

# Survival and Inflammatory Response in Adipose-derived Mesenchymal Stem Cell-enriched Mouse Fat Grafts

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**Background:** Adipose tissue-derived mesenchymal stem cells (ATMSCs) are currently used in grafting procedures in a number of clinical trials. The reconstructive role of such cells in fat graft enrichment is largely unclear. This study was undertaken to assess survival and inflammatory response in fat grafts enriched with ATMSCs in mice.

**Methods:** ATMSC-enriched adipose tissue was grafted subcutaneously in a clinically relevant manner in mice, and survival and inflammatory response were determined by bioluminescence imaging of transgenic tissue constitutively expressing luciferase or driven by inflammation in wild-type animals.

**Results:** Only a minor fraction of ATMSCs transplanted subcutaneously were found to survive long term, yet fat grafts enriched with ATMSCs showed improved survival for a limited period, compared with no enrichment. NF- $\kappa$ B activity was transiently increased in ATMSC-enriched grafts, and the grafts responded adequately to a pro-inflammatory stimulus. In one animal, cells originating from the subcutaneous graft were found at a site of inflammation distant from the site of engraftment.

**Conclusion:** ATMSCs display limited subcutaneous survival. Still, ATMSC enrichment may improve the outcome of adipose tissue grafting procedures by facilitating short-term graft survival and adequate inflammatory responses. Migration of cells from grafted adipose tissue requires further investigation. (*Plast Reconstr Surg Glob Open* 2016;4:e1110; doi: 10.1097/GOX.0000000000001110; Published online 22 December 2016.)

fat possesses the desired softness for many reconstructive purposes<sup>1-4</sup> including breast and facial malformations, repair of scars, and as filler for aesthetic purposes. Fat grafting is, however, hampered by unpredictability of results by formation of oil cysts, necrosis, and resorption of grafted tissue.<sup>5</sup> Recent development of automated stem cell isolation techniques has made it feasible to enrich adipose tissue grafts

with adipose-derived mesenchymal stem cells (MSCs), with or without stem cell expansion before grafting.<sup>6</sup> Stem cells of mesenchymal origin possess inflammation-modulatory properties<sup>7</sup> related to their expression of cytokines that may influence recipient tissue responses in grafting procedures.<sup>8</sup> Moreover, they have been shown to improve vascularization through their ability to facilitate angiogenesis.<sup>9</sup> Clinical application of stem cell enrichment requires, however, consistent and predictable results regarding long-term survival of both grafts and stem cells. Such data are currently scarce, although histological evaluation of fat grafts has been performed.<sup>10</sup> We have here utilized reporter mice expressing luciferase and optical imaging to assess stem cell and fat graft survival, and also inflammation-modulatory effects of adipose tissue-derived mesenchymal stem cells (ATMSCs). We used fat from 2 transgenic reporter mice models, one in which all cells possess a genomic sequence coding for luciferase driven by 3 DNA binding sites for the inflammation regulatory transcription factor NF- $\kappa$ B and other where luciferase expression is driven

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by a constitutively active promoter. The former mouse model has been shown to faithfully report NF- $\kappa$ B transcriptional activity in a number of tissues including adipose tissue.<sup>11</sup>

## MATERIALS AND METHODS

### Cells and Transgenic Mice

Transgenic mice with luciferase driven by NF- $\kappa$ B have been described.<sup>12</sup> ATMSCs were isolated from wild-type C57BL/6 mice or transgenic mice for luciferase in the same genetic background driven by a constitutive promoter (see Document, Supplemental Digital Content 1, which displays generation of transgenic mice, <http://links.lww.com/PRSGO/A344>). ATMSCs were isolated from visceral adipose tissue as described by Yu et al<sup>13</sup> and differentiated, briefly described in Supplemental Digital Content 1. Skin fibroblasts were isolated by outgrowth from ear biopsies.

### Flow Cytometry

ATMSCs were characterized by incubation with 10  $\mu$ l primary antibody against Sca-1, CD105, CD106, CD44, CD29, CD73, CD11b, or CD45 (R&D Systems; Minneapolis, Minn.) for 30 minutes on ice. Cells were washed and incubated with phycoerythrin-labeled secondary Goat F(ab')<sub>2</sub> Anti-rat IgG (R&D Systems; Minneapolis, Minn.). Cells were washed and analyzed by flow cytometry using rat IgG2A (R&D Systems; Minneapolis, Minn.) as isotype control. At least 10,000 events were counted for each sample.

