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Biological Alchemy: Engineering Bone and Fat From Fat-Derived Stem Cells

[Original Articles]

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Outline

Graphics

Abstract

Adipose tissue contains a population of pluripotent stem cells capable of differentiating along multiple mesenchymal cell lineages. In this study the authors isolated these fat-derived stem cells successfully from Lewis rats and induced differentiation along adipogenic and osteogenic lineages in vitro and in vivo. Induction was stimulated by exposing stem cells to lineage-specific induction factors. Adipocyte-inducing media contained dexamethasone, insulin, and isobutyl-methylxanthine. Osteoblast inducing media contained dexamethasone, [beta]-glycerophosphate, and ascorbic acid. Undifferentiated stem cells were maintained in minimal essential media alpha and fetal bovine serum. At 10 days, cells cultured in adipogenic media differentiated into adipocytes in vitro, as evidenced by positive Oil red O staining of lipid vacuoles. At 21 days, cells cultured in osteogenic media differentiated into osteoblasts in vitro as demonstrated by Alizarin red staining of a calcified extracellular matrix and immunohistochemical staining for osteocalcin. Differentiated cells were seeded at a density of 5×10^6 cells onto 15 × 15-mm polyglycolic acid grafts and implanted subcutaneously into three groups of Lewis rats: Group I contained undifferentiated stem cell grafts, group II contained adipocyte grafts, and group III contained osteoblast grafts. At weeks 4 and 8, in vivo fat formation was demonstrated in group II rats, as confirmed by Oil red O staining. At 8 weeks, group III rats demonstrated in vivo bone formation, as confirmed by the presence of osteocalcin on immunohistochemistry and the characteristic morphology of bone on hematoxylin–eosin staining. Group I rats demonstrated no in vivo bone or fat formation at either time interval. These results demonstrate the ability to isolate pluripotent stem cells from adipose tissue, to induce their differentiation into osteoblasts and adipocytes in vitro, and to form bone and fat subsequently in vivo. This is the first published report of in vivo bone formation from fat-derived stem cells. These cells may eventually serve as a readily available source of autologous stem cells for the engineering of bone and fat.

The quest for a readily available, viable substance to fill soft-tissue and bony defects in patients has challenged reconstructive surgeons for decades. The current available options all fall short of the ideal, with such adverse side effects as unnatural texturing, inflammation, extrusion, resorption, and even rejection. Autologous fat and bone grafts can also fail to meet the need because they are limited in availability and may demonstrate poor viability. Innovative tissue culture techniques using stem cells may allow for an elegant solution to this problem. By differentiating stem cells into bone and fat, one can create the building blocks for an autologous, nonimmunogenic graft.

There are two major sources of the pluripotent cells necessary for this type of tissue engineering: embryonic stem cells and adult autologous stem cells. Embryonic stem cell use is limited severely by governmental regulation and the surrounding ethical debate related to embryonic tissue research. On the other hand, adult stem cells are readily available, have been harvested successfully from bone marrow, [1–4] and can be differentiated into adipocytes, [4–7] chondrocytes, [4–8] myoblasts, [5,6,9] and osteoblasts. [4–7,10] However, bone marrow harvesting is painful, has a relatively low yield of usable cells, and is associated with a small but not negligible risk of infection and marrow suppression. A few groups have had success in isolating stem cells from adipose tissue. [11–15] These pluripotent cells have been differentiated successfully into different mesenchymal cell lines in vitro by exposing them to specific growth conditions. [11–15] However, an extensive review of the literature shows that no groups have yet been able to translate this idea successfully into in vivo experiments.

In our novel model, we procure adult stem cells from autologous fat, and differentiate these fat-derived stem cells into adipocytes and osteoblasts in vitro. After the period of in vitro differentiation, we seed them on polyglycolic acid (PGA) scaffolds, implant them subcutaneously, and grow them in vivo. The ease with which fat can be collected makes this technique eminently practical and cost-effective. The potential applications of such a system are myriad, including the design of completely nonimmunogenic grafts for bone reconstruction, breast reconstruction, and soft and hard-tissue augmentation.

