Dermal fillers include a variety of specialized chemical and biologic substances that are administered to improve the appearance of the skin surface. In dermatology and aesthetic medicine, dermal fillers may be utilized when patients wish to improve their appearance or for numerous other clinically appropriate instances, such as nonsurgical rhinoplasty or treatment for acne scarring. Treatment with dermal fillers is a type of soft-tissue augmentation that comprises 2 clinical categories, depending on the type of application: (1) operative (invasive) applications of filling substances or small implants, commonly performed by plastic or craniofacial surgeons, and (2) injectable preparations administered by dermatologists. The latter category is the subject of the present study.

The first administration of a dermal filler was described by a German physician, Gustav Adolf Neuber, at the 22nd meeting of the Deutschen Gesellschaft für Chirurgie in April 1893. Neuber presented an innovative method of filling deep facial scars with fat grafted from the upper arm. Numerous dermal fillers have been introduced since then, but their clinical effects have varied. Some fillers have been controversial and associated with complications, whereas others have produced insufficient or unsatisfactory clinical results. Paraffin and beeswax were...
administered as fillers in the beginning of the 20th century, and silicone formulations emerged in the mid-1950s. Collagen fillers were employed in the late 1970s, and the revolutionary hyaluronic acid (HA) products were introduced in the early 1980s. Hyaluronic acid currently is regarded as a first-line filling product.\textsuperscript{10-15} However, several investigators have noted significant limitations of dermal fillers, including foreign body reaction and longevity, and have suggested that the ideal dermal filler does not yet exist.\textsuperscript{16-19}

Innovations in regenerative medicine and tissue engineering have advanced numerous branches of science and medicine and are applicable to many popular medical procedures. The implementation of stem cells has evoked tremendous enthusiasm among clinicians.\textsuperscript{20-24} In the present study, we aimed to determine the local and systemic effects and utility of adipose-derived stem cells (ADSCs) as a component of dermal fillers in an in vivo rat model.

METHODS

Animals

This study included twenty 10-week-old inbred Wistar rats and 5 donor Wistar rats, from which ADSCs were isolated. All experiments were approved by and conducted in accordance with the local ethical committee associated with the University of Technology and Life Sciences in Bydgoszcz, Poland, which is a legally established official committee for the Bydgoszcz district. Our work involving experimental animal models was conducted in compliance with the guidelines of the European Union (Directive 2010/63/EU) and other representative recommendations such as the Guide for the Care and Use of Laboratory Animals of the National Research Council.\textsuperscript{25}

Animals were divided into 4 groups according to the dermal filler received, as follows: ADSCs combined with HA (ADSC-HA; n = 7), ADSCs combined with an experimental filler prepared from fish collagen (ADSC-COL; n = 7), HA alone (CONTROL-HA; n = 3), or COL alone (CONTROL-COL; n = 3).

Isolation, Validation, and Labeling of ADSCs

Adipose-derived stem cells were isolated from adipose tissue harvested from the retroperitoneal space of 5 donor rats, as described previously.\textsuperscript{26} Briefly, adipose tissue (1 g) was digested in collagenase type I solution (1 mg/mL; Sigma-Aldrich, St Louis, Missouri) for 30 minutes at 37°C with shaking. The reaction was stopped by the addition of Dulbecco’s modified Eagle’s medium (DMEM; PAA, Austria) supplemented with 10% fetal bovine serum (FBS; PAA) and antibiotics (PAA). The cell suspension was filtered through a 100-µm cell strainer (BD Biosciences, Franklin Lakes, New Jersey) and was centrifuged at 350g for 5 minutes. Viable cells were counted by trypsin blue exclusion and seeded on a 25-cm² flask at a density of 15 000 cells/cm². The ADSCs were cultured according to standard protocols in DMEM supplemented with 10% FBS, fibroblast growth factor (10 ng/mL; Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) (PAA) at 37°C, 5% CO₂, and 95% humidity until the third passage.

To confirm the phenotype of ADSCs, flow cytometric analysis of cellular antigens was performed. Detached cells from the third passage were washed and resuspended with phosphate-buffered saline (PBS). Approximately 1 × 10⁶ cells were incubated for 30 minutes with monoclonal antibodies against CD34 (Santa Cruz Biotechnology, Santa Cruz, California; catalog no. sc-7324 PE; 20 µL/sample), CD44 (Millipore, Billerica, Massachusetts; catalog no. CBL1508F; 10 µL/sample), CD45 (BD Biosciences; catalog number 554877; 0.06 µg/sample), or CD90 (Millipore; catalog number CBL1500F; 10 µL/sample) and conjugated with phycoerythrin (PE) or fluorescein (FITC).

