated to mature adipocytes. After 10 days of differentiation, expression of adipocyte genes was quantitated (* $P < 0.05$ relative to undifferentiated cells; $n = 3$).

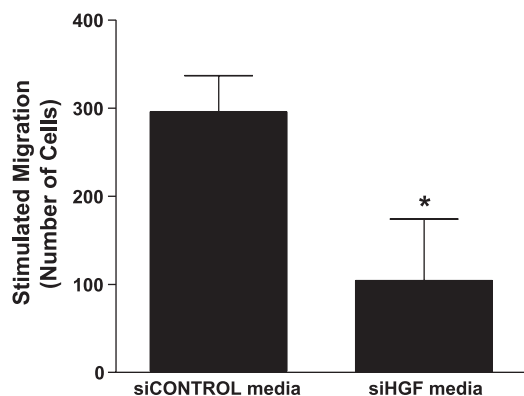


Fig. 4. Migration of endothelial progenitor cells (EPCs) to medium conditioned by 3T3-F442A preadipocytes with HGF knockdown is attenuated. Conditioned medium from 3T3-F442A cells with (siHGF) and without (siCONTROL) HGF silencing was used to stimulate Transwell migration of EPCs in vitro. Migration in the absence of medium conditioning was determined in each experiment and subtracted from the conditioned medium values. (* $P = 0.01$; $n = 6$).

injected preadipocytes (Fig. 6B). These data suggest that endothelial cell gene expression in developing fat pads at 72 h is the result of endothelial cells that are just entering the developing fat pad and have not yet formed vascular structures.

The effects of reduced neovascularization on 3T3-F442A preadipocyte differentiation were also explored (Table 3). Expression of the adipocyte-specific genes *C/EBP α* , *PPAR γ* , and *LpL* was increased significantly at 72 h postinjection compared with that in preadipocytes before injection (see Fig. 3 for reference). However, there was no significant difference in expression of adipocyte differentiation markers between developing fat pads with and without HGF silencing 72 h following injection. These results demonstrate that lack of HGF decreases endothelial cell migration in developing fat pads and suggest that attenuated neovascularization of the fat pad did not impair early events in 3T3-F442A preadipocyte differentiation.

Elevated HGF results in accelerated neovascularization and increased early differentiation of fat pads in vivo. To examine if elevated HGF expression could accelerate fat pad neovascularization in vivo, 3T3-F442A preadipocytes were injected under the skin of HGF-overexpressing mice and wild-type littermates. At 72 h postinjection, expression of *TIE-1* was increased significantly by 16.5-fold, and *PECAM-1* by 21.4-fold, in fat pads from HGF-overexpressing mice compared with wild-type littermates (Fig. 7). The significant elevation in these endothelial cell markers was maintained to 144 h postinjection.

Table 2. Gene expression in undifferentiated 3T3-F442A preadipocytes grown in culture and in developing fat pads in vivo

	Undifferentiated 3T3-F442A	24 h	4 day	6 day	Epididymal Adipose Tissue
<i>TIE-1</i>	Undet	0.5	12	32	25 \pm 11
<i>PECAM-1</i>	Undet	11	44	183	184 \pm 48
<i>LpL</i>	10	63	46	1017	2309 \pm 937

Relative mRNA expression normalized to 36B4; $n = 1$ experiment for each time point and $n = 4$ for epididymal adipose tissue.

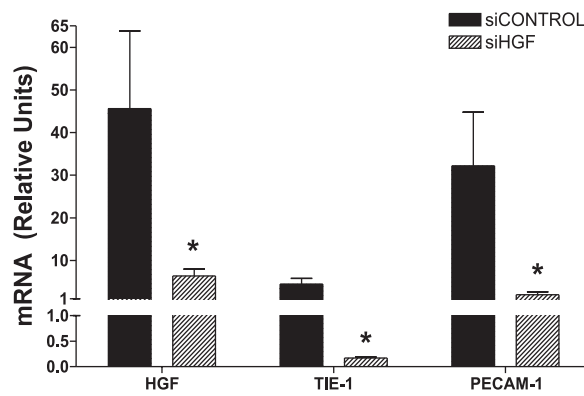


Fig. 5. Silencing of HGF results in impaired endothelial cell migration in developing fat pads in vivo. 3T3-F442A preadipocytes with (siHGF) and without (siCONTROL) HGF silencing were injected under the skin of nude mice, and fat pads were allowed to develop for 72 h. Expression of the endothelial cell-specific genes *TIE-1* and platelet endothelial cell adhesion molecule (*PECAM-1*) was quantitated in the excised fat pads (* $P < 0.05$; $n = 4$).

