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(54) **METHODS FOR OPTIMIZED EXPANSION AND IMPLANTATION OF MESENCHYMAL STEM CELLS**

VERFAHREN FÜR OPTIMIERTE EXPANSION UND IMPLANTATION MESENCHYMALER STAMMZELLEN

PROCEDES POUR L'EXPANSION ET L'IMPLANTATION OPTIMISEES DE CELLULES SOUCHES MESENCHYMATEUSES

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Description

TECHNICAL FIELD

5 **[0001]** The invention generally relates to methods for expansion of stem cells of a host in need thereof. More specifically, the invention relates to ex-vivo culture expansion of mesenchymal stem cells (MSCs) under optimized growth conditions.

BACKGROUND OF THE INVENTION

10 **[0002]** Mesenchymal stem cells are pluripotent blast or embryonic-like cells located in blood, bone marrow, dermis and periosteum. In general these cells are capable of renewing themselves over extended periods of time as well as, under various environmental conditions, differentiating into cartilage, bone and other connective tissue. Recently, various investigators have researched the potential for using these cells to repair or regenerate target tissues, *e.g.*, bone, cartilage, etc. In this manner MSCs have been reported to have regenerative capabilities in a number of animal models.
 15 See Acosta et al. (2005) Neurosurg Focus 19(3):E4; Barry (2003) Novartis Found Symp. 249:86-102, 170-4, 239-41; Brisby et al. (2004) Orthop Clin. North Am. 35(1):85-89; Buckwalter and Mankin (1998) Instr Course Lect. 47:487-504; Caplan (1991) J Orthop Res. 9(5):641-650. Further, these findings are being extended in clinical trials to humans, however, most of these trials require *in vitro* expansion of isolated, non-autologous MSCs using highly concentrated recombinant cytokines and growth factors. For example, most human studies have utilized isolated MSCs from bone marrow (or
 20 peripheral blood), followed by *ex-vivo* expansion of the cells in a laboratory setting using a fetal bovine serum (FBS) based culture medium spiked with various recombinant growth factors. These supplemented FBS-based culture mediums have shown the capacity to support MSC expansion but also include the risk of cross-contamination of infectious vectors, use of non-Food and Drug Administration (FDA) approved drugs/factors, *e.g.*, recombinant TGF- β , FGF, cross species reactions, and possible increased potential for forming cancerous progenitors.

25 **[0003]** In addition, most of the MSC-based human studies have required trained laboratory staff and laboratory equipment to perform the expansion of the isolated MSCs. These techniques are not amenable to performance by physicians and/or hospital staff, especially given that physicians are legally bound by FDA protocols and procedures concerning non-FDA approved drugs. Therefore, it is difficult for MSC based therapies to be performed in a pragmatic manner, *i.e.*, in a hospital setting with hospital employees. Given these numerous concerns, most MSC based research is directed
 30 at non-autologous cells that have been isolated and cultured into permanent cell lines.

[0004] Doucet (Doucet, Ernou et al. 2005 J. Cell Physiol 205(2):288-36) has recently described a technique for expanding MSCs of young healthy donors using a 5% platelet lysate enriched culture medium. However, Doucet's investigations did not determine effectiveness of these procedures on elderly patients, patients with degenerative joint diseases (for example osteoarthritis), or other patient specific characteristics. Nor was the study performed using any expansion
 35 conditions except for 5% platelet lysate enriched culture medium. In this light, it has been shown that there is a wide variation in MSC growth in patients with and without osteoarthritis, with age, with gender, and based on certain genetic phenotypes. Therefore the Doucet study has very limited applicability to real life situations, where most patients in need of MSC-based therapy are generally either older, or have degenerative joint, organ, or spinal diseases. The Doucet data also does not apply to other disease states of bony metabolism such as avascular necrosis or osteonecrosis. In addition,
 40 the generalized findings in Doucet are not gender or age specific, having little guidance on how to treat the different sexes or how to expand MSC's from patients of advanced age.

[0005] MSC's can readily differentiate in culture depending on cytokine exposure, environmental conditions (pressure, attachment opportunities, passage treatment, etc...), or other chemical exposure. For example, exposure to varying levels of TGF-beta, FGF, and/or PDGF can all have impacts on the final cell phenotype produced in culture. In addition,
 45 leaving cells in culture longer has impacts on differentiation potential. Cells can be cultured for a certain visual morphology, confluence, or density, all of which impacts the final cell product produced and its potential for certain types of tissue repair. As a result, this invention focuses on controlling factors/parameters so as to produce a homogeneous cell product with certain restorative properties.

[0006] In replacing or repairing tissue with MSC's, one concern is the use of non-autologous cells. While MSC's have
 50 been traditionally considered immune privileged, recent investigations have demonstrated their activation of the natural killer cell system in a foreign host. (Spaggiari, Capobianco et al. 2006 Blood 107(4):1484-90) This makes the use of non-autologous cells difficult, as it is anticipated that the host's immune system will attack these foreign cells and potentially decimate the population of transplanted MSCs, thus severely limiting their repair capabilities. In addition, a recent work published by Ueda may have other far reaching implications for the use of non-autologous cells. (Ueda, Inaba et al. 2007 Stem Cells 25(6): 1356-63) This study demonstrated that senile mice with osteoporosis transferred that disease into normal mice through a bone marrow vector. This suggests that the MSC's of the senile mice with osteoporosis once transferred to normal healthy mice were able to transfer that disease state into normal healthy mice. This genetic vector for disease transmission is concerning, as any donor MSC's would theoretically need to be screened

for all known genetic susceptibilities and diseases that may be transferred by the donor.

[0007] There is a need in the art for MSC expansion techniques that do not use drugs or growth factors which are not FDA approved and can be effectively used to replace tissue in a patient in need thereof. This replacement should be with autologous cells that have been optimally expanded based on the patient's medical condition, age, gender and other relevant replacement conditions. In addition, there is a need for autologous techniques to yield a homogeneous cell line with known regeneration capabilities and rigid quality control.

[0008] The present invention is directed toward overcoming one or more of the problems discussed above.

SUMMARY OF THE INVENTION

[0009] Embodiments of the invention provide methods for *ex-vivo* expansion of human mesenchymal stem cells (MSCs).

[0010] In particular, the present invention provides a method for *ex-vivo* culture expansion of mesenchymal stem cells (MSCs) from patients having osteoarthritis, osteoporosis, avascular necrosis, or other diseases of bone or cartilage, whereby the method comprises:

a) expanding the patient's MSCs with a culture medium comprising platelet lysate at a concentration of about 10% of the culture medium;

b) determining whether the patient's MSCs of step a) are within a growth channel characterized by the following parameters:

(i) a doubling time of 1 to 3 days;

(ii) a cell density within a range of about 18 to about 23 calculated according to the equation $\text{Surface area} \times (\% \text{ confluence})$ divided by cell number;

(iii) even distribution of MSCs as a monolayer culture; and

(iv) a spindle-shaped cellular morphology in monolayer culture; and

c) adjusting the culture medium in the following passages, wherein the MSCs are outside of the scope of at least one parameter of step b) by increasing the amount of platelet lysate in the culture medium to between 15-20% platelet cell lysate; and wherein the patient's MSCs are expanded for no more than 10 *ex-vivo* passages.

[0011] In one embodiment of the invention, the required dose of MSCs are between 1 million and 100 million cells. Alternatively, red blood cells are used in the growth medium to improve attachment and colony formation of the MSCs.

[0012] In another embodiment, the MSCs are expanded to have optimized growth by a 10th *ex-vivo* passage or by a 7th *ex-vivo* passage.

[0013] In still another embodiment, the patient is a patient who has been diagnosed with osteoarthritis.

[0014] These MSCs are optimized for their capacity to implant and replace/regenerate target tissue, *e.g.*, regenerate cartilage in a knee joint. As discussed above, they are also optimized to produce a homogeneous cell line with rigid quality control which expresses one or more of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD166, and CD105 and in some embodiments express two, three, four, five, six or seven of the above cell surface antigens. In addition, some optimized cells described herein do not express one or more of the following cell surface antigens: CD14, CD31, CD45, and/or CD106.

[0015] Aspects of the invention include novel expansion compositions that do not require purified or recombinant growth factors, cytokines, or non-naturally occurring human factors. In particular, expansion compositions are designed to include varying amounts (and varying timing) of the introduction of platelet lysate to optimize cell growth, especially optimize cell growth for cells at time of implantation into a target patient. Optimization in some instances includes expanding cells in a controlled manner to facilitate the cells capability of successfully implanting in a patient in need thereof; in some cases cell growth and cell growth conditions are monitored and modified to keep the cells within a predetermined "growth channel", *i.e.*, expansion of cells to a required number prior to a limited number of cell passages. These platelet lysate based growth conditions provide for consistent and autologous release of the necessary factors for facilitating *ex-vivo* MSC expansion within this growth channel. In addition, in order to ensure homogeneity and strict quality controls, the "growth channel" has multiple other embodiments such as cell density, morphology, and culture pattern. Again, these are designed to produce a consistent cell expansion with known cell reparative properties, as small changes to this formula for cell growth will result in a wholly different cell product with different differentiation and repair properties.

[0016] Further, described herein is preparing a patient for receipt of optimally cultured MSCs by implanting the cells with determined amounts of platelets or platelet lysate. Implantation of cells and platelets can occur simultaneously or subsequent to each other. Typically, the MSCs and platelets and platelet lysate are from the patient into which the MSCs

and platelets/platelet lysate will be implanted.

[0017] Also described herein is a method for isolation of MSC's from a patient in need of MSC-based restoration therapy, optimized expansion of these isolated MSCs using cell specific expansion data obtained using varying amounts of platelet lysate and culture decisions based on cell properties (such as confluence, morphology, cell culture pattern, etc...) necessary to ensure proper and homogeneous growth for a target need (*i.e.*, growth in the growth channel), and implantation of the expanded cells with or without context dependent MSC growth facilitator materials.

[0018] Aspects of the invention are particularly useful where cells are harvested and replaced into patients having osteoarthritis or other diseases of cartilage or bone metabolism (such as avascular necrosis or osteoporosis) given that the cells harvested from these patients conventionally show little prospect of use in replacement therapy. However, this statement is not meant to limit the scope or use of this invention to one application.

[0019] Aspects of the invention also provide a growth channel for ensuring that harvested MSCs are maintained in a natural manner that facilitates cell implantation back into the patient. These cells are autologous and optimized for potential growth in the target using only natural factors from the same host that the cells were harvested from, *i.e.*, no synthetic or recombinant factors used to facilitate cell growth. In typical growth channel embodiments the cells are expanded and ready for implementation before their 10th passage and in other embodiments the cells are expanded and ready for implementation by their 3rd, 4th, 5th, 6th, 7th, 8th or 9th passage (post harvest). Cells that are implanted after about the 10th passage show an increasing tendency to be ineffective for clinical use.