Materials and Methods

Isolation of Stem Cells

Epididymal adipose tissue was collected in a sterile fashion from inbred male Lewis rats (300–350 g; Charles River Laboratories, Wilmington, MA). The rat scrotum was shaved and prepared with povidone–iodine, a 15-mm incision was made along the scrotum, and the testes were pulled outward exposing the epididymal fat pad. The outer layer of connective tissue was incised and the adipose tissue was then excised from the epididymis and was placed on a sterile glass surface. The adipose tissue was minced finely under sterile conditions. The tissue was then transferred to a sterile 50-ml conical tube and was washed in Hanks Balanced Salt Solution (Life Technologies, Gaithersburg, MD). Stem

cells were isolated as described previously. [16,17] Briefly, the minced tissue was placed in a sterile, vented T75 flask containing 2 mg per milliliter tissue culture grade collagenase (Sigma–Aldrich, St. Louis, MO) and 5% bovine serum albumin (Sigma–Aldrich). The flask was incubated at 37°C for 45 minutes, shaking every 5 minutes. The flask contents were filtered through a sterile 250-µm nylon mesh to remove large, undigested debris. The digested tissue was then centrifuged at 250 *g* for 10 minutes and the mature adipocytes were removed by aspirating the supernatant, leaving a pellet of stem cells behind. The pellet was then resuspended in Hanks Balanced Salt Solution and filtered twice through a 25-µm nylon mesh. The filtrate was then centrifuged at 250 *g* for 10 minutes. To eliminate erythrocytes, the filtrate was incubated with erythrocyte lysis buffer (Sigma–Aldrich) for 5 minutes at 37°C. The suspension was centrifuged at 250 *g* for 10 minutes and the cells were counted using a hemacytometer.

Cell Culture and Expansion

The collected fat-derived stem cells were plated in 75-cm^2 vented tissue culture flasks at a density of 1.5×10^6 cells per flask in minimal essential media alpha with 10% fetal bovine serum and 1% antibiotic/antimycotic (Life Technologies). The flasks were maintained in a tissue culture incubator at 37°C, 5% carbon dioxide conditions. The media were replaced the day after the initial stem cell harvest and then every third day. The cells were monitored for confluence on a daily basis.

The cells were subcultured when the flasks reached 80% confluence. The media were removed and 3 ml trypsin was added to each flask for 5 minutes to allow the cells to deplate. The cell layer was then collected into a 50-ml conical tube and was centrifuged at 250 *g* for 10 minutes. The pellet was resuspended with 10 ml control media and the cells were counted. The cells were split into flasks at a density of 1.5×10^6 cells per flask.

Preparation of Differentiation Media and Differentiation of Fat-Derived Stem Cells

At the third pass, the fat-derived stem cells were analyzed for their capacity to differentiate into adipogenic and osteogenic lineages. To induce differentiation, the stem cells were cultured in tissue-specific induction media, as described in the Table. The culture media were prepared under sterile conditions in a dual-flow tissue culture hood. The media used in this experiment have been described in previous experiments and have been proved to induce differentiation of mesenchymal stem cells. [5,6,10,11,13,18,19]

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Table. Lineage-Specific Differentiation MediaMEM = minimal essential media; FBS = fetal bovine serum.

At pass 3, the cells were divided into three groups: group I, stem cells in control media; group II, stem cells in adipogenic media; and group III, stem cells in osteogenic media. All cells were plated at a density of 1.5×10^6 cells per flask with 15 ml appropriate media added to each flask. The media in each flask were replaced every 3 days and the cells were monitored for growth and confluence on a daily basis. When the cells became 80% confluent they were split using the previously described protocol. Following set in vitro induction periods, these cells were assessed for successful differentiation via histology and immunohistochemistry.

In Vitro Adipocyte Differentiation

Adipogenic differentiation was induced by culturing the fat-derived stem cells for 10 to 14 days in adipogenic media. Assessment of adipogenesis was performed with Oil red O staining, which demonstrates intracellular lipid accumulation.

