Induction of Adipogenesis by External Volume Expansion

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Background: External volume expansion by suction is used to prepare the recipient site for fat grafting by increasing its compliance and vascularity. The authors previously developed a mouse model for external volume expansion and demonstrated its pro-proliferative and angiogenic effects. Increased thickness of the subcutaneous tissue was also observed. This study was thus designed to assess the adipogenic potential of external volume expansion stimulation.
Methods: A miniaturized external volume expansion device consisting of a rubber dome connected to a −25 mmHg suction source was applied to the dorsum of mice for a single 2-hour stimulation or for 2 hours daily for 5 days. Tissues were harvested up to 48 hours after the last stimulation and analyzed for edema, inflammation, and adipocyte content by staining for hematoxylin and eosin, CD45, and perilipin-A. Expression of peroxisome proliferator-activated receptor-γ (proadipogenic factor) and preadipocyte factor 1 (preadipocyte marker) was evaluated by Western blot analysis.
Results: Both a 2-hour stimulation and cyclical 2-hour stimulation for 5 days induced 1.5- and 1.9-fold increases in the number of adipocytes per millimeter. Edema was present in the immediate poststimulation period, and inflammation was seen 2 days later. Peroxisome proliferator-activated receptor-γ was increased at the end of stimulation.
Conclusions: Stretch is known to stimulate proliferation, whereas edema and inflammation are both emerging proadipogenic factors. Their combination in external volume expansion seems to produce proadipogenic effects, seen even after a single 2-hour stimulation. (*Plast. Reconstr. Surg.* 137: 122, 2016.)

External volume expansion refers to a noninvasive tissue expansion system recently gaining popularity in plastic surgery.[1] By the use of vacuum (created within rigid plastic domes) applied to the skin, it expands tissue compartments and increases their vascularity. As such, it has emerged in clinical practice to provide an adjuvant preparatory technique to improve outcomes of autologous fat grafting to the breast.[2,3]

The development and introduction of external volume expansion devices into the plastic surgery and breast augmentation markets was guided by the idea that three-dimensional tensile forces could be used to directly stimulate breast growth and induce nonsurgical breast enlargement (i.e., Brava Breast Enhancement and Shaping System; Brava LLC, Miami, Fla.).[4] In the initial clinical experience with the device, most users achieved an average increase

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of one cup size after several months of external volume expansion use alone, by receiving stimulation for up to 10 hours daily. Although these findings were in agreement with accumulating in vitro[5,6] and in vivo evidence[7–9] of the role played by mechanical forces in tissue expansion, the device demanded high patient compliance and did not achieve widespread use for breast augmentation alone because of inconsistent results. There is now significant clinical interest in adopting the device as a preparatory approach for fat grafting to the breasts.[10]

Previous work in our laboratory focused on the development of a miniaturized animal model for external volume expansion to investigate the mechanism through which it clinically optimizes fat graft outcomes.[11,12] We demonstrated that external volume expansion causes an increase in cell proliferation rate and vascular density, creating a local environment from which fat grafts potentially benefit. We showed how edema, ischemia, and inflammation occurring early after external volume expansion likely play a role in facilitating these effects. In addition, we also noticed an increase in thickness of the subdermal fat compartment in external volume expansion–treated sites after prolonged stimulation, suggestive of new adipocytes accumulating in the stimulated area.[11] The idea that external volume expansion stimulation may have adipogenic effects is intriguing. Because mature adipocytes generally are believed not to proliferate, adipogenesis is thought to result from stem cells' commitment to the adipocyte lineage, proliferation of preadipocytes, their recruitment, and terminal differentiation into mature adipocytes. Building on the evidence collected in our previous work, we designed a study to assess the hypothesis that external volume expansion stimulation can directly induce adipogenesis, independent from fat grafting.

MATERIALS AND METHODS

Animal Model

A miniaturized external volume expansion device, consisting of a rubber dome with an inner diameter of 1 cm and connected to a vacuum pump (VAC Instill; KCI, San Antonio, Texas) set to a constant −25 mmHg, was applied to the dorsa of mice, 1 cm lateral to the spine. The nontreated contralateral side was used as the matched control (not significant). No fixatives were used other than suction power.[12]

Under a protocol approved by the local Institutional Animal Care and Use Committee, 28 male SKH1-E hairless, euthymic mice (Charles River Laboratories, Wilmington, Mass.) were stimulated for 2 hours/day for either 1 or 5 consecutive days. These patterns were designed based on our previous studies with external volume expansion models[11,12] and on a pilot study, to establish single-stimulation effects and explore the kinetics of cyclical stimulation, mimicking the clinically adopted daily wearing of the external volume expansion system. Half of the animals receiving either treatment pattern were sacrificed immediately after completing stimulation (+0h) to evaluate the early effects of external volume expansion, and the other half were sacrificed 48 hours after stimulation (+48h) (Fig. 1).