The expression of cell-surface markers was quantified using an Epics XL flow cytometer (Beckman Coulter, Fullerton, California). The ADSCs were induced to differentiate into adipogenic, osteogenic, or chondrogenic lineages by culturing in the appropriate media according to the manufacturer’s instructions (Invitrogen, Carlsbad, California). Negative-control cells were maintained in DMEM/Ham’s F-12 medium supplemented with 10% FBS.
and antibiotics. Adipogenesis was assessed as the accumulation of neutral lipids in fat vacuoles stained with Oil Red O (Sigma-Aldrich). Osteogenesis was confirmed by von Kossa staining (Bio-Optica, Milan, Italy). Chondrogenic differentiation was assessed by anti–collagen type II immunocytochemical staining (clone 6B3, 1:100; Millipore) for 16 hours at 4°C.

Prior to injection, ADSCs were labeled with the fluorochrome PKH26 (labeling kit from Sigma-Aldrich) to assess migration in vivo.

**Preparation and Injection of Dermal Fillers**

To prepare the dermal fillers, 10⁶ ADSCs were suspended by reciprocal mixing in 1 mL of cross-linked HA with high viscosity (Revanesse Ultra, Prollenium Medical Technologies, Inc, Ontario, Canada) or in an experimental filler containing fish collagen (Collife, Poznań, Poland). The ADSCs were omitted from control fillers (CONTROL-HA, CONTROL-COL).

Fillers and control substances were injected according to current dermatologic and aesthetic medical standards of practice and representative guidelines. Animals were prepared for injection by shaving and disinfecting the skin site, delineating the injection area with a red marker, and applying EMLA cream per the manufacturer’s instructions (lidocaine 2.5%, prilocaine 2.5%; AstraZeneca, Waltham, Massachusetts). One researcher (M.N.) performed all injections at the same time of day. Animals were injected through a syringe connected to a sterilized 13-mm, 30-gauge needle into the glabella, dorsum, and chest regions. All animals were administered the same dose of filler (1 mL) and received injections at all 3 anatomic sites. Fillers were injected slowly into the dermis, via the linear tracking technique, with overcorrection for improved macroscopic assessment (Figure 1). Overcorrection enabled us to obtain accurate, noninvasive measurements with calipers. To our knowledge, this is the most appropriate approach for evaluating this type of rat model.

**Assessment of Filling Effects**

All animals were sacrificed by anesthesia overdose with ketamine and xylazine (Sedazin and Bioketan, Biowet, Pulawy, Poland) after 3 months of follow-up. The monitoring period was selected to maximize detection of PKH26 fluorescence, which has a half-life in vivo of approximately 100 days. The filling effects (FEs) of the injected substances were observed macroscopically upon injection and at 1.5 months and 3 months after injection. FEs were represented as the largest width of the overcorrected area measured with standard calipers.

**Histologic Analysis**

Tissue specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Cross sections of the analyzed areas were prepared and stained for histologic analysis with hematoxylin and eosin.

**Cell Migration Assay**

Migration of ADSCs in the skin, brain, lungs, kidneys, and spleen was determined immediately after animals were sacrificed. Tissues were frozen in an optimal cutting temperature compound (Tissue-TeK; Sakura, Torrance, California), and 5-µm-thick sections were obtained. Sections were washed with PBS, stained with DAPI (Sigma-Aldrich) for visualization of cell nuclei, and mounted to slides with Aqua-Poly/Mount coverslipping medium (Polysciences, Warrington, Pennsylvania). Slides were examined immediately after preparation by means of a C1 laser-scanning confocal microscope (Nikon Corp, Tokyo, Japan) with a ×20 objective, a 405-nm diode laser, and a 543-nm HeNe laser. Double-labeled images were collected with EZ-C1 software (v3.80; Nikon) at the brightest PKH26 signals. PKH26 is a red fluorochrome with excitation (551 nm) and emission (567 nm) characteristics similar to rhodamine or phycoerythrin detection systems.