### Grafts and Cell Enrichment

Male mice transgenic for luciferase driven by the EF1 $\alpha$ -based constitutive promoter or promoter with NF- $\kappa$ B-binding sites were used as donors of adipose tissue. Donor mice were killed by CO<sub>2</sub> or cervical dislocation; abdomen was washed with 70% ethanol and opened surgically. Visceral adipose tissue was dissected and washed extensively with phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin. The tissue was minced and washed by settling connective tissue at 1g, before the supernatant containing adipocyte aggregates was collected and washed once with PBS. Before tissue enrichment, all cells were expanded in vitro in DMEM:F12 with 10% FCS, detached by trypsin treatment and centrifuged for 10 minutes at 300g, then washed once in PBS (0.1 M, pH 7.4, Sigma; St. Louis, Mo.) and resuspended at 1  $\times$  10<sup>7</sup> cells per ml in PBS with 1% penicillin-streptomycin.

### Grafting and Cell Injection

A dorsal area on the side of the vertebral column of anesthetized recipient female mice was shaved and washed with 70% ethanol. Each wild-type recipient animal received 166  $\mu$ l adipose tissue without enrichment, or enriched with 2  $\times$  10<sup>6</sup> mouse skin fibroblasts or ATMSCs, by a single subcutaneous injection 10 mm away from grafting area, deposited in a fan-shaped manner under the shaved area (approximately 1 cm<sup>2</sup>) using a 0.9 mm fat injector (Tulip Medical Products; San Diego, Calif.) and secured with metal staples. Animals were housed and used in accordance with the Guide for Care and Use of Laboratory

Animals and handled according to procedures approved by the National Ethics Committee for animal research (approval #FOTS 6476). No adverse phenotypes were observed.

### Histochemistry and Immunohistochemistry

Grafts were dissected from animals and fixed in buffered 4% formaldehyde for 24 to 48 hours. Tissues were embedded in paraffin and sectioned at 4- $\mu$ m thicknesses in a microtome. Consecutive sections were used for hematoxylin and eosin (HE) (DAKO; Glostrup, Denmark) staining. HE-stained slides were assessed blindly by a histopathologist, and the most central cross section of the affected area was selected for immunohistochemistry (IHC). Sections were deparaffinized for CD31 IHC labeling as described in Supplemental Digital Content 1.

### Quantification of Adipocytes and Capillaries

Images of IHC labeled tissue sections were acquired on a DMRE microscope fitted with a DFC 500 CCD camera (Application Suite [LAS] v4.1–4.4) to obtain 4080  $\times$  3072 pixel color images (all from Leica Microsystems; Heidelberg, Germany). Overview images were acquired with an N Plan 2.5  $\times$  /0.07 objective, and images to be used for quantitative analysis were acquired with a Plan Fluotar 10  $\times$  /0.3 objective. Image processing was adapted for analyses of both adipocyte cellular profiles and CD31 immunoreactive vascular structures in the same images (optimized and standardized contrast). For the computerized quantitative analyses, the open source software ImageJ (<http://rsbweb.nih.gov/ij/>) and Adobe Photoshop (CS5 or CC) were used. All tissue areas histologically separated from recipient tissues were analyzed, avoiding regions of necrosis or severe tissue repair. All samples' analyses of adipocytes were performed using a specifically designed script: (1) total area, (2) number of adipocyte profiles, (3) area of each adipocyte, (4) total adipocyte area, and (5) adipocyte area relative to the total area were analyzed. In each image, CD31 immunoreactive tubular or circular (i.e., capillary vessel) structures were identified visually, uniquely labeled, and counted.

### In vivo Optical Imaging

At the indicated time points after grafting, mice with luciferase positive (Luc<sup>+</sup>) grafts or cells were anesthetized with isoflurane, the fur in the grafted area was removed, and 130 mg/kg luciferin (Biosynth; St. Gallen, Switzerland) was injected intraperitoneally. After 10 minutes, the mice were placed in an IVIS Spectrum  $\mu$ CT instrument (Perkin Elmer; Waltham, Mass.), and photons were counted during 300-second exposures and analyzed with LivingImage v. 4.3.1 software.