The treated site and the nontreated contralateral skin were harvested with a 10-mm punch biopsy tool. One half was snap-frozen and preserved at −80°C for protein analysis; the remaining half was mounted on cardboard, fixed in 10% formaldehyde for 24 hours for histologic evaluation, and stored in 70% ethanol at 4°C.

Histologic Evaluation and Immunohistochemistry

Samples were embedded in paraffin and cut into 5-μm sections. Hematoxylin and eosin staining was performed according to standard protocols. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval for perilipin-A was accomplished by microwaving in 10 mM sodium citrate (pH 6.0). Sections for pan-leukocyte marker CD45 were treated with 40 μg/ml proteinase K (Roche Diagnostics Corp., Branchburg, N.J.) for 30 minutes at 37°C. Perilipin-A and CD45 primary antibodies were incubated at 4°C overnight. (**See Table, Supplemental Digital Content 1,** which shows the primary and secondary antibodies used in immunohistochemistry and Western blotting, ***http://links.lww.com/PRS/B510***.) Signal was intensified using the tyramide amplification system (Perkin-Elmer, Boston, Mass.), and positive staining was detected with diaminobenzidine (Dako North America, Inc., Carpinteria, Calif.). Slides were counterstained with hematoxylin. Images were acquired using an Aperio ScanScope XT slide scanner (Aperio, Vista, Calif.) and a Nikon E200 microscope (Nikon Corp., Tokyo, Japan). One animal in the external volume expansion-1 +48h group was excluded from examination because of sample labeling issues, and one in the external volume expansion-5 +48h group because of sample loss in processing.

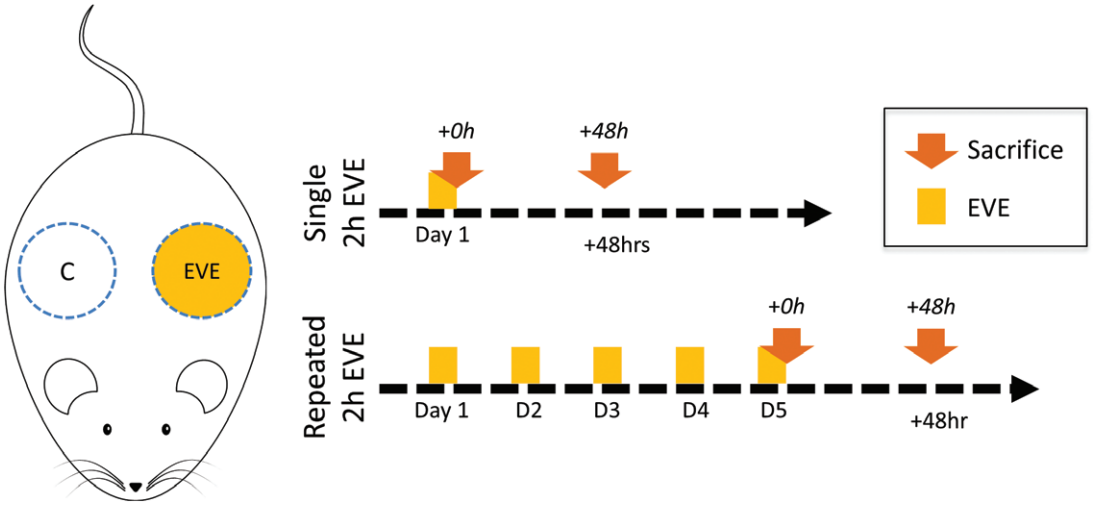

Fig. 1. (*Left*) A selected area of mouse dorsum lateral to the spine was stimulated (*EVE*); the contralateral area was left untreated as the internal control (*C*). (*Right*) Animals were distributed into two groups. Group 1 (EVE1) received a single stimulation of 2 hours. Group 2 (EVE5) received a daily stimulation of 2 hours repeated for 5 consecutive days. The animals were sacrificed immediately after the last session of stimulation *(+0h, n* = 7 per group) or 48 hours after the last session *(+48h, n* = 7 per group). *EVE*, external volume expansion.

