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(19) **United States**(12) **Patent Application Publication**
Slavin et al.(10) **Pub. No.: US 2004/0170610 A1**(43) **Pub. Date: Sep. 2, 2004**(54) **COMPOSITIONS COMPRISING BONE
MARROW CELLS TOGETHER WITH
DEMINERALIZED AND/OR MINERALIZED
BONE MATRIX AND USES THEREOF IN
THE INDUCTION OF BONE AND
CARTILAGE FORMATION**(76) Inventors: **Shimon Slavin**, Jerusalem (IL); **Olga
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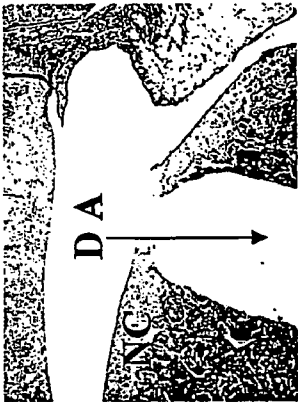
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1185 Avenue of the Americas
New York, NY 10036 (US)(21) Appl. No.: **10/471,031**(22) PCT Filed: **Mar. 5, 2002**(86) PCT No.: **PCT/IL02/00172**(30) **Foreign Application Priority Data**

Mar. 5, 2001 (IL) 141813

Publication Classification(51) **Int. Cl.⁷ A61K 45/00; A61K 38/18**(52) **U.S. Cl. 424/93.7; 424/423; 514/12**(57) **ABSTRACT**

A composition comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM) and optionally comprising bone morphogenetic protein/s (BMP) and/or other active agents, particularly for use in the transplantation of mesenchymal progenitor cells into a joint and/or a cranio-facial-maxillary bone, for restoring and/or enhancing the formation of a new hyaline cartilage and subchondral bone structure. The composition of the invention and method of treatment employing the same may be used for the treatment of hereditary or acquired bone disorders, hereditary or acquired cartilage disorders, malignant bone or cartilage disorders, metabolic bone diseases, bone infections, conditions involving bone or cartilage deformities and Paget's disease. The composition and method may further be used for the correction of complex fractures, bone replacement and formation of new bone in plastic or sexual surgery, for support of implants of joints, cranio-facial-maxillary bones, or other musculoskeletal implants, including artificial implants. The method of the invention may further be used for treating damaged joints or degenerative arthropathy associated with malformation and/or dysfunction of cartilage and/or subchondral bone. A kit is provided for performing transplantation into a joint or a cranio-facial-maxillary bone of a mammal of the composition of the invention.



Def. D. 0

Fig. 1D



Norm. Cart.

Fig. 1C



Osteoch. Comp.

Fig. 1B



Typ. Kn. J.

Fig. 1A



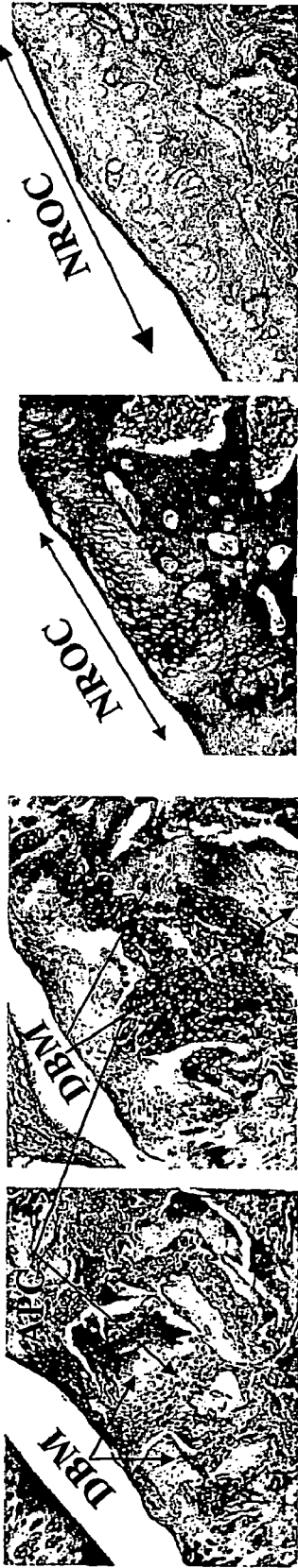
Fig. 1E

Fig. 1F

Fig. 1G

Fig. 1H

Def. + DBM + BMC



24 We.

8 We.

4 We.

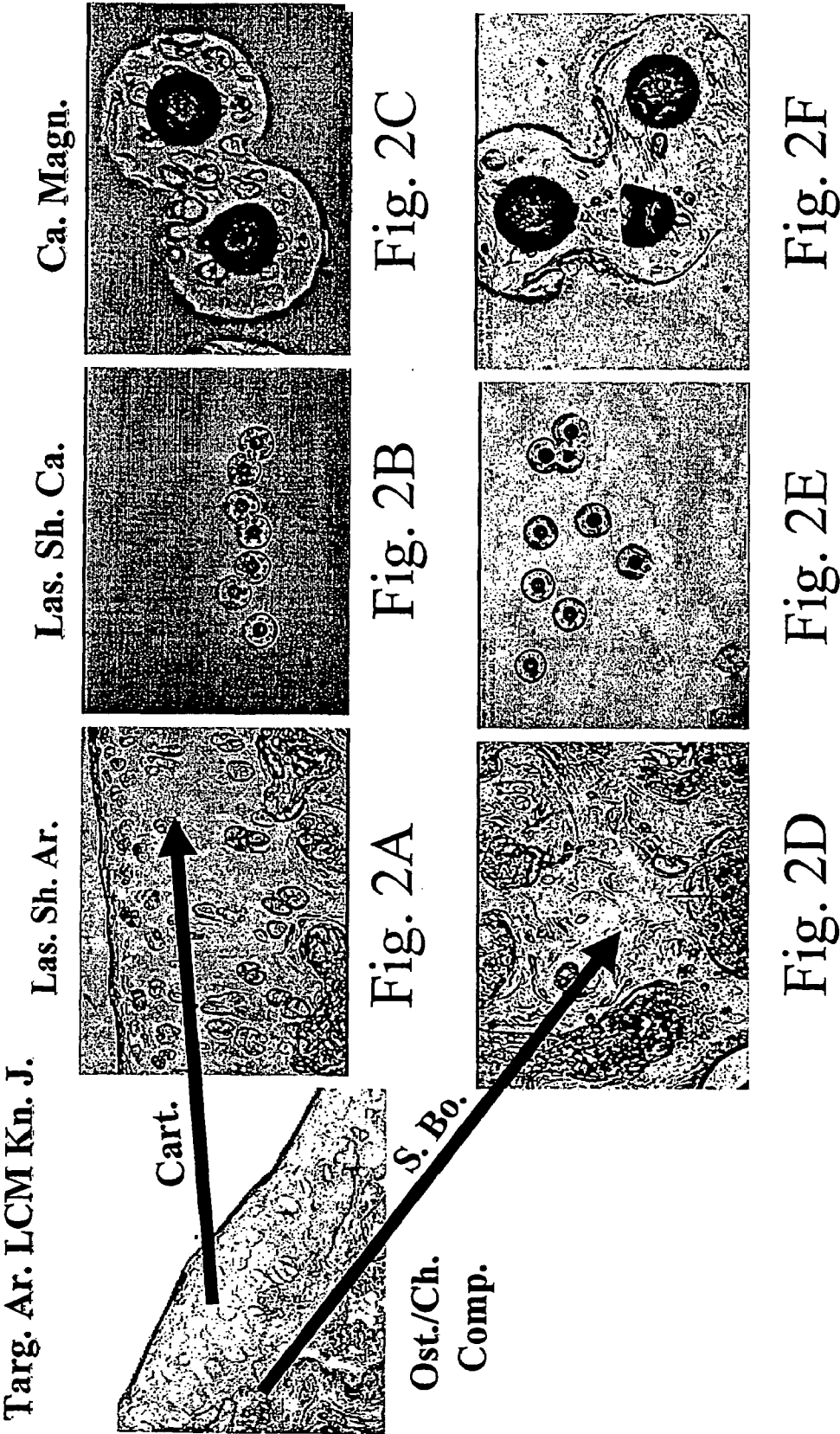
2 We.

Fig. 1L

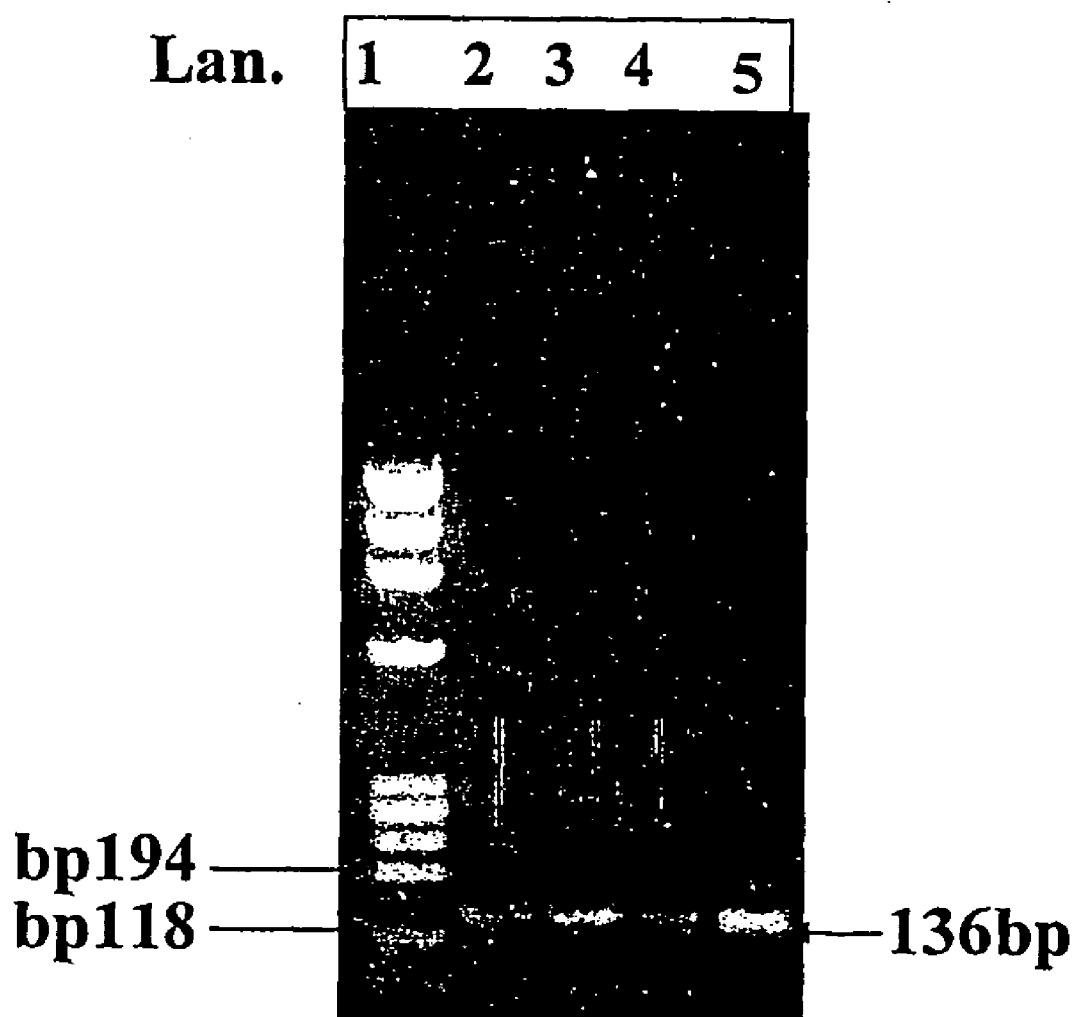
Fig. 1K

Fig. 1J

Fig. 1I



Det. Don.
Der. Cel. PCR Anal.