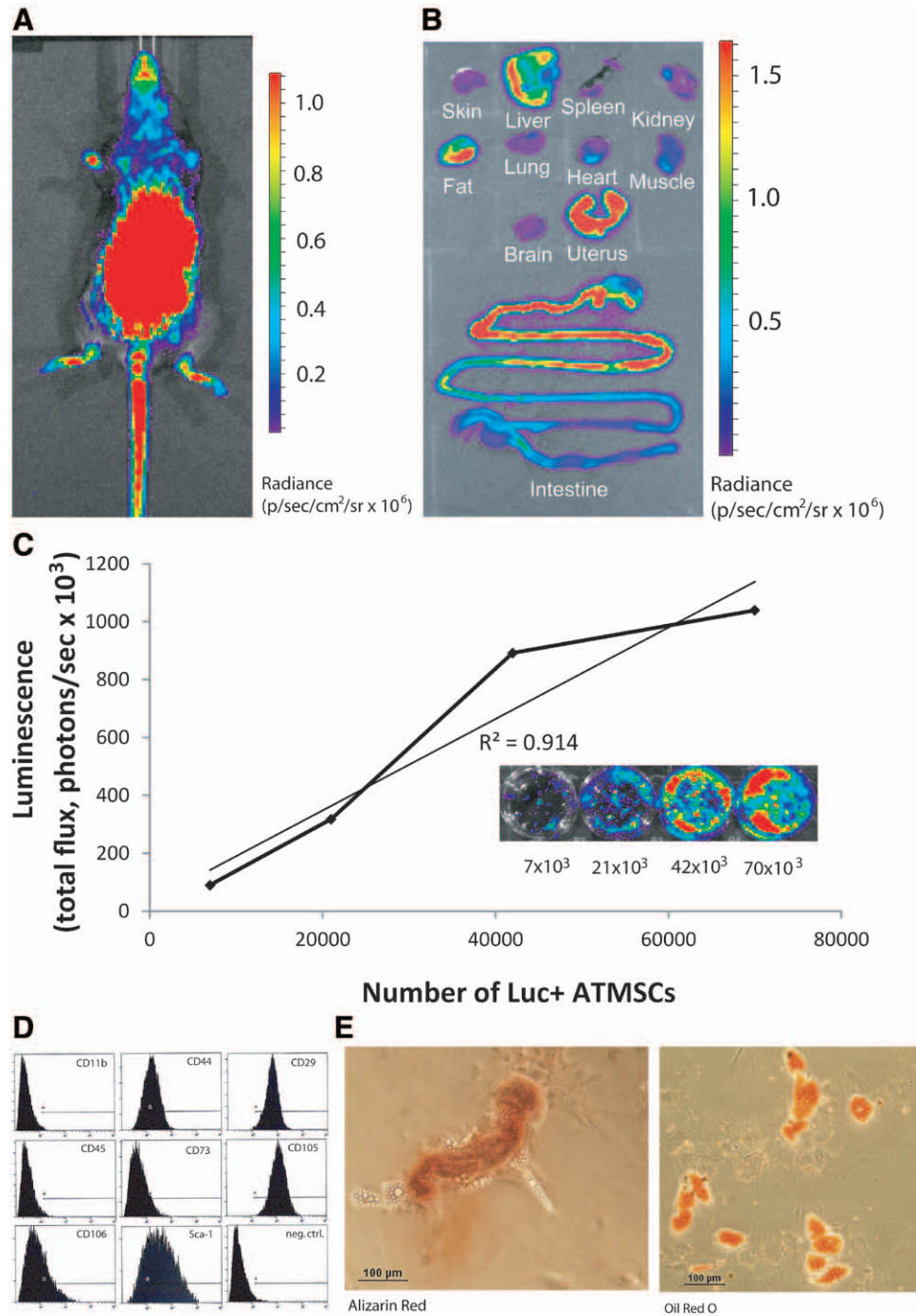
### Statistical Analysis

Benjamini–Hochberg correction was used for correction of multiple testing (R software), and the power of 2-sample comparison was calculated with Sampsiz software (<http://sampsiz.sourceforge.net/>).

**RESULTS**

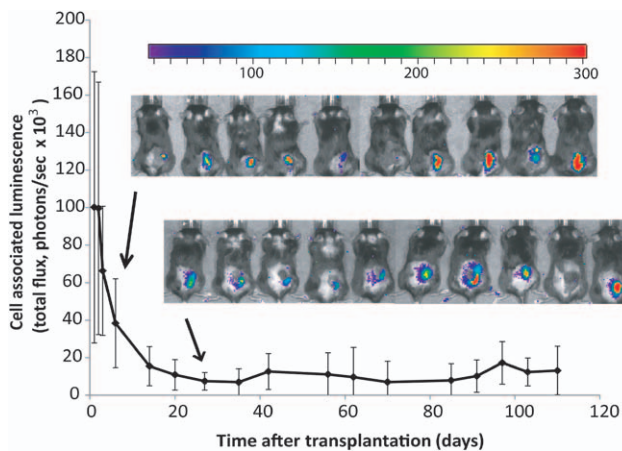
To assess viability of fat grafts and cells, we generated a transgenic mouse strain that expresses Fire Fly luciferase in all tested tissues including fat (Fig. 1B) and subsequently isolated Luc<sup>+</sup> ATMSCs from visceral fat and characterized the cells regarding bioluminescence (Fig. 1C), cell surface

markers (Fig. 1D), and differentiation capacity (Fig. 1E). Isolated Luc<sup>+</sup> ATMSCs were then injected subcutaneously on the back of isogenic wild-type animals. Bioluminescence from cells at the injection site was then measured at intervals for up to 15 weeks (Fig. 2) and found to decline rapidly during the first 2 weeks before reaching a stable