Adipocyte Quantification

Adipocyte number was selected as the primary outcome of our study. Viable adipocytes were identified in sections stained for the specific marker perilipin-A (PLIN-A), which is a protein coating lipid droplets in adipocytes that protects them against lipases.[13] Oval structures with positivity for antigen expression around the membrane were considered as mature adipocytes.

Quantification of the number of adipocytes in the subdermal adipose layer, superficial to the panniculus carnosus muscle, was performed in the external volume expansion-1 +48h and external volume expansion-5 +48h groups. Counting was performed on entire sections scanned at 40× magnification with the aid of ImageScope software (Aperio) by three investigators and confirmed by blinded manual counting by independent investigators. The results were normalized as the number of adipocytes per millimeter based on section length as measured in the scanned slides, and expressed as fold increase of treated sides over internal controls. Adipocyte counts in animals from the +0h groups was not performed, as edema and tissue distortion impeded adequate staining and identification of structures.

Qualitative Assessment of Tissue

The architecture of epidermis, dermis, and subcutaneous tissue was analyzed on hematoxylin and eosin–stained slides, paired with the macroscopic appearance, and reviewed by two independent examiners.

Inflammation

$CD45^+$ cell density in the external volume expansion-1 +48h and external volume expansion-5 +48h groups was quantified over three representative 10× fields of each stained section by three independent observers with the aid of ImageJ software (National Institutes of Health, Bethesda, Md.).

Protein Expression

Western blot analysis was performed on samples of animals receiving stimulation for 5 days and sacrificed immediately and after 48 hours (n = 4 each) for peroxisome proliferator-activated receptor-γ (PPAR-γ) and preadipocyte factor 1 (Pref-1, also called DLK1) under similar protocols. Tissue samples were placed in T-PER Pierce/Thermo scientific Protein Extraction Buffer (Thermo Scientific, Grand Island, N.Y.) that contained complete Mini, EDTA–free Proteinase Inhibitor Cocktail (Roche). Samples were then lysed on ice by means of motorized pestle.

Protein concentrations were measured using the standard bicinchoninic acid protein assay method (Pierce, Waltham, Mass.) and ultraviolet spectrophotometer (NanoDrop ND-2000; Thermo Scientific). Gel electrophoresis was performed using precast Ready Gel Tris-HCl 4–12% Gels (Bio-Rad, Hercules, Calif.). Tris/glycine/

sodium dodecyl sulfate buffer (Bio-Rad) served as running buffer. Equal amounts of protein (15 to 30 μg) were loaded into each lane, and the gel was run at 180 V for 1 hour. Polyvinylidene difluoride membranes (Bio-Rad) were briefly incubated in 100% methanol, quickly rinsed, and then incubated in 20% methanol containing Tris-glycine transfer buffer. Next, proteins were transferred onto polyvinylidene difluoride membranes at 25 V for 50 minutes using transfer buffer and a Trans-Blot SemiDry System (Bio-Rad). Polyvinylidene difluoride was then blocked with 5% milk in Tris-buffered saline with Tween for 90 minutes at room temperature. The primary antibody was diluted in 5% milk in Tris-buffered saline with Tween. Polyvinylidene difluoride membranes were incubated in the primary antibody solution at 4°C overnight on an orbital shaker. Polyvinylidene difluoride membranes were washed three times with Tris-buffered saline with Tween and then incubated in the secondary antibody solution (also in 5% milk in Tris-buffered saline with Tween) for 45 minutes at room temperature followed by three washes. Thermo Scientific Pierce ECL 2 Western Blotting Detection reagents (Thermo Scientific) served as the chemiluminescence kit. Semiquantitative imaging analysis was performed using ImageJ.

Statistical Analysis

Results are expressed as mean ± SD in text and figures. A paired two-tailed Student's t test was performed to compare means of external volume expansion–treated versus control tissues within the same animals and at the same time points using Prism software (GraphPad Software, Inc., La Jolla, Calif.). Means between groups at different time points were compared with a two-tailed t test for independent samples with equal variance. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Adipocyte Quantification

An increased density of subdermal adipocytes in external volume expansion–treated sites was detected at microscopic evaluation of hematoxylin and eosin–stained slides, and confirmed by quantitative analysis of slides stained for perilipin-A. Two days after a single stimulation for 2 hours (external volume expansion-1 +48h), tissues presented a 1.5 ± 0.25-fold increase in number of adipocytes per linear millimeter of tissue compared with nonstimulated tissue ($p < 0.01$). Treatment for 5 consecutive days (external volume expansion-5 +48h) yielded a 1.9 ± 0.6-fold increase in adipocytes in the subcutaneous tissue after 2 days compared with nonstimulated skin on the control side ($p < 0.01$) (Fig. 2). The difference in fold increase between time points was not significant.