Immunohistochemical staining for *PECAM-1* (CD31) in fat pads removed 72 h after injection under the skin of HGF-overexpressing mice is shown in Fig. 8. At 72 h postinjection, there was a larger band of injected cells, and more extensive *PECAM-1* staining, in the fat pad from the HGF-overexpressing mouse (Fig. 8B) compared with that in the fat pad from the wild-type mouse (Fig. 8A). Interestingly, at 72 h postinjection, endothelial cells in both wild-type and HGF-overexpressing mice appear to be organizing into vessel-like structures to a much greater extent than observed at 72 h in nude mice (Fig. 6). This could be because of the presence of mature adipose tissue under the skin of C57BL/6J mice secreting angiogenic factors that facilitate vessel development in the injected cell mass. In contrast, in nude mice, the injected cells rest directly on the muscle layer (Fig. 6) and are therefore exposed to a different in vivo milieu. Overall, these data suggest that endothelial cell gene expression detected in developing fat pads in HGF-overexpressing and wild-type mice is derived from endothelial cells that have sprouted from existing blood vessels and have migrated into the injected cell mass.

The effect of accelerated angiogenesis on adipogenesis was also evaluated. As shown in Table 4, expression of the adipocyte differentiation markers *C/EBP α* and *PPAR γ* was increased significantly at early time points (24 and 48 h postinjection) in fat pads excised from HGF-overexpressing mice. However, there was no significant difference in adipocyte differentiation markers at 72 h postinjection. To test for a direct effect of HGF to promote differentiation of 3T3-F442A preadipocytes, cells were differentiated in vitro in the presence and absence of 100 ng/ml HGF. Treatment with HGF had no effect on the expression of *C/EBP α* (130.3 \pm 7.5 vs. 167.7 \pm 53.0 relative units, $n = 3$) or *PPAR γ* (67.3 \pm 7.4 vs. 119.1 \pm 39.2 relative units, $n = 3$) quantitated after 72 h of differentiation. These studies suggest that overexpression of HGF accelerates migration of endothelial cells into developing fat pads.

DISCUSSION

It was first shown by Green and Kehinde (8) that fat pads develop from 3T3-F442A preadipocytes injected under the skin of BALB/c nude mice. This model has subsequently been

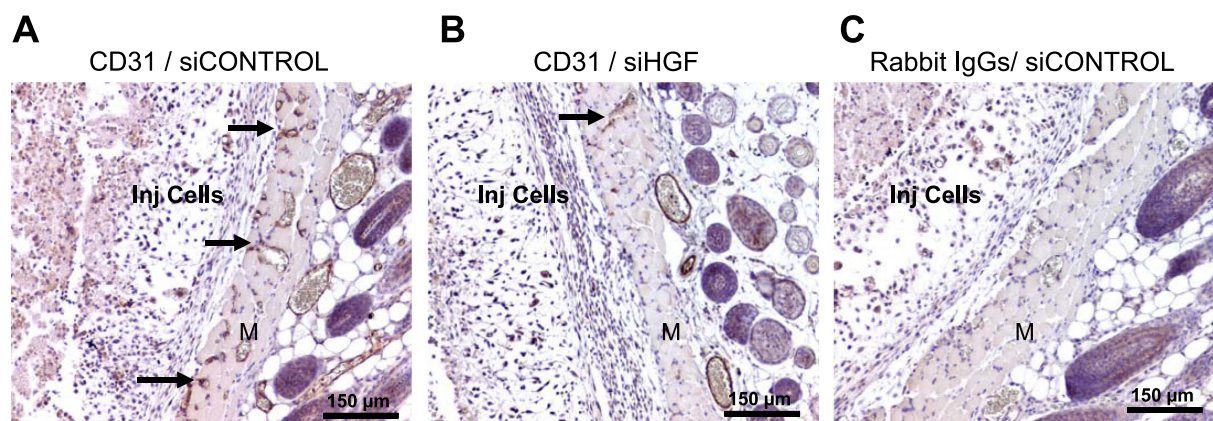


Fig. 6. Immunohistochemical evaluation of vascular development in fat pads formed by 3T3-F442A cells with HGF knockdown. Fat pads attached to the underside of the skin from nude mice were stained with antibodies to the endothelial cell marker PECAM-1 (CD31). Endothelial cells were not present in the center of the injected control preadipocytes (A), or those with HGF knockdown (B), at 72 h. There was greater staining for endothelial cells in the muscle layer adjacent to the control 3T3-F442A preadipocytes (siCONTROL; A) compared with that in the muscle layer adjacent to injected cells with HGF knockdown (siHGF; B). Black arrows indicate endothelial cells. Inj Cells, injected cells; M, muscle.