[0020] Aspects of the invention provide for ensuring that expanded MSCs are within a growth channel by manually counting the cells. In other aspects of the invention MSCs are visually inspected for characteristic indication that the cells are within the growth channel, proper indicators include culture morphology, culture pattern and culture density. In some aspects both the number of cells and the visual inspection of cells are used to indicate whether cells are within a growth channel of the invention. Note that visual inspection can be performed on site of the cultured MSCs, *e.g.*, a blood bank in a hospital that has been contracted to expand the cells, or via a remote site, where experienced tissue culture personnel view cultures via digital camera microscopy (live video or updated pictures or other like technology) and provide feedback to distant personnel on-site regarding conditions of cultured cells.

[0021] Finally, also described herein are cell populations that are enriched for identified phenotypes using the methods described herein, the phenotype including one or more of the following cell surface antigens: CD29, CD44, CD59, CD90, CD166, CD73 and CD105. Cells identified herein further are typically negative for the CD14, CD31, CD45 and CD106 cell surface antigens. The optimized and expanded cell populations may express CD29 and CD44 with at least one other of the following cell surface antigens: CD59, CD90, CD105, and CD66. The optimized cell populations may express at least: CD29, CD44 and CD59; express CD29, CD44 and CD90; express CD29, CD44 and CD105 or express CD29, CD44 and CD66.

[0022] Cells prepared using the methods described herein having the above described phenotype show optimal growth characteristics for implantation into a patient in need thereof.

[0023] These and various other features and advantages of the invention will be apparent from a reading of the following detailed description and a review of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]

Figures [Figure] 1A-E shows various illustrative aspects of the "Growth Channel" hereindescribed including Cell Growth Rate, Cell Density, Cell Morphology, and Cell Culture Pattern. A: Cell Growth Rate to Optimize Expansion for Repair. Doubling time is defined as the number of days to double the count of cells in monolayer culture. This is a key metric, as cells which are capable of repair are readily able to exponentially grow in culture. The % platelet lysate required to allow doubling time within the acceptable growth channel also determines the amount of platelet lysate that will be needed to support cell expansion and engraftment *in-vivo*. Cell Culture Actions:

Above expected channel (>3 days): Increase platelet lysate concentration until growth rate is in the expected channel; Reseed at higher density until growth rate is in the expected channel;

Below expected channel (<1 day): Decrease platelet lysate concentration until growth rate is in the expected channel; Reseed at lower density until growth rate is in the expected channel.

B: Exemplar of two 10 cc marrow draws at the PSIS yielding >100 nucleated cells into colony formation culture and approximately 700,000 MSC's out of colony formation culture (after 7-10 days in 20% lysate). In this example, the growth channel is shown in that any lag of doubling time results in a cell culture action as already described. Cell Culture Action:

Above expected channel as shown: Increase platelet lysate concentration until growth rate is in the expected channel; Reseed at higher density until growth rate is in the expected channel;
Below expected channel as shown: Decrease platelet lysate concentration until growth rate is in the expected channel; Reseed at lower density until growth rate is in the expected channel.

C: Cell Confluence is defined as the percentage of free space between cells in monolayer culture. As shown here, cells that are too tightly packed will quickly move toward a differentiated state, while cells that are too loosely packed will fail to hit growth rate targets. Spatial distribution of cells can be quantified by the following equation: $\text{Surface area} \times (\% \text{ Confluence}) / \text{Cell number}$. This figure should be in the range of 18-23. Cell Culture Actions:

Below expected channel (<18): Decrease platelet lysate concentration until confluence is in the expected channel; Reseed at lower density until confluence is in the expected channel;

Above expected channel (>23): Reseed at higher density until confluence is in the expected channel (from 12×10^3 cells/cm² to 15×10^3 cells/cm²);

Significantly above expected channel (>27); Increase platelet lysate concentration.

D: Cell Distribution is defined as the randomness of cells in two dimensional space (monolayer culture). Randomly distributed cells are within the growth channel, clumped or unevenly distributed cells are out of the growth channel. Cell Culture Actions:

Out of expected channel (unevenly distributed): Reseed at higher density until evenly distributed; Passage cells sooner than expected.

E: MSC Morphology type associated with this invention and types not associated. MSC's can be grown with various environmental stimuli and conditions to prefer one phenotype over another. Displayed here are phenotypes not associated with this invention. The phenotype associated with this invention is spindle shaped. Or type 1 as shown. Any deviation from this morphology requires a cell culture action as described.

TYPE 1: Photomicrograph of Type 1 MSC at 10x taken from the iMSC's grown according to the growth channel considerations described in this invention.

TYPE II: Photomicrograph of Type II MSC at 10x taken from: Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system Denitsa Docheva *, Cvetan Popov, Wolf Mutschler, Matthias Schieker J. Cell Mol. Med. Vol 11, No 1, 2007 pp. 21-38.

TYPE III: Photomicrograph of Type III MSC at 20x taken from: Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells Raquel Talens-Visconti, Ana Bonora, Ramiro Jover, Vincente Mirabet, Francisco Carbonell, Jose Vincente Castell, Maria Jose Gomez-Dechon World J. Gastroenterol 2006 September 28; 12(36): 5834-5845.

TYPE IV: Photomicrograph of Type IV MSC at 10x taken from: Autologous Bone Marrow-Derived Cultured Mesenchymal Stem Cells Delivered in a Fibrin Spray Accelerate Healing in Murine and Human Cutaneous Wounds, V FALANGA, S IWAMOTO, M CHARTIER, T YUFIT, J BUTMARC, N KOUTTAB, D SHRAYER, P; CARSON TISSUE ENGINEERING Volume 13, Number 6, 2007.

Cell Culture Actions:

Out of expected channel (> 30% Type II cells): Increase platelet lysate concentration until morphology is type I or transplant if passage > 5;

Significantly out of expected channel (> 30% Type III of IV cells); Passage or transplant cells sooner than expected; Cells may not have desired effect.

Figure 2 is a cell expansion plot illustrating differences in yield and rate of growth between eight osteoarthritic patient's cells when those cells were grown *in vitro* in 5-10% platelet lysate.

Figures 3A-E illustrate bar graphs for 5 patient isolated MSC populations over a course of 1 to 6-16 days using from 5-20% platelet lysate.

Figure 4 illustrates a bar graph for 6 different patient MSC expansions using from 5-20% platelet lysate where patients cells showed either slow growth or fast growth.

Figure 5 is a stem cell growth channel overlay for 5 different patients.

Figures 6A and B are before and after "fast spin proton density images" for a MSC implantation using cells optimized

by embodiments of the present invention.

Figure 7 is a bar plot illustrating 4 patients cell growth using 10 or 20% platelet lysate as introduced either at a first passage, second passage or third passage.

Figure 8 shows cell expansion for a patient with stage 3-4 Avascular Necrosis under two different marrow draw conditions. Condition 1: Two 10 cc marrow draws yielded 48 million nucleated cells which failed to expand in 10% platelet lysate. Culture was aborted after 2 weeks. Condition 2: six small aliquots of 1-2 cc of marrow taken from the bilateral PSIS area yielded 164 million nucleated cells. The expansion plot for MSC's grown in 20% platelet lysate is shown.

Figures 9A and 9B are before and after "fast spin proton density MRI images" for a MSC implantation using cells optimized by embodiments of the present invention. Partial regeneration of the anterior-medial knee meniscus is shown. Expansion of MSC's isolated from patient with Avascular Necrosis of the hip. Continuing matching image sequence of the right knee before cells (left image in January 2007), then 3 months after cells (right image in June 2007).

Figures 10A and 10B are before and after radiographs for a MSC implantation using cells optimized by embodiments of the present invention. Partial healing of humerus non-union fracture is shown.

Figures [Figure] 11A and 11B are before and after sagittal proton density images of an osteoarthritis patient with a severely degenerated medial meniscus and subsequent regeneration of parts of that meniscus using MSC's expanded using the embodiments of this invention.

DETAILED DESCRIPTION OF THE INVENTION

[0025] Embodiments of the invention provide methods for the expansion of mesenchymal stem cells under optimal growth conditions. Expansion conditions are based on individualized growth characteristics for the patient's particular MSC population, not requiring synthetic or recombinant growth factors. In typical embodiments the optimal growth conditions are at least partially provided by platelet lysate from the same patient. These platelet lysate compositions provide a consistent and effective release of the patient's own combination of growth factors. Note that aspects of the invention equally apply to other cell types besides MSCs, e.g., stem cells, chondrocytes, etc., but for convenience embodiments described herein will be directed toward MSCs. Further, optimally grown cells can be implanted in combination with autologous factors to enhance the cells capacity for enhanced therapeutic results, for example in combination with platelets or platelet lysate, *i.e.*, platelets harvested from the same patient that will receive the MSCs. Finally, embodiments of the invention described herein include MSCs enriched for a homogeneous phenotype that results from the optimized growth conditions described herein, such cells are identified as cells optimized for use in regenerative MSC-based therapy.

Definitions:

[0026] The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

[0027] "Individualized growth characteristic" refers to individual specific *ex vivo* growth characteristics of harvested cells. For example, cells typically harvested from many individuals having osteoarthritis show slow growth, requiring modified growth characteristics to ensure that the cells have optimal growth and therefore are prepared for implantation back into a patient.

[0028] "Mesenchymal stem cell" or "MSCs" refers to multipotent stem cells capable of differentiating into osteoblasts, chondrocytes, myocytes, adipocytes, neuronal cells, pancreatic islet cells, and the like (see below).

[0029] "Natural expansion factor" refers to factors that are native to a patient in need thereof as opposed to synthetic or recombinant expansion factors that are prepared from *in vitro* sources. Natural expansion factors are typically associated with and released from a platelet lysate.

[0030] "Platelet lysate" refers to the combination of natural growth factors contained in platelets that has been released through lysing those platelets. This can be accomplished through chemical means (*i.e.* CaCl_2), osmotic means (use of distilled H_2O), or through freezing/thawing procedures. Platelet lysates of the invention can also be derived from whole blood and can be prepared as described in US Patent No. 5,198,357.

[0031] "Protein," "peptide," and "polypeptide" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

[0032] "Stem cells" refers to any cell having the characteristic of being unspecialized and able to renew for extended periods of time through cell division and being inducible to become cells with specialized function.

[0033] "Cell Culture Action" refers to a change in platelet lysate concentration, reseeding of cells in culture at a different density, or a change in planned passaging time (*i.e.* leave in culture a longer or shorter time before medium change).

[0034] "Passage" refers to changing spent medium in a monolayer culture or otherwise changing medium to improve

the cell culture microenvironment.

Source of MSCs and Platelet Lysates

[0035] Mesenchymal stem cells are multipotent stem cells located in the bone marrow, peripheral blood, adipose tissue and other like sources. MSCs have the capacity to differentiate into a number of cell types, including osteoblasts, chondrocytes, myocytes, adipocytes, and beta-pancreatic islet cells.

[0036] Source MSCs of the invention are typically harvested from the iliac crest of the patient in need of the restorative/replacement therapy (or a suitable donor), such patient is referred to herein as a "patient in need thereof" (note that other sources, such as adipose tissue, synovial tissue, and connective tissue have recently been identified and are also considered as MSC sources within the scope of the present invention). As described herein, approximately 10-20 cc of bone marrow is harvested and "isolated" using methods described in US Patent Application 60/761,441 to Centeno or through adherence to plastic, as described in US Patent No. 5,486,359 to Caplan et al.