Qualitative Assessment of Tissue

Immediately after external volume expansion, both with single 2-hour stimulation (external volume expansion-1 +0h) and with a 5-day course (external volume expansion-5 +0h), tissues appeared macroscopically swollen (Fig. 3). Histologic evaluation of hematoxylin and eosin–stained slides confirmed a large amount of interstitial fluid having accumulated, in particular, into the hypodermis. A normal histologic architecture was restored in samples collected 48 hours after treatment, when tissue swelling was no longer evident at examination

Inflammation

External volume expansion stimulation induced an inflammatory response, with a 2.2 ± 0.9-fold ($p < 0.01$) and 1.8 ± 0.7-fold ($p = 0.01$) increase over controls in $CD45^+$ cell density in both the external volume expansion-1 +48h and external volume expansion-5 +48h, respectively (Fig. 4).

Western Blot Analysis

Western blot analysis revealed that immediately after 5 days of stimulation (external volume expansion-5 +0h), relative PPAR-γ expression was significantly increased in stimulated skin compared with nonstimulated skin (1.0 ± 0.17 versus 0.24 ± 0.05; $p < 0.01$; $n = 4$). This effect, though to a lesser degree, was still detectable 48 hours later (0.75 ± 0.04 versus 0.43 ± 0.08; $p < 0.05$; $n = 4$). In samples analyzed for Pref-1, no difference was found between external volume expansion and nonstimulated sites after 5 days in the immediate sacrifice group (external volume expansion-5 +0h) ($p = 0.88$). The samples from animals sacrificed 48 hours later (external volume expansion-5 +48h) showed no difference in Pref-1 expression (control, 0.81 ± 0.2; external volume expansion, 0.40 ± 0.01; $p = 0.12$; $n = 4$) (Fig. 5).

DISCUSSION

The results from this study support the hypotheses that external volume expansion possesses an intrinsic proadipogenic potential, showing in our model an increased number of

