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# Molecular Mechanisms of Adipose Tissue Survival during Severe Hypoxia: Implications for Autologous Fat Graft Performance

Sheri Wang, BS
Jeffrey A. Gusenoff, MD
J. Peter Rubin, MD, FACS
Lauren Kokai, PhD

**Background:** Variable retention outcomes remain a significant issue in autologous fat grafting procedures. Among seemingly similar patients, using identical harvesting procedures, variability in graft retention is noted. Recent data suggest that the inherent characteristics of donor adipose tissue dictate graft healing outcomes. The goal of this study was to elucidate intrinsic qualities of human adipose tissue that confer resistance to ischemic stress to therapeutically target such mechanisms and improve overall results of fat grafts.

**Methods:** Whole fat from 5 female patients was cultured in vitro under severe (1% $O_2$) and mild (8% $O_2$) hypoxic conditions. Microarray analysis of 44 hypoxia-related genes was performed. Perilipin was used to visualize viable adipocytes. Macrophage phenotypes were identified using PCR.

**Results:** Analysis of adipocyte survival with perilipin suggested improved viability for tissue obtained from high BMI donors. Microarray data revealed a significant positive correlation for induced expression of ANGPTL4, a survival gene, and subject BMI ($P = 0.0313$) during hypoxic conditions whereas HIF1α and HIF2α genes were negatively correlated with donor BMI ($P = 0.0003$ and $0.0303$). Interestingly, induced differentiation of proinflammatory M1 macrophages was negatively correlated with BMI under hypoxia ($P = 0.0177$).

**Conclusions:** The innate resilience of adipocytes to hypoxia and relative macrophage activation play a crucial role in fat graft retention. This study suggests that adipose tissue from high BMI donors demonstrates greater resistance to hypoxia-induced apoptosis associated with an increased expression of ANGPTL4. Therefore, therapeutic interventions that target this factor may improve clinical adipose graft survival. (*Plast Reconstr Surg Glob Open 2019;7:E2275; doi: 10.1097/GOX.0000000000002275; Published online 27 June 2019.*)

## INTRODUCTION

Fat grafting is a safe and minimally invasive procedure that has become a staple in plastic surgery, especially in breast and craniofacial reconstruction. Unfortunately, this versatile technique is limited by unpredictable graft retention, resulting in variable clinical outcomes. This variability was initially attributed to the differences in technique; however, researchers, such as Smith et al[1] and Fisher et al,[2] have found that harvesting and processing techniques do not significantly impact graft retention in animal models.[3] Similarly in the clinic, some patients experience unusually high fat resorption despite undergoing the same technical procedure as patients who experience excellent retention.[4] Our recent experience in craniofacial fat grafting suggests that the greatest predictor of a patient's fat graft retention is their retention from a previous procedure.[5] Therefore, we hypothesize that the inherent make-up of donor adipose tissue dictates graft performance. To better understand adipose mechanisms of survival, in this study, we developed an in vitro culture model of mild and severe hypoxia to explore molecular changes that occur under hypoxic stress and correlate changes in gene expression with overall tissue viability.

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Over the past decade, the process of adipocyte survival, cellular infiltration, and fat repopulation of grafted adipose has been described in detail.[6] After surgically induced ischemia, adipocytes farther than 200–300 µM from a blood source begin to necrotize and lyse 3 days postgrafting, releasing intracellular lipids and other cellular components into the milieu.[7] Necrotic cell debris is comprised endogenous inflammatory signals, such as heat-shock proteins, nuclear proteins (including high-mobility group box 1 protein), histones, DNA, and other nucleotides.[8] Recruitment of macrophages intensifies after cell necrosis and continues until inflammation is resolved. In this study, we aim to determine if interpatient differences exist in the response of adipose tissue to hypoxia and, if such differences exist, determine if intrinsic properties of fat provide protection from hypoxia-induced apoptosis or reduce inflammation and macrophage recruitment. To achieve this aim, we have examined whole fat tissue particles under mild and severe hypoxia, focusing on the interactions between adipocytes and macrophages.

## SUBJECTS AND METHODS

### Sample Procurement and Processing

Discarded surgically resected subcutaneous adipose tissue was obtained from 5 healthy, nondiabetic, never-smoker, female subjects under the University of Pittsburgh IRB (exemption PRO13090506). Samples were immediately processed into uniformly sized fat particles under sterile conditions using surgical instruments. Our preliminary work demonstrated that media nutrient (ie, glucose) depletion occurs when particles larger than 300 mg are used; thus, particle size was standardized to 200±20 mg. Control particles from each subject was placed into formalin for histology (n = 5) or snap frozen in lysis buffer for molecular analysis (n = 5).