**Statistical Analysis**

Filling effects were represented as mean ± standard deviation (SD) for each group. The t test was applied to compare differences in FEs between groups and over time. Differences among more than 2 groups were ascertained by 1-way analysis of variance followed by Tukey’s post hoc test for multiple comparisons. Statistical significance was defined as P < .05.

**RESULTS**

Following injection, no topical or systemic complication was noted in any animal, and no atypical situations occurred to necessitate medication or exclusion of any animal from the study. We did not observe any changes in animal behavior or circadian rhythm. No pathologic changes or lesions of the skin were noted.

Filling effects were calculated by averaging the caliper measurements at all 3 anatomic sites in all animals of each group. For both the ADSC-HA and CONTROL-HA groups, the mean FE immediately after injection was 0.50 cm. After 1.5 months and 3 months, the mean FEs in the ADSC-HA group were 0.40 cm and 0.31 cm, respectively (Table 1). In the CONTROL-HA group, the mean FEs at 1.5 and 3 months were 0.37 cm and 0.30 cm, respectively (Table 1). For both the ADSC-COL and CONTROL-COL
Figure 1. Injection of dermal fillers. (A) Delineation of glabella, dorsum, and (B) chest sites for injection. (C) Application of EMLA cream to glabella before filler injection. (D) Injection of dermal filler into dorsum. (E) Overcorrected filling effects immediately after injection into the dorsum, (F) chest, and (G) glabella. (H) Persistence of filling effects 1.5 months after injection with a formulation of adipose-derived stem cells and hyaluronic acid.
groups, the mean FE immediately after injection was 0.38 cm. The FEs for the ADSC-COL group were 0.27 cm at 1.5 months and 0.14 cm at 3 months. In the CONTROL-COL group at 1.5 and 3 months, the FEs were 0.12 cm and 0.08 cm, respectively (Table 1). Animals injected with a mixture of ADSCs and HA demonstrated significantly larger FEs at 1.5 and 3 months (P < .001 vs all other groups).

Histologic analysis confirmed that cell morphology was well preserved. The ADSCs incorporated into HA were stacked immediately below the thin layer of muscle cells, and no inflammatory response was observed (Figure 2).

Results of the cell migration assay indicated that the proportions of cells exhibiting PKH26 fluorescence differed in skin and organ specimens across groups. Among animals receiving ADSC-HA, 68.61% of cells were positive for PKH26 at the injection sites 3 months after treatment (Figure 3A). In contrast, PKH26 fluorescence was observed in 60.62% of cells sampled from ADSC-COL animals (Figure 3B). PKH26 fluorescence was noted in the spleens of both groups (Figure 3C) but could not be detected in the lungs, kidney, or brain (Figure 3D-F), suggesting that ADSCs do not migrate chaotically after injection into a dermal filler preparation.

**DISCUSSION**

Currently, 80% to 85% of all aesthetic procedures are minimally invasive, many of which are dermal filler treatments. Despite the growing popularity of these fillers, they have been associated with limited effectiveness and other problems in aesthetic medicine and dermatology.27-29

The development of novel dermal fillers has involved the incorporation of specific cell types as primary or supplementary constituents. However, experimental research
Figure 3. Migration of adipose-derived stem cells (ADSCs) labeled with PKH26 and injected as a dermal filler formulation. Images were obtained 3 months after injection. (A) Compared with other dermal fillers, PKH26-positive cells were most abundant in animals treated with ADSCs and hyaluronic acid (HA), comprising 68.61% of detectable fluorescence at the injection sites. (B) In specimens collected from the injection sites of animals treated with ADSCs and fish collagen, PKH26-positive cells comprised 60.62% of detectable fluorescence. (C) PKH26 fluorescence comprised 5.86% of detectable fluorescence in spleen specimens from animals treated with ADSCs and HA. PKH26 expression was not detected in (D) lung, (E) kidney, or (F) brain specimens of animals treated with ADSCs and HA. Images were obtained by light microscopy (original magnification × 20).
is scarce regarding fibroblasts or other types of stem cells as dermal fillers, and few investigators have assessed cell migration or described the practical and clinical applications of this type of cell therapy.\textsuperscript{30-33} We maintain that the in vivo effects of cell-based dermal fillers cannot be established until the persistence and cell migration of these fillers are understood.\textsuperscript{34-36}