**Fig. 1.** Characterization of HLuc transgenic mouse and adipose-derived stromal cells. A, In vivo and (B) ex vivo imaging of transgenic mouse and internal organs expressing Fire Fly luciferase driven by an EF1 $\alpha$ -derived constitutive promoter. C, Bioluminescent imaging of indicated number of Luc<sup>+</sup> ATMSCs isolated from visceral fat from HLuc mouse in (A). D, Flow cytometric analysis of ATMSCs for expression of stem cell-related surface markers. E, Osteogenic (left) and adipogenic differentiation (right) of isolated ATMSC stained with Alizarin Red and Oil Red O, respectively.



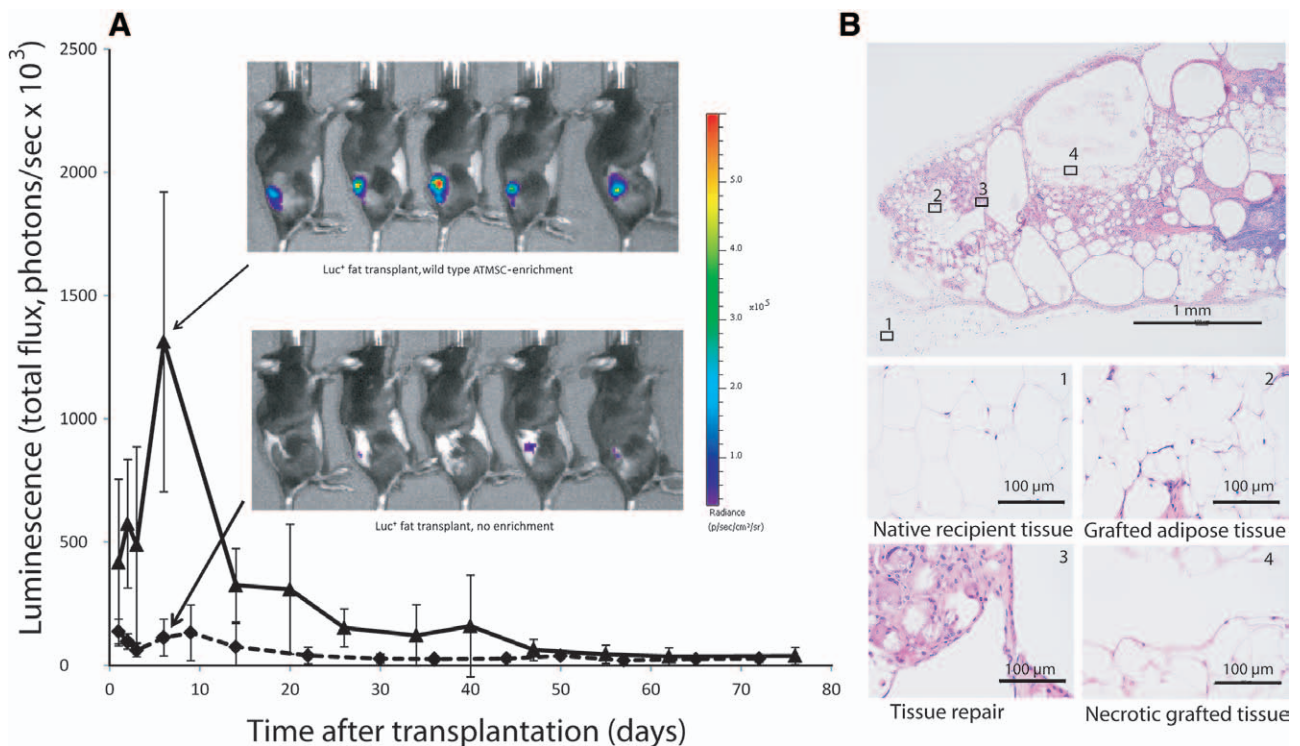


**Fig. 2.** Survival of subcutaneously grafted Luc<sup>+</sup> ATMSC. ATMSCs were isolated from HLuc transgenic mice and cultured for 3 passages before 5 × 10<sup>5</sup> cells were injected subcutaneously on the back of wild-type isogenic animals. At the indicated days after injection, luciferin was injected and animal imaged for counting of photons from the injected cells. Inset shows images of all animals at 2 selected time points. Bars indicate ±SD, n = 10.