### Tissue Culture under Hypoxia

Adipose particles were immediately placed into well plates containing preconditioned media (n = 6 per treatment per timepoint). Preconditioned media was generated by incubating media containing DMEM (Corning MT10013CV), 10% fetal bovine serum (Thermo Scientific 10082139, Thermo Fisher Scientific, Waltham, MA), and 1% antibiotics (Invitrogen 15140-122, 15710-072, 15290-018, Thermo Fisher Scientific, Waltham, MA) in a 12-well plate for at least 1 hour under either 1% (severe hypoxia) or 8% oxygen (mild hypoxia). Particles were covered with permeable screens for complete submersion in media. At days 2 and 7, cultured samples were placed in formalin for histology (n = 3 per treatment) or snap frozen in lysis buffer (n = 3 per treatment). Conditioned culture media was stored at –80°C.

### Histology with H&E and Perilipin

Tissue morphology was analyzed by haematoxylin and eosin stain (H&E) Samples were placed in 10% formalin for 24–28 hours, embedded in paraffin, and sectioned at 6 µM (2 slides/sample). Immunohistochemistry for adipocyte viability was performed with antibody against perilipin-A (Progen GP29) after antigen retrieval with 10 mM sodium citrate buffer pH 6.0 at 80°C for 30 min.

### Microarray Assay

Equal amounts of RNA from 3 control or 3 experimental samples (day 2, 1% oxygen) were isolated for each subject and, respectively, pooled for gene expression using a Taq-Man Array Human Hypoxia 96-well Plate (Thermo Scientific 4414090, Thermo Fisher Scientific, Waltham, MA) containing 44 assays for hypoxia associated genes and 4 assays for control genes. Relative gene expression was determined using the $\Delta\Delta C_t$ method. RNA was extracted from samples using a Qiagen RNEasy Silica column system (Qiagen 74034).

### RNA Isolation

Adipose particles were cut into small pieces, transferred to a 5-mL round bottom tube in 1 mL RLT lysis + buffer, homogenized with a homogenizer (Bio-Gen PRO200), and digested in Proteinase K (Thermo Scientific AM2548, Thermo Fisher Scientific, Waltham, MA) for 15 min at 55°C. RNA was then isolated according to the *RNeasy Plus Mini Handbook*. RNA quantity and purity were assessed using a plate-reader (Tecan Infinite M200, Tecan Life Sciences, Switzerland) at 260 nm/280 nm ratio. cDNA was reverse transcribed using 25 ng/µL RNA with M-MLV Reverse Transcriptase (200 U/µL) and TaqMan array plates.

### Statistical Analysis

Data are presented as mean ± SD for all groups. Analysis of variance and post hoc comparisons were performed in Graphpad Prism with α = 0.05.

## RESULTS

### Tissue Procurement

Age, sex, and body mass index (BMI) of the 5 donors were recorded (1). Particle weight for each sample was recorded. No subject's average particle size differed significantly from 200 mg (Fig. 1). Although subjects 4 and 5 had significantly different average partial weights ($P = 0.002$), the results were normalized using the sample weight before analysis.
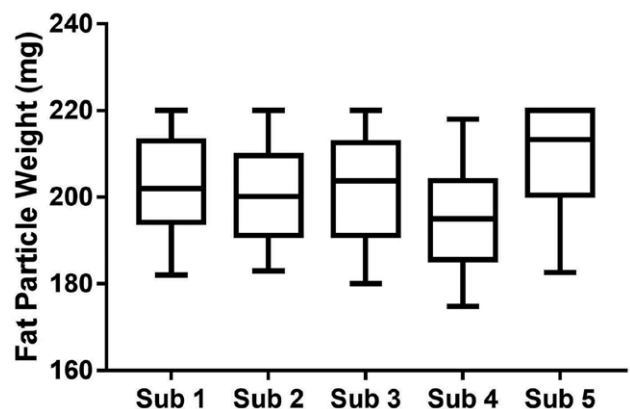