Norm. Ra. Cran.

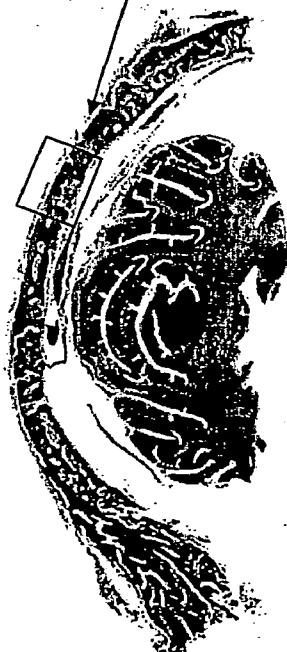


Fig. 3A

Def.

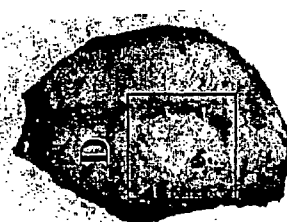


Fig. 3B

Def. Ar. (DA)

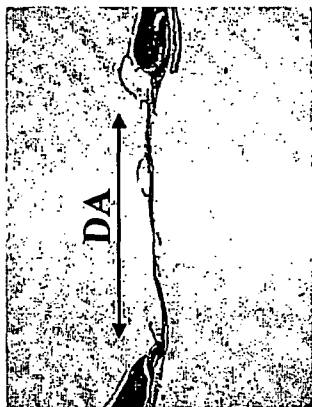


Fig. 3C

D.-8-Po. Transpl.

Def. Al.



Fig. 3D

Def. + DBM



Fig. 3E

Def. + DBM + BMC



Fig. 3F

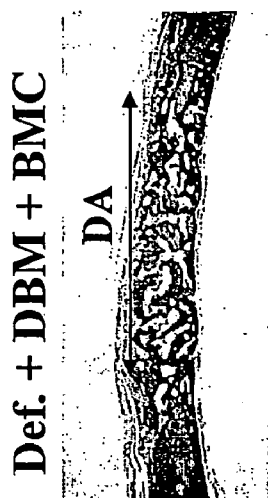


Fig. 4C

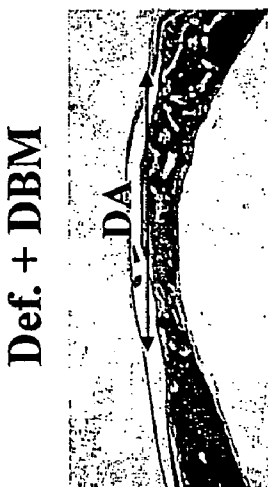


Fig. 4B

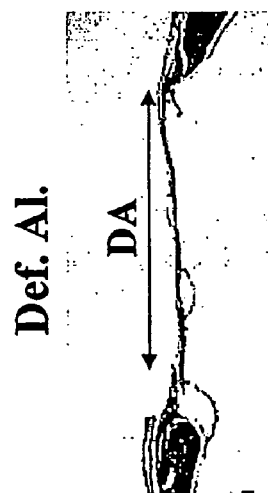


Fig. 4A

D.15 {



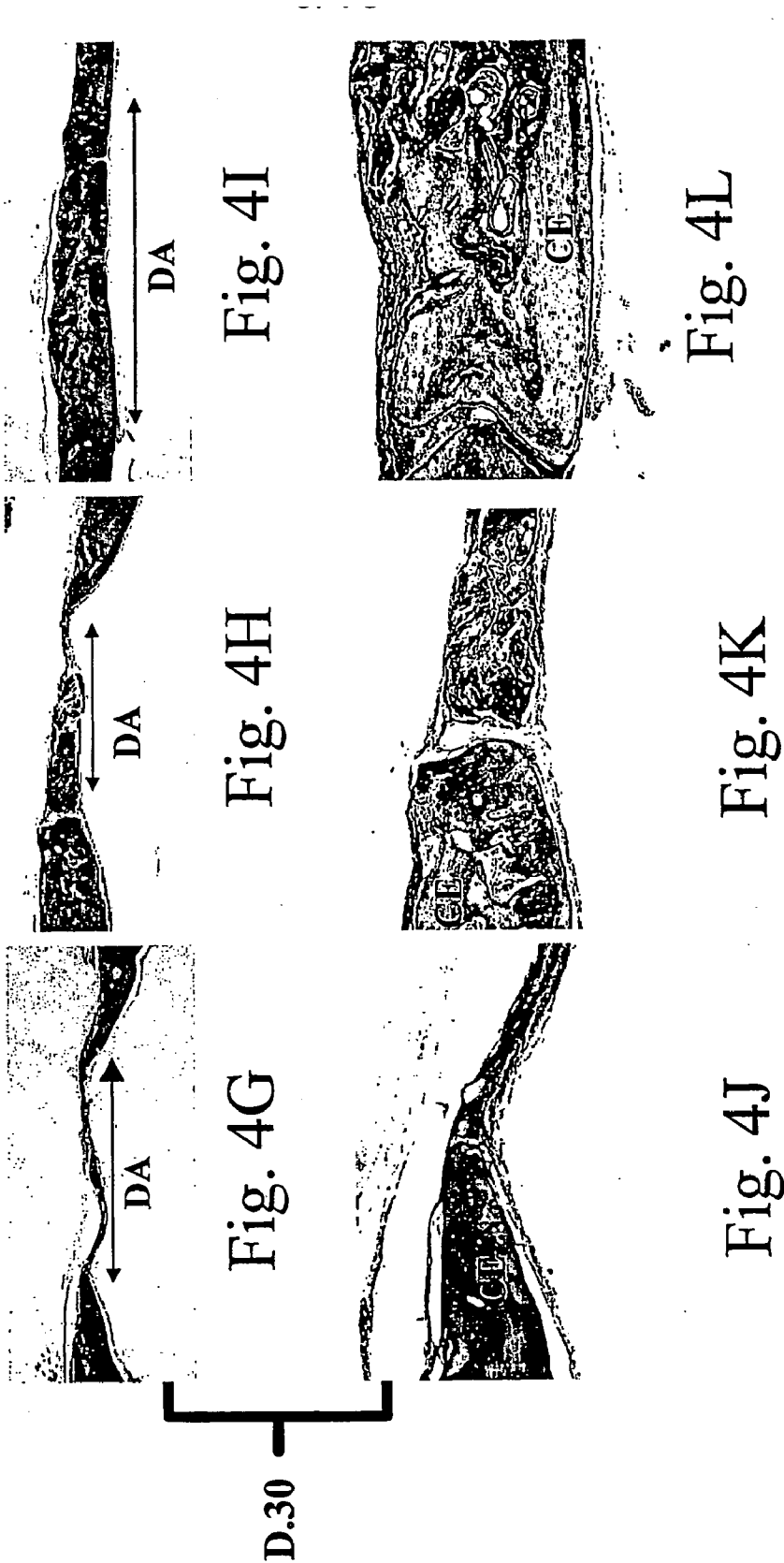
Fig. 4F



Fig. 4E



Fig. 4D



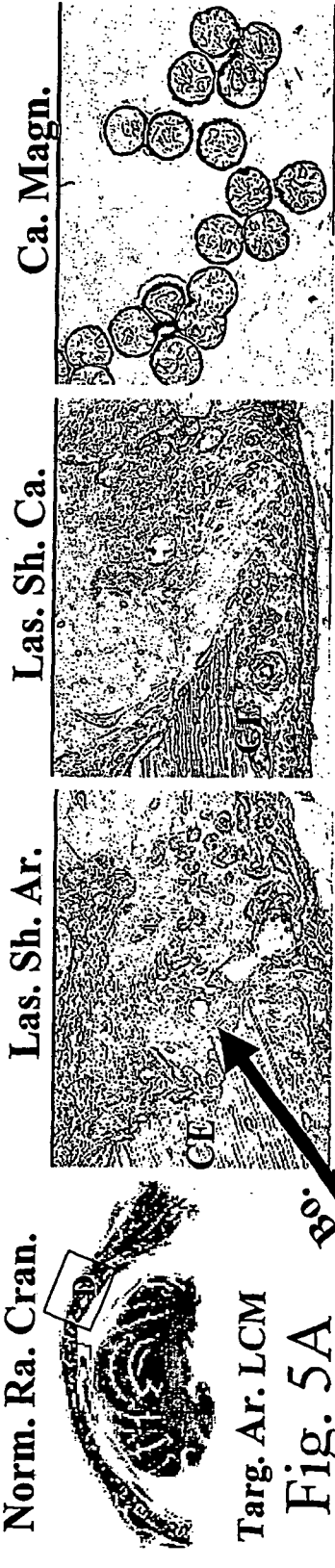


Fig. 5A

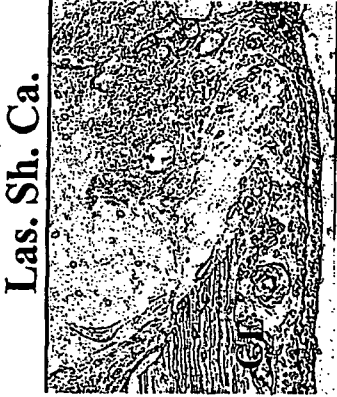


Fig. 5D

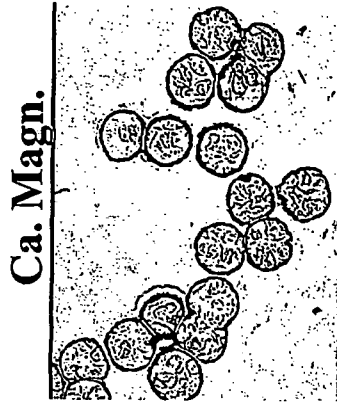


Fig. 5E



Fig. 5B

Det. Don. Der. Cel. PCR Anal.