used to study control of leptin synthesis *in vivo* (14) and investigate the relationship between angiogenesis and adipogenesis during fat pad development (7, 16). Our work now extends the use of this model to study the role of HGF in the early angiogenic response in adipose tissue. We show that silencing of HGF mRNA expression in 3T3-F442A preadipocytes reduced the ability of the cells to secrete HGF protein and to stimulate EPC migration *in vitro*. Injection of preadipocytes with HGF knockdown under the skin of nude mice significantly impaired recruitment of endothelial cells into the developing fat pad. In contrast, elevated HGF in transgenic mice accelerated endothelial cell migration into developing fat pads. Collectively, these findings support a central regulatory role for HGF in adipose tissue angiogenesis.

As we have observed for human preadipocytes and adipocytes (1, 17, 18), 3T3-F442A cells express HGF mRNA and secrete HGF protein. HGF mRNA levels do not differ between 3T3-F442A preadipocytes and mature adipocytes differentiated *in vitro*, but preadipocytes secrete significantly more HGF protein than mature adipocytes. This finding suggests that HGF protein synthesis is regulated differentially at the translational level or that the packaging of the hormone for secretion differs between the two cell states. Of importance *in vivo*, this finding may suggest that preadipocytes, rather than adipocytes, are primarily responsible for regulating the formation of capillaries to support adipose tissue growth.

Secretion of HGF from 3T3-F442A preadipocytes is significantly greater than that of VEGF *in vitro*. This observation is in agreement with earlier studies from our laboratory and

others showing that synthesis of HGF in human adipose tissue is ~10-fold greater than that of VEGF or basic fibroblast growth factor (6, 18). In contrast, differentiated 3T3-F442A cells appear to secrete more VEGF than HGF, a finding that is

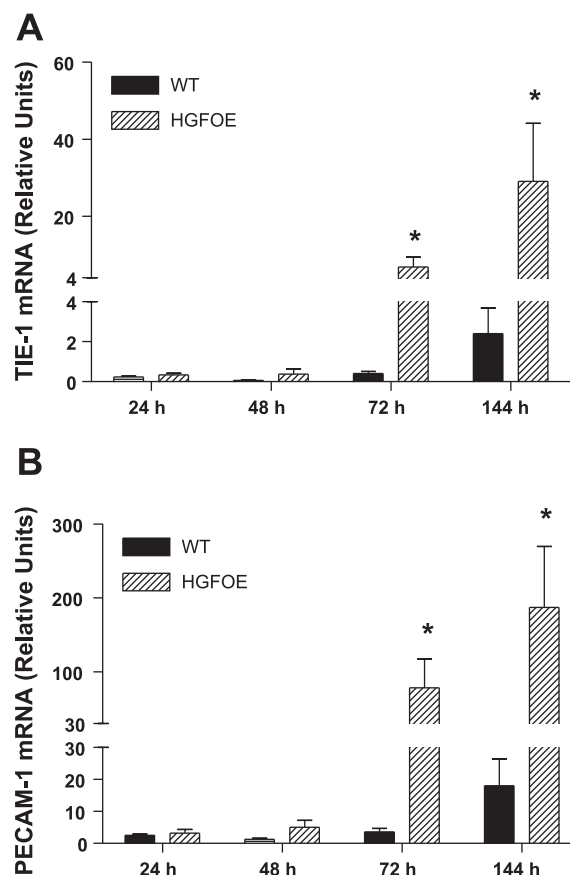


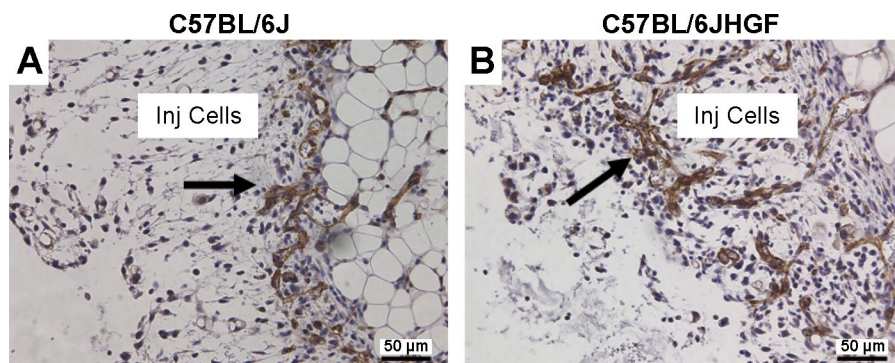
Fig. 7. Overexpression of HGF increases endothelial cell gene expression in developing fat pads *in vivo*. 3T3-F442A preadipocytes were injected under the skin of transgenic mice overexpressing HGF (HGFOE) or wild-type littermates (WT), and expression of the endothelial cell genes TIE-1 (A) and PECAM-1 (B) was quantitated in developing fat pads. Values represent means \pm SE for 4–7 independent experiments at each time point (* $P < 0.05$ relative to WT).