**Fig. 1.** No subject's average particle size was significantly different from 200 mg.
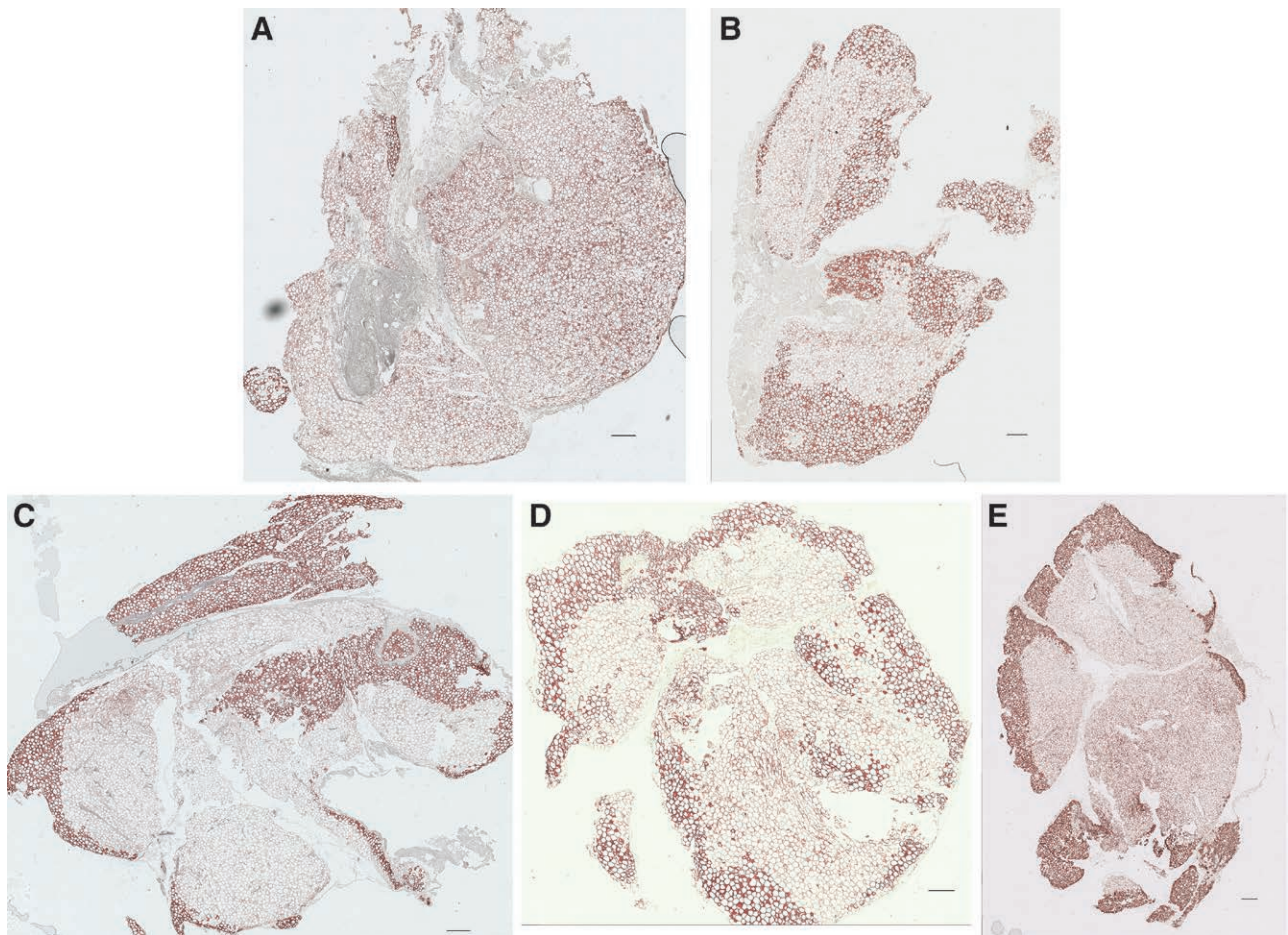
**Fig. 2.** Perilipin staining of adipose tissue cultured in 1% hypoxia for 7 days. A, Subject 1. B, Subject 2. C, Subject 3. D, Subject 4. E, Subject 5. Scale bar at 500 μm.

### Hypoxia Model Validation

We used polymerase chain reaction (PCR) to measure hypoxia markers to validate our model before analysis. The reversible hydration of $CO_2$ was measured using CA9. Under both mild and severe hypoxic conditions, CA9 increased significantly from baseline to day 2 and from day 2 to day 7 ($P < 0.0001$). Measuring acute hypoxia, hypoxia-inducible factor (HIF) 1 increased from baseline to days 2 and 7 under both oxygen conditions. The increase was not statistically significant from baseline to day 7 under 1% oxygen ($P = 0.071$). All other increases in HIF1 from baseline were significant ($P < 0.0001$). Under both oxygen conditions, chronic hypoxia was measured using HIF2a, which decreased from baseline to both days 2 and 7. Aerobic activity was measured by pyruvate dehydrogenase kinase (PDK1), which significantly increased from baseline to both days 2 and 7 under 1% oxygen ($P < 0.0001$, 0.0003). PDK1 also significantly increased from baseline to day 2 under 8% oxygen but not day 7 ($P = 0.0003$, 0.096).

*Perilipin imaging* of the subjects was performed (Fig. 2). Percent viability was assessed by a blinded researcher and was used to visually confirm the microarray analysis. The lowest BMI subject sample had <10% viability. Intermediate BMI subject sample had 15% viability. Highest BMI subject sample had 40% viability.

*Microarray analysis* of 44 hypoxia signaling associated genes was normalized to the housekeeping gene for each subject (Fig. 3). Changes in 4 genes highly correlated with subjects' BMI under hypoxic conditions at day 2 (Fig. 4). PIK3CA, an antiapoptosis gene in the HIF1a pathway, was positively correlated with BMI without significance ($P = 0.1292$). Apoptosis survival factor ANGPTL4 was also positively correlated with subject BMI ($P = 0.0313$). HIF1α and HIF2α genes were downregulated in subjects with higher BMIs, demonstrating that cells are under less stress with higher BMIs ($P = 0.0003$, 0.0303).