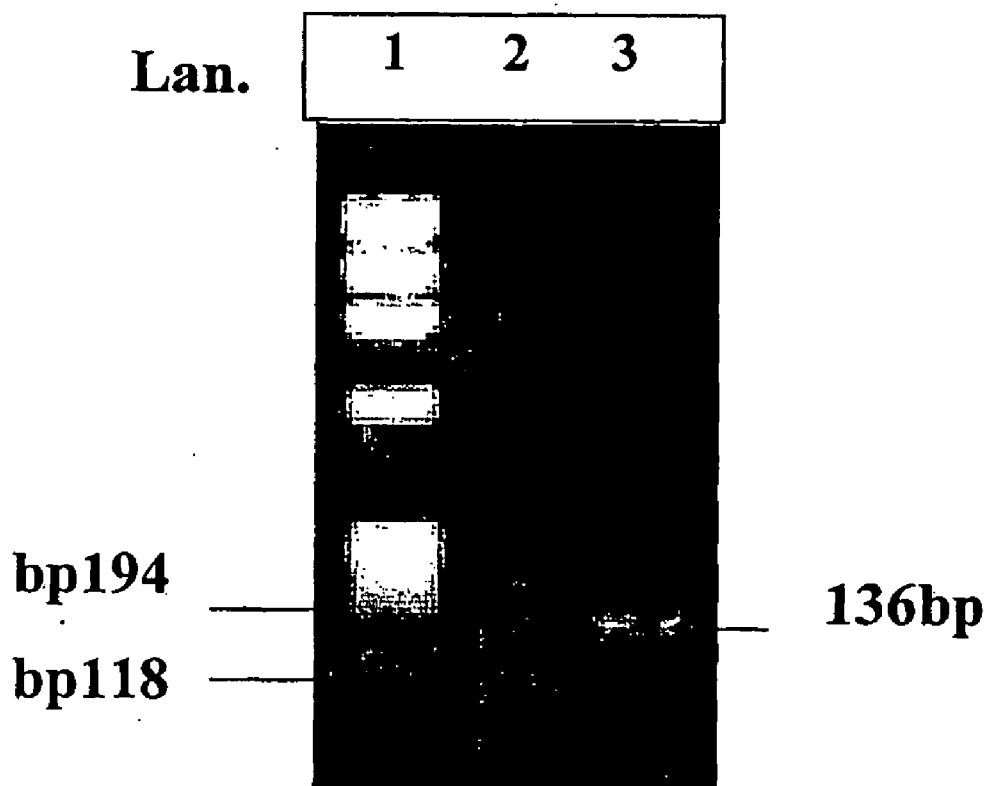


Fig. 5F

COMPOSITIONS COMPRISING BONE MARROW CELLS TOGETHER WITH DEMINERALIZED AND/OR MINERALIZED BONE MATRIX AND USES THEREOF IN THE INDUCTION OF BONE AND CARTILAGE FORMATION

FIELD OF THE INVENTION

[0001] The present invention relates to compositions comprising bone marrow cells (BMC) and demineralized and/or mineralized bone matrix (DBM and MBM, respectively) and to their novel uses in induction of new bone and cartilage formation in mammals.

BACKGROUND OF THE INVENTION

[0002] New bone formation, such as in the case of damage repair or substitution of a removed part of the bone in postnatal mammals, can only occur in the presence of the following three essential components, (i) mesenchymal progenitor cells; (ii) a conductive scaffold for these cells to infiltrate and populate; and (iii) Bone Morphogenetic Proteins. Unfortunately, local conditions usually do not satisfy the requirements of osteogenesis, and thus substitution of removed, damaged or destroyed bones does not occur spontaneously.

[0003] Previous research has already uncovered somewhat about these three components.

[0004] It was shown that multipotent mesenchymal stem cells, which are capable of extensive proliferation and differentiation into cartilage, bone, tendon, muscle, fat and etc. are present in the bone marrow [Caplan, A. I. (1991) *J Orthop Res* 9:641-650; Prockop J. D. (1997) *Science* 276:71-74; Pittenger, M. F. et al. (1999) *Science* 284:143-147; Wakitani, S. W. et al. (1995) *Muscle & Nerve* 18:1417-1426].

[0005] DBM (and/or MBM) has been shown to play the role of supportive material or structure that is essential for promoting engraftment of mesenchymal progenitor cells and their proliferation and differentiation in the course of bone and cartilage development, whenever mesenchymal cells are introduced as a cell suspension (Inventor's unpublished results). It serves as a conductive scaffold for cartilage and bone regeneration, while providing a natural source for inducing both chondro- and osteogenesis, thus combining all the essential inductive and conductive features. DBM also has additional advantageous, that can be summarized as follows: (i) it is mechanically flexible and slowly biodegradable, with the degradation time compatible with the period of de novo chondro- and osteogenesis; (ii) it is strong enough to provide at least partially biomechanical properties of the flat bone and joint surface during the period of new bone and cartilage formation; (iii) it can be provided as an amorphous powder that can be inserted locally, without major surgical intervention, while avoiding iatrogenic damage; (iv) it is a low immunogenic material even when used as a xenograft, and when used in an allogeneic combination, it is practically non-immunogenic [Block, J. E. and Poser, J. (1995) *Med Hypotheses* 45(1):27-32; Torricelli, P. et al. (1999) *Int Orthop* 23(3):178-81; Hallfeldt, K. K. et al. (1995) *J Surg Res* 69(5):614-20].

[0006] BMPs are growth factors that play an important role in the formation of bone and cartilage [Ducy, P. and

Karsenty, G. (2000) *Kidney Int* 57(6):2207-14; Schmitt, J. M. et al. (1999) *J Orthop Res* 17(2):269-78]. Most importantly, DBM is a natural source of BMPs. Moreover, induction of cartilage and bone may be enhanced by additional exogenous supply of BMPs that are not even species-specific [Sampath, T. K. and Reddi, A. H. (1983) *Proc Natl Acad Sci USA* 80(21): 6591-5; Bessho, K. et al. (1992) *J Oral Maxillofac Surg* 50(5):496-501], together with DBM [Niederwanger, M. and Urist, M. R. (1996) *J Oral Implantol* 22(3-4):210-5].

[0007] Arthropathies are a group of chronic progressive joint diseases that can result from degenerative changes in the cartilage and hypertrophy of bone at the articular margins. Arthropathies can be secondary to trauma, inflammatory (autoimmune or infectious), metabolic or neurogenic diseases. Hereditary and mechanical factors may be an additional factor involved in the pathogenesis of arthropathies.

[0008] Restoration of a healthy joint surface in a damaged or degenerative arthropathy requires addressing the treatment both towards the cartilage and the subchondral bone.

[0009] Various attempts have been made to replace damaged cartilage, including:

[0010] 1. Stimulation of bone marrow from subchondral bone to form a fibrotic repair tissue;

[0011] 2. Osteochondral transplantation (allogeneic and autologous);

[0012] 3. Transplantation of autologous cultured chondrocyte or mesenchymal cells;

[0013] 4. Combined transplantation of chondrocytes with different kinds of matrices; and

[0014] 5. Artificial implantation of mechanical joints.

[0015] Each of these methods has limitations and disadvantages and most of them are expensive, cumbersome, ineffective and rather impractical. Autologous osteochondral graft is restricted to a small area of damaged cartilage, up to 2 cm², and could cause discomfort, infection and morbidity in the donor site. Allogeneic osteochondral graft is immunogenic, hence requires life-long use of undesired, hazardous immunosuppressive agents, which would be an impractical approach for routine orthopedic practice. Transplantation of cultured chondrocytes is cumbersome and very expensive, involving a two-stage procedure. The hyaline-like tissue which is produced after transplantation has sub-optimal biomechanical properties [Gilbert, J. E. (1998) *Am J Knee Surg* 11(1):42-6; Temeno, J. S. and Mikos, A. G. (2000) *Biomaterials: Tissue Engineering for Regeneration of Articular Cartilage*, 21:431-440; Buckwalter, J. A. and Mankin, H. J. (1998) *Instr Course Lect* 47:487-504; Stocum, D. L. (1998) *Wound Repair Regen.* 6(4):276-90]. Hence, adequate restoration of cartilage remains an unsolved problem.

[0016] Currently, autologous grafts are the most commonly used bone and cartilage graft material. However, the use of autografts has limitations, such as donor site discomfort, infection and morbidity and limited sizes and shapes of available grafts. Even if enough tissue is transplanted there is an acute limitation in the number of mesenchymal stem

cells with high proliferative potential present in the differentiated bone tissue implanted.

[0017] In theory, the most promising approach should involve the combined transplantation of cells capable of hyaline cartilage formation and a matrix, providing means for induction/conduction and support of cartilage development and maintenance.

[0018] It is widely accepted that, for successful application of combined cell-matrix graft, the basic requirements are the following:

- [0019] 1. Rich source of progenitor cells capable of differentiation into chondrocytes, for continuous repair of "wear and tear" of weight bearing joints.
- [0020] 2. Conductive scaffold for cell attachment should be maintained, leading to development of hyaline cartilage.
- [0021] 3. Conductive scaffold should be non-immunogenic, non-toxic and susceptible to biodegradation simultaneously with the development of new cartilage.
- [0022] 4. Conditions for stimulating development of chondrocytes from mesenchymal precursor cells.

[0023] So far, most of the matrices that were tried in combined cell-matrix grafts were either immunogenic or non-biodegradable, and the remaining others did not possess conductive or inductive properties needed to support formation of biomechanical strong cartilage. Cells used in combined cell-matrix grafts were in most of the cases chondrocytes, which were already fully differentiated cells, with relatively low metabolic activity and limited self-renewal capacity. Whereas the proliferative capacity of such cells may be sufficient to maintain healthy cartilage, it is certainly insufficient for the development of large areas of hyaline cartilage. In addition to being immunogenic, mesenchymal progenitor cell allografts were not combined with optimal supportive matrix. Thus, unfortunately, none of the available options fulfill all basic requirements, and all options are far from being satisfactory for reliable routine clinical application.

[0024] The composition of the invention comprising BMC and DBM and/or MBM overcomes the above shortcomings and provides, upon administration into a damaged joint, replacement and/or restoration of hyaline cartilage together with subchondral bone, in a one-step transplantation procedure, without any preliminary cultivation of mesenchymal progenitor cells.

[0025] Thus, it is the major object of the present invention to provide a mixture of bone marrow cells and demineralized or mineralized bone matrix, for use as a graft in patients in need of restoration of damaged joints and cranio-facial-maxillary bones. This and other objects of the invention will be elaborated on as the description proceeds.

SUMMARY OF THE INVENTION

[0026] The present invention relates to compositions comprising a mixture of bone marrow cells (BMC) and demineralized and/or mineralized bone matrix (DBM and MBM, respectively) and to their novel uses in the transplantation of mesenchymal progenitor cells into joints and cranio-facial-maxillary bones.

[0027] Thus, in a first aspect, the present invention relates to a composition comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM).

[0028] In a second aspect, said composition comprising BMC and DBM and/or MBM is for use in transplantation of mesenchymal progenitor cells present in the bone marrow into a joint and/or a cranio-facial-maxillary bone of a subject in need, wherein said subject is a mammal, preferably a human.

[0029] In a first embodiment, the DBM and MBM comprised within the composition of the invention are of vertebrate origin, and they may be of human origin.