[0037] Also described herein are changes to standard marrow draw procedures to allow appropriate nucleated cell number yield to use the platelet lysate techniques described. Since the vast majority of the published research is again performed in healthy humans or animals, the application of this technique to humans with various disease states has never been tested. An example is shown in Figure 8, where a marrow draw from a patient with AVN (in need of bony repair at an AVN site) and using the bilateral 10 cc draw technique described above produced a failed culture expansion in platelet lysate. However, the use of an altered technique drawing three small 2-3 cc marrow aliquots on each side (total of 6 aliquots), produced the required nucleated cell yield which was successfully expanded in 20% platelet lysate.

[0038] Platelet lysate for use herein is prepared from the bone marrow harvest using the method of Doucet. Typical lysates include from about tens of millions to 100's of billions platelets. As shown by Martineau et al., *Biomaterials*, 2004 25(18) p4489-503, platelet lysates inherently include the growth factors required to facilitate consistent MSC growth. In typical embodiments the platelet lysate and MSC are autologous and are in amounts useful for effective and consistent expansion of the MSCs (described more fully below). In particular, it should be noted that while the levels of growth factors such as TGF-beta are much lower in platelet lysate than those commonly used to expand MSC's, it is believed that there are significant synergistic effects when all of the low level growth factors contained in platelet lysate are used together.

Growth Channel Considerations

[0039] As discovered by the inventors herein, harvested MSCs of the invention provide optimal restorative/regenerative therapy when implanted back into a patient by no later than the 10th *ex-vivo* passage, and preferably no later than the 5th *ex-vivo* passage (one passage being equivalent to harvest and plating of cells to allow for enhanced cell numbers for medium and/or tissue culture housing/substrate). As such, each patient's MSCs must be expanded to the necessary numbers, for their therapeutic use, in a limited number of passages without drugs or growth factors that are not FDA approved. Embodiments of the invention provide growth channel conditions for ensuring that harvested MSCs are expanded to the required amount using platelet lysates and are therefore optimized for implantation and use in the target patient.

[0040] Various considerations exist for determining a patient's MSC growth potential (for ensuring the required number of cells), *i.e.*, individualized growth characteristics (see definition above). These considerations include the source of the MSCs, *i.e.*, age, gender, hereditary restraints and presence of degenerative disorders like osteoarthritis.

[0041] With regard to MSC's harvested from patients having osteoarthritis, the inventors have identified two different cell growth types: "slow growth" and "fast growth." The presence of slow growth cells in a patient presents a practical problem, the ability to expand cells quickly and keep maximum differentiation potential within this group of cells. Cells that are not in maximum differentiation potential are likely to fail during implantation. Crisostomo et al., *Shock*, 2006 26(6): p 575-80. Therefore, higher levels of the patient's platelet lysate are required to stimulate the required MSC growth. As a result, these cells must be treated quite differently from MSCs isolated from a young, healthy individual. Note that cells showing limited *ex vivo* expansion potential, *i.e.*, cells increase in number by less than 100% over the course of a passage time (<3 days) are considered slow growth for purposes of this invention.

[0042] In one embodiment of the invention, the amount of platelet lysate required for optimum MSC *ex-vivo* culture expansion is determined from monitoring harvested cells for growth under various growth conditions. This is particularly important where the cells are associated with a patient having osteoarthritis, osteoporosis, AVN, or other diseases of bone, cartilage, or connective tissue. MSC expansion is dependent on a number of variables: amount of growth factors in patients' platelet lysate (therefore modifying the % lysate required to maximize cell growth), the bioavailability of those growth factors (*i.e.*, effect of these factors on the patient's cells), the relative concentration of those growth factors, and quality/quantity of patients' starting source cells. In one embodiment of this invention, to optimize the MSC growth under these variables a "growth channel" has been developed herein, *i.e.*, the targeted expansion rate of a patients' cells in

relation to a predetermined amount of time and/or cell passages (not more than 10 passages for optimal growth conditions). This growth channel takes into account all of the necessary cell culture decisions needed to produce a specific homogeneous cell population.

[0043] In one embodiment of the invention, the amount of platelet lysate required for targeted MSC *ex-vivo* culture expansion is combined with visual parameters to determine the optimum growth conditions. In particular, this embodiment requires that platelet lysate considerations discussed above be combined with consideration of colony formation of the harvested MSC and monolayer expansion of the MSCs. In one aspect, during colony formation the MSCs must be prevented from overgrowth, *i.e.*, cells on the edge of the colony enclosing the colony must be prevented. In another aspect, during colony formation the MSCs must be prevented from undergrowth, *i.e.*, cells not expanding. If MSCs during colony formation overgrow, they must be removed from the colony formation culture and placed into monolayer culture and if MSCs during colony formation undergrow, the medium should be partially removed (approximately half) and replaced with fresh medium (plus at least the previously used platelet lysate concentration). In another aspect, cells in monolayer expansion must be viewed for overgrowth, *i.e.*, high density, and reseeded at, for example, 10,000-12,000 cells/cm or undergrowth where cell morphology shows rounded, flag, or bloated cell shape. When cells show this morphology the platelet lysate concentration should be increased to at least 10-15% platelet lysate.

[0044] More specifically, the "Growth Channel" herein described for maximum expansion and repair capabilities for cells grown in platelet lysate encompasses four different aspects:

1. Cell Growth Rate in Monolayer Culture;
2. Cell Density in Monolayer Culture;
3. Cell Culture Pattern in Monolayer Culture; and
4. Cell Morphology in Monolayer Culture.

[0045] These concepts are also explained further in graphical format in Figure 1.

[0046] "Cell Growth Rate in Monolayer Culture"-Since platelet lysate has variable levels of growth factor from patient to patient, there is no method to determine the biologic activity of those factors until their impact on culture expansion rates are accessed. As such, one key component of expansion within the "Growth Channel" is a minimum rate of growth. This is defined as adjusting the parameters of platelet lysate concentration and/or seeding density until doubling time is between 1 and 3 days. This will accomplish approximately a 50-100 fold increase in cells before the 5th-7th passage (Figure 1, Graph 1). Also shown in Figure 1 (graph 2) is an exemplar of a growth rate channel beginning with a bilateral 10 cc marrow draw (used for illustration purposes only). Deviation from the growth channel discussed above would require a cell culture action as described in Figure 1, graph 1 and associated descriptions. "Cell Density in Monolayer Culture:" -- Cell density can impact cell growth and differentiation capacity. Doucet (referenced above) described a very low seeding density of approximately several thousand cells per milliliter (in otherwise healthy controls). However, we have found that patients with diseases such as osteoarthritis, AVN, and fracture non-union require a much higher seeding density and that maintaining that density during passage is critical to producing expanded cells capable of repair. As such, Figure 1, graph 3 shows the acceptable cell confluence channel for an embodiment of this invention (to optimize the in-vivo repair capabilities of the cells). Spatial distribution of MSC's described by this invention can be quantified by the following equation: $\text{Surface area} \times (\% \text{ Confluence}) / \text{Cell number}$. This Figure should be in the range of 18-23. If the value <18, then the cells can be seeded at a lower seeding density as they are growing exceptionally well. If this value falls between 23-27, then the cells should be seeded at a higher seeding density (from 12×10^3 cells/cm² to 15×10^3 cells/cm²). And if this value is >27 then the platelet lysate concentration also should be increased.

[0047] Cell Culture Pattern in Monolayer Culture- The pattern of cell growth is important in this invention as evenly distributed distance between cells is required to promote continued expansion and maintain cells in an undifferentiated state. To ensure this, evenly distributed MSC's are the part of the growth channel described herein. This is further illustrated in Figure 1, graph 4. Any deviation from the cell culture pattern discussed above would require a cell culture action as described in Figure 1, graph 4 and associated descriptions.

[0048] Cell Morphology in Monolayer Culture: The cell morphology in monolayer culture is important to insure a specific MSC phenotype associated with this invention. Only as it applies to mesenchymal stem cells, the preferred morphology is spindle shaped (fibroblastic) in monolayer culture. It should be noted that other mesenchymal stem cells lines often appear to have a polygonal or flag shaped morphology, which is not the phenotype in the growth channel as described. Note that a classification system has been established for the purposes of this invention which encompasses types 1-4, with the preferred cell type associated with this invention as type 1. This is further illustrated in Figure 1, graph 5. One should note that grades 2-4 displayed as part of Figure 1, graph 5 are from the prior art and each of the cited authors considered these to be acceptable morphology for their MSC lines. Again, the preferred morphology to stay within the described growth channel is spindle shaped with the cell occupying little surface area, unlike the MSC's in types 2-4 which occupy approximately 50% more surface area than the optimal type 1 cells. Once the culture has >30% of grade 2-4 MSC's, the concentration of platelet lysate should be increased from 10% to 15-20%. The cell population should be

transplanted at this point to avoid the potential deviation from the ideal morphology.

[0049] Any deviation from the cell morphology channel discussed above would require a cell culture action as described in Figure 1, graph 5 and associated descriptions.

[0050] In one embodiment, a growth channel represents the growth characteristics of autologous MSCs in *ex vivo* culture required to obtain 10 million to 100 million cells for implantation into a target site, a knee joint for example. The number of expanded cells is somewhat dependent on the target site in need of regeneration, for example regeneration of a vertebral disc requires approximately 1-10 million cells per ml whereas the number for a knee surface requires approximately tens to hundreds of millions of cells per ml. Harvested cells are monitored and growth modified through the use of varying amounts of autologous platelet lysate.

[0051] *Ex vivo* expanded MSCs of the invention can be monitored for growth via cell counting techniques and/or through visual cell culture parameters. Cell counting techniques are based on harvesting and passing cells from a tissue culture housing or substrate when the number of cells has exceeding the available space/density for those cells. Cell counting techniques must be performed on site by a qualified technician who can harvest and count the cells in a counting chamber device, such as a hemocytometer, or spectrophotometrically. As described elsewhere as part of this invention, cell counting can also be performed remotely through transmission of digital images via the internet to an experienced technician.

[0052] As discussed above, visual cell culture parameters disclosed herein include the capacity to visually inspect the cells of the invention to determine the readiness of cells to be harvested and re-plated. Visual parameters include cell culture morphology, cell culture pattern and cell culture density. In particular, the following specific qualitative parameters can be viewed: colony formation overgrowth, colony formation undergrowth, monolayer expansion culture overgrowth, monolayer expansion culture undergrowth, images of the hemacytometer for platelet and stem cell count; number of cells sticking when first seeded into a flask after the marrow draw; colony formation and later developed colonies to determine when the cells should be picked (including overgrowth and undergrowth); how evenly the cells are seeded into flasks, *i.e.*, cells should be uniform within flask during monolayer expansion; how densely cells are seeded; stages of confluence as well as visualizing cell division to determine when the cells are ready for passage; how bloody the flask looks when the cells are in a colony; separation of blood after it is spun down to obtain platelets; what the separation in the marrow should look like after spinning it down to separate the nucleated cells from the red blood cells; and cell morphology, *i.e.*, bloated, bright balls, spread out.