*Activated macrophages* were identified using CD5L, which is associated with recruitment of inflammatory macrophages into adipose tissue and with inhibition of macrophage apoptosis (Fig. 5). In 8% oxygen group, there was no increase in macrophage activation at days 2 and 7 compared with baseline. However, in 1% oxygen group, there was a significant increase in macrophage activation at day 2 ($P = 0.0001$), which tapered off at day 7. *Pan-macrophage* expression was identified using CD68, which is expressed by human monocytes and tissue macrophages. Compared with baseline, relative macrophage expression increased
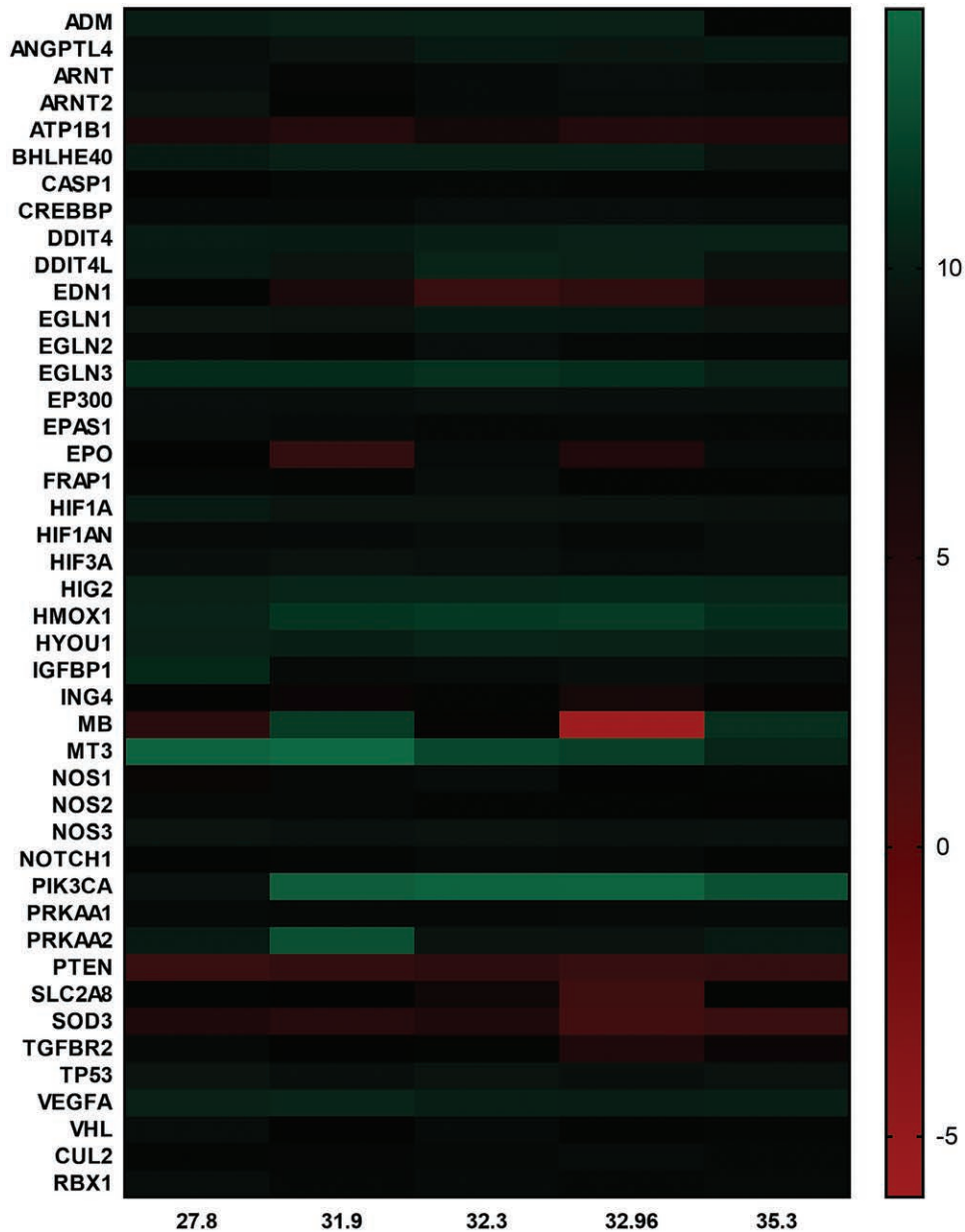
**Fig. 3.** This heatmap shows variable fold induction at day 2 in 1% oxygen for each subject.

significantly at day 2 under 1% oxygen and at day 7 for both oxygen concentrations ($P < 0.05$). While day 2 under 8% oxygen also experienced relative increase in macrophage expression compared with baseline, the change was not significant ($P = 0.061$). There was no correlation between subject BMI and either macrophages activation or pan-macrophage expression.