[0030] In a second embodiment, the DBM or MBM comprised within the composition of the invention are in powder or slice form. The particle size of the DBM may be about 50 to 2500 μ . Preferably, said particle size is about 250 to 500 μ . The most preferable particle size will depend on the specific needs of each case.

[0031] In another embodiment, the composition of the invention is for restoring and/or enhancing the formation of a new hyaline cartilage and subchondral bone structure.

[0032] In a further embodiment, the composition of the invention is intended for the treatment of a patient suffering from any one of a hereditary or acquired bone disorder, a hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, conditions involving bone or cartilage deformities and Paget's disease. Additionally, the invention is also intended for the treatment of a patient in need of any one of correction of complex fractures, bone replacement and formation of new bone in plastic or sexual surgery.

[0033] In a yet further embodiment, the composition of the invention may further optionally comprise a pharmaceutically acceptable carrier or diluent, as well as additional active agents.

[0034] In another aspect, the present invention relates to a method for transplantation of a mixture comprising BMC with DBM and/or MBM and optionally further comprising pharmaceutically acceptable carrier or diluent, into a joint and/or a cranio-facial-maxillary bone of a subject in need, wherein said method comprises introducing into said joint or bone the composition of the invention.

[0035] In a first embodiment of the method of the invention, the mixture is administered by any one of the following procedures injection, minimally invasive arthroscopic procedure, or by surgical arthroplasty into the site of implantation, wherein said method is for support or correction of congenital or acquired abnormalities of the joints, cranio-facial-maxillary bones, orthodontic procedures, bone or articular bone replacement following surgery, trauma or other congenital or acquired abnormalities, and for supporting other musculoskeletal implants, particularly artificial and synthetic implants.

[0036] Thus, in a further aspect, the invention relates to a method of treating a damaged or degenerative arthropathy associated with malformation and/or dysfunction of cartilage and/or subchondral bone in a mammal in need of such treatment, comprising administering into an affected joint or bone of said mammal a mixture comprising BMC with DBM and/or MBM, said mixture optionally further com-

prising a pharmaceutically acceptable carrier or diluent and/or additional active agents.

[0037] In one embodiment, the BMC which are present in the administered mixture are either allogeneic or said mammal's own.

[0038] In another embodiment, the DBM or MBM which is present in the administered mixture is in a slice, powder, gel, semi-solid or solid form embedded in or encapsulated in polymeric or biodegradable materials.

[0039] In a yet further aspect, the present invention relates to a non-invasive (through injection), minimally invasive (through arthroscopy) or surgical transplantation method for support of implants of joints or other musculoskeletal implants, comprising introducing a graft into a joint or a cranio-facial-maxillary bone of a subject in need, wherein said graft comprises a mixture of BMC and DBM or MBM.

[0040] In an even further aspect, the present invention relates to the use of a composition comprising BMC and DBM and/or MBM as a graft of mesenchymal and/or mesenchymal progenitor cells for transplantation/implantation into a mammal, wherein said mammal is preferably a human. The transplantation is to be performed into a joint or into a cranio-facial-maxillary bone, for the development of new bone and/or cartilage.

[0041] Furthermore, the composition used in said transplantation is intended for the treatment of a patient suffering from any one of a hereditary or acquired bone disorder, a hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, conditions involving bone or cartilage deformities and Paget's disease. In addition, said composition is intended for the treatment of a patient in need of any one of correction of complex fractures, bone replacement and formation of new bone in plastic or sexual surgery.

[0042] In one embodiment, the composition used in the invention further comprises an active agent.

[0043] In another embodiment, the DBM and MBM comprised within the composition used in the invention are of vertebrate origin, and they may be of human origin. Moreover, said DBM and MBM may be in powder, strips, thin layers, or slice form.

[0044] In an additional aspect, the present invention concerns the use of a mixture of BMC with DBM and/or MBM in the preparation of a graft for the treatment of a bone or cartilage disorder.

[0045] Lastly, the present invention provides a kit for performing transplantation into a joint or a cranio-facial-maxillary bone of a mammal of BMC in admixture with DBM and/or MBM, wherein said kit comprises:

- [0046] (a) DBM and/or MBM in a compacted form;
- [0047] (b) a BM aspiration needle;
- [0048] (c) an intra-osseous bone drilling burr;
- [0049] (d) a needle with a thick lumen for infusion of viscous bone marrow-DBM mixture;
- [0050] (e) a 2-way lumen connector for simultaneous mixing of BMC with DBM and diluent;
- [0051] (f) a medium for maintaining BMC; and optionally

[0052] (g) cryogenic means for handling and maintaining BMC or BMC together with DBM.

[0053] The kit of the invention may optionally further comprise a carrier and/or a diluent for the BMC and DBM and/or MDM mixture.

BRIEF DESCRIPTION OF THE FIGURES

[0054] FIGS. 1A-L: Photomacrographs and micrographs of sagittal knee joint sections 2 to 24 weeks after the experimentally created microfracture drilling defect (Picro-indigocarmin, PIC, staining).

[0055] FIG. 1A: Photomacrograph of a normal rat knee joint section.

[0056] FIG. 1B: Photomicrograph of the entire normal osteo-chondral complex in the interchondylar region of the femur.

[0057] FIG. 1C: Photomicrograph of the articular cartilage in the normal osteo-chondral complex shown in FIG. 1B.

[0058] FIG. 1D: Microfracture drilling (full thickness defect), immediately after damage.

[0059] FIG. 1E: Micro-fracture left without the implant, two weeks after damage. The drilled hole, filled with connective tissue, can be seen.

[0060] FIG. 1F: Micro-fracture left without the implant, 24 weeks after damage. Regenerated subchondral bone and damaged joint surface constituted of fibro-cartilaginous tissue can be seen.

[0061] FIG. 1G: DBM particles alone were transplanted into defect area, two weeks after transplantation. DBM particles are clearly seen in the site of transplantation surrounded mostly with connective tissue.

[0062] FIG. 1H: DBM particles alone were transplanted into defect area, 24 weeks after transplantation. Regenerated sub-chondral bone and damaged joint surface covered with connective tissue together with fibro-cartilage could be observed.

[0063] FIG. 1I: DBM particles together with BMC were transplanted into defect area, 2 weeks after transplantation. Extensively developing hyaline cartilage surrounding the implanted DBM particles could be seen.

[0064] FIG. 1J: DBM particles together with BMC were transplanted into defect area, 4 weeks after transplantation. Extensively developing hyaline cartilage, as well as considerably degraded DBM particles can be seen.

[0065] FIG. 1K: DBM particles together with BMC were transplanted into defect area, 8 weeks after transplantation. Almost complete regeneration of subchondral bone; surface of the damaged area is built of a continuous layer of extensively developing young hyaline cartilage.

[0066] FIG. 1L: DBM particles together with BMC were transplanted into defect area, 24 weeks after transplantation. The histological structure of the regenerated osteo-chondral complex is indistinguishable from normal. Abbreviations: Typ. Kn. J., typical knee joint; Osteoch. Comp., osteochondral complex; Norm. Cart., normal cartilage; Def., defect;

D., day(s); Al., alone; We., week(s); NROC, newly reconstituted osteochondral complex.

[0067] FIGS. 2A-G: Laser Capture Microdissection and PCR analysis of cells captured from the newly reconstituted osteochondral complex of the knee joint (6 months after transplantation of DBM with donor male BMC into female recipient).

[0068] FIG. 2A: Laser shot general area, new cartilage formation.

[0069] FIG. 2B: Laser shot cap, new cartilage formation.

[0070] FIG. 2C: Magnification ($\times 20$) of laser shot cap, new cartilage formation.

[0071] FIG. 2D: Laser shot general area, new subchondral bone formation.

[0072] FIG. 2E: Laser shot cap, new subchondral bone formation.

[0073] FIG. 2F: Magnification ($\times 20$) of laser shot cap, new subchondral bone formation.

[0074] FIG. 2G: Detection of donor-derived cells by PCR analysis. Lanes: 1, DNA size markers (ϕ X714 cut with HaeIII), arrows point to the 194 bp-long and 118 bp-long bands, respectively; 2, Amplification of male rat DNA derived from cartilage area of female rat knee joint; 3, Amplification of male rat DNA derived from subchondral bone area of female rat knee joint; 4, Male rat DNA derived from hematopoietic marrow area of female rat knee joint; 5, Internal positive control DNA from male blood. The PCR results confirm the expression of donor derived cells in all the three tissues composing the newly reconstituted osteochondral complex. Abbreviations: Targ. Ar. LCM Kn. J., target area for LCM in the knee joint; Las. Sh. Ar., laser shot area; Las. Sh. Ca., laser shot cap; Ca. Marn., caps magnified; Cart., cartilage; S. Bo., subchondral bone; Ost. Ch. Comp., osteo-chondral complex; Det. Don. Der. Cel. PCR Anal., detection of donor-derived cells by PCR analysis; Lan., lanes.

[0075] FIGS. 3A-F: Correction of the calvarial defect by transplantation of demineralized bone matrix (DBM) and bone marrow cells (BMC) in rats, shown by sagittal sections stained with Picroindigocarmin (PIC).

[0076] FIG. 3A: Photomacrographs of a normal rat cranium. Region marked by a square (D) is shown in FIG. 3B in higher magnification.

[0077] FIG. 3B: Site of the artificial defect (D) in the parietal region of the cranium.

[0078] FIG. 3C: Photomacrograph of the defect area (DA) between the two cut edges.

[0079] FIG. 3D: Photomicrograph of cranial section 8 days after the experimentally created calvarial defect (PIC staining). Defect left untreated. Cut edge (CE) and the fibrous connective tissue can be seen.

[0080] FIG. 3E: Photomicrograph of cranial section 8 days after the experimentally created calvarial defect (PIC staining). DBM particles alone were transplanted into defect area. Actively proliferating fibroblastic cells surrounding the cut edge and DBM particles could be seen.

[0081] FIG. 3F: Photomicrograph of cranial section 8 days after the experimentally created calvarial defect (PIC staining). DBM particles together with BMC were transplanted into defect area. Active remodeling of the transplanted DBM particles, areas of new bone formation are clearly visible. Abbreviations: Norm. Ra. Cran., normal rat cranium; Def., defect; Def. Ar., defect area; Def. Al., defect alone; D., day(s); Po. Transpl., post-transplantation.

[0082] FIGS. 4A-L: Photomacro- and micrographs of cranial sections 15 and 30 days after the experimentally created calvarial defect (Sagittal sections, Picroindigocarmin, PIC, staining).

[0083] FIG. 4A: 15 days post-operation, control (no transplant).

[0084] FIG. 4B: 15 days post-transplantation, transplantation of DBM alone.

[0085] FIG. 4C: 15 days post-transplantation, transplantation of DBM and BMC.

[0086] FIG. 4D: 15 days post-operation, control (no transplant), $10\times$ magnification. There is no new bone formation in the area of defect.