Table 3. Impaired neovascularization does not alter 3T3-F442A differentiation *in vivo*

	mRNA Level (relative units)	
	siControl	siHGF
C/EBP α	95.8 \pm 45.7	58.8 \pm 31.2
PPAR γ	13.7 \pm 10.9	12.5 \pm 9.7
LpL	197.7 \pm 108.5	183.2 \pm 119.5

Values are means \pm SE for 4 experiments. si, Small interfering. Relative mRNA expression normalized to 36B4.

Fig. 8. Immunohistochemical evaluation of vascular development in fat pads formed by 3T3-F442A cells in wild-type and HGF-overexpressing C57BL/6J mice. There are a greater number of endothelial cells and capillaries present within preadipocytes injected under the skin of HGF-overexpressing mice (B) than in wild-type mice (A) after 72 h of fat pad development. Black arrows indicate endothelial cells. C57BL/6J, wild-type mice; C57BL/6J-HGF, HGF-overexpressing mice.



not in line with observations in human cells and that requires additional investigation. Several studies have documented that HGF and VEGF have similar efficacy in stimulating endothelial cell proliferation, migration, and tubule formation (12, 22, 24). Thus the greater secretion of HGF from 3T3-F442A preadipocytes and human adipose tissue suggests that HGF is a principal angiogenic factor in adipose tissue.

siRNA constructs were used to silence HGF gene expression in 3T3-F442A preadipocytes, resulting in a significant reduction in HGF protein secretion. HGF knockdown had no effect on the ability of the cells to differentiate into mature adipocytes *in vitro*. However, there was a significant reduction in EPC migration to medium conditioned by 3T3-F442A preadipocytes lacking HGF, establishing a role for HGF in preadipocyte-regulated angiogenesis at the level of endothelial cell chemotaxis. Our *in vitro* findings are in agreement with the recent report of Saiki et al. (21) in which silencing of HGF in 3T3-L1 adipocytes suppresses the ability of the cells to stimulate human umbilical vein endothelial cell tubule formation by 61% compared with control siRNA-treated cells (21).

In the current study, we quantitated endothelial cell recruitment into developing fat pads by measuring expression of the endothelial cell genes TIE-1 and PECAM-1. Neels et al. (16) have previously shown that endothelial cell mRNA in developing fat pads correlates with histological evidence of endothelial cells and formation of new microvasculature. We demonstrate that cultured 3T3-F442A preadipocytes do not express TIE-1 and PECAM-1; thus, the presence of TIE-1 and PECAM-1 mRNA in the developing fat pad is the

result of endothelial cell recruitment. We detected low levels of endothelial cell mRNA in developing fat pads as soon as 24 h following injection, and that expression significantly increased by 72 h. Histological examination of the developing fat pad shows that, at 72 h postinjection, endothelial cells are sprouting from established vessels and associating with the fascia surrounding the injected cell mass. Thus measures of endothelial cell mRNA reflect endothelial cells that are attached to or have migrated just inside the fascia, when the fat pad is removed from the mouse. Knockdown of HGF secretion from 3T3-F442A preadipocytes before injection resulted in a significant reduction in endothelial cell gene expression in the developing fat pad and less evidence of endothelial cell staining in the tissue surrounding the injected preadipocytes. In combination with our *in vitro* observations of reduced EPC migration to medium conditioned by 3T3-F442A cells with HGF silencing, these *in vivo* data strongly support a role for HGF in neovascularization of developing adipose tissue.

We also tested the hypothesis that elevated HGF would accelerate neovascularization. For this experiment, 3T3-F442A preadipocytes were injected under the skin of HGF-overexpressing transgenic mice in which HGF mRNA is elevated 3- to 50-fold, depending on the tissue examined (23). Endothelial cell migration into 3T3-F442A fat pads was increased significantly in the presence of elevated HGF at 72 and 144 h postinjection of the preadipocytes. However, it is important to note that HGF in this model is elevated in the tissue surrounding the injected cells. Therefore, the HGF-activated endothelial cells migrated in the developing fat pad in response to a chemotactic signal other than HGF. Interestingly, endothelial cell migration in the 3T3-F442A preadipocyte mass appears greater when injected under the skin of wild-type C57BL/6J mice (Fig. 8A) than when injected under the skin of BALB/c nude mice. One explanation for this difference may be the presence of mature adipose tissue under the skin of the C57BL/6J mice, which promotes and supports angiogenesis to a greater extent than the muscle tissue upon which the 3T3-F442A cells rest under the skin of the BALB/c mice. Overall, our findings with HGF overexpression compliment our knockdown studies, and together both experiments strongly support a role for HGF in regulating adipose tissue vascular development.