*M1 macrophage* relative expression was identified using inducible nitric oxide synthase (iNOS). In both oxygen concentrations, relative iNOS expression increased from baseline to days 2 and 7. In the group day 7 under 8% oxygen, there was a statistically significant increase from baseline ($P = 0.002$). M1 macrophage expression also appeared to be correlated to subject BMI (Fig. 6). Under

8% oxygen concentrations, subjects with higher BMI had significantly lower iNOS expression at day 7 compared with baseline ($P = 0.018$). This correlation was not significant at day 2. Under 1% oxygen, subjects with higher BMI trend toward decreased iNOS expression at days 2 and 7 ($P = 0.059, 0.186$).

*M2 macrophage* expression was measured using Arg1 and IL10 (Fig. 6). There was no significant difference in Arg1 or IL10 expression across either timepoints or oxygen concentration. Under 8% oxygen on day 7, both M2a matrix generating macrophages and M2c regenerative macrophages trended higher in subjects with greater BMI ($P = 0.197, 0.271$) (Fig. 6).
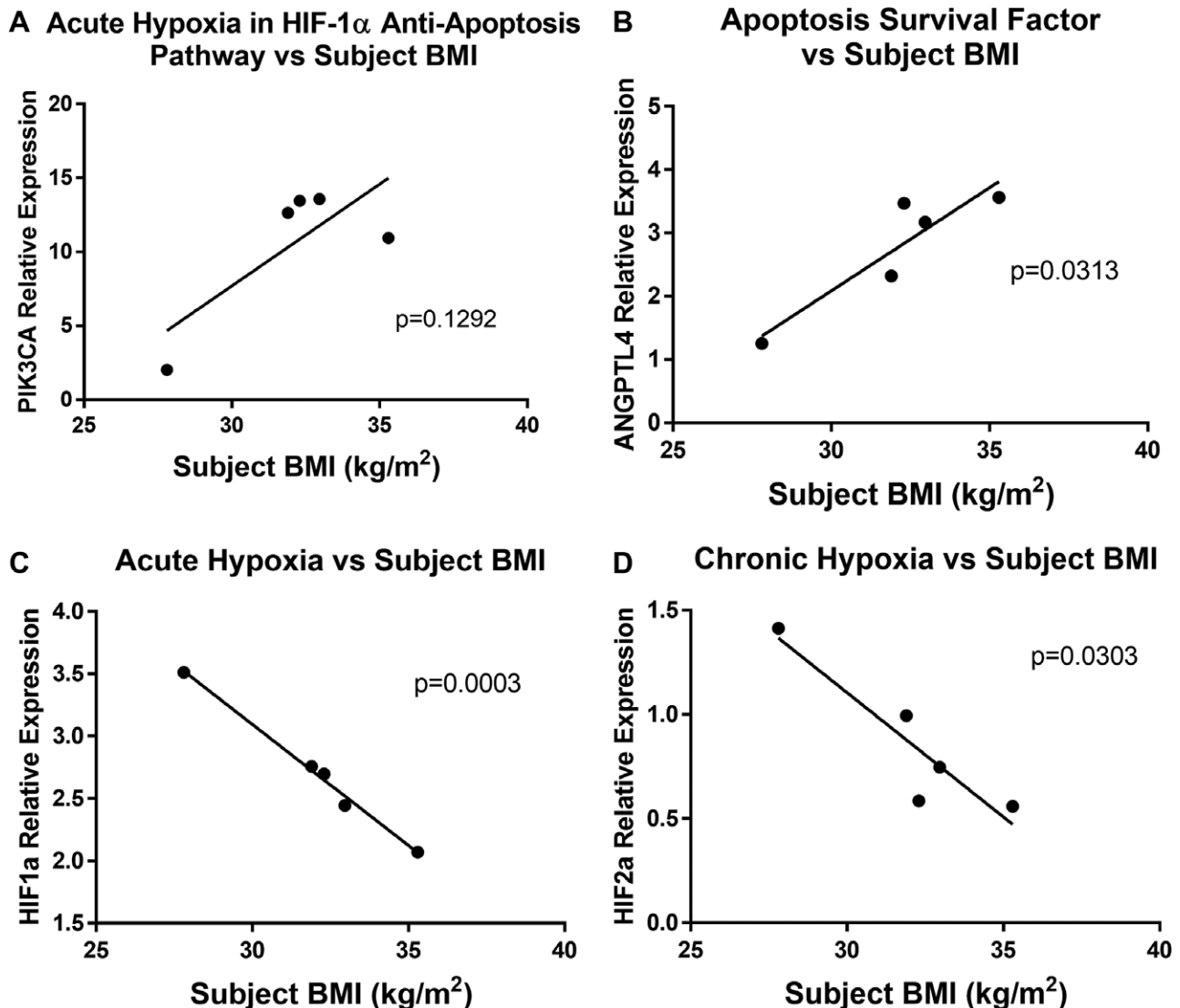
**Fig. 4.** Relative expression of hypoxia pathway marker was assessed. A, PIK3CA was used to assess the activation of antiapoptosis genes in the HIF1a pathway. B, ANGPTL4 was used to assess resistance to apoptosis. C, HIF1α was used to assess induction of the acute hypoxia pathway. D, HIF2α was used to assess induction of the chronic hypoxia pathway.