To perform this staining, on day 8 of differentiation, cells cultured in adipogenic media were trypsinized, collected, and plated onto eight-well chamber slides (Fisher–Scientific, Hanover Park, IL) at a density of 5×10^4 cells per well. The slides were then placed in the incubator (37°C, 5% carbon dioxide) and were used for staining procedures at days 10 to 14 of differentiation. The same procedure was performed with the cells in the control media. At 10 to 14 days, the cells on the chamber slides were fixed for 5 minutes at room temperature in 10% formalin and washed with 1× phosphate-buffered saline for 5 minutes. The cells were then incubated in 2% (weight per volume) Oil red O reagent

(Polyscientific Corporation, Bay Shore, NY) for 30 minutes at room temperature. They were then washed with 1× phosphate-buffered saline three times for 3 minutes each, followed by several changes of distilled water to remove excess staining. The cells were counterstained for 1 minute with hematoxylin. The same procedure was performed on the control cells. The percentage of cells committed to the adipogenic lineage was quantified under 20× microscopic power.

In Vitro Osteoblast Differentiation

Osteogenic differentiation was induced by culturing the fat-derived stem cells for 21 days in osteogenic media. Assessment of osteogenesis was performed with Alizarin red staining, which demonstrates extracellular matrix calcification and immunohistochemical staining of osteocalcin—a protein specific for osteoblasts and osteoblasts.

To perform this staining, on day 17 of differentiation, cells cultured in osteogenic media were trypsinized, collected, and plated onto eight-well chamber slides at a density of 5×10^4 cells per well. The slides were then placed in the incubator (37°C, 5% carbon dioxide) to be used for staining procedures. The same procedure was performed with the cells in the control media. To detect extracellular matrix calcification with Alizarin red staining, at 21 days the osteogenic media-cultured cells were fixed with 10% formalin for 30 minutes at room temperature. The cells were washed with phosphate-buffered saline for 5 minutes. A 1% Alizarin red solution (Polyscientific Corporation) was placed on each slide and incubated for 10 minutes at room temperature. Slides were then washed with running tap water for 5 minutes and left to dry. Manual counts of calcium nodules were performed. The same procedure was performed for undifferentiated control stem cells.

Immunohistochemistry for osteocalcin was performed on the osteoblasts and the control stem cells prepared on chamber slides in the same manner as those for Alizarin red staining. The goat antirat osteocalcin primary antibody (Biomedical Technologies, Stoughton, MA) was diluted to a concentration of 5 µg per milliliter. Antigen was unmasked via heat treatment with 10 mmol per liter sodium citrate buffer (pH, 6.0) for approximately 20 minutes. Visualization of osteocalcin was achieved by exposing the primary antibody to biotinylated immunoglobulin G and chromogen from the Avidin–HRP kit (Santa Cruz Biotechnology, Santa Cruz, CA).

Seeding of PGA Grafts

After demonstration of in vitro adipogenesis and osteogenesis, the three groups of cells were then collected from their respective flasks via trypsinization and centrifugation. The cells from each flask were counted with a hemacytometer. The three groups of cells were seeded separately into sterile, resorbable PGA scaffolds (Albany International, Mansfield, MA) at a density of 5×10^6 cells per graft. PGA scaffolds were 15 × 15 mm, 2 mm thick, with a pore size of 200 µm, and were composed of 12 to 14-µm fibers at a density of 55 to 65 mg per cubic centimeter, with a resorptive half-life of 2 weeks. Grafts were incubated for 3 hours at 37°C, allowing the cells to adhere to the fibers of the PGA. After this incubation period, the control, adipocyte-, and osteoblast-seeded fat-derived stem cells grafts were incubated in control, adipogenic, and osteogenic media respectively. The media were replaced on day 4 of incubation and the grafts were ready for implantation on day 7 of incubation.

Surgical Procedures

The seeded PGA grafts were implanted into inbred adult Lewis rats (350–400 g). The rats were divided into three groups: group I, fat-derived stem cell seeded grafts; group II, adipocyte-seeded grafts; and group III, osteoblast-seeded grafts. The rats were weighed and anesthetized with 80 mg per kilogram ketamine and 6 mg per kilogram xylazine. Their heads were shaved and prepared with povidone–iodine and alcohol. A 15-mm incision was made on the rat's head immediately posterior to the occiput. A 15 × 15 mm subcutaneous pocket was then dissected bluntly overlying the skull. The PGA grafts were then placed inside this pocket. The incision was then sutured with a running 5-0 nylon suture.

The rats were killed at either 4 weeks (groups I and II, N = 4; group III, N = 3) or 8 weeks (groups I and II, N = 4; group III, N = 5). The implantation sites were harvested and bisected. Half the bisected specimens from group I and group II rats were frozen with liquid nitrogen and the other half were placed in 10% formalin for fixation. All the bisected specimens from group III rats were placed in 10% formalin for fixation.