level at 10% to 20% of the initial luminescence at the end of the experiment, suggesting long-term survival of a reasonable fraction of the cells. To test the hypothesis that ATMSCs provide conditions favorable for adipose tissue survival in a cotransplantation setting, we grafted Luc<sup>+</sup> fat

subcutaneously on the back of wild-type animals without ATMSC enrichment (control) or enriched with wild-type ATMSCs before grafting. Bioluminescence was then measured from both groups of animals at intervals for several weeks (Fig. 3A). Bioluminescence from ATMSC-enriched and control Luc<sup>+</sup> adipose tissue followed different trajectories; it was substantially higher in enriched adipose tissue already the first day after transplantation and increased during the first 6 days, notwithstanding variations between individuals (Fig. 3A). Bioluminescence from grafts without ATMSC enrichment was only marginally increased in the same period. This suggests that the presence of ATMSCs improved survival of the fat graft although it cannot be ruled out that improved access to the luciferase substrate through increased vascularization contributed to the increased luminescence. Either way, our results provide evidence for a supportive effect of ATMSCs on the adipose tissue graft survival for the first week. From day 6 and onwards, there was a steady decline in luminescence from enriched grafts until 48 days (Fig. 3A).

Three months after transplantation, the animals were killed, and the grafted material together with surrounding recipient tissue was subjected to histological assessment. Histologically, areas of cystic necrosis and repair tissue dominated all grafts (Fig. 3B, representative image from 1 animal). In specimens from 7 animals, 15% to 47% of central cross sections consisted of viable adipose tissue, and the highest ratios were seen in the enriched grafts, al-



**Fig. 3.** Survival of subcutaneous Luc<sup>+</sup> fat grafts. A, The graph shows average total flux from fat grafts at the indicated time points after being grafted at day 0 with no or with ATMSC enrichment. The inset shows images of representative animals 6 days after transplantation. Dashed line, no enrichment; straight line, ATMSCs enrichment. Bars indicate ±SD, n = 10. The statistical power of comparisons of the 2 groups at days 3, 6, and 14 was calculated to be 78%, 99%, and 94 %, respectively. B, Upper panel shows HE-stained section of graft dissected from representative animal 101 days after transplantation. Middle and lower panels show higher magnification of selected areas indicated in the upper panel.

though no correlation between the ratios and enrichment protocol was observed (Table 1). The density of cytologically viable adipocytes in each individual graft varied between 97 and 336 per mm<sup>2</sup>, the highest numbers observed in the stromal cell-enriched group (336 per mm<sup>2</sup>) and the skin fibroblast group (259 per mm<sup>2</sup>), but no statistically significant correlation to enrichment protocol was observed (Table 1). To explore the possibility that vascularization is more pronounced in ATMSC-enriched grafts, we analyzed immunohistochemically the grafts for the endothelial marker CD31. The density of CD31-positive capillaries varied considerably (10.1 to 57.6 per mm<sup>2</sup>), without any detectable trend toward improved vascularization in transplanted tissue enriched with ATMSCs (Table 1).

ATMSCs have been shown to modulate inflammatory processes in vitro and in vivo.<sup>7</sup> We therefore tested whether ATMSCs affect inflammatory state of the fat grafts. To this end, we harvested adipose tissue from a transgenic mouse where adipocytes harbor a NF-κB-luciferase reporter construct. NF-κB-driven luminescence, with or without ATMSC enrichment, was then monitored regularly for 46 days. Initially, luminescence from the 3 groups of mice was found to vary substantially and inconsistently between time points after grafting (the first 3 wk, Fig. 4), suggesting variable degree of inflammation in the grafts, possibly because of mechanical tissue damage activating NF-κB signaling pathways. Contribution from cell proliferation is most likely low, as most cells in the graft are terminally differentiated adipocytes that have exited the cell cycle, and ATMSCs do not seem to proliferate after transplantation as judged from the drop in luminescence to a stable level seen in Figure 2. It cannot be ruled out, however, that integrated Luc<sup>+</sup> immune cells cotransplanted with the adipose tissue enter the cell cycle on inflammatory cues and contribute to variation of luminescence. After the initial variations and approximately 30 days after transplantation, the grafts enriched with ATMSCs displayed increased luminescence as compared to those enriched with skin fibroblasts or without enrichment (Fig. 4, *p* value corrected for multiple testing), suggesting increased NF-κB activity in grafts enriched with ATMSCs.