[0087] FIG. 4E: 15 days post-transplantation of DBM alone, $10\times$ magnification. Remodeling of DBM particles results in bridging the area of defect with the newly formed bone tissue.

[0088] FIG. 4F: 15 days post-transplantation of DBM and BMC, $10\times$ magnification. The cut edge of the parietal bone could hardly be distinguished in the continuous uniform layer of actively remodeling bony tissue.

[0089] FIG. 4G: 30 days post-operation, control (no transplant).

[0090] FIG. 4H: 30 days post-transplantation of DBM alone.

[0091] FIG. 4I: 30 days post-transplantation of DBM and BMC.

[0092] FIG. 4J: 30 days post-operation, control (no transplant), $10\times$ magnification. There is no new bone formation in the area of defect.

[0093] FIG. 4K: 30 days post-transplantation of DBM alone, $10\times$ magnification. Remodeling of DBM particles results in bridging the area of defect with the newly formed bone tissue.

[0094] FIG. 4L: 30 days post-transplantation of DBM and BMC, $10\times$ magnification. The cut edge of the parietal bone could hardly be distinguished in the continuous uniform layer of actively remodeling bony tissue. Abbreviations: Def. Al., defect alone; Def., defect; D., day(s); DA, area of defect; CE, cut edge.

[0095] FIGS. 5A-F: Laser Capture Microdissection (LCM) and PCR analysis of cells captured from the newly developing bony tissue in the area of the experimentally created calvarial defect after transplantation of DBM together with donor male BMC to female recipient.

[0096] FIG. 5A: General view of the normal rat cranium with the place where the defect was inflicted highlighted (D).

[0097] **FIG. 5B:** Regenerating bony tissue, area target for LCM.

[0098] **FIG. 5C:** Higher magnification, area target for LCM.

[0099] **FIG. 5D:** Laser shot caps; cells captured from this area were used for PCR analysis.

[0100] **FIG. 5E:** Laser shot caps, 10× magnification, cells captured for PCR analysis.

[0101] **FIG. 5F:** Detection of donor-derived cells by PCR analysis. Lanes: 1, DNA size markers (ϕ X714 cut with HaeIII), arrows point to the 194 bp-long and 118 bp-long bands, respectively; 2, male rat DNA derived from bone area of female knee cranium; 3, internal positive control, DNA from male blood. The results of the PCR analysis confirm the expression of donor derived cells in the newly forming bony tissue. Abbreviations: Norm. Ra. Cran., normal rat cranium; Bo., bone; Las. Sh. Ar., laser shot area; Las. Sh. Ca., laser shot caps; Ca. Magn., caps magnified; Targ. Ar. LCM, target area for LCM; CE, cut edge; D, area of defect; Det. Don. Der. Cel. PCR Anal., detection of donor-derived cells by PCR analysis; bp, base-pair(s).

DETAILED DESCRIPTION OF THE INVENTION

[0102] The following abbreviations are utilized throughout this specification:

[0103] BM: bone marrow

[0104] BMC: bone marrow cell(s)

[0105] BMP: bone morphogenetic protein

[0106] DBM: demineralized bone matrix

[0107] LCM: Laser Capture Microdissection

[0108] MBM: mineralized bone matrix

[0109] PCR: polymerase chain reaction

[0110] PIC: Picroindigocarmin, a dye used in histological staining.

[0111] In search for improving regeneration of damaged osteochondral complex in joint and cranio-facial-maxillary areas, the inventors have found that using a composition comprising BMC and DBM and/or MBM as a graft results in the development of bone and cartilage according to the local conditions of the site of transplantation. New tissue formation follows a differentiation pathway producing different types of bone and cartilage, depending on the local conditions. Thus, the newly formed tissue meets precisely the local demands.

[0112] The present invention relates to compositions comprising a mixture of bone marrow cells (BMC) and demineralized and/or mineralized bone matrix (DBM and MBM, respectively) and to their novel uses in the transplantation of mesenchymal progenitor cells into joints and cranio-facial-maxillary bones.

[0113] Thus, in a first aspect, the present invention relates to a composition comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM).

[0114] DBM is a preferable essential ingredient in the composition of the invention in view of its advantageous ability to combine all the features needed for making it an excellent carrier for mesenchymal progenitor cells. The properties of DBM can be summarized as follows:

[0115] 1. DBM can be a conductive scaffold essential for the engraftment, proliferation and differentiation of mesenchymal progenitor cells, in the course of bone and cartilage formation.

[0116] 2. DBM is the natural source of BMPs, which are active in stimulating osteo- and chondrogenesis, thus also fulfilling the inductive function.

[0117] 3. DBM is slowly biodegradable, the degradation time being compatible with the period of de novo chondro- and osteogenesis.

[0118] 4. DBM has very low immunogenicity when used as a xenograft, and it is practically non-immunogenic when used in allogeneic combinations.

[0119] 5. DBM is sufficiently flexible and strong to provide biomechanical properties to the joint surface during the period of new cartilage formation.

[0120] 6. DBM can be provided as an amorphous powder that can be injected locally, without major surgical intervention, thus avoiding iatrogenic damage to complex joints.

[0121] In a second aspect, said composition comprising BMC and DBM and/or MBM is for use in transplantation of mesenchymal cells and/or mesenchymal progenitor cells into a joint and/or a cranio-facial-maxillary area of a subject in need, wherein said subject is a mammal, preferably a human.

[0122] It is an object of the present invention to provide the said composition for transplantation of BMC into damaged joints for the replacement and/or restoration of hyaline cartilage as well as of subchondral bone, originating from the mesenchymal precursor cells existing in the transplanted BMC.

[0123] In a first embodiment, the DBM and MBM comprised within the composition of the invention are of vertebrate origin, and they may be of human origin.

[0124] In a second embodiment, the DBM and MBM comprised within the composition of the invention are in powder or slice form. The particle size of the DBM may be about 50 to 2500 μ . Preferably, said particle size is about 250 to 500 μ . The most preferable particle size will depend on the specific needs of each case.

[0125] In another embodiment, the composition of the invention is for restoring and/or enhancing the formation of a new hyaline cartilage and subchondral bone structure.

[0126] The idea underlying the present invention is that bone marrow cells (BMC) may provide a source for mesenchymal stem cells, which are capable of inducing osteo- and chondrogenesis. Thus, as described in the following examples, when a BMC suspension in admixture with DBM and/or MBM powder was administered directly into either a joint bearing a damage in the osteo-chondral complex, or in the cranium of an animal with a partial bone defect in the parietal bone, significant restoration occurred. Treated

recipients were mobile with no need for fixation of the joints, and full restoration of the anatomic structure of the treated joint was accomplished. Likewise, newly reconstituted parietal bone replacing surgically removed parietal bone in the skull showed normal remodeling. In the damaged joint, there was formation of subchondral bone structure and hyaline cartilage, and in the cranial defect, new flat bone was formed, both originating from the mesenchymal cells present in the transplanted BMC, as confirmed by the LCM-PCR analysis.

[0127] In a further embodiment, the composition of the invention is intended for the treatment of a patient suffering from any one of a hereditary or acquired bone disorder, a hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, metabolic bone diseases, bone infections, conditions involving bone or cartilage deformities and Paget's disease. Said disorders are listed in detail in Table 1. Additionally, the invention is also intended for the treatment of a patient in need of any one of correction of complex fractures, bone replacement, treatment of damaged or degenerative arthropathy and formation of new bone in plastic or sexual surgery.

TABLE 1

Congenital and Hereditary Bone Disorders	Bone Infections	Metabolic Bone Diseases	Non-neoplastic Disorders of the Bone
Achondroplasia	Hematogenous (Pyogenic) Osteomyelitis	Osteoporosis	Fibrous Dysplasia of the Bone
Osteogenesis Imperfecta (Brittle Bones, Fragilitas Ossium)	Osteomyelitis from a Contiguous Infection	Rickets and Osteomalacia	Fibrous Cortical Defect and Non-ossifying Fibroma
Osteopetrosis (Marble Bone Disease, Osteosclerosis)	Osteomyelitis from an Introduced Infection	Bone Changes in Hyperparathyroidism (Generalized Osteitis, Cystic Fibrosis, Von Recklinghausen's Bone Disease)	Solitary Bone Cyst (Unicameral Bone Cyst)
Hereditary Multiple Exotosis (Osteochondromatosis)	Bone Tuberculosis	Renal Osteodystrophy	Aneurysmal Bone Cyst
Enchondromatosis (Ollier's Disease)	Bone Syphilis	Paget's Disease of Bone (Osteitis Deformans)	Eosinophilic Granuloma of Bone
	Bone Fungus Infections		Bone Lesions of Gaucher's Disease

[0128] In a yet further embodiment, the composition of the invention may further optionally comprise a pharmaceutically acceptable carrier or diluent, as well as additional active agents.

[0129] A pharmaceutically acceptable (or physiologically acceptable) additive, carrier and/or diluent mean any additive, carrier or diluent that is non-therapeutic and non-toxic to recipients at the dosages and concentrations employed, and that does not affect the pharmacological or physiological activity of the active agent.

[0130] The preparation of pharmaceutical compositions is well known in the art and has been described in many articles and textbooks, see e.g., Remington's Pharmaceutical Sciences, Gennaro A. R. ed., Mack Publishing Company, Easton, Pa., 1990, and especially pages 1521-1712 therein.

[0131] Active agents of particular interest are those agents that promote tissue growth or infiltration, such as growth factors. One example is BMPs, which may enhance the activity of the composition of the invention. Other exemplary growth factors for this purpose include epidermal growth factor (EGF), osteogenic growth peptide (OGP), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factors (TGFs), parathyroid hormone (PTH), leukemia inhibitory factor (LIF), insulin-like growth factors (IGFs), and growth hormone. Other agents that can promote bone growth, such as the above-mentioned BMPs, osteogenin [Sampath et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7109-13] and NaF [Tencer et al. (1989) *J. Biomed. Mat. Res.* 23: 571-89] are also preferred.

[0132] Other active agents may be anti-rejection or tolerance inducing agents, as for example immunosuppressive or immunomodulatory drugs, which can be important for the success of bone marrow allografts or xenografts transplantation.

[0133] Alternatively, said active agents may be for example antibiotics, provided to treat and/or prevent infections at the site of the graft. On the same token, anti-inflammatory drugs can also be added to the composition of the invention, to treat and/or prevent inflammations at the site of the graft. Said inflammations could be the result of for example rheumatoid arthritis, or other conditions.

[0134] Polymeric or biodegradable materials are pharmaceutically acceptable carriers and diluents. Biodegradable films or matrices, semi-solid gels or scaffolds include calcium sulfate, tricalcium phosphate, hydroxyapatite, polylactic acid, polyanhydrides, bone or dermal collagen, fibrin clots and other biologic glues, pure proteins, extracellular matrix components and combinations thereof. Such biodegradable materials may be used in combination with non-biodegradable materials, to provide desired mechanical, cosmetic or tissue or matrix interface properties.