Angiogenesis and adipogenesis are interdependent events in adipose tissue growth (4, 9, 10), and we hypothesized that HGF has a central role in coordinating both processes. We found that differentiation of 3T3-F442A preadipocytes *in vitro* was not

Table 4. Expression of adipocyte differentiation markers in fat pads excised from HGFOE and WT littermates

	Hours			
	24 (n = 4)	48 (n = 5)	72 (n = 6)	144 (n = 7)
C/EBP α				
WT	14.2 \pm 4.1	20.4 \pm 4.1	140.7 \pm 59.7	87.4 \pm 22.0
HGFOE	40.8 \pm 7.5*	48.5 \pm 10.0*	105.3 \pm 33.12	213.8 \pm 116.9
PPAR γ				
WT	7.9 \pm 3.2	8.5 \pm 1.2	44.9 \pm 17.6	31.5 \pm 12.3
HGFOE	11.0 \pm 5.4	16.7 \pm 4.3*	32.0 \pm 10.0	29.4 \pm 15.0
LpL				
WT	20.8 \pm 7.6	55.8 \pm 14.4	489.6 \pm 161.8	269.5 \pm 86.7
HGFOE	32.0 \pm 13.1	115.5 \pm 32.3	315.8 \pm 92.5	531.7 \pm 290.2

Values are means \pm SE; n, no. of experiments. WT, wild type; HGFOE, HGF overexpressing. Relative mRNA expression normalized to 36B4. *P < 0.05 relative to WT.

altered by HGF knockdown or treatment with the hormone, ruling out a direct effect of HGF to promote preadipocyte differentiation. However, HGF could influence preadipocyte differentiation by regulating endothelial cell migration in the developing fat pad. Using a dorsal skinfold chamber for fat pad development, Fukumura et al. (7) found that blocking angiogenesis with an anti-VEGF receptor antibody impaired differentiation of 3T3-F442A preadipocytes when assessed at 21 days postcell implantation. Thus we hypothesized that silencing HGF expression in preadipocytes and impairing endothelial cell migration in the developing fat pad might slow 3T3-F442A preadipocyte differentiation. However, we found instead that markers of adipocyte differentiation were not different between fat pads with and without HGF gene silencing. Furthermore, in developing fat pads from HGF-overexpressing mice, there was also no difference in adipocyte differentiation markers compared with that in fat pads from wild-type mice at 72 h, despite an early (48 h), transient increase in C/EBP α and PPAR γ expression. Therefore, our findings suggest that preadipocyte-endothelial cell interactions are not required for initiation of preadipocyte differentiation, which is more likely regulated by such signals as insulin-like growth factor I and/or insulin. Furthermore, although the findings of Fukumura et al. (7) support the notion of an interaction between endothelial cells and preadipocytes that promotes preadipocyte differentiation, this work does not provide insight into the timing of such an interaction. Thus it may be that paracrine factors from endothelial cells are important at later time points in the differentiation process. Future experiments with a detailed time course will be needed to fully investigate this possibility.

Several limitations of our study require mention. HGF silencing in 3T3-F442A preadipocytes was achieved by electroporation of siRNA in the cells. Because of the transient nature of HGF knockdown achieved with this technique, we were unable to examine time points later than 72 h postinjection in a reliable manner. Future experiments using stable lentiviral-mediated knockdown of HGF in 3T3-F442A preadipocytes will eliminate this problem. Differences in the number of preadipocytes that survive implantation is an important additional consideration in evaluating the extent of fat pad development, especially at later time points. Future experiments using preadipocytes within a collagen gel placed under the skin (5) will allow for more precise quantitation of recovered cells and their extent of differentiation.

In summary, our data support a role for HGF in adipose tissue angiogenesis. We show that silencing of HGF expression impairs the ability of developing fat pads to recruit endothelial cells for neovascularization and that excess HGF accelerates endothelial cell migration in the fat pad. Increased endothelial cell migration in developing fat pads in the presence of elevated HGF appears to accelerate early differentiation of the injected preadipocytes. Understanding the role of HGF in regulating adipose tissue angiogenesis may provide insight into a possible target to inhibit expansion of adipose tissue and obesity.

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