## DISCUSSION

Immediately after grafting, transplanted fat survives by plasmatic imbibition until neovascularization occurs. According to Yoshimura et al Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K. Cell-assisted lipotransfer for cosmetic breast augmentation: Supportive use of adipose-derived stem/stromal cells. *Aesthetic Plast Surg.* 2008;32:48-55. doi:10.1007/s00266-007-9019-4, adipocytes located 100–300 μm from the graft edge remain alive while the rest of adipocytes die within 24hrs after grafting.[9–13] The regeneration of new adipocytes relies on adipose derived stem cells (ASCs) following macrophage clearance of cellular debris. The interim hypoxic condition results in adipocyte death, release of multiple injury-associated factors, volume loss, and replacement by fibrotic scar tissue and oil cysts.[11] Therefore, it is assumed that adipose tissue grafted as small particulates, such as nanofat, have

increased adipocyte viability and improved retention compared to excised whole fat or lipoaspirate with larger globule size. However, in practice, grafting with small adipose particles does not improve procedure outcomes for all patients.[5,14] Therefore, more research is needed on adipose mechanisms that lead to graft resorption such that we can improve our ability to predict patients that will have poor procedure outcomes and either surgically account for anticipated volume losses or defer use of fat grafting all together.

Hypoxia is not a problem unique to adipocytes. For instance, the myocardium is susceptible to ischemic insult. However, in the heart, short periods of repeated ischemia serve as a protective factor against subsequent ischemic injury. We hypothesize ischemic preconditioning, first described by Murry et al,[15] applies to adipocytes as well. We hypothesize that chronically hypoxic fat, as observed in