ATMSC enrichment could possibly facilitate a more robust inflammatory response to an adequate stimulus as compared to nonenriched grafts. To test this possibility, mice grafted with or without ATMSC enrichment of the adipose tissue were challenged with lipopolysaccharide (LPS), a powerful inducer of NF-κB, by injection into a visceral fat pad at day 76 after grafting. Mice were imaged before and after LPS injection, and luminescence was quantified by photon counting (Fig. 5A). Grafts enriched with ATMSCs responded with a higher induction of luminescence than grafts without enrichment after the proinflammatory stimulus, suggesting that ATMSC enrichment contributed to a robust NF-κB response in the grafts.

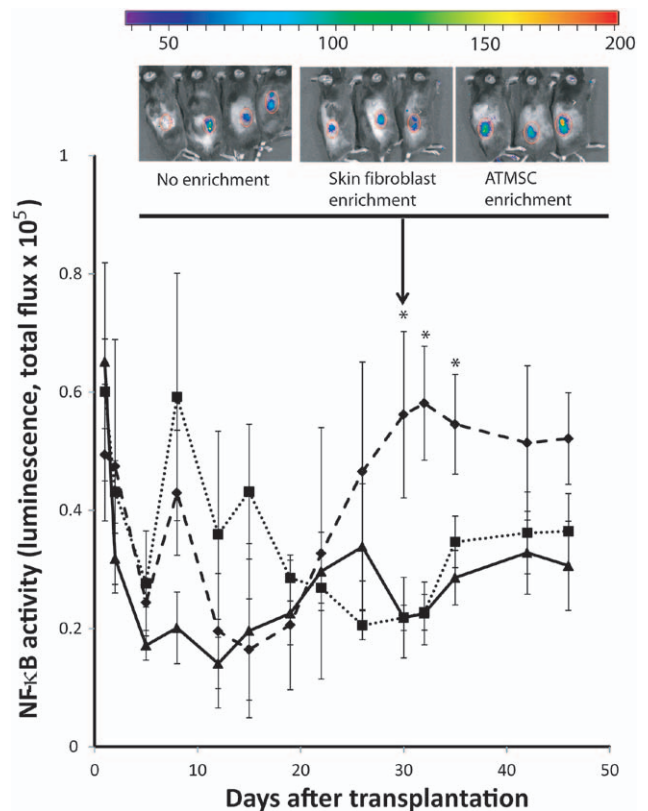
It has been demonstrated that ATMSCs can detach from the surrounding tissue and migrate to sites of inflammation.<sup>14</sup> Along this line, it is reasonable to suspect that ATMSCs in ectopically transplanted adipose tissue can also respond to a proinflammatory stimulus and migrate to

**Table 1. Histological, Immunohistochemical, and Image Analyses of HE-stained Sections of Dissected Grafts**

Treatment	Viable Adipocyte Area*	Adipocytes/sq. mm	Capillaries/sq. mm
No enrichment	17%	105	26
	27%	105	58
	26%	128	16
Skin fibroblasts	47%	259	14
	15%	97	28
Stromal cells	27%	207	22
	46%	336	10