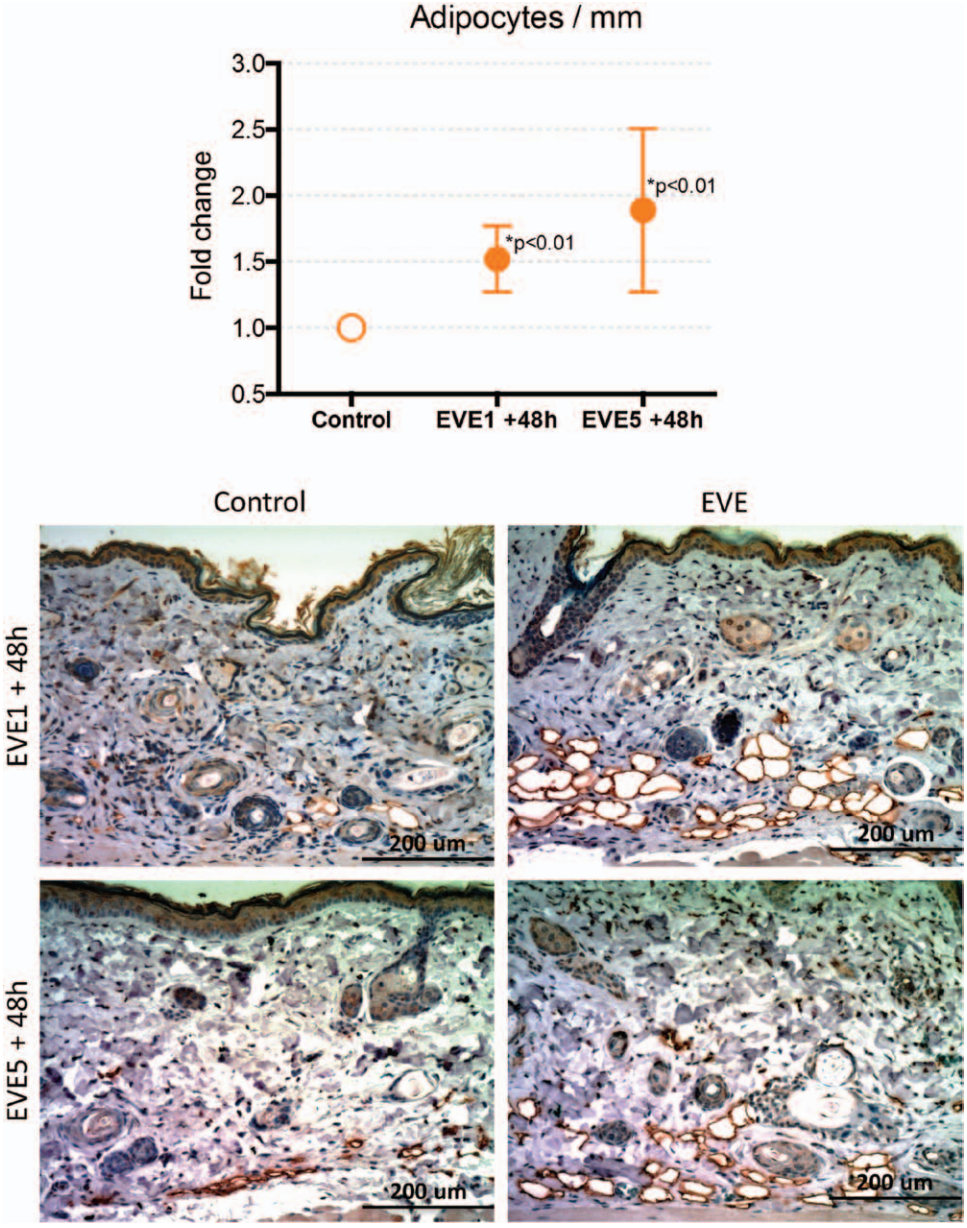

Fig. 2. Adipocytes (perilipin-A–positive oval-shaped structures) were identified in the subdermal layer above the panniculus carnosus muscle, and their number per millimeter over the entire section and surface percentage in standard 40× fields were quantified. Results are expressed as fold increase over controls. The *p* values refer to the comparison of each stimulated group with its internal controls from the same animals. *EVE*, external volume expansion; *EVE1+48h*, animals that received a single stimulation of 2 hours and were sacrificed after 48 hours; *EVE5+48h*, animals stimulated for 5 days and sacrificed after 48 hours.

adipocytes after a period of recovery of 48 hours, even from a single 2-hour stimulation. This seems to elicit its effect by means of the establishment of a proadipogenic, edematous, inflammatory environment.

PPAR-γ is a ligand-activated transcription factor expressed chiefly in adipocytes and macrophages.[14,15] This transcription factor has been demonstrated to be the main initiator and orchestrator of adipogenesis.[15,16] PPAR-γ activation initiates preadipocyte expansion in vitro[17] and induces adipogenesis in vivo,[18] whereas in its absence, adipose tissue fails to develop.[19] In our study, we found that a proadipogenic environment is induced, as demonstrated by increased expression of the PPAR-γ signaling pathway.