## A  Activated Macrophages
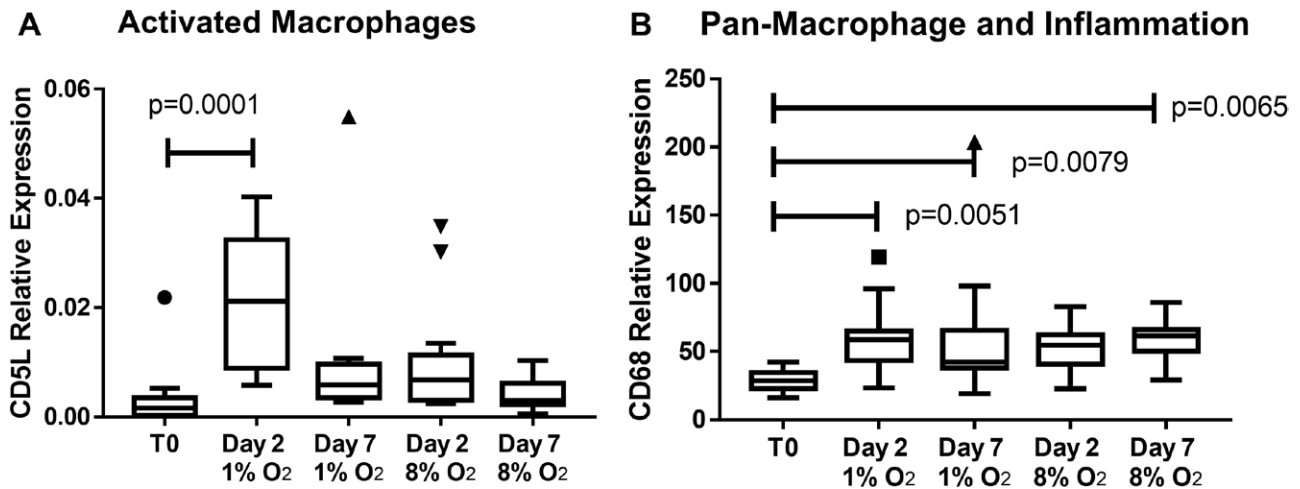


## B  Pan-Macrophage and Inflammation



**Fig. 5.** Relative macrophage expression was assessed. A, CD5L was used to assess relative macrophage activation. B, CD68 was used to assess relative pan-macrophage expression.
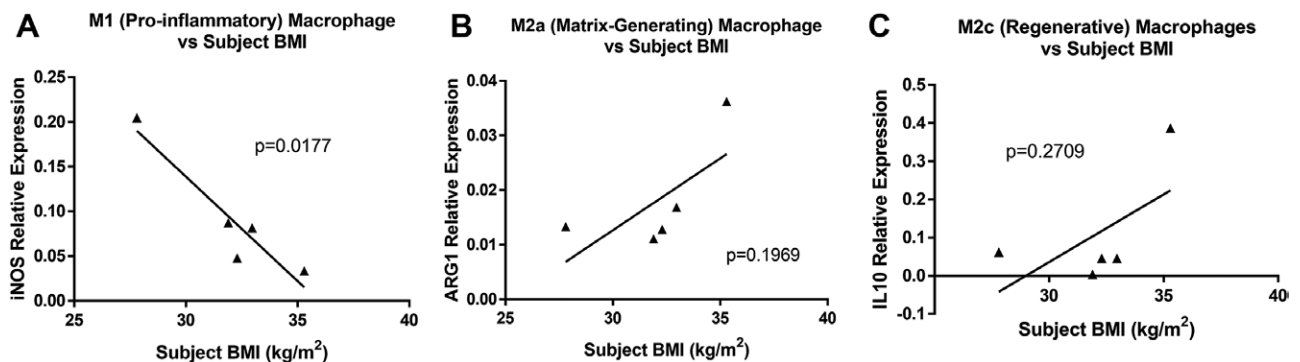
## A  M1 (Pro-inflammatory) Macrophage vs Subject BMI



## B  M2a (Matrix-Generating) Macrophage vs Subject BMI



## C  M2c (Regenerative) Macrophages vs Subject BMI



**Fig. 6.** Gene expression at day 7 in 8% oxygen. A, iNOS expression was negatively correlated with BMI. B, M2a macrophages trended toward increase with increase in BMI. C, M2c macrophages trended toward increase with increase in BMI.

very high BMI subjects, experience less stress under hypoxic conditions following fat grafting due to low baseline oxygen levels at the donor site.

Obesity is associated with decreased $pO_2$ in both human and animal studies. Obese patients have fewer capillaries per volume in their white fat, which has a lower oxygen requirement.[12] Using direct $O_2$ sensors and the Hypoxyprobe System, Ye et al[16] and Hosogai et al[17] found that adipose tissue in obese mice has lower $pO_2$ compared with their healthy counterparts. Furthermore, Pasarica et al[18] found that obese subjects had the same PDK1, a target of the hypoxia pathway, as healthy subjects despite having lower $pO_2$, lower capillary density, and lower vascular endothelial growth factor (VEGF).[19] These studies indicate that hypoxic preconditioning increases adipocytes survive and results in less hypoxic shock immediately following fat grafting.

We tested this hypothesis by culturing the same size particles of 5 subjects with different BMIs under mild and severe hypoxic conditions. We used members of the HIFs pathway to evaluate cellular response to hypoxia. HIF-1 and HIF-2 are transcription factors heavily implicated in response to low $pO_2$ and are regularly used in cancer research.[20–22] Our microarray assay demonstrated that hypoxia-related genes, HIF-1a and HIF-2a, are over expressed in subjects with lower BMI compared with higher

BMI ($P < 0.05$). Meanwhile, the apoptosis survival factor ANGPTL4 was positively correlated with subject BMI ($P < 0.05$) (Fig. 4). Together these assays indicate that the fat particles from obese subjects tend to tolerate hypoxia better and that lower oxygen levels are required to achieve induction of the hypoxic pathway compared with healthy subjects. Perilipin visualization also supports the finding that higher BMI is correlated with increased adipocyte survival (Fig. 2). Thus, adipocyte survival depends on the adipocytes' ability to withstand hypoxia-induced apoptosis in addition to size of the fat particles.

We also investigated macrophage activity in our study. Our results with human tissue samples confirm results previously published in mice[7,9] demonstrating an early and critical participation of blood-derived macrophages in phagocytosis of necrotic adipocyte debris. Macrophage activity is concentrated in hypoxic regions in fat tissue, breaking down cellular debris, particularly after cell death, and signaling for vascular regeneration.[12,23] Under low pO2 conditions, macrophages upregulate the release of cytokines and other inflammation-related factors including IL-6.[16,24,25] Consistent with the current literature, we found that there was a significant increase in relative activated macrophage expression from baseline to day 2 under severe hypoxic conditions ($P < 0.0001$). There was also
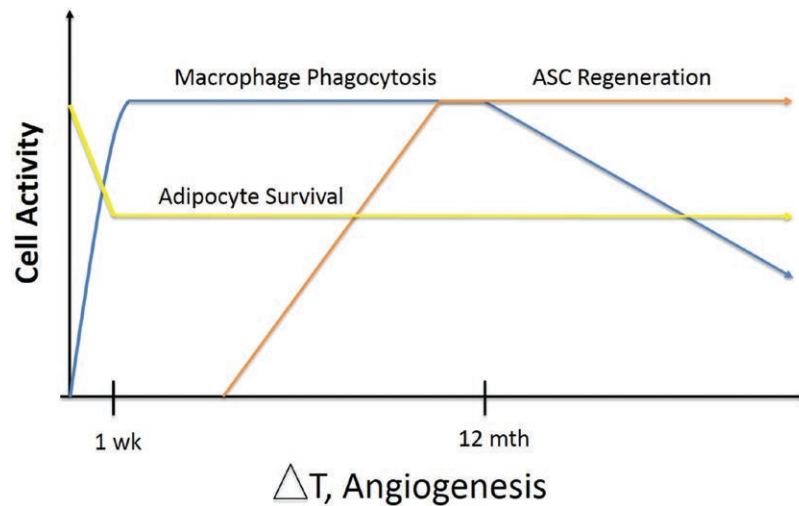
**Fig. 7.** Relative increase in macrophage activity precedes relative increase in ASC activity.

a significant increase at day 7 in macrophage-associated inflammation under both oxygen conditions ($P < 0.05$). This relative increase in macrophage expression may be due to the macrophages' ability to tolerate hypoxia compared with other cell types.