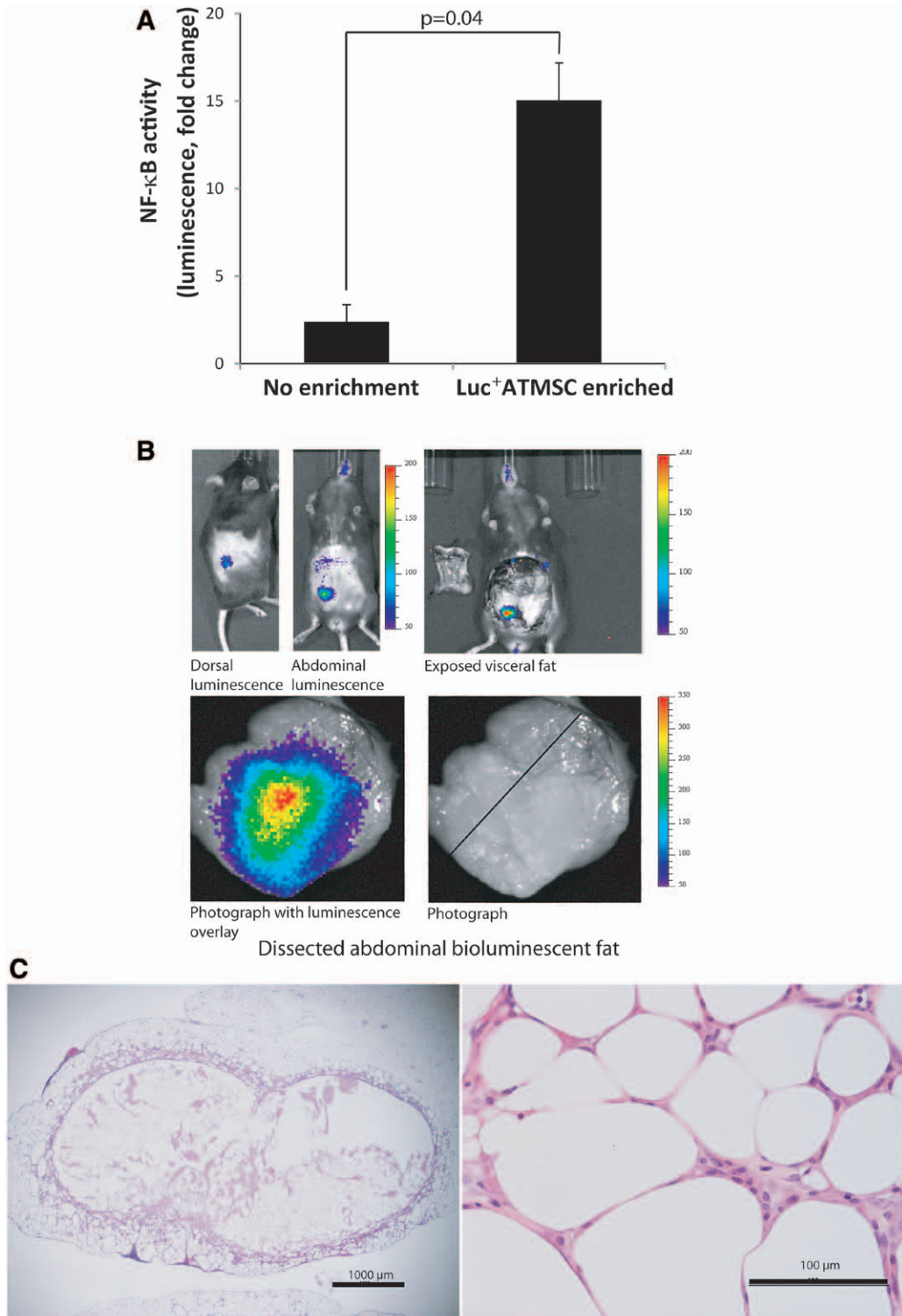
Measurements were made on a single, central cross section of the graft from each animal, as described in Materials and Methods.

\*Viable adipocyte area refers to the total area of all counted adipocytes.



**Fig. 4.** NF-κB activity in subcutaneous Luc<sup>+</sup> fat grafts. The graphs show average total flux (photons/s) from grafts where luciferase expression is driven by NF-κB activity at the indicated number of days after transplantation. Grafts were either enriched with ATMSCs (dashed line), skin fibroblasts (dotted line), or no enrichment (straight line). The inset shows images of representative animals 30 days after transplantation. Bars indicate ±SD, cell enrichment *n* = 3, no enrichment *n* = 4. Student *t* \* = Benjamini-Hochberg corrected *P* < 0.05.

other sites. To explore this, whole-body bioluminescence from LPS-injected animals was monitored for another 4 weeks. Figure 5B shows that luminescence appeared at the site of LPS injection 25 days after injection in 1 of 6 animals with ATMSC-enriched NF-κB-responsive fat graft. Guided by luminescence, the Luc<sup>+</sup> structure was dissected and sectioned along the indicated line and evaluated histologically after HE staining (Fig. 5C). The interior lacks



**Fig. 5.** Inflammatory response in subcutaneous NF-κB-Luc<sup>+</sup> fat grafts. **A**, Seventy-six days after transplantation of NF-κB-Luc<sup>+</sup> fat grafts subcutaneously on the back, with and without ATMSC enrichment, 3 mice were injected with 2 mg/kg LPS intraperitoneally and imaged 5 h after LPS injection; bars represent ±SD, *P* value from Student *t* test, *n* = 3. **B**, LPS-injected animals were imaged regularly for another 31 days after which 1 animal displayed luminescence from an abdominal structure near the site of previous LPS injection. The mouse was killed, and the luminescent structure was dissected and sectioned before HE staining, **(C)** left micrograph: 40x magnification of the whole section; right micrograph, 400x magnification of the neighboring section.



recognizable structures congruent with necrosis of adipose tissue, possibly because of extensive inflammation caused by the injected LPS. Histologically, the cyst rim contains adipocytes, fibroblasts in a collagenous matrix, chronic and acute inflammatory cells, and a tight network of capillaries visible in the upper corner (Fig. 5C, right micrograph). This is interpreted as a migrated population of transplanted adipocytes that merge with surrounding native adipose tissue with no distinct front or border. These results suggest that inflammation and necrotic tissue have attracted NF- $\kappa$ B–Luc<sup>+</sup> cells from the ectopic subcutaneous graft.