[0135] In preferred embodiments, the composition of the invention contains BMC suspensions at cell concentrations ranging from $1 \times 10^6/\text{ml}$ to $1 \times 10^8/\text{ml}$ and DBM at a ratio of from 1:1 to 20:1, preferably between 2:1 to 9:1, most preferably the composition of the invention is at a ratio of 4 parts BMC concentrate to 1 part of DBM in powder form (volume:volume). The absolute number of BMC and DBM is dependent on the size of the joint that needs to be corrected or the size (surface, shape and thickness) of the bone that needs to be replaced

[0136] In another aspect, the present invention relates to a method for transplantation of a mixture comprising BMC with DBM and/or MBM and optionally further comprising pharmaceutically acceptable carrier or diluent, into a joint and/or a cranio-facial-maxillary bone of a subject in need, wherein said method comprises introducing into said joint or bone the composition of the invention.

[0137] The composition of the invention, which possesses all the essential features for accomplishing local bone formation wherever it is implanted, could be efficiently applied

for all kinds of bone repair or substitution, especially in places lacking or deprived of mesenchymal stem cells. Amongst the most problematic places in this sense are joints, cranio-facial-maxillary areas and different kinds of segmental bony defects. Thus, the present invention may be explained as a complex graft, comprising all necessary components, and which its implantation into a damaged joint or bone is sufficient for regeneration or substitution of removed, damaged or destroyed cartilage and/or bone.

[0138] In a first embodiment of the method of the invention, the mixture is administered by any one of the following procedures, injection, minimally invasive arthroscopic procedure, or by surgical arthroplasty into the site of implantation, wherein said method is for support or correction of congenital or acquired abnormalities of the joints, cranio-facial-maxillary bones, orthodontic procedures, bone or articular bone replacement following surgery, trauma or other congenital or acquired abnormalities, and for supporting other musculoskeletal implants, particularly artificial and synthetic implants.

[0139] Thus, in a further aspect, the invention relates to a method of treating a damaged or degenerative arthropathy associated with malformation and/or dysfunction of cartilage and/or subchondral bone in a mammal in need of such treatment, comprising administering into an affected joint or bone of said mammal a mixture comprising BMC with DBM and/or MBM, said mixture optionally further comprising a pharmaceutically acceptable carrier or diluent and/or additional active agents.

[0140] As demonstrated in the following examples, the process of induced development (i.e. proliferation and differentiation) of mesenchymal progenitor cells present within the BMC/DBM mixture can accomplish bone and cartilage formation wherever the mixture is transferred to. The findings presented by the inventors indicate that administration of the composition of the invention into a damaged area of the joint, results in generation of new osteochondral complex consisting of articular cartilage and subchondral bone. When administered into an experimentally created calvarial defect, the composition of the invention results in generation of full intramembranous bone development at the site of transplantation. New tissue formation follows a differentiation pathway, producing different types of bone and cartilage, depending on the local conditions. Thus, the newly formed tissue meets precisely the local demands.

[0141] In one embodiment, the BMC which is present in the administered mixture are either allogeneic or said mammal's own.

[0142] In another embodiment, the DBM or MBM which is present in the administered mixture is in a slice, powder, gel, semi-solid or solid form embedded in or encapsulated in polymeric or biodegradable materials.

[0143] The procedure of applying the composition of the invention into a damaged joint or cranial area comprises the following steps:

[0144] 1. Selecting the source for BMC. The donor may be allogeneic or the BMC may be obtained from the same treated subject (autologous transplantation).

[0145] 2. Selecting the source of DBM and/or MBM. The DBM may be supplied commercially and since it

is not immunogenic, there are no limitations for a specific donor. DBM and/or MBM may be in powder, granules or in slice form. The particle size of the DBM may be about 50 to 2500 μ . Preferably, said particle size is about 250 to 500 μ . The most preferable particle size will depend on the specific needs of each case.

[0146] 3. Preparing a composition comprising a suspension of BMC, at a cell concentration ranging from 1 $\times 10^6$ /ml to 1 $\times 10^8$ /ml and mixing it with DBM at a ratio of from 1:1 to 20:1, preferably between 2:1 to 9:1, most preferably the composition of the invention is at a ratio of 4 parts BMC concentrate to 1 part of DBM in powder form (volume:volume). DBM may be used instead of DBM. If so desired, BMP may optionally be included in the composition.

[0147] 4. Administering said composition into a subject in need either through a syringe (non-invasive injection), closed arthroscopy or open surgical procedure. Alternatively, the composition may be administered so that it is encapsulated within normal tissue membranes. Still alternatively, the composition may be contained within a membranous device, made of a selective biocompatible membrane that allows cells, nutrients, cytokines and the like to penetrate the device, and at the same time retains the DBM and/or MBM particles within the device. Such a membranous device, bone strips or additional scaffolds are preferably surgically introduced. Or, still alternatively, the composition may be administered within a biocompatible and biodegradable polymeric device retaining the DBM and/or MBM particles within the device and suitable to create the needed shape of the transplanted complex.

[0148] 5. Providing glue, preferably consisting of fibrinogen and thrombin, this may be used for fixation of the implant composition at the site of implantation, if necessary.

[0149] In a yet further aspect, the present invention relates to a non-invasive transplantation method comprising introducing a graft into a joint or a cranio-facial-maxillary bone of a subject in need, wherein said graft comprises a mixture of BMC and DBM or MBM.

[0150] In the examples presented herein (see Examples), the inventors show that administration of the composition of the present invention (e.g. BMC in admixture with DBM, as in Example 1) into a damaged area of the joint is essential and sufficient for the generation of new osteochondral complex, consisting of articular cartilage and subchondral bone, at the site of transplantation. The newly formed donor-derived osteochondral complex was capable of long-term maintenance, remodeling and self-renewal, as well as carrying out specific functions of joint surface, such as motion and weight bearing.

[0151] In an even further aspect, the present invention relates to the use of a composition comprising BMC and DBM and/or MBM as a graft of mesenchymal and/or mesenchymal progenitor cells for transplantation into a mammal, wherein said mammal is preferably a human. The transplantation is to be performed into a joint or into a cranio-facial-maxillary bone, for the development of new bone and/or cartilage. The graft of said transplantation may also be for supporting orthodontical procedures for bone augmentation caused by aging, or by congenital, acquired or degenerative processes.

[0152] Furthermore, the composition used in said transplantation is intended for the treatment of a patient suffering from any one of a hereditary or acquired bone disorder, a hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, conditions involving bone or cartilage deformities and Paget's disease. In addition, said composition is intended for the treatment of a patient in need of any one of correction of complex fractures, bone replacement, treatment of damaged or degenerative arthropathy and formation of new bone in plastic or sexual surgery.

[0153] The method of the invention may also be used to induce or improve the efficiency of bone regeneration in damaged cranio-facial-maxillary areas, for therapeutic and cosmetic purposes.

[0154] In one embodiment, the composition used in the invention further comprises an additional active agent.

[0155] In another embodiment, the DBM and MBM comprised within the composition used in the invention are of vertebrate origin, and they may be of human origin. Moreover, said DBM and MBM is in powder or slice form.

[0156] In an additional aspect, the present invention concerns the use of a mixture of BMC with DBM and/or MBM in the preparation of a graft for the treatment of a bone or cartilage disorder, and/or for support of musculoskeletal implants, as a 'glue' to enforce metal implants, joints, etc. that may become loose with time, or to provide a constantly adapting "biological glue" to support such non-biological implants. Alternatively, the invention could be for the support of limb transplants, especially in the articular/bone junction.

[0157] Lastly, the present invention provides a kit for performing transplantation into a joint or a cranio-facial-maxillary bone of a mammal of BMC in admixture with DBM and/or MBM, wherein said kit comprises:

[0158] (a) DBM and/or MBM in a compacted form;

[0159] (b) a BM aspiration needle;

[0160] (c) an intra-osseous bone drilling burr;

[0161] (d) a needle with a thick lumen for infusion of viscous bone marrow-DBM mixture;

[0162] (e) a 2-way lumen connector for simultaneous mixing of BMC-DBM and diluent;

[0163] (f) a medium for maintaining BMC; and optionally

[0164] (g) cryogenic means for handling and maintaining BMC or BMC together with DBM.

[0165] The kit of the invention may optionally further comprise a carrier and/or a diluent for the BMC and DBM and/or MDM mixture.

[0166] The present inventors have concluded that transplantation of multipotent mesenchymal stem cells, and not of differentiated bone or chondrocytes, for remodeling and restoration of a healthy joint or cranio-facial-maxillary structure in arthropathy, is especially important for the following reasons:

[0167] (1) Chondrocytes, as well as the cells transferred within a bone transplant are already fully differentiated cells, with relatively low metabolic activity and limited

self-renewal capacity that may be sufficient to maintain healthy cartilage or bone, but is certainly insufficient for the development of large areas of bone or of hyaline cartilage de novo.

[0168] (2) Most frequently in joints, both cartilage and subchondral bone are damaged. Thus, even a successfully developed new hyaline cartilage is unlikely to be maintained for long if the subchondral bone is left damaged. Based on these findings, it was observed in the following examples that mesenchymal stem cells present in bone marrow, if transplanted under the appropriate conditions, will create a self-supporting osteochondral complex providing healthy joint surface.

[0169] It is not yet clear what makes multipotent mesenchymal stem cells, under the influence of DBM, to choose between an osteogenic and a chondrogenic differentiation pathway. It has however been reported that the ratio of cartilage to bone production depends in particular on the site of DBM implantation, which is naturally influenced by the local conditions [Inoue, T. et al. (1986) *J Dent Res* 65(1):12-22], such as the local source of mesenchymal cells and blood supply [Reddi, A. H. and Huggins, C. H. (1973) *P.S.E.B.M.* 143:634-637]. Low oxygen tension favors chondrogenesis [Bassett, C. A. L. (1962) *J Bone Joint Surg* 44A:1217], most likely due to the low O₂ tension in poorly vascularized cartilage [Sledge, C. B. and Dingle, J. T. (1965) *Nature* (London) 205: 140]. Interestingly, a successful substitution of anterior cruciate ligament (ACL) by demineralized cortical bone matrix has been reported in a goat model [Jackson, D. W. et al. (1996) *Amer J Sports Medicine* 24(4):405-414]. The remodeling process included new bone formation within the matrix in the osseous tunnels and a ligament-like transition zone developing at the extra-articular tunnel interface [Jackson, D. W. et al. (1996) *id ibid.*]. Taking into consideration that hyaline cartilage is naturally developed and maintained only in the joints, where contact with synovial membranes and lubrication with synovial fluid is available and probably essential, it seems reasonable to assume that the environmental conditions in the joint play a major role in enhancing chondrogenesis.