[0053] Visual inspection of target cultures can be performed either on-site via trained tissue culture personnel, remotely via a digital microscopy video technology (live feed) or via updated pictures taken by a digital microscope camera over the course of the growth channel procedures discussed herein. Remote monitoring of cell cultures can be performed where more limited control over multiple cell culture sites are required. For example, where a highly trained specialist of specialists are provided visual data from a number of off-site cultures. The highly trained specialist would have access to information that would validate the use of certain visual parameters, *e.g.*, a particular cell density, culture pattern, or morphology that has provided good clinical results for knee joint regeneration, avascular necrosis stabilization, healing of bony non-union fractures, etc. The specialist would then know to associate that culture morphology with all such cultures (whereas a high number of on-site personnel may not make this correlation for many months or years, if ever). In one embodiment, cells having consistent, non-overgrown, cell culture morphology would be considered optimal and within the growth channel and therefore ready for harvest and re-plating. used in

[0054] Platelet lysate compositions used in the invention include a number of growth factors known to be necessary for cell mitosis, including: hFGF, PDGF-BB, TGF- β , and VEGF. Platelet lysate compositions are added to serum free growth medium to obtain the targeted amount of lysate in the medium, for example, a 10% platelet lysate includes by volume 10% platelet lysate composition. In preferred embodiments the serum free basal medium is DMEM, Hams F12, MEM, or other like medium. Amounts of useful growth factors are inherent to a patient's platelet lysate and will typically vary from patient to patient.

[0055] In typical embodiments, cells from a patient are initially cultured in a medium having 10% platelet lysate for 7-10 days in colony formation and then recounted (colony formation). If the patient has osteoarthritis, the cells are transferred to monolayer culture using a starting 10 % platelet lysate. Cells are grown for 2-4 days and compared to the total number of cells required for the particular patient's therapeutic procedure. In some embodiments the cells are visually inspected. Cells that are not within the growth channels described above will have their culture medium modified to an enhanced amount of platelet lysate (for example 15-20%), whereas cells that are within the channel will be allowed to proceed for at least 2-4 days before the procedure is repeated. Other cell culture actions that depend on channel conditions include reseeding cells at a lower or higher density, changing medium more or less frequently, or transplanting cells sooner or later. Note that visual parameters can also be utilized to determine whether the cells are within the growth channels described (see above).

[0056] Note that variability of MSC expansion rates are dependent both on the patient's harvested MSCs as well as based on the levels of growth factors within the patient's platelets. As such, in certain instances, higher levels of required platelet lysate to keep a patient's MSC within the growth channel are due to, not only the cells growth characteristics,

but also on needing more platelets to provide the required levels of growth factors, *i.e.*, where the patient's platelet lysate may have a lower concentration of growth factors as compared to other platelet lysates. Surprisingly, the inventors herein have determined that cells grown under optimal conditions are much more capable of achieving a therapeutic result as compared to a same number of cells grown under non-optimal (non-growth channel) conditions (for example a culture of cells that require 15 passages to have a sufficient number to perform the required therapeutic result). For example, the inventors have discovered that cells grown for less optimal expansion using a 10 cc bilateral PSIS marrow draw (total 20 cc marrow) have produced poorer clinical results (no or less cartilage regeneration noted on follow-up MRI, no or less meniscus regeneration) as compared to cells optimally expanded using the growth channels methodology described herein.

Method for Autologous MSC Replacement

[0057] Further described herein are methods for the therapeutic restoration of a site in a patient in need thereof. For example, therapeutic restoration of a degenerative disc or cartilage of a joint in need thereof. Other examples include the expansion of MSC's for cardiac muscle regeneration, cutaneous wound healing, healing of fracture or bony non-unions, neural repair, treatment of graft vs. host and other immune applications, and other uses, replacement of pancreatic islet cells, treatment of osteoporosis, treatment of hearing loss, and other uses. Methods described herein utilize autologous MSC restorative therapy where the MSCs are treated with natural (non-synthetic/non-recombinant) growth factors (typically obtained by culture with varying percent platelet lysate).

[0058] Initially, a MSC source is harvested from a patient in need of stem cell therapeutic restoration. The harvested source is maintained in a sterile environment and under sterile conditions throughout the procedure. As discussed previously herein, the harvested cells are seeded and *ex vivo* cultured under conditions to maintain the cells within the growth channel embodiments of the invention (MSC isolation from a source is described above). This requires that platelet lysate from the patient be obtained and prepared, for example as described in Example 1. Autologous MSCs grown under optimal expansion conditions are monitored and prepared for implantation prior to the cells being passaged 10 times with a target yield of 10-100 million cells (a total number of cells for the target site are identified, see above). In some embodiments the autologous MSCs are grown and monitored for implantation prior to the cells being passaged 5 times with a target yield of 10-40 million cells. In other embodiments the autologous MSCs are grown and monitored for implantation prior to the cells being passaged 6, 7, 8 or 9 times with a target yield of 10-40 million cells. The prepared MSC composition is then implanted into the target site and monitored for effectiveness over the next several months. The procedure can be repeated dependent upon desired result. Cells that have been treated under conditions that result in 10 to 40 million cells within 4-7 passages, are optimal cells for implantation into a target site in a patient in need thereof.

[0059] In particular, in some embodiments, this invention also encompasses a novel method of seeding red blood cells with isolated marrow nucleated cells in initial colony formation and attachment culture. Since red blood cells also contain growth factors, this further supplements the cell growth environment and has other differentiating effects on the isolated MSC's.

[0060] Note that embodiments of this method are performed with autologous cells and growth factors thereby avoiding a number of immunologic and infectious issues inherent in other non-autologous MSC replacement therapies. This is of significant importance at this juncture, due to recent research demonstrating that non-autologous MSC's activate the natural killer cell system in the host. Spaggiari et al., Blood, 2006 107(4):1484-90; Rasmusson et al., Transplantation, 2003 76(8):1208-13. These embodiments also optimize the potential for the implanted cells to expand at the site and differentiate into the required cells (chondrocytes at a joint surface, osteoblast in a bone defect, etc). As described herein platelet lysate compositions (or platelets themselves) can also be injected into the site concurrently or subsequently to the MSC implantation.

[0061] Using the cell growth embodiments described herein, optimally grown cells are prepared that may be used in a target patient. Cells grown in accordance to embodiments described herein were tested using FACS to determine cell phenotype, *i.e.*, determine the cells surface antigen profile. A cell population may be selected and expanded to be positive for at least one of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD105 and CD166. Note that a positive result is one where at least 90% of the tested cells are positive by FACS analysis for the particular cell surface antigen.

[0062] As described herein typical identified cell populations are positive for at least two of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD105 and CD166. More typical cell populations are positive for at least three of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD105 and CD166. Even more typical cell populations are positive for at least four of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD105 and CD166. Still more typical cell populations are positive for at least five of the following cell surface antigens: CD29, CD44, CD59, CD73, CD90, CD105 and CD166. Finally, some cell populations described herein are positive for six or all seven of the CD29, CD44, CD59, CD73, CD90 and CD105 cell surface antigens. Cell populations having these potential cell surface antigens are shown herein to be optimal for purposes of therapeutic use. In addition, cell populations

described herein have the above cell surface antigens but lack at least one of the following antigens: CD14, CD31, CD45 and CD106. Alternatively, the cell populations have the above cell surface antigens but lack two or more of the following antigens: CD14, CD31, CD45 and CD10, or the cell populations are positive for CD29, CD44, CD59, CD73, CD90, CD105 and CD166 cell surface antigens but negative for CD14, CD31, CD45 and CD106 cell surface antigens.

Direct Platelet Injection into Target Site

[0063] As described herein, a platelet composition may be directly injected into a patient's target site. Platelet injections can be performed prior to MSC replacement, combined with MSC replacement (contemporaneous), or after MSC replacement to help optimize the MSC growth environment. In typical embodiments, MSCs expanded using the platelet lysate based medium and growth channel considerations of the invention are harvested (while they are in the growth channel). As described herein, they may be injected into a target site with either autologous platelets or platelet lysate.

[0064] In order to determine the number of platelets required to inject directly into a site the following calculation can be performed. The first issue is to determine the average platelet number/cc required to sustain maximum *ex vivo* MSC growth. For example, if a patient's cells were shown to require a 10% platelet lysate for 3 days, a 20% platelet lysate for 3 days and a 30% platelet lysate for 3 days, the highest platelet lysate concentration is utilized in determining how much platelet supplementation is required at the site. The maximum usage of platelets per cc per day was 30% (for three days) in this example. If the starting volume of platelets per cc of volume for the patient was 1.0×10^9 , during this time period 3×10^8 platelets used per cc of volume over three days or 1×10^8 platelets per cc per day is necessitated.

[0065] Marineau et al., Biomaterials, 2004 25(18): p 4489-502 provides insight into the levels of thrombin and calcium necessary to promote *in vivo* MSC growth via release of growth factors from platelets. This is a natural occurrence during the first weeks of development to promote tissue growth and angiogenesis. From these studies it can be deduced that activated platelets will release most of their cargo of growth factors upon activation with thrombin and calcium over a 7 day period. As such, the 1×10^8 platelets per cc per day is multiplied by 7 to provide the final amount of platelets required per cc - 7×10^8 platelets per cc. The final volume for injection into the patient is added to the initial fluid joint volume (IFJV), which represents the final fluid joint volume (FFJV). Therefore, using the above calculation, 7×10^8 platelets per cc is multiplied by 12.5 to yield 8.75×10^9 platelets. This number represents the final platelet dose (see formula 1):

[0066] (Average Platelet per cc to sustain MSC growth/Days at this level before medium change) x (Days at this level before medium change required ((7) (Final fluid joint volume)) = PHC Platelet Dose. (Formula (I)).

[0067] Alternatively, the supplementation can be carried out using platelet lysate equivalent to the highest *ex-vivo* percentage required to promote expansion, adjusted for joint volume, and supplemented more frequently.

Site Monitoring of MSC Implantation

[0068] Implanted cells described herein can be monitored to ensure that these cells are surviving *in-vivo* and that the cells ultimately differentiate into the cell type required to obtain the needed repair. As described herein, MRI labeling is performed to allow for non-invasive monitoring of the patient's site (however, note that this procedure requires magnetic particles and only provides cell location, not cell expansion or differentiation state).

[0069] Real-time cell monitoring is therefore preferred. After transplantation of the optimally expanded cells, a percutaneous fluid wash is taken from the implantation site. Free floating cells and minimally adherent MSCs are obtained and examined for number of cells, type of cells, differentiation state of MSCs (if any), MSC appearance and for the MSCs state of proliferation. The joint wash can also be monitored or assayed for the expression of key substances such as glycoaminoglycans, key proteins, gene expression, or other important chemical or genetic indicators of improvement in the joint microenvironment.

[0070] Two types of real-time monitoring can be performed: a random sampling of site fluid and/or a high pressure "knock-off of site fluid. The high pressure "knock-off" is performed using a needle or catheter (typically equivalent to a 14-22 gauge) where high pressure fluid is pushed through to knock off minimally adherent MSCs.

[0071] Alternatively, needle arthroscopy or traditional arthroscopy to obtain tissue samples for analysis can also be used.

[0072] Percutaneous sampling is performed at baseline prior to the MSCs being transplanted (which form a sample which can be compared to all future samples). Sampling is also performed at 1 week, potentially at 2 weeks and potentially at 3 weeks post implantation of MSCs into the target site.