## DISCUSSION

Here we show what, to our knowledge, is the first demonstration of the survival rate of ATMSC in real time after a clinically relevant grafting procedure. After an initial surge of graft-associated luminescence that may be associated with the grafting procedure itself or rapidly proliferating endothelial cells or macrophages, we demonstrate a long-term survival of a small fraction of transplanted Luc<sup>+</sup> ATMSCs as judged by stable luminescence from the site of ATMSC transplantation. Luc<sup>+</sup> fat grafts enriched with wild-type ATMSCs showed a trend toward higher luminescence than nonenriched fat for up to 7 weeks after grafting, suggesting that ATMSC enrichment has a beneficial effect on cotransplanted grafts. Also, ATMSC enrichment increased NF- $\kappa$ B activity in fat grafts in the same period as ATMSC improved survival. Additionally, when animals with NF- $\kappa$ B–Luc<sup>+</sup> fat grafts were challenged with a proinflammatory stimulus, we found that ATMSC enrichment resulted in increased NF- $\kappa$ B response in the grafts. Of note, in 1 of 6 animals treated with a proinflammatory stimulus, NF- $\kappa$ B–Luc<sup>+</sup> cells were found at an anatomical location different from the site of grafting, suggesting migration of fat-derived cells. Taken together, these results suggest that ATMSC enrichment improves the integrity of the grafts although only for a limited period. Notably, there was still detectable luminescence above background when the animals were killed for histological assessment, suggesting long-term survival of a fraction of the graft regardless of ATMSC enrichment. We cannot, however, distinguish between cell types in the grafts regarding survival because the grafts were produced by crude disruption of adipose tissue and likely contained various cell types, i.e., fibroblasts, mesenchymal stromal cells, endothelial cells, and leukocytes in addition to mature adipocytes, this requires further investigation. There are several possible reasons why the effect is time limited: (1) ATMSCs enter into apoptosis or necrosis due to insufficient nutrient supply or lack of appropriate cell contacts; (2) ATMSCs attain properties that no longer support adipocyte survival; (3) adipocytes no longer respond to survival factors from ATMSCs. Discrimination between these explanations requires further studies.

ATMSCs, graft-resident macrophages, and mature adipocytes may all be responsible for the observed NF- $\kappa$ B activation by LPS as they express Toll-like receptor 4 (the LPS receptor).<sup>15–17</sup> The amplitude of the response indicates, however, that it comes from a large popula-

tion of cells, suggesting that LPS induces NF- $\kappa$ B activation in the mature adipocytes. Alternatively, increased NF- $\kappa$ B activity could be caused by increased proinflammatory infiltration and thus be responsible for graft destruction at later time points. The increased response to a proinflammatory stimulus can be beneficial for tissue integration of the grafted material since an inflammatory response is considered necessary for regeneration of tissue in wound healing.<sup>18</sup> Noteworthy is also the observation by Asterholm et al<sup>19</sup> that expression of inflammatory factors in adipocytes is required for adequate adipose tissue remodeling in response to energy intake. Noteworthy is also the observation by Guneta et al<sup>20</sup> who demonstrated marked differences between MSCs from breast fat and abdominal fat and it cannot be ruled out that ATMSCs-dependent graft survival depends on depot of origin.<sup>21</sup>

In a recent study, K olle et al<sup>22</sup> used enrichment of adipose tissue grafts in humans to demonstrate that stromal cell enrichment contributed to higher amounts of adipose tissue and newly formed connective tissue. Complementary to this, we detected considerable areas of tissue repair (mononuclear inflammatory cells, fibroblast-like cells, and capillaries) that in time would be expected to result in collagenous connective tissue.

MSCs, including those found in adipose-derived stroma-vascular fraction, have been shown to facilitate angiogenesis.<sup>23</sup> It can be speculated that stromal cells contributed to vascularization of the grafts in our experiments and thereby provided improved access to luciferin. However, when grafts were sectioned and analyzed histologically for tubular CD31-positive structures, i.e., capillaries, we could not detect any differences between sections from grafts with stromal cells, fibroblasts, or no enrichment. Although the number of animals was limited and the protocols were significantly different, our analyses confirm the observation by K olle et al<sup>22</sup> that stromal cell enrichment of grafts does not increase vessel density.

The identity of the migrated cells and mode of migration are unknown. However, we find it unlikely that cells located outside the site of grafting arrived at the site by being accidentally injected into the blood stream. MSCs are large in size and have been shown to be trapped in the lung.<sup>24</sup>

We conclude that stromal cell enrichment may be used for reconstructive purposes to improve survival and modulate inflammatory responses in adipose tissue grafts although only for a limit period after grafting. However, the safety of the procedure needs further studies regarding migratory properties of MSCs to evaluate the risk of hyperplasia at unwanted anatomical locations.

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