We evaluated ADSCs combined with 2 filler substances because cells suspended in standard saline or PBS failed to produce any FEs (data not shown). Our approach enabled assessment of the utility of HA and COL as ADSC carriers. We evaluated ADSCs rather than stromal vascular fraction (SVF) cells because ADSCs are established homogeneous cells with well-known properties.\textsuperscript{37} Adipose-derived stem cells proliferate rapidly with a few passages and exhibit a stable phenotype after the third passage. These properties allowed us to obtain a large number of ADSCs with a low risk of culture-induced chromosomal abnormalities or teratoma formation because the latter typically is not associated with mesenchymal stem cells.\textsuperscript{38,39} The ADSCs also have the potential to differentiate into multiple lineages.\textsuperscript{30,41} Although SVF cells are easier to obtain, these cells have been associated with adverse events in humans, such as cyst formation and microcalcifications.\textsuperscript{38} Our ADSC filler preparations allowed for precise control of types and amounts of filler components. Although ADSCs have been evaluated in several clinical trials, peer-reviewed data on ADSCs have been limited in the field of aesthetic medicine.\textsuperscript{37} Adipose-derived stem cells have been applied successfully to maxillary reconstruction and to treat fistulas of cryptoglandular origin with or without Crohn disease.\textsuperscript{42-45}

In the latter application, ADSCs were more effective than stromal vascular cells.\textsuperscript{43}

Our findings regarding the persistence of dermal fillers are consistent with those of other in vivo studies. We observed decreases in overcorrected FEs with time in all study groups. Similarly, other investigators have reported that the effects of experimental dermal fillers diminish with time in humans and animal models.\textsuperscript{46,47} Our results indicate that ADSC-based formulations of dermal fillers produce greater FEs that persist significantly longer than dermal fillers prepared without ADSCs. Other researchers have observed trophic changes and modulation of the tissue environment when mesenchymal stem cells were applied to a reconstructed bladder wall in a rat model or injected into human skin as a filler component.\textsuperscript{48-50} ADSCs and their soluble factors function in protective and regenerative roles in the skin, inducing collagen synthesis, inhibiting melanogenesis, and recruiting and protecting dermal fibroblasts.\textsuperscript{51}

Our cell migration assays and histologic analyses indicate that ADSCs in HA were maintained in the vicinity of the injection sites during the 3-month observation period, supporting their localized activity. This phenomenon may be explained by the incorporation of stem cells into the cross-linked HA. The absence of uncontrolled pathologic cell migration to other organs (brain, kidneys, or lungs) supports the potential safety of ADSC-based dermal fillers for clinical applications. When mesenchymal stem cells were injected into the circulatory system, cell migration to sites of inflammation and injury was observed.\textsuperscript{52} However, cell migration is minimized when cells are implanted directly into the injury site,\textsuperscript{48} as demonstrated in the present study.

Mesenchymal stem cells secrete various bioactive trophic and proangiogenic factors that enhance tissue regeneration and neoangiogenesis, downregulate the inflammatory response,\textsuperscript{53-55} and inhibit apoptosis.\textsuperscript{56} Minimal ADSC migration combined with extended viability after implantation could prolong release of these factors, thereby increasing the therapeutic effects on surrounding tissues. In clinical practice, ADSC-based fillers could potentially stimulate tissue regeneration, obviate frequent treatments, and extend cosmetic effects.

Although the Wistar rat model utilized in our in vivo study was appropriate for our present objectives, a nude animal model might be preferable for subsequent studies due to better continuous observation and measurement possibilities of FEs. We demonstrated that ADSC-based dermal fillers were safe in our rat model; however, we did not examine biologic effects on the skin layers. This topic should be evaluated in a larger series of animals to obtain greater statistical power and validate the effectiveness of our methods. If safety and effectiveness are confirmed in a large study of animals, a prospective clinical study of ADSC-based dermal fillers should be conducted.

Our research complements existing data regarding the biologic effects of ADSC implantation, and our novel formulation for a dermal filler may be applicable to dermatology and aesthetic medicine. We expect that the development and evaluation of stem cell–based procedures will be vital to improvements in aesthetic surgery and dermatology, especially when noninvasive methods are preferred.

### Conclusions

Stem cells have the potential to become an essential component of dermal fillers, improving and prolonging cosmetic results and decreasing the complications and ineffectiveness associated with minimally invasive aesthetic procedures. Our findings in a rat model support the safety and effectiveness of fillers formulated with ADSCs and HA. Subsequent investigations are needed to assess the immunohistologic properties of these fillers.

### Disclosures

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