[0073] In particular, at one week post implant, a joint wash or tissue sample is taken and examined. While it is taken for granted that the cell population can be easily examined *ex-vivo* and adjustments to growth media made, without an *in-vivo* sampling method, the same needed adjustments can not be made to ensure *in-vivo* growth and engraftment. However, based on this real-time monitoring, changes in platelet and/or platelet lysate supplementation can be performed into the target site. This process can be repeated based on need. In addition, additional autologously cultured MSCs can be implanted into the site.

[0074] Finally, upon a determination that the implanted MSCs are alive and capable of proliferation and or that the joint microenvironment is appropriate for cell survival and engraftment, a differentiation agent can be contacted to the patient's site. In addition, cells obtained from the real-time monitoring analysis can be cultured in the presence of various differentiation agents to determine which agent is best suited for the required result. Illustrative agents include bone morphogenetic protein 2, dexamethasone, hyaluronic acid, and the like. In addition, where inflammatory cells are recovered in the real-time monitoring, an antiinflammatory agent can be included in the treatment or where the site is dehydrated a hyaluronic acid can be added.

[0075] In addition, *in-vivo* post implantation of cells can be monitored using assay methods for other secondary effects of tissue regeneration such as production of glycoaminosglycans (GAG's), reduction in known degradative enzymes, and other factors. The important aspect described herein is that direct or indirect monitoring of the cells continues after implantation. Again, this allows for real time changes in post-transplantation protocols to ensure cell survival and engraftment as well a function once differentiated. As an illustrative example, MSC's once differentiated into an early stage chondrocytes would be monitored for GAG production so that they would be considered fully functional and biologic equivalent of a mature and health chondrocyte. In addition, certain differentiating or supplemental substances may be introduced into the joint for the purposes of increasing the monitored GAG production. This same example could also be applied to other areas of tissue regeneration such as the replacement of pancreatic islet cells and the monitoring of insulin production from MSC's differentiating into islet cells *in-vivo*.

Therapeutic Applications

[0076] Methods and compositions described herein can be used to treat, *i.e.*, repair or maintain a target site in a patient in need of a MSC application. Conditions that can be treated include osteoarthritis, degenerative disc disease, cartilage replacement in joints, stabilization of a bony avascular necrosis site, healing of bony fracture or other bony non-unions, cardiac muscle regeneration, cortex repair, cutaneous wound healing, neural repair, cell therapy for immunosuppression or regulation, replacement of beta islet cells, replacement of cells and structures involved in hearing, treatment of osteoporosis, and other disorders where MSC's can differentiate into cells for repair and replacement of injured, missing, or degenerated cells.

EXAMPLES

[0077] The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1: Patients' MSC Growth In Relation to Growth Channel

[0078] Nucleated cells were harvested from a patient's posterior iliac crest and separated from the RBCs using centrifugation (serum is a gradient).

[0079] Approximately 10 ml of bone marrow was harvested from the target patient and transferred to a cell culture lab in a 15 ml centrifuge tube. The marrow sample was then spun down at 100g for 2-3 minutes. The RBC pellet was checked to ensure that the bulk of the RBCs were in the lower half of the sample and that a clear zone exists between the RBC pellet and top fraction of material. Note that if the top fraction is not 40-50% of the total volume, the spin step will need to be repeated. The top fraction was then removed and placed in a 15 ml centrifuge tube and spun at 1000g for 10 minutes. Note that the nucleated cell pellet may appear red and perhaps loosely packed, dependent on the number of RBCs present. The serum supernatant is removed and added back to the RBC pellet. The nucleated cell pellet is re-suspended in 1 ml of saline. The steps above were repeated to obtain additional nucleated cells.

[0080] The nucleated cells were then counted by diluting the suspension 1:20 in water (lysis RBCs) and counting nucleated cells. The RBCs were counted using a 1:2000 dilution.

[0081] Nucleated cells were then seeded for monolayer growth. For each cm^2 seed approximately $0.66 - 1.25 \times 10^6$ nucleated cells and 0.16×10^9 RBCs were combined (supplement RBCs in nucleated suspension with cells from RBC suspension). The combined cell mix was then spun at 1000g for 10 minutes and re-suspended in DMEM + Cipro + heparin + 10% platelet lysate (10% was chosen as a starting dose based on our data demonstrating a significant suboptimal expansion rate with 5% lysate as described by Doucet). The suspension was then warmed for 30 minutes at 37°C . The warmed suspension was plated into an appropriate sized tissue culture flask and fresh medium added. Cell culture was incubated for 7-12 days at 5% CO_2 , 37°C .

[0082] As shown in Fig. 1, MSC growth from 8 patients were plated under the above conditions and plotted for growth as a function of cell number versus days. An acceptable growth channel has been overlaid onto the cell number expansion. As of day 7, Gi cells are sufficient and optimal for implantation, at day 8 St cells are sufficient and optimal for implantation, and as of day 10 CI cells are sufficient and optimal for implantation. All other cells are not within an acceptable growth

rate and would need to have increased levels of platelet lysate added to the culture medium to ensure optimal growth.

Example 2: Patient MSC Growth is Dependent on Patient's Health

[0083] MSCs were harvested from individuals having osteoarthritis and grown as described in Example 1. Growth rates for each patient's cells were determined as well as overall yield. Harvested MSCs were grown on varying amounts of platelet lysate (5-10%) and plotted over the course of 11 days (500% concentration of patient native platelet concentration and lysis using freezing). The data is shown graphically in Figure 2, where MSC yield and rate of growth varied significantly. This Example illustrates the variability of growth found between patients and the need to optimize MSC growth rates.

Example 3: 5% Platelet Lysate is Ineffective At Optimizing MSC Growth

[0084] MSC harvested from patients having osteoarthritis (slow growth MSC), as described in Example 1, were expanded using either 5% platelet lysate, 10% platelet lysate or 20% platelet lysate. As shown in Figures 3A-E, many cell lines grown with 5% platelet lysate were unable to show maximum expansion as compared to cells grown on 10-20% platelet lysate. Note that for most cell lines grown on 20% platelet lysate conditions showed only minimal expansion benefit as compared to 10% platelet lysate. However, it must be stressed that our experimental data shows that patients with OA have extremely variable expansion rates, with a few patients able to hit growth channel targets at 5%, most at 10%, and some requiring 20% supplementation. In addition, patients with other conditions such as avascular necrosis of the femoral head, fail expansion in even 10% lysate and require multiple modifications only determined via experimental protocol (see following example).

[0085] Figure 4 further illustrates that MSCs harvested from osteoarthritis patients typically require higher levels of platelet lysate (10%+) to obtain optimum expansion. However, MSCs from healthy individuals having normal growth showed little variation under growth conditions of 5% or 10% platelet lysate.

[0086] The data in this Example illustrates that the conditions described by Doucet et al did not produce optimum expansion conditions under conditions where cells were harvested from osteoarthritic patients. These cells required higher percent platelet lysate in the growth medium to show optimum expansion.

[0087] However, under conditions where the cells are harvested and show "fast growth type," the cells were able to grow under conditions of 5 or 10% platelet lysate.

Example 4: Illustrative Growth Channel For 5 Different Patients

[0088] Five patients donated MSCs as harvested in Example 1. Cells were grown using varying lysate concentrations and cell number calculated. Cell growth data is shown in Figure 5, where a cell growth channel of the present invention is overlayed. Cells able to expand within the cell growth channel parameters are considered optimal and ready for use in a target patient, whereas the cell growth for patient 5 would be a poor yield with minimal chances of clinical success. As such, platelet lysate based moderation of the cells' growth conditions should be used to obtain cell growth within the recited growth channel. In addition, the culture decisions already described based on cell density, culture pattern, and morphology would also need to be applied.;

Example 5: MSC Cell Surface Antigens Present When Cells Are Grown Within Growth Channel Parameters

[0089] MSC were harvested and expanded using embodiments described herein and in Examples 1 and 2. To determine the phenotype of the cultured cells they were incubated with fluorescently labeled monoclonal antibodies (mAbs) directed against known stem cell surface antigens (MAbs used are listed in Table 1 and 2).

[0090] The expression level of the cell surface antigens on the cultured cells from 2 subjects was analyzed using FACSCalibur flow cytometer. Results are provided in Tables 1 and 2.

Table 1:

Mean Fluorescence Intensity			
Surface Antigen	Subject 1	Subject 1+ HA	Subject 2
CD14	5.78	4.66	5.05
CD29	216.56	221.47	243.44
CD31	7.18	5.68	5.21

(continued)

Mean Fluorescence Intensity			
Surface Antigen	Subject 1	Subject 1+ HA	Subject 2
CD44	775.36	828.8	543.91
CD45	5.46	5.73	5.4
CD59	1712.53	1684.87	1412.54
CD90	1222.7	1149.04	764.27
CD106	22.87	21.31	19.06
CD166	149.1	144	93.69
CD73	1653.22	1688.02	1438.74
CD105	1178.26	1169.62	1671.74

Table 2:

% Positive			
Surface Antigen	Subject 1	Subject 1 + HA	Subject 2
CD14	0.66	0.36	0.5
CD29	99.93	99.49	99.51
CD31	2.17	1.18	0.75
CD44	99.9	99.44	99.49
CD45	0.77	0.64	0.64
CD59	99.34	99.76	99.5
CD90	99.96	99.96	99.99
CD106	42.93	41.03	27.73
CD166	99.76	99.22	98.05
CD73	99.94	99.84	99.76
CD105	99.94	99.63	99.65

[0091] Table 1 shows the mean fluorescence intensity (MFI) for each target cell surface antigen. The percent positive value for each cell surface antigen is listed in Table 2. As shown in Table 2, greater than 99% of the cells optimized for implantation into a target patient expressed CD29, CD44, CD59, CD73, CD90, CD105 and CD166. Conversely, few cells expressed CD14, CD31, CD45 and CD106 which are not considered cell surface antigens generally present on the cells having optimal capacity for implantation into a target patient.

Example 6: MSC Implantation Using Embodiments Herein Provide For *In Vivo* Osteochondral Cartilage Replacement

[0092] A bone marrow sample was obtained from a 57 year old patient needing cartilage replacement to correct a defect in the knee. The bone marrow was harvested and MSCs isolated using the methods described in Example 1. Cells were grown on 10-20% platelet lysate over the course of 6 passages to obtain 10 million cells. Cells were maintained in the growth channel of the present invention. Cells were then implanted into the site using the autologous MSCs.

[0093] Figure 6 (A&B) show sagittal Fast Spin Proton Density images from a 3.0 T MRI. The cartilage defect in the posterior weight bearing surface of the lateral femoral condyle is shown (A). After 39 days the image was re-taken and the cartilage defect has been filled (see Fig. 6 B). The data from the Example shows the effectiveness of using the methods and compositions described herein to correct large cartilage defects at target sites.