Histological Analysis of In Vivo Studies

Group II (adipocyte) collected wounds frozen in liquid nitrogen were stained with Oil red O to determine in vivo

adipogenesis via intracellular lipid accumulation. Frozen sections were cut at 10 µm and collected on coated glass slides. Slides were fixed in 10% formalin and then washed well in tap water, rinsed in distilled water, and the excess water was drained off. Slides were placed in propylene glycol for two changes, 5 minutes each. Oil red O solution was made by combining 0.7 g Oil red O in 100 ml propylene glycol, heated at 100°C for 3 minutes while stirring and then filtered through Whatman no. 2 filter paper twice. Samples were then placed in Oil red O for 7 minutes and then 85% propylene glycol for 3 minutes. Samples were rinsed in distilled water, placed in hematoxylin for 2 minutes, then in running tap water for 1 minute, and rinsed with distilled water. Cells were mounted with aqueous mounting media. The same process was performed for the group I frozen sections.

Group III (osteoblast) wounds were stained with hematoxylin–eosin (H&E) stain to evaluate for the presence of the characteristic features of bone formation, and immunohistochemistry was performed for the presence of osteocalcin as outlined previously. The same staining was performed with group I rats as a control.

All the samples were analyzed microscopically and compared with controls. Digital photographs were recorded.

Results

Successful Isolation and Culture of Fat-Derived Stems Cells

The fat-derived stem cells were isolated successfully from the Lewis rats with approximately 4×10^6 stem cells collected from the epididymal adipose tissue of each 300 to 350-g rat. These cells were expanded readily in vitro, with the average population doubling time of 72 hours, and cells reached confluence in 6.5 days on average. The cells demonstrated fibroblastlike morphology and grew in a monolayer in vitro before and during differentiation.

Successful In Vitro Adipogenesis and Osteogenesis

In vitro histological analysis of fat-derived stem cells grown in adipogenic media demonstrated successful differentiation into adipocytes at 14 days. The adipocytes were stained with Oil red O, which revealed an accumulation of lipid-rich vacuoles within the differentiated cells (Fig 1B). The fat-derived stem cells grown in control media demonstrated no intracellular lipid vacuoles by Oil red O staining (Fig 1A). The percentage of fat-derived stem cells grown in adipogenic media that differentiated into adipocytes was 92%.

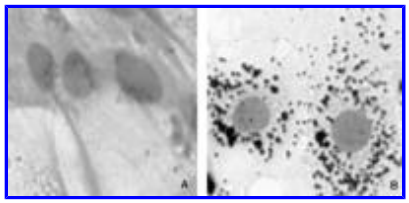

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Fig 1. In vitro adipogenesis. Cells stained for lipid vacuoles with Oil red O stain. (*A*) Cells grown in control media demonstrate the absence of fat vacuoles (Oil red O, original magnification ×40 before XX% reduction). (*B*) Cells grown in adipogenic media demonstrate abundant fat vacuoles (Oil red O, original magnification ×40 before XX% reduction).

In vitro osteogenesis was demonstrated with Alizarin red staining and osteocalcin immunohistochemistry. Cells cultured in osteogenic media demonstrated a dramatic change in cell morphology from day 5 of induction, with the cells changing from a spindle-shaped morphology to a polygonal, spiculated morphology. The nondifferentiated cells remained spindle shaped. The cells grown in osteogenic media demonstrated intense staining of calcium nodules with Alizarin red whereas the fat-derived stem cells grown in control media demonstrated no calcium nodule staining (Figs 2A, B). Immunohistochemical staining for osteocalcin revealed intensely positive staining in stem cells cultured with osteogenic media and no staining in stem cells cultured in control media (Figs 2C, D).