We hypothesize that like adipocytes, macrophages in higher BMI subjects behave differently than those in lower BMI subjects. As lower BMI subjects are not as accustom to hypoxia, the adipocytes will signal for an increase in inflammatory macrophages, those naturally concentration in hypoxic tissue, resulting in poor outcomes. To test this theory, we assessed the relative expression of macrophages types versus subject BMI. We found that lower BMI subjects had significantly more relative proinflammatory M1 Macrophage expression during day 7 in 8% oxygen ($P = 0.0177$). From these relative macrophage expressions, it appears that lower BMI subjects undergo a more inflammatory response after fat grafting, potentially inhibiting angiogenesis and decreasing blood flow to newly grafted fat.[26,27]

Higher BMI subjects trended towards higher expression of M2a Matrix Generating Macrophages and M2c Regenerative Macrophages ($P = 0.197, 0.271$). The expression of M2 macrophages are implicated in angiogenesis; therefore, the prevalence of M2 macrophages in higher BMI subjects suggests that these subjects are better facilitated to make new vessels post fat grafting.[28,29] Further studies will investigate the specific ratio of inflammatory markers versus regenerative markers excreted by macrophages from subjects of different BMIs.

The innate resilience of adipocytes to hypoxia and relative macrophage activation play a crucial role in fat graft retention. From our study, we can obtain a better understanding of the relative interactions between different cell types over time (Fig. 7). The initial hypoxic insult causes adipocyte death. Some preconditioned adipocytes, such as those in obese subjects, tolerate the hypoxia better and have a higher rate of survival. Next, in lower BMI subjects, the preferential activation of M1 macrophages inhibits neovascularization, which is required to support the sur-

**Table 1. Age, Sex, and BMI (kg/m²) of Donors**

| Subject ID | Age (y) | Sex | BMI |
|---|---|---|---|
| 1 | 59 | Female | 31.9 |
| 2 | 62 | Female | 27.8 |
| 3 | 50 | Female | 35.3 |
| 4 | 46 | Female | 32.3 |
| 5 | 40 | Female | 33.0 |

vival of remaining adipocytes. Although not discussed in this article, we hypothesize that the final player in fat graft retention is the ASCs. Unlike adipocytes, ASCs are less susceptible to hypoxia and can proliferate after macrophage degradation of cells into accessible nutrients.[12] After the initial necrosis of adipocytes, ASCs differentiate and replace the dying adipocytes. Using ki67 staining, Suga et al[7] demonstrated that ASCs are both resistant and can proliferate under hypoxia. This has been demonstrated in both human and animal model: ASC enhanced fat grafts had higher retention and decreased necrosis compared to fat graft alone.[30–33] Therefore, the next step in our investigation is to link the adipocyte and macrophage activity to ASC regeneration of fat.

This study has several limitations. The use of a limited sample size (5 subjects) renders the study more likely to commit Type II Error. This subject number was chosen to preliminarily test the underlying hypothesis that significant differences in patient response to hypoxia exist; however, gene expression studies with more patients are needed to confirm correlations with subject BMI. Furthermore, due to the demographics in our body contouring practice, all 5 subjects are female. Considering the many known differences between female and male accumulation of adipose, results from this study cannot be assumed to apply to both sexes. Finally, our study was conducted with tissue samples from a limited donor age group, ranging from 40 to 62 years old and subject BMI was limited to 27.8–35.3 kg/m². The limited range in BMIs studies poses a challenge as we cannot extrapolate the results for

healthy patients. Further studies with leaner subjects are warranted. In addition, as this is an in vitro study, we are unable to assess the migration of immune cells to the graft.

## CONCLUSIONS

Fat grafting is a versatile and powerful procedure with a low rate of complication and high patient satisfaction. The greatest drawback is the unpredictability of volume retention. Our study suggests that adipocytes from high BMI subjects have improved survival under hypoxic stress, potentially increasing tissue retention. We also found increased expression in M1 macrophages in lower BMI patients. Further investigation in this pathway will allow us to obtain a better understanding of molecular mechanisms of fat graft retention, resulting in ability to predict and surgically plan for patient specific differences in retention.

*Lauren Kokai, PhD*
Scaife Hall, Suite 6B
3550 Terrace Street
Pittsburgh, PA 15213
E-mail: kokail@upmc.edu

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