Example 7: Timing of Higher Level Platelet Lysate Can Add Growth Momentum To Allow Slow Grower Types of MSCs to Hit Growth Channel Targets

[0094] The following Example illustrates the concept that altering platelet lysate concentration during the course of ex vivo expansion dramatically improves MSC yield. A 20% platelet lysate concentration ("lysate boost") was used in the initial expansion culture as cells emerged out of colony formation. Four patient cell populations were tested, symbolized as Re, Gi, Ve and Ca. Growth conditions were monitored and are shown in Table 3 and Fig. 7.

[0095] Of the four patients listed in Table 3, note that for Gi and Ve, the MSC fold increase in cell growth per passage was dramatically improved. For subject Gi the sum of fold increase per passage increased from 9.74 to 12.96 when the 20% lysate boost was used. For Re this metric almost doubled from 6.88 to 10.34. Of note, both of these patients are in the target demographic for regenerative therapies with MSCs (diagnosed osteoarthritis (OA) and in 5th and 6th decades of life). For the younger subject, Ve (without known OA), there was only a slight improvement in the sum of the fold increase per passage (5.0 to 5.7). In the oldest subject in the 7th decade of life and with severe multi-joint OA, there was also only a mild improvement in this measure (5.08 to 5.13).

[0096] Since half of the four subjects had a marked improved yield and the other two subjects had no decrement in yield and saw very slight yield improvement, the methods and compositions described herein are valid for improving MSC yield in a cohort of OA patients in need of regenerative medicine.

Example 8: Growth Conditions For A Patient In Need of MSC Implantation

[0097] 44 year old white female with avascular necrosis had 10 cc of marrow drawn from each PSIS and the cells processed per this invention. Her nucleated cell yield was very poor and her cells were grown in monolayer with 10% lysate, but failed to expand beyond the second passage. The patient was returned and an altered marrow draw technique was used to draw three small aliquots of marrow from the left and right PSIS and then cells were boosted with 20% platelet lysate while still in colony formation culture and then grown in 20% lysate. Figure 8 demonstrates both cell expansion graphs and highlights that many patients with this disorder require a altered marrow draw techniques to improve nucleated cell count, a lysate boost during colony formation culture, and much higher platelet lysate concentrations while in the monolayer culture expansion phase.

Example 9: Direct Injection of Cells

[0098] 37 year old white female with a 9 month old fracture of the humerus treated with Open Reduction and External Fixation and a bone stimulator. This fracture went onto significant non-union as demonstrated in figure 10a. The patient had 100 cc of marrow drawn from each PSIS and the MSC's were isolated and grown in 10% platelet lysate. These were then percutaneously implanted into the fracture non-union site via sterile trocar under fluoroscopic guidance. Figure 10b shows significant healing of the non-union at 5 weeks post injection of cells. This example illustrates the in-vivo osteogenic capabilities of the MSC's expanded with this invention.

Example 10: Knee Cartilage Replacement

[0099] 43 year old white male with severe degenerative disease of the medial compartment of the knee showing significant degradation of the medial anterior meniscus. Figure 11A shows a 3.0T proton density sagittal MRI image where the significantly degenerated medial meniscus is almost entirely absent anteriorly. Figure 11B is 3 months post after percutaneous implantation of MSC's obtained and expanded per the growth channel methods described herein. Figure 11B shows regeneration of the meniscus and subsequent 3-D image volume analysis demonstrated a 32.5% increase in meniscus volume. Note that meniscus regeneration occurred in the inner portion of the meniscus, in what is known as the "white" or "avascular" zone. For this to occur, vessels would have to be brought to the area. This occurred either as a property of this specific cell line or more likely due to the platelet lysate injections provided to the joint after implantation of cells. For example, it is well known that platelet lysate has significant level of VEGF capable of causing neovascularization.

Table 3: Fold Increase in Cell Growth Per Passage

Fold Increase In Cell Growth/Passage				
Passage No.	Re	Gi	Ve	Ca
	10%PL20%PL	10%PL20%PL	10%PL20%PL	10%PL120%PL
P1	3.6/5	5.6/5.4	2.6/2.6	3.1/1.95

(continued)

Fold Increase In Cell Growth/Passage				
Passage No.	Re	Gi	Ve	Ca
P2	1.48/2.19	2.18/3.36	2.4/3.1	1.98/3.18
P3	1.8/3.15	1.96/4.2		

[0100] While the invention has been particularly shown and described with reference to a number of embodiments, it would be understood by those skilled in the art that changes in the form and details may be made to the various embodiments disclosed herein.

Claims

1. A method for ex-vivo culture expansion of mesenchymal stem cells (MSCs) from patients having osteoarthritis, osteoporosis, avascular necrosis, or other diseases of bone or cartilage, the method comprising:

a) expanding the patient's MSCs with a culture medium comprising platelet lysate at a concentration of about 10% of the culture medium;

b) determining whether the patient's MSCs of step a) are within a growth channel **characterized by** the following parameters:

(i) a doubling time of 1 to 3 days;

(ii) a cell density within a range of about 18 to about 23 calculated according to the equation Surface area * (% confluence) divided by cell number;

(iii) even distribution of MSCs as a monolayer culture; and

(iv) a spindle-shaped cellular morphology in monolayer culture; and

c) adjusting the culture medium in the following passages, wherein the MSCs are outside of the scope of at least one parameter of step b) by increasing the amount of platelet lysate in the culture medium to between 15-20% platelet cell lysate;

and wherein the patient's MSCs are expanded for no more than 10 ex-vivo passages.

2. The method of claim 1, wherein

(a) the required dose of MSCs are between 1 million and 100 million cells; or

(b) red blood cells are used in the growth medium to improve attachment and colony formation of the MSCs.

3. The method of claim 1, wherein the MSCs are expanded to have optimized growth by a 10th ex-vivo passage or by a 7th ex-vivo passage.

4. The method of claim 1, wherein the patient is a patient who has been diagnosed with osteoarthritis.

Patentansprüche

1. Verfahren zur ex-vivo Kulturexpansion von mesenchymalen Stammzellen (MSCs) von Patienten mit Arthrose, Osteoporose, avaskulärer Nekrose oder anderen Erkrankungen von Knochen oder Knorpel, wobei das Verfahren Folgendes umfasst:

a) Expansion der MSCs des Patienten mit einem Kulturmedium umfassend Blutplättchenlysate in einer Konzentration von etwa 10% des Kulturmediums;

b) Bestimmen, ob die MSCs des Patienten von Schritt a) sich in einem Wachstumsprogramm befinden, **gekennzeichnet durch** die folgenden Parameter:

- (i) eine Verdopplungszeit von 1 bis 3 Tagen;
- (ii) eine Zelldichte innerhalb eines Bereichs von etwa 18 bis etwa 23, berechnet gemäß der Gleichung $\text{Oberflächenbereich} \cdot (\% \text{ Konfluenz})$ geteilt **durch** Zellzahl;
- (iii) gleichmäßige Verteilung von MSCs als Einschichtkultur; und
- (iv) eine spindelförmige zelluläre Morphologie in der Einschichtkultur; und

c) Anpassen des Kulturmediums in den folgenden Passagen, in denen die MSCs außerhalb des Bereichs von wenigstens einem Parameter von Schritt b) liegen, **durch** das Erhöhen der Menge von Blutplättchenlysat im Kulturmedium auf zwischen 15-20% Blutplättchenzelllysat;

und wobei die MSCs des Patienten nicht mehr als 10 Ex-vivo-Passagen lang expandiert werden.

2. Verfahren nach Anspruch 1, wobei

- (a) die benötigte Dosis von MSCs zwischen 1 Millionen und 100 Millionen Zellen liegt; oder
- (b) die roten Blutzellen in dem Wachstumsmedium verwendet werden, um die Anheftung und Koloniebildung der MSCs zu verbessern.

3. Verfahren nach Anspruch 1, wobei die MSCs für ein optimiertes Wachstum in einer 10. x-vivo-Passage oder in einer 7. Ex-vivo-Passage expandiert werden.

4. Verfahren nach Anspruch 1, wobei der Patient ein Patient ist, bei welchem Arthrose diagnostiziert wurde.

Revendications

1. Procédé de développement, en culture *ex vivo*, de cellules souches mésenchymateuses (CSM) de patients souffrant d'arthrose, d'ostéoporose, de nécrose avasculaire, ou d'autres maladies des os ou du cartilage, le procédé comprenant:

- a) le développement des CSM du patient avec un milieu de culture comprenant un lysat plaquettaire à une concentration d'environ 10 % du milieu de culture;
- b) la détermination du fait que les CSM du patient de l'étape a) se trouvent ou non dans un canal de croissance **caractérisé par** les paramètres suivants:

- (i) un temps de doublement de 1 à 3 jours;
- (ii) une densité cellulaire dans une plage d'environ 18 à environ 23 calculée selon l'équation $\text{Aire de surface} \times (\% \text{ de confluence})$ divisé par le nombre de cellules;
- (iii) une distribution homogène des CSM sous la forme d'une culture mono-couche; et
- (iv) une morphologie cellulaire en forme de fuseau en culture monocouche; et

c) l'ajustement du milieu de culture dans les repiquages suivants, dans lequel les CSM sont hors de la portée d'au moins un paramètre de l'étape b) par augmentation de la quantité de lysat plaquettaire dans le milieu de culture à 15 à 20 % de lysat de cellules plaquettaires;

et dans lequel les CSM du patient ne sont pas développées pendant plus de 10 repiquages *ex vivo*.

2. Procédé selon la revendication 1, dans lequel

- (a) la dose requise de CSM est de 1 million à 100 millions de cellules; ou
- (b) les globules rouges sont utilisés dans le milieu de croissance pour améliorer la fixation et la formation de colonies des CSM.

3. Procédé selon la revendication 1, dans lequel les CSM sont développées pour présenter une croissance optimisée par un 10ème repiquage *ex vivo* ou par un 7ème repiquage *ex vivo*.