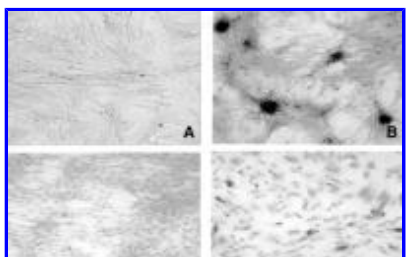

Fig 2. In vitro osteogenesis. (*A*) Cells grown in control media lack formation of calcium nodules (Alizarin red, original magnification ×20 before reduction. (*B*) Cells grown in osteogenic media demonstrate numerous calcium nodules (Alizarin red, original magnification ×20 before reduction. (*C*) Cells grown in control media do not stain for osteocalcin with immunoperoxidase staining (original magnification ×10 before reduction). (*D*) Cells grown in osteogenic media have intense staining for osteocalcin with immunoperoxidase staining (original magnification ×10 before reduction).

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Successful In Vivo Bone and Fat Formation

In vivo histological studies demonstrated in vivo bone and fat formation from the differentiated fat-derived stem cells. Specimens implanted with grafts containing differentiated adipocytes demonstrated the characteristic dissolution of intracellular lipid vacuoles at 4 and 8 weeks with H&E staining (Figs 3A, B). Staining of 4 and 8-week samples with Oil red O demonstrated large, red-stained lipid vacuoles within the tissue (Figs 3C, D). The grafts containing control cells

did not reveal H&E or Oil red O evidence of fat formation.

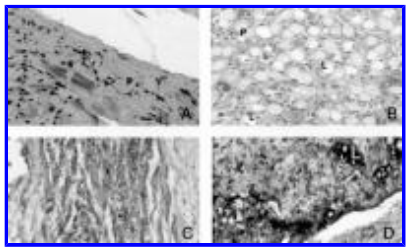

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Fig 3. In vivo adipogenesis at 8 weeks. (*A*) Polyglycolic acid grafts seeded with control cells demonstrate no adipose tissue (H&E, original magnification ×20 before reduction). (*B*) Grafts seeded with differentiated adipocytes demonstrate abundant adipose tissue by the presence of numerous lipid vacuoles (L) (H&E, original magnification ×20 before reduction). (*C*) Grafts seeded with control cells lack staining for lipid vacuoles (Oil red O, original magnification ×20 before reduction). (*D*) Grafts seeded with differentiated adipocytes demonstrate extensive formation of fat vacuoles (Oil red O, original magnification ×20 before reduction).

Grafts containing differentiated osteoblasts were analyzed by H&E and immunohistochemistry for osteocalcin. The samples at 4 weeks did not yet show characteristic bone formation but stained positively for osteocalcin, with the edges of the graft staining more intensely. The 4-week control samples did not stain positively for osteocalcin. At 8 weeks, the wounds containing differentiated osteoblasts showed characteristic signs of bone formation by H&E analysis, with abundant eosinophilic, acellular matrix with occasional intensely basophilic, flattened nuclei (Fig 4). The 8-week osteoblast samples also stained positively for osteocalcin. The 8-week control samples showed no such bone formation and did not stain positive osteocalcin.

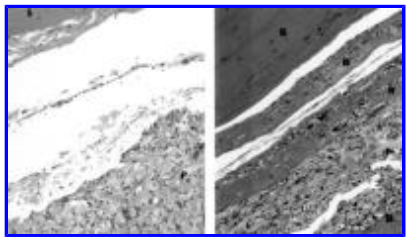

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Fig 4. In vivo osteogenesis. (*A*) Grafts seeded with control cells demonstrate a lack of new bone formation. (*B*) Grafts seeded with differentiated osteoblasts demonstrate growth of new bone (B) surrounding and growing with the polyglycolic acid matrix (P). The new bone appears morphologically similar to the native skull (S). H&E, original magnification ×20 before reduction.

Discussion

Adipose tissue develops from the embryonic mesoderm and has been shown to have a population of pluripotent stem cells. 11,12,15 These fat-derived stem cells exhibit the same characteristics as other mesenchymal stem cells, including a fibroblastlike morphology and the ability to differentiate into multiple mesenchymal cell lines including bone and fat. In this study we have demonstrated that we can isolate these fat-derived stem cells, differentiate them into adipocytes and osteoblasts, and then grow them in vivo. In vitro differentiation was accomplished successfully by exposing the fat-derived stem cells to lineage-specific growth factors.