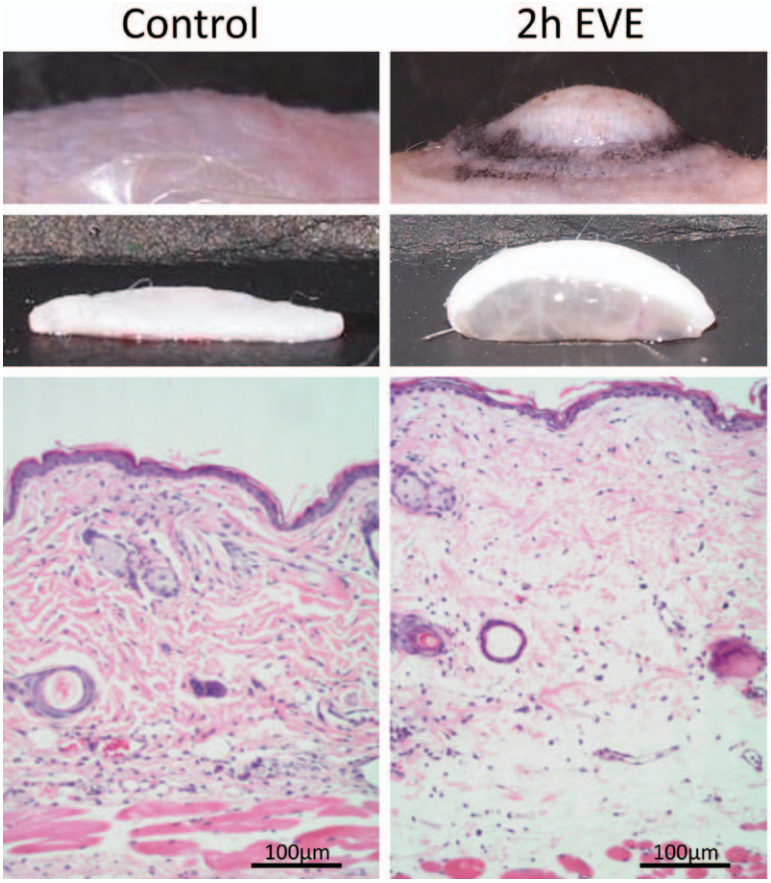

Fig. 3. Acute effects of external volume expansion (*EVE*) on stimulated tissues. Immediately after ceasing stimulation, tissues were macroscopically swollen (*above*). This was confirmed by cross-sectional biopsy (*center*) and at histologic analysis, which showed interstitial fluid accumulated in the dermis and hypodermis (*below*).

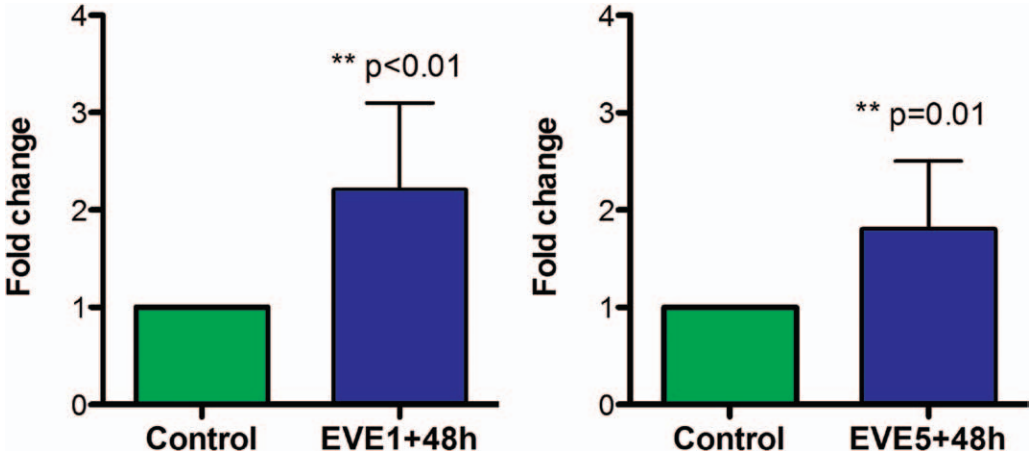

Fig. 4. Inflammatory cells ($CD45^+$) in external volume expansion-1 and external volume expansion-5, 48 hours after treatment. Results are expressed as fold increase over controls.

In parallel, Pref-1 is a specific marker of preadipocytes, acting as a gatekeeper in the process of adipogenesis. Pref-1 acts as an autocrine/paracrine factor, inhibiting adipocyte differentiation.[20] As differentiation occurs, Pref-1 expression decreases to undetectable levels, reflecting the degree of maturation and differentiation.[21] We did not find a difference of expression of this marker