4. Procédé selon la revendication 1, dans lequel le patient est un patient chez qui un diagnostic d'arthrose a été posé.

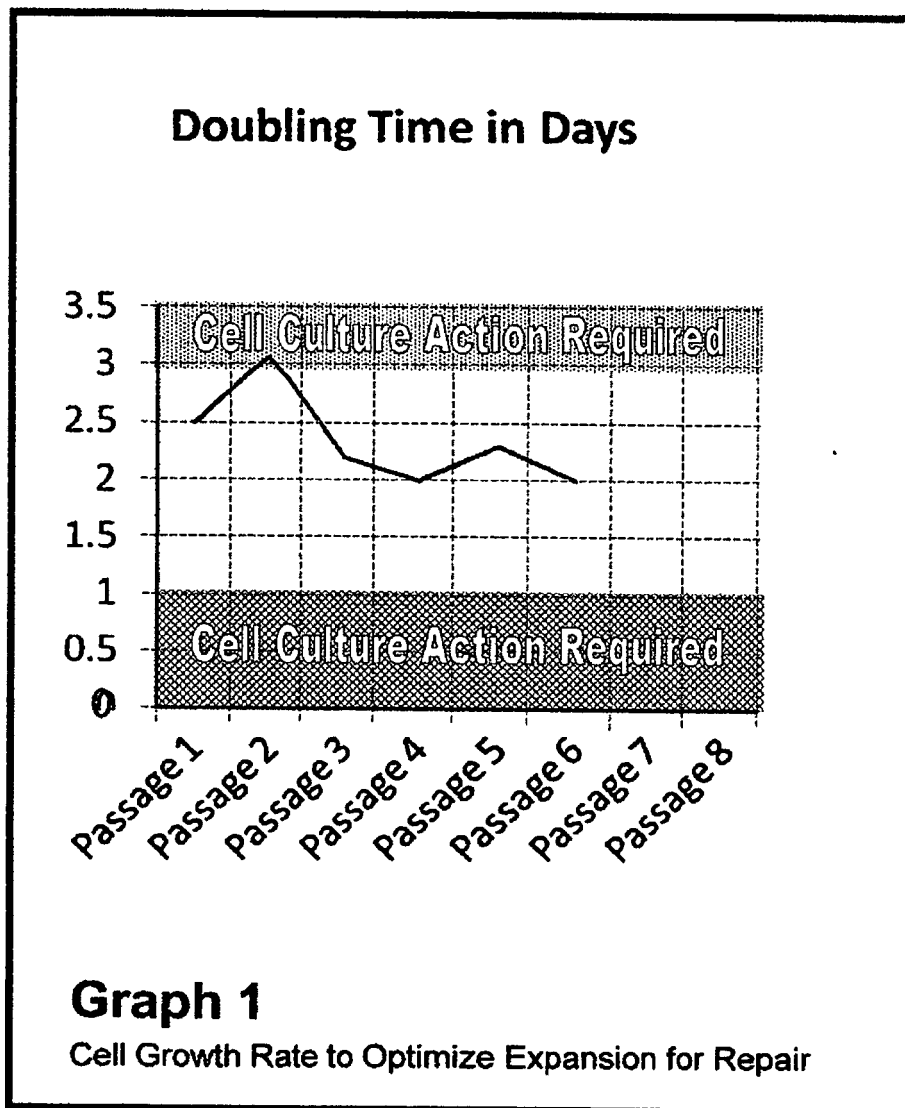


Fig. 1A

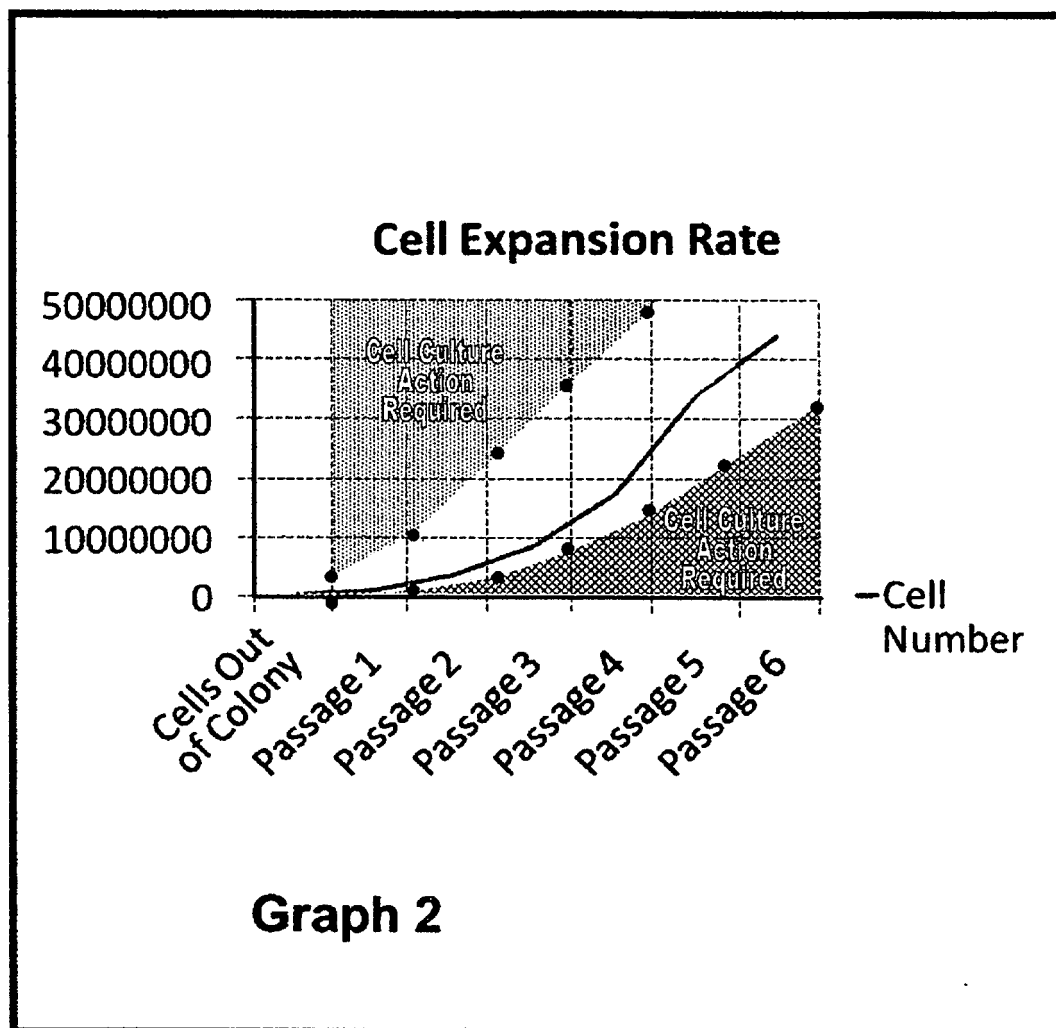


Fig. 1B

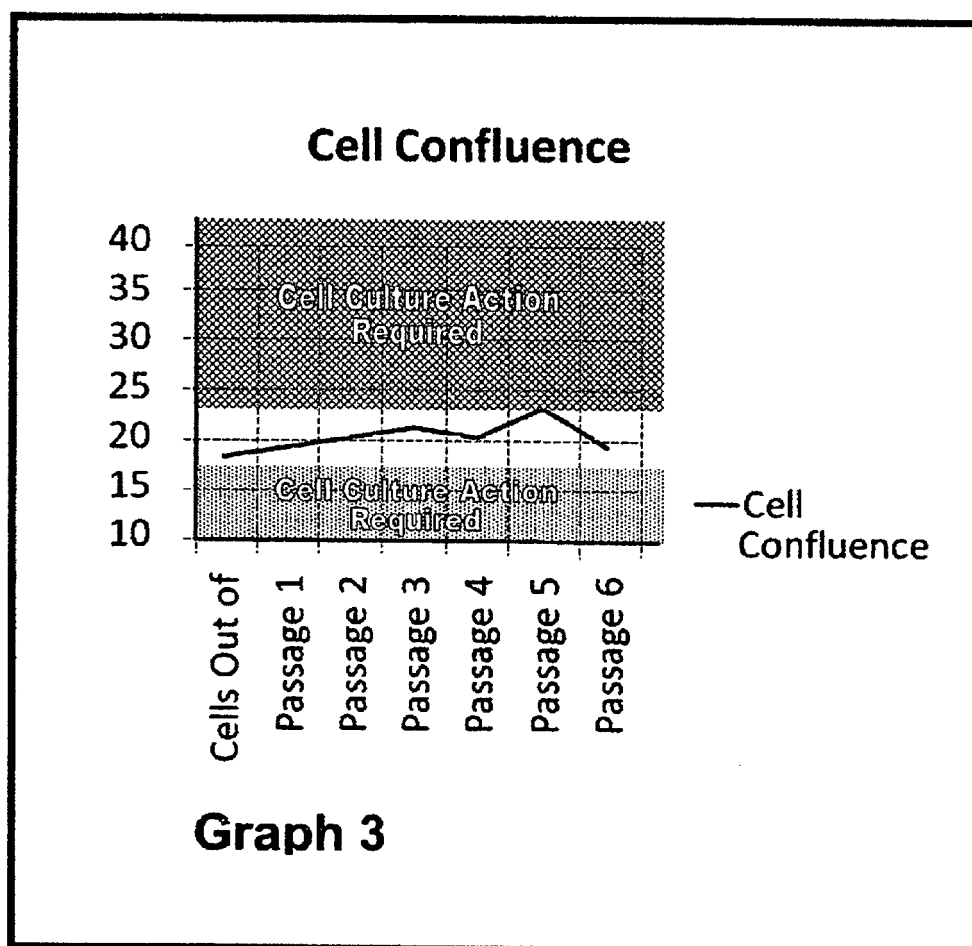


Fig. 1C

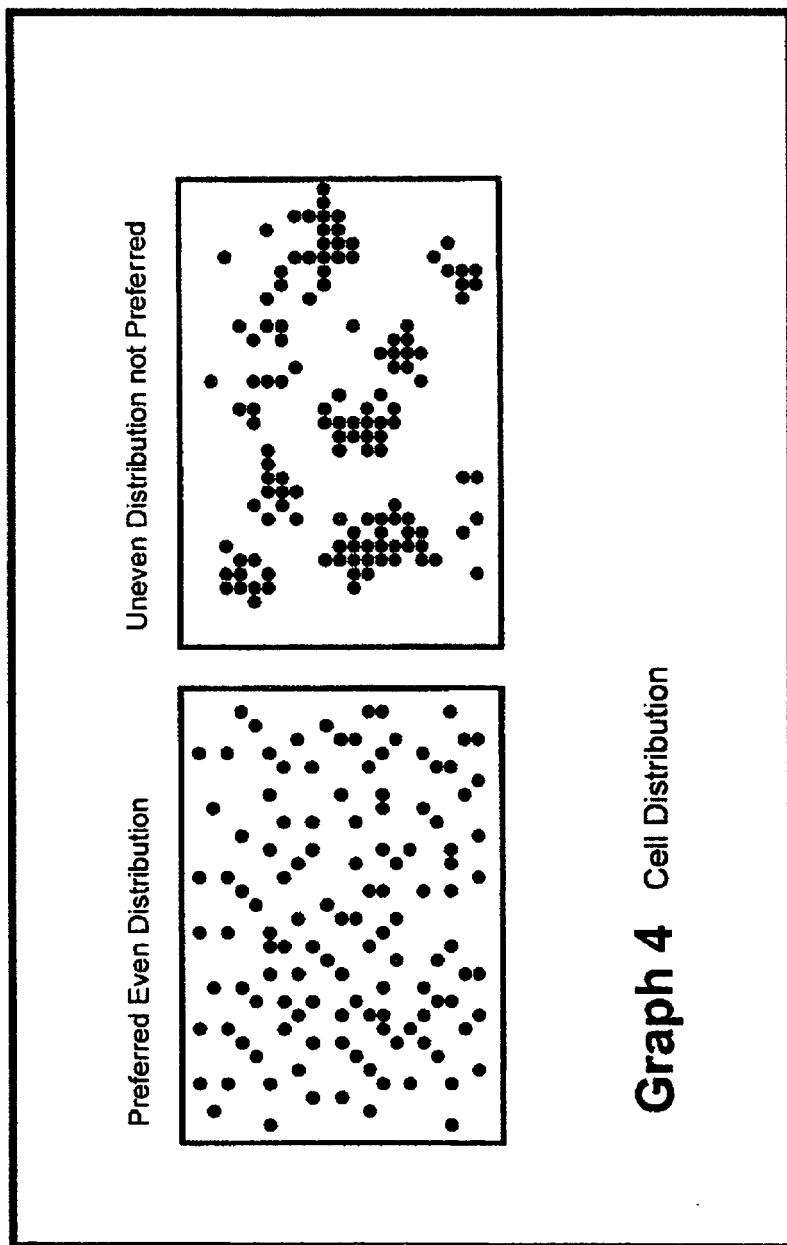