Adipogenesis was accomplished by adding isobutyl-methylxanthine, insulin, and dexamethasone to the adipogenic media. Insulin has been shown to promote differentiation of stem cells into adipocytes using serum or physiological concentrations of insulin. 13,18,20 It has been shown that glucocorticoids at physiological concentrations, in the presence of insulin, promote differentiation of mesenchymal stem cells toward the adipogenic lineage. 13,18,20 Successful differentiation was confirmed by positive Oil red O staining, which showed the presence of intracellular lipid vacuoles. The fat-derived stem cells exposed to the control media did not demonstrate the presence of lipid vacuoles. These data support the conclusion that factors in the adipogenic media induced the differentiation of fat-derived stem cells into adipocytes.

Osteogenesis was accomplished by adding ascorbic acid, [beta]-glycerophosphate, and dexamethasone to the osteogenic media. Dexamethasone has been shown to be an absolute requirement for in vitro osteogenesis of stem cells and bone nodule formation. 6,10,19–21 Its exact mechanism of action in osteoblast differentiation is unknown, however. Ascorbic acid functions as a cofactor in the hydroxylation of proline and lysine residues in collagen 22 and, in addition, increases the synthesis of noncollagenous bone matrix proteins. 23,24 [beta]-Glycerophosphate has been shown to be important in the calcification of the extracellular matrix. 24 Successful in vitro differentiation of stem cells to

osteoblasts was demonstrated by staining with Alizarin red and immunohistochemistry for osteocalcin. The fat-derived stem cells treated with osteogenic media developed a calcified extracellular matrix that was evident with Alizarin red staining. This calcified bone matrix did not develop in the nondifferentiated cells. The differentiated osteoblasts demonstrated the presence of osteocalcin via immunohistochemical staining. Osteocalcin, also known as BGP (or bone *gla* protein), is produced exclusively in bone and is a biochemical indicator of bone turnover. Positive staining for osteocalcin in the differentiated osteoblasts indicates the presence of metabolically active bone cells. There was no evidence of osteocalcin in the undifferentiated cells. These data support the conclusion that factors in the osteogenic media induced the differentiation of fat-derived stem cells into osteoblasts.

Having corroborated that in vitro differentiation of fat-derived stem cells into osteoblasts and adipocytes is possible, we developed a novel model to demonstrate that in vivo growth of the differentiated cells was possible. We seeded the fat-derived stem cells, differentiated osteoblasts, and differentiated adipocytes onto three-dimensional scaffolds made of PGA fibers and implanted them into a subcutaneous pocket above the skull in Lewis rats. The Lewis rat is extensively inbred and therefore genetically identical, making the model one of autologous transplantation. After periods of 4 and 8 weeks, the animals were killed and the grafts were collected.

The grafts containing the differentiated adipocytes showed the presence of intracellular lipid vacuoles on staining with Oil red O. The grafts seeded with fat-derived stem cells did not demonstrate intracellular lipid vacuoles on Oil red O staining. This result proved that fat-derived stem cells differentiated into adipocytes in vitro could be grown successfully in vivo. Furthermore, these differentiated cells remained adipocytes even when the high levels of isobutyl–methylxanthine, dexamethasone, and insulin found in the adipogenic media were no longer present.

The grafts containing the osteogenically differentiated fat-derived stem cells demonstrated the characteristic findings of bone formation on H&E staining and the presence of osteocalcin on immunohistochemistry. The grafts seeded with undifferentiated control fat-derived stem cells did not demonstrate any evidence of bone formation on Alizarin red staining or immunohistochemistry for osteocalcin. These results proved that fat-derived stem cells differentiated into osteoblasts in vitro could be grown successfully in vivo and that they remained committed to the osteoblast line even in the absence of high levels of growth factors. Furthermore, the H&E staining of the osteoblasts grown for 8 weeks showed that the engineered cells formed an ultrastructure nearly identical to native bone. This study represents the first demonstration of in vivo bone formation from fat-derived stem cells.

The results from this study prove that it is possible to differentiate fat-derived stem cells into osteoblasts and adipocytes in vitro and then grow them in vivo. The success of this in vivo model shows that the three-dimensional grafts may create solutions to multiple reconstructive problems, including craniofacial and orthopedic bone reconstruction, breast reconstruction, and soft- and hard-tissue augmentation. It has been demonstrated previously that such stem cells are isolated easily from human liposuction aspirates, making fat-derived stem cell harvest safer and more efficient than bone marrow harvest. [11] The potential uses of this technique are seemingly limitless.

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