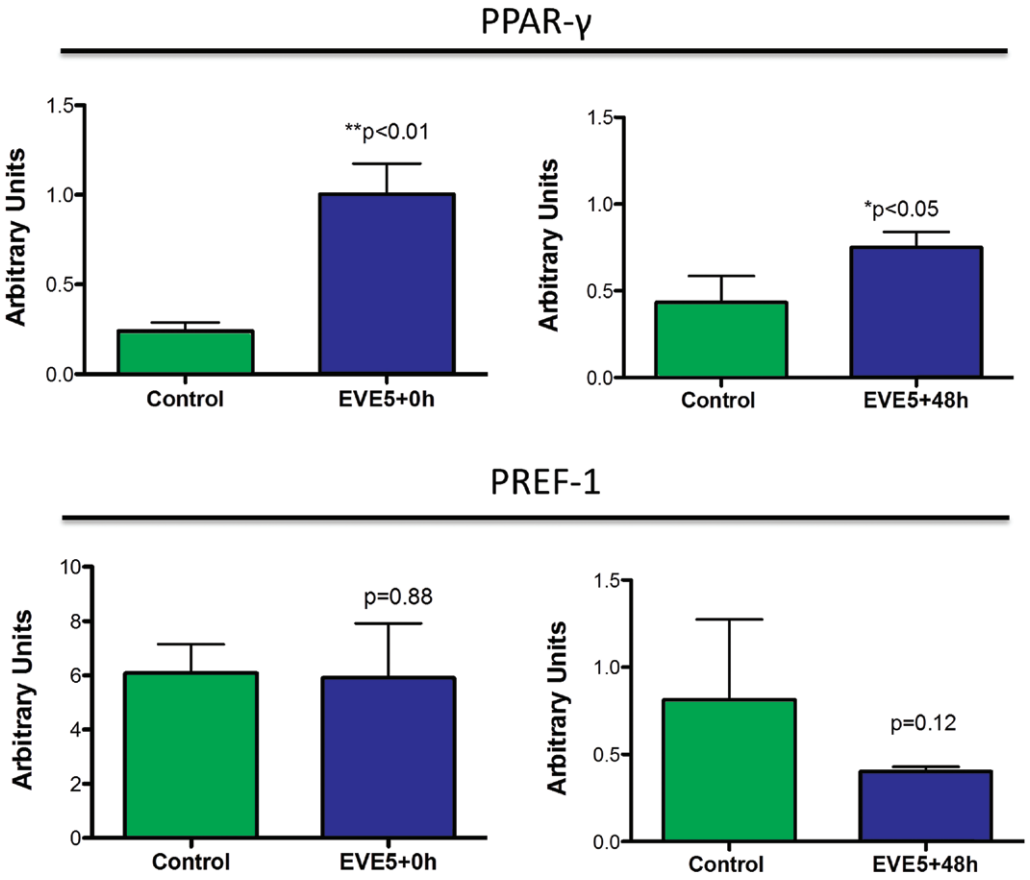

Fig. 5. Expression of *PPAR-γ* and *PREF-1* evaluated by Western blot analysis in animals treated with 2 hours' stimulation repeated for 5 days, immediately after the animals were sacrificed (*EVE5+0h*) and 48 hours later (*EVE5+48h*).

in external volume expansion–treated tissue after 48 hours. However, this may be attributable to our small sample size resulting from technical problems and tissue loss.

External volume expansion creates mechanical tension and induces edema and inflammation, each of which, independently, has been found to trigger adipogenesis (Fig. 6). Inflammation up-regulates PPAR-γ[22] and promotes M2 macrophage activation.[23] In the Matrigel (Corning, Inc., Corning, N.Y.) chamber with the arteriovenous loop model, inflammation was key in formation of adipose tissue, which could be blunted by macrophage depletion.[22,24–26] Furthermore, adipose tissue formation has been noted in lymphedema as a result of chronic inflammation in patients[27–29] and replicated in animal models.[30,31] Similarly, we found in our recently published model that inflammation was significantly increased by the end of a 2-hour external volume expansion treatment[12] and remained elevated for at least 2 days. In the aforementioned studies, inflammation and edema are chronic elements that remain consistently present, whereas in our present study, they are acute phenomena, which regress over the course of hours for edema and days for inflammation.[12]

Mechanical stimulation is also a potential independent player in the observed adipogenic effects. We and others have established its pro-proliferative and proangiogenic effects in vitro[5,6] and in vivo.[8,9] Moreover, static mechanical traction induces a proadipogenic effect.[32–34] Our model is in agreement with these observations, as the stimulation applied resembles periods of static tension through an externally applied source repeated over several days.

In contrast, hypoxia is known as an antiadipogenic factor, as hypoxia-inducible factor-1 down-regulates PPAR-γ expression.[35] We previously showed transitory presence of hypoxia and ischemia during external volume expansion treatment.[12] The transitory inhibitory effect of hypoxia is likely overcome by the other proadipogenic factors, but its potentially negative role should be taken into account when developing clinical protocols of stimulation with external volume expansion.