Fig. 1D

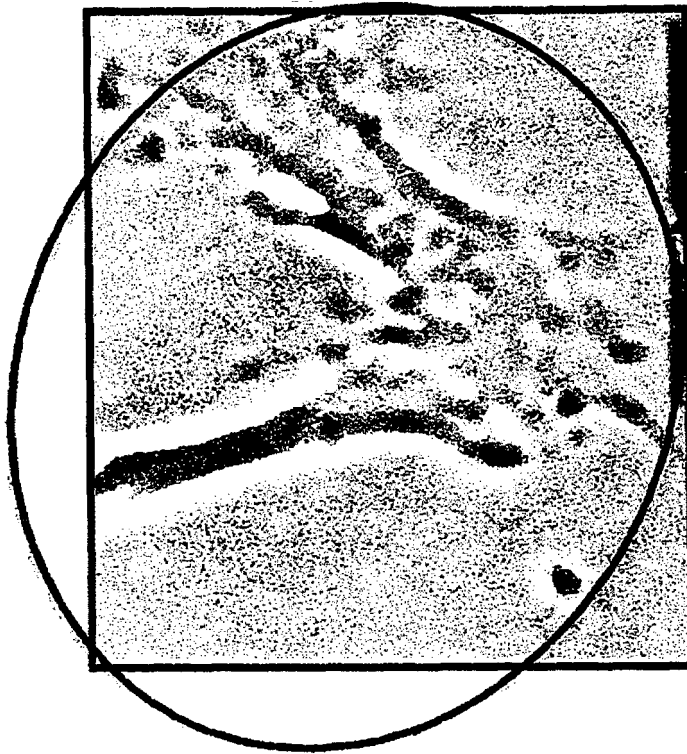


Fig. 1E-TYPE II



Fig. 1E-TYPE I



Fig. 1E-TYPE IV

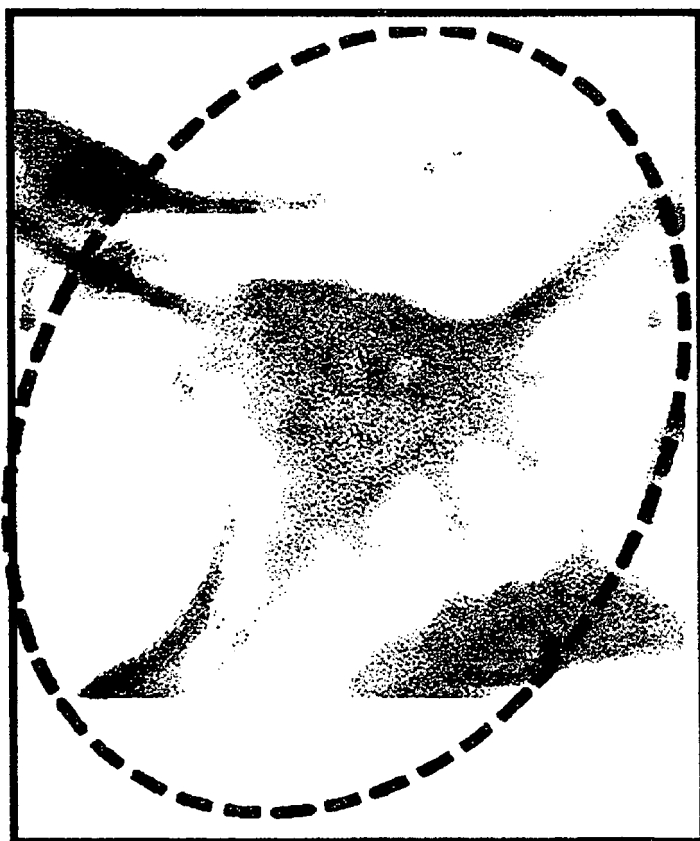


Fig. 1E-TYPE III

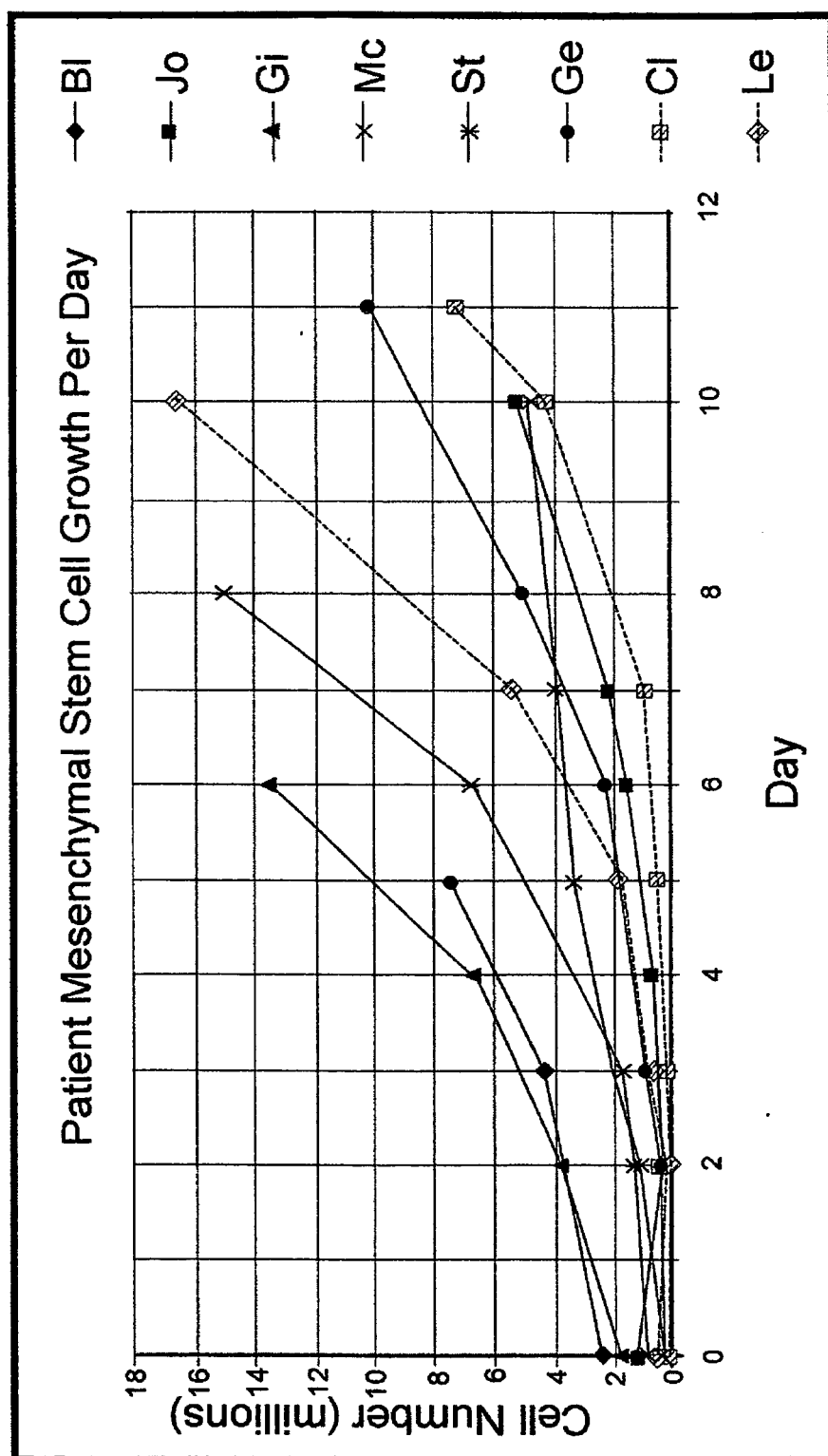
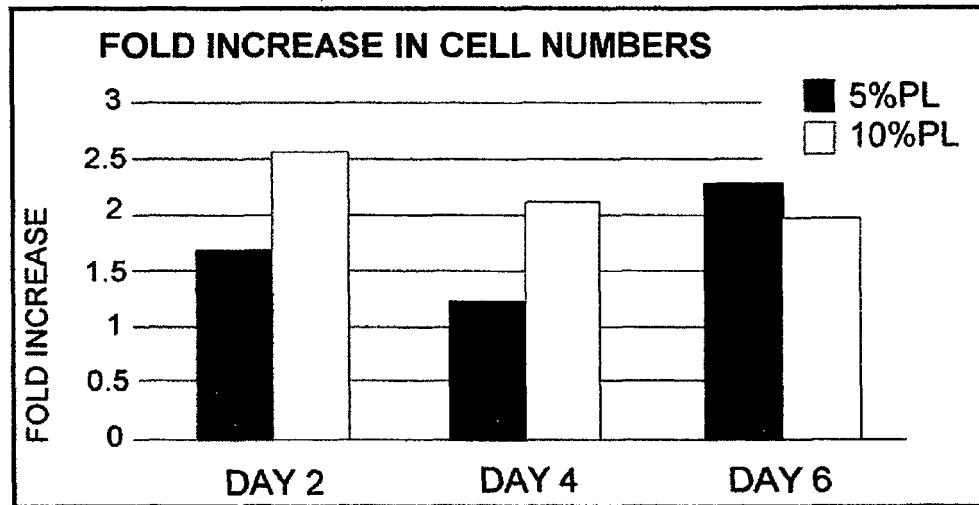
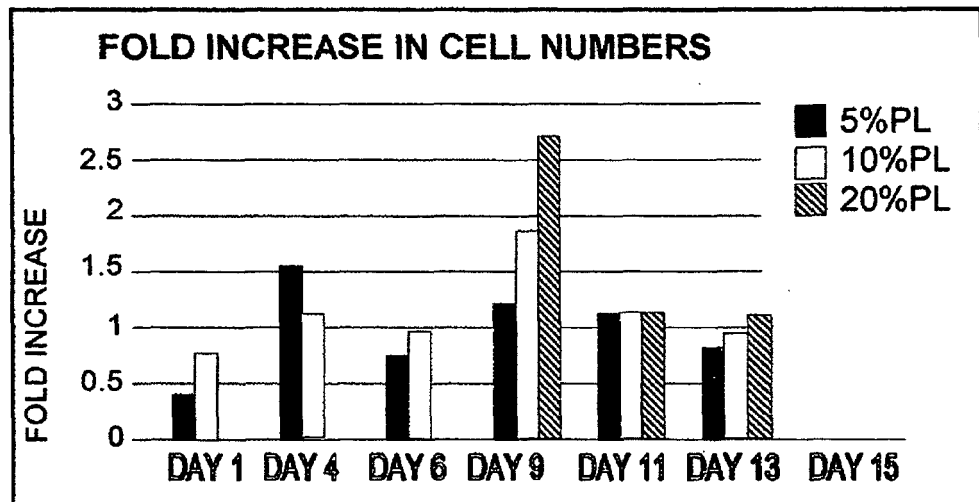