[0170] In the following examples the inventors have shown, for the first time, that a graft composed of DBM and/or MBM and bone marrow cells transplanted into a damaged joint or cranial bone, led to successful replacement of damaged cartilage and subchondral bone. This was the result of osteogenesis on the side of contact with bone and chondrogenesis on the free joint surface, thus the physiological environmental conditions favored osteogenesis or chondrogenesis, respectively. The same kind of a graft composed of DBM and/or MBM and bone marrow cells transplanted into experimentally created partial bone defect in the parietal bone of the cranium led to successful replacement of the removed part of the bone. Thus, the new tissue formation follows a differentiation pathway, producing different types of bone and cartilage depending on the local conditions, such that the newly formed tissue meets precisely the local demands.

[0171] Many publications are referred to throughout this application. The contents of all of these references are fully incorporated herein by reference.

[0172] Throughout this specification and the claims which follow, unless the context requires otherwise, the word

“comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0173] It must be noted that, as used in this specification and the appended claims, the singular forms “a”, “an” and “the” include plural referents unless the content clearly dictates otherwise.

[0174] The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

EXAMPLES

Experimental Procedures

[0175] 1. Animals

[0176] 8 weeks old C57BL/6 male mice and Lewis male rats with body weight of 180-200 g were used as the donors of bones (for matrix preparation) and BMC. Rats from the same batches were used as graft recipients.

[0177] 2. Preparation of Demineralized Bone Matrix (DBM)

[0178] Demineralized bone matrix (DBM) was prepared as described [Reddi and Huggins (1973) *id ibid.*] with the inventors' modification. Diaphyseal cortical bone cylinders from Lewis rats were cleaned from bone marrow and surrounding soft tissues, crumbled and placed in a jar with magnetic stirring. Bone chips were rinsed in distilled water for 2-3 hrs; placed in absolute ethanol for 1 hr and in diethyl ether for 0.5 hr, then dried in a laminar flow, pulverized in a mortar with liquid nitrogen and sieved to select particles between 400 and 1,000 μ . The obtained powder was demineralized in 0.6M HCl overnight, washed for several times to remove the acid, dehydrated in absolute ethanol and diethyl ether and dried.

[0179] Mineralized bone matrix (MBM) was prepared according to the same procedure but the stage of demineralization with HCl was omitted.

[0180] With the exception of the drying step, all steps of the procedure were performed at 4° C.; to prevent degradation of Bone Morphogenetic Proteins (BMP) by endogenous proteolytic enzymes. The matrices were stored at -20° C.

[0181] 3. Preparation of the Implanted Material

[0182] Preparation of Donor BMC Suspensions for Transplantation:

[0183] The femurs of donor mice or rats were freed of muscle. Marrow plugs were mechanically pressed out of the femoral canal by a mandrin. Highly concentrated single cell suspensions of BMC were prepared by dissolving 4-5 femoral plugs into 100 μ l of RPMI 1640 medium (Biological Industries, Beit Haemek, Israel), and passing the cells through the needle several times to dissolve the bone marrow tissue into a single-cell suspension. The number of

nucleated cells per femoral bone marrow plug is rather stable (about 10⁷ cells/plug for a C57BL/6 male, 8 week old mouse). Several reproducible verifications have shown that BMC prepared for transplantation in a form of a single cell suspension contains an approximate concentration of 3 $\times$ 10⁸ cells/ml.

[0184] Composition of the Grafts:

[0185] Grafts were composed of the following ingredients, in different combinations:

[0186] 1. 20 μ l of BMC suspension (concentration 3 $\times$ 10⁸ cells/ml);

[0187] 2. 4 mg of DBM (or MBM);

[0188] 3. 0.5 μ g BMP-2 (R & D systems, USA), optionally.

[0189] The exogenous BMP that is optionally added to the composition of the invention is not a mandatory ingredient. DBM exhibits conductive properties essential for the engraftment, proliferation and differentiation of mesenchymal progenitor cells transplanted within BMC suspension, in the course of bone and cartilage formation. At the same time, DBM is the natural source of BMPs (bone morphogenetic proteins) active in stimulating osteo- and chondrogenesis, thus fulfilling also the inductive function. Addition of exogenous BMPs may enhance the efficiency of the induction.

[0190] 4. Implantation of a Mixture of BMC and Demineralized (or Mineralized) Bone Matrix Into the Area of Local Damage in the Articular Cartilage of the Knee Joint

[0191] A standard artificial damage in the articular cartilage and subchondral bone in the rat knee joint was induced as described. Following anesthesia, the knee joint was accessed by a medial parapatellar incision, and the patella was temporarily displaced towards the side. A microfracture drilling (for a full thickness defect) of 1.5 mm in diameter and 2.0 mm in depth was made in the intercondylar region of the femur.

[0192] The defect was filled with DBM (or MBM) in the form of powder (with particle size of 300-450 micron, or in the form of slice), alone (control) or together with the BMC suspension, prepared as described above. Another control consisted of transferring only BMC into the damaged area. The transplanted material was fixed in place with fibrinogen-thrombin tissue adhesive glue, the patella was returned into its place and the incision was sutured with bioresorbable thread. The skin was closed with stainless clips. In another control group, the damaged area was closed with fibrinogen-thrombin tissue adhesive glue only, without the addition of any of DBM, MBM or BMC.

[0193] 5. Implantation of a Mixture of BMC and Demineralized Bone Matrix Into the Experimentally Created Calvarial Defect.

[0194] Male Lewis rats were anesthetized by intraperitoneal injection of Ketamine. An incision was performed in the frontal region of the rat cranium. The muscular flap was removed from the parietal bone area and a bony defect (0.4 $\times$ 0.5 mm²) was made lateral to the sagittal suture using a dental burr. The defect was then filled with DBM in powder form (particle size of 300-450 micron) together with BMC suspension, prepared as described above. In one control group, only DBM particles were transferred into the

damaged area. The transplanted material was fixed in place with fibrinogen-thrombin tissue adhesive glue. In another control group the damaged area was only closed with fibrinogen-thrombin tissue adhesive glue, without addition of the transplanted material. The skin was closed with stainless clips.

[0195] 6. Laser Capture Microdissection (LCM) and Polymerase Chain Reaction (PCR) Analysis of the Reconstituted Bone Articular Cartilage and Hematopoietic Tissue After Implantation of BMC and Demineralized Bone Matrix Into Damaged Intracondylar Region of Femoral Bone or Into the Cranial Defect

[0196] In the experiments in which BMC of male donors was transplanted either into the damaged intracondylar region of femoral bone, or into the cranial defect, both in female recipients, the newly formed articular cartilage, cranial and subchondral bone were checked by PCR analysis for the origin of the donor. The new technology of Laser-Capture Microdissection (LCM) (service provided by the Common Facility Unit of Hadassah University Hospital, Jerusalem, Israel) allows the isolation of individual cells from tissue sections under precise microscopic control, and was used to harvest isolated cells from newly formed articular cartilage, cranial and subchondral bone. PCR analysis of the harvested cells (about 70-100 cells per test) was performed using a set of primers specific to the Sry gene—the sex determination region of the Y chromosome [An, J. et al. (1997) *J Androl.* 18(3):289-93].

[0197] 7. Histological Evaluation

[0198] The autopsied material was fixed in 4% neutral buffered formaldehyde, decalcified, passed through a series of ethanol grades and xylene, and then embedded in paraffin. Serial sections (5-7 microns thick) were obtained. One set of representative serial sections of each sample was stained with Hematoxylin & Eosin (H&E), and another one with Picroindigocarmine (PIC).

Example 1

[0199] Transplantation of BMC Into the Joint Together with Demineralized (or Mineralized) Bone Matrix

[0200] **FIG. 1** presents the results of experiments carried out to test whether the mesenchymal stem cells present within the bone marrow cells of the composition of the invention could be induced to develop hyaline (articular) cartilage and subchondral bone, when transplanted into the damaged areas of the knee joints.

[0201] Male Lewis rats were anesthetized by intraperitoneal injection of Ketamine. Microfracture drilling (full thickness defect) was inflicted in articular cartilage and subchondral bone in the intercondylar region of the femur. The defects were then filled with DBM (or MBM) together with BMC. In separate groups of experimental animals with defects in articular cartilage and subchondral bone, said defects were filled with DBM (or MBM) or BMC alone. The optional addition of BMPs was also tested (data not shown). The implanted material was fixed in place with fibrinogen-thrombin tissue adhesive glue. In one group of control animals, only glue (and no DBM, MBM or BMC) was grafted into the defect area.

[0202] **FIGS. 1A, 1B and 1C** show healthy undamaged knee joint of the rat with osteo-chondral complex in the

intercondylar region of the femur. In **FIG. 1D**, the microfracture drilling (full thickness defect) can be seen immediately after damage. When DBM powder mixed with BMC was transplanted into the drilled hole, areas of extensively developing hyaline cartilage surrounding implanted DBM particles were observed already two weeks after transplantation, when slight degradation of DBM particles could be observed (**FIG. 1I**). One month after transplantation the DBM particles were already considerably degraded, and areas of extensively developing hyaline cartilage surrounding implanted DBM particles were still present (**FIG. 1J**). After two months, regeneration of the subchondral bone was almost complete, while the surface of the damaged area was built of a thick and continuous layer of extensively developing young hyaline (articular) cartilage (**FIG. 1K**). Six months after transplantation of DBM powder, mixed with BMC, into the microfracture drilling defect in the intercondylar region of the femur, the histological structure of the regenerated osteo-chondral complex was indistinguishable from normal (**FIG. 1L**).

[0203] PCR analysis of isolated cells from different tissues composing newly developed osteo-chondral complex, captured by LCM techniques after implantation of DBM together with BMC into the micro-fracture drilling was performed (**FIGS. 2A-F**). The results of the PCR showed the presence of donor derived cells within newly formed articular cartilage and subchondral bone (**FIG. 2G**). This is strong evidence that active mesenchymal progenitor cells, transplanted within the donor BMC suspension, took an active part in the development of a new osteo-chondral complex.

[0204] Most importantly, bone and cartilage regeneration, to the same extent as that of the experimental group that received a DBM together with BMC graft, were not observed in the control groups. Thus, the inventors concluded that the DBM+BMC mixture included the entire array of components essential for the successful regeneration of the osteo-chondral complex in the damaged joint.

[0205] The specificity of the artificial defect model used in the present experiments resided in the penetration of the microfracture drill into the subchondral bone, thus supplying the damaged area with locally existing bone marrow containing mesenchymal progenitor cells potentially capable of restoring both subchondral bone and articular cartilage, when local conditions stimulating osteo- and chondrogenesis were supplied.

[0206] However, without the implant, regeneration of micro-fracture was incomplete, and two weeks after drilling the hole was filled with connective tissue (**FIG. 1E**). During the following month, the subchondral bone was repaired, although with no regeneration of the articular cartilage on the surface of the damaged area (data not shown). Six months after the micro-fracture had been inflicted, the regenerating surface of the damaged joint constituted of fibro-cartilaginous tissue (**FIG. 1F**). Implantation of exogenous BMC does not bring any considerable changes in the pathway of regeneration, i.e. the regenerating surface of the damaged joint constituted of fibro-cartilaginous tissue usually deteriorating over time. It is important to note that, unfortunately, despite the restricted efficiency of this procedure, as also shown by these results, this is the most used procedure for damaged joint surface repair and for the treatment of osteoarthritis in the current clinical practice.