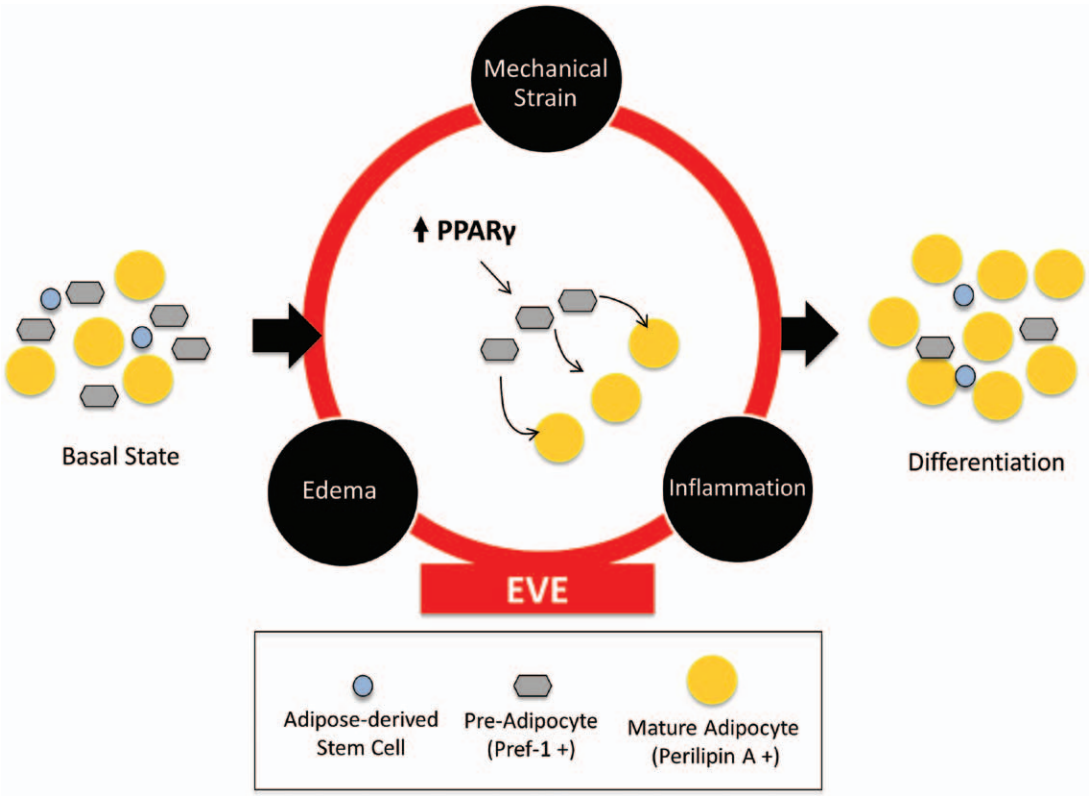

Fig. 6. A theoretical model for adipogenesis induction by external volume expansion. The combined effects of mechanical stimulation, edema, and inflammation may stimulate differentiation of committed preadipocytes present in the tissue. This overall results in accumulation of mature adipocytes. *PPAR-γ*, peroxisome proliferator-activated receptor-γ; *Pref-1*, preadipocyte factor 1; *EVE*, external volume expansion.

Although our results demonstrate the capacity of external volume expansion alone to induce a proadipogenic environment and increase adipocyte number, previous clinical experience attempting to increase breast mass with this strategy alone has been insufficient. Moreover, our results are from histologic analysis at the microscopic level, whereas volume augmentation of a larger mass likely takes many more factors to create a macroscopic change. However, our model is suggestive of a trend toward a superior number of adipocytes gained as the number of sessions of stimulation increases. Cyclical external forces have demonstrated a greater regenerative effect than constant forces.[9] Therefore, the kinetics of repeated stimulations should still be assessed for maximum gains. The mouse and human models present significant differences[36,37]; however, similar mechanisms, even of different magnitudes, could reasonably be expected to be at play.

According to recent theories that are gaining sound experimental support, much of the retained fat mass after fat grafting may be attributable not to adipocyte survival but to repopulation by surviving adipose-derived stem cells in the empty scaffold left by dead mature adipocytes.[38–40] As we demonstrated in earlier work, application of external volume expansion before fat grafting expands the recipient site, reducing the compression on the graft and stimulating the formation of a rich vascular network. All of these are key elements for fat graft survival.[41] Another scenario suggested by our results may be to clinically use external volume expansion after fat grafting as well, with the intention not of "stabilizing the graft"[10,42] but rather of specifically stimulating a proadipogenic environment that may increase stem cell commitment to adipocytic differentiation in the scaffold repopulation phase. Such a strategy may help to reduce scarring and reabsorption of the fat grafts, leading to overall quantitatively and qualitatively improved take. Moreover, external volume expansion may find application in tissue engineering to drive the development of new adipose tissue within a scaffold, combining its adipogenic and angiogenic potentials. This study has demonstrated the adipogenic potential of external volume expansion mediated by inflammation, edema, and mechanical tension. Further studies are needed to

identify the nuances of the mechanical forces and the specific timeframe in which an external volume expansion–based adipogenic induction can be of most benefit to fat grafting and potentially tailored to other uses.

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