[0207] When DBM particles alone were transplanted into the drilled hole, it seems that the number of locally available mesenchymal progenitor cells was not sufficient for effective

regeneration of osteo-chondral complex. No extensive developing hyaline cartilage could be seen among the implanted DBM particles two weeks after transplantation (**FIG. 1G**). In most of the cases the degradation and remodeling of DBM particles as well as the process of new bone and cartilage formation was not efficient enough and the damaged area was finally covered with connective tissue together with fibro-cartilage (**FIG. 1H**).

Example 2

[0208] Transplantation of BMC Together with DBM Into the Experimentally Created Calvarial Defect.

[0209] Experiments were carried out to test whether the mesenchymal stem cells within the BMC comprised in the composition of the invention could initiate and accomplish the intramembranous development of bone, when transplanted together with DBM into the experimentally created calvarial defect. The results of these experiments are shown in **FIGS. 3 and 4**. This method could then be extended to treat facial-maxillary defects.

[0210] An incision was performed in the frontal cranium region of anesthetized Lewis rats (8-12 weeks old) and the skin flap was moved aside. The muscular flap was removed from the parietal bone area and a bony defect ($0.4 \times 0.5 \text{ mm}^2$) was created laterally to the sagittal suture using a dental burr. The defect area was either left empty, filled with DBM alone, or filled with DBM together with BMC, as described above. In all the groups (experimental and control) the defect area was finally covered with fibrin glue. Lastly, the skin flap was returned to place and fixed with stainless clips.

[0211] The utilization of non-healing cranial defects allows for the observation of both osteo-conductive and osteo-inductive components of the healing process. Thus, the non-healing cranial defect represents an appropriate model for evaluating the ability of the composition of the present invention (in this Example, BMC together with DBM) to accomplish intramembranous bone formation when transplanted into a damaged area of the crania.

[0212] **FIG. 3A** shows the normal (undamaged) cranium of a rat. **FIGS. 3B and 3C** show the experimental defect in the parietal bone area.

[0213] 15 and 30 days after the operation, absence of bony tissue regeneration could be observed when the site of removed bone was left empty (**FIG. 4A, 4D, 4G and 4J**), suggesting that the size of the defect sufficiently large, compatible with the definition of non-healing cranial defect.

[0214] Filling of the experimental cranial defect with DBM alone resulted in the gradual degeneration and remodeling of transplanted DBM particles, and blood vessels and new bone formation on different stages of maturity could be observed (**FIG. 3E, FIG. 4B, 4E, 4H and 4K**). However, the process of intensive new bone formation was not presented uniformly in the defect area. New bone formation was considerably more active in the periphery, close to the edges of the cut bone, suggesting that the number of mesenchymal progenitor cells that could be induced and conducted to osteogenesis was limited in the central area of the defect.

[0215] When DBM powder together with BMC was transplanted into the site of the experimental cranial defect, extensive remodeling of the transplanted DBM particles and developing areas of new bone could be observed as early as 8 days after transplantation (**FIG. 3F**). 15 and mostly 30 days after transplantation, the cut edge of the parietal bone

could hardly be distinguished from the surrounding new bony tissue (**FIGS. 4F and 4L**). The defect area was reconstituted with a continuous layer of newly developing bone (**FIG. 4C, 4F, 4I and 4L**). It should be especially stressed that extensive remodeling of transplanted DBM particles and active new bone formation were presented uniformly throughout the defect area, suggesting that the quantity of available mesenchymal progenitor cells capable of being induced and conducted to osteogenesis was sufficient when the implant consisted of DBM particles mixed with BMC.

[0216] PCR analysis of the cells isolated by LCM from the newly developed bone tissue, in the site of experimental calvarial damage, 8 and 30 days after transplantation of DBM together with BMC (from male donor to female recipient) showed the presence of donor derived cells (**FIG. 5**). This is strong evidence that the mesenchymal progenitor cells transplanted within the donor BMC suspension play an active role in the development of this new cranial bone, and are subject to the osteo-inductive and osteo-conductive properties of DBM.

[0217] These findings indicate that administration of the composition of the present invention (in this case, DBM together with BMC) into an experimentally created calvarial defect was sufficient for active and complete intramembranous bone formation at the site of transplantation. This procedure could be extended to treat facial-maxillary defects.

[0218] Pilot experiments utilizing BMC in combination with MBM rather than DBM also showed positive results. Mainly, the difference between employing DBM and MBM lies on delayed bone and cartilage formation with MBM. Also, since MBM particles are much more dense and hard, as compared to DBM particles, they are more useful when weight bearing or shape preservation of the transplant are needed. Transplantation of a mixture of both DBM and MBM together with BMC should enable the best of the advantages of both: (a) significantly prolonging the period of osteo- and chondrogenic activity (with DBM acting fast and MBM after a delay); (b) improving the shape preservation of the implant throughout the whole period of new tissue formation.

[0219] Addition of BMP to the mixture of BMC and DBM has considerably accelerated formation of new tissue both in the osteochondral complex of the knee joint and in the flat bones of the skull.

1. Use of a mixture comprising bone marrow cells (BMC) and demineralized bone matrix (DBM) and/or mineralized bone matrix (MBM) as a graft of mesenchymal progenitor cells for transplantation into a joint and/or a cranio-facial-maxillary bone of a subject in need.

2. The use according to claim 1, wherein said transplantation is for restoring and/or enhancing the formation of a new hyaline cartilage and subchondral bone structure.

3. The use according to any one of claims 1 or 2, wherein said transplantation is for generation of new osteochondral complex consisting of articular cartilage and subchondral bone.

4. The use according to any one of claims 1-3, wherein said BMC are allogeneic or said subject's own.

5. The use according to any one of claims 1 to 4, further comprising active agents, preferably selected from bone

morphogenetic proteins (BMPs), immunosuppressants, immunomodulators, antibiotics and anti-inflammatory agents.

6. The use according to any one of claims 1-5, wherein said subject is a mammal.

7. The use according to any one of claims 1 to 6, wherein the DBM and/or MBM are of vertebrate origin.

8. The use according to any one of claims 1 to 7, wherein the DBM and/or MBM are of human origin.

9. The use according to any one of claims 1 to 8, wherein the DBM or MBM are in powder or slice form.

10. The use according to any one of claims 1 to 9, wherein the particle size of the DBM is about 50 to 2500 μ .

11. The use according to claim 10, wherein the particle size of the DBM is about 250 to 500 μ .

12. The use according to any one of the claims 1 to 11, wherein the ratio between BMC and DBM is between 1:1 and 20:1 (volume:volume).

13. The use according to claim 12, wherein the ratio between BMC and DBM is between 2:1 and 9:1 (volume:volume).

14. The use according to claim 13, wherein the ratio between BMC and DBM is 4:1 (volume:volume).

15. The use according to any one of claims 1 to 14, wherein said mammal is a human.

16. The use according to any one of the preceding claims, wherein said transplantation is for the treatment of a patient suffering from any one of hereditary or acquired bone disorder, hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, metabolic bone diseases, bone infections, conditions involving bone or cartilage deformities and Paget's disease.

17. The use according to any one of claims 1 to 16, wherein said transplantation is for the treatment of a patient in need of any one of correction of complex fractures, bone replacement and formation of new bone in plastic or sexual surgery.

18. The use according to any one of claims 13 to 17, wherein the number of bone marrow cells in the mixture is from about 10⁶ to 10⁸ cells/ml.

19. The use according to any one of the preceding claims, further optionally comprising a pharmaceutically acceptable carrier or diluent.

20. The use according to any one of claims 1 to 19, further comprising additional active agents.

21. Use of a mixture comprising BMC and DBM and/or MBM, in the preparation of a composition for the treatment of a damaged joint or degenerative arthropathy, and/or for the treatment of a patient suffering from any one of hereditary or acquired bone disorder, hereditary or acquired cartilage disorder, a malignant bone or cartilage disorder, metabolic bone diseases, bone infections, conditions involving bone or cartilage deformities and Paget's disease.

22. Use of a mixture comprising BMC and DBM and/or MBM, in the preparation of a composition to be used for transplantation, for restoring and/or enhancing the formation of a new hyaline cartilage and subchondral bone structure.

23. A method for transplantation of a mixture comprising BMC with DBM and/or MBM and optionally further comprising pharmaceutically acceptable carrier or diluent, into a joint or a cranio-facial-maxillary bone of a subject in need, wherein said method comprises introducing into said joint or

bone a mixture comprising BMC and DBM and/or MBM, or a composition as prepared in claim 21.

24. The method according to claim 23, wherein said mixture is administered non-invasively by a syringe, an arthroscopic procedure or by open surgery into the site of implantation.

25. The method according to claim 24, for support of implants of joints, cranio-facial-maxillary bones, or other musculoskeletal implants.

26. A method of treating a damaged joint or degenerative arthropathy associated with malformation and/or dysfunction of cartilage and/or subchondral bone in a mammal in need of such treatment, comprising administering into an affected joint or bone of said mammal a mixture comprising BMC with DBM and/or MBM, said mixture optionally further comprising a pharmaceutically acceptable carrier or diluent and/or additional active agents.

27. The method according to claim 26, wherein the BMC are either allogeneic or said mammal's own.

28. The method according to any one of claims 26 or 27, wherein said DBM or MBM are in a slice, powder, gel, semi-solid or solid form embedded in or encapsulated in polymeric or biodegradable materials.

29. The method according to claim 28, wherein the particle size of said DBM is about 50 to 2500 μ .

30. The method according to claim 29, wherein the particle size of said DBM is about 250 to 500 μ .

31. The method according to any one of the preceding claims, wherein the ratio between the transplanted BMC and DBM is between 1:1 and 20:1 (volume:volume).

32. The method according to claim 31, wherein the ratio between the transplanted BMC and DBM is between 2:1 and 9:1 (volume:volume).

33. The method according to claim 32, wherein the ratio between the transplanted BMC and DBM is 4:1 (volume:volume).

34. A non-invasive implantation method for support of implants of joints or other musculoskeletal implants, comprising introducing a graft into a joint or a cranio-facial-maxillary bone of a subject in need, wherein said graft comprises a mixture of BMC and DBM and/or MBM.

35. A kit for performing transplantation into a joint or a cranio-facial-maxillary bone of a mammal of a mixture as defined in any one of claims 1-20, wherein said kit comprises:

- (a) the mixture as defined in any one of claims 1-20;
- (b) a BM aspiration needle;
- (c) an intra-osseous bone drilling burr;
- (d) a needle with a thick lumen for infusion of viscous bone marrow-DBM mixture;
- (e) a 2-way lumen connector for simultaneous mixing of BMC-DBM and diluent;
- (f) a medium for maintaining BMC; and optionally
- (g) cryogenic means for handling and maintaining BMC or BMC together with DBM.

36. The kit according to claim 35, optionally further comprising a carrier and/or diluent for the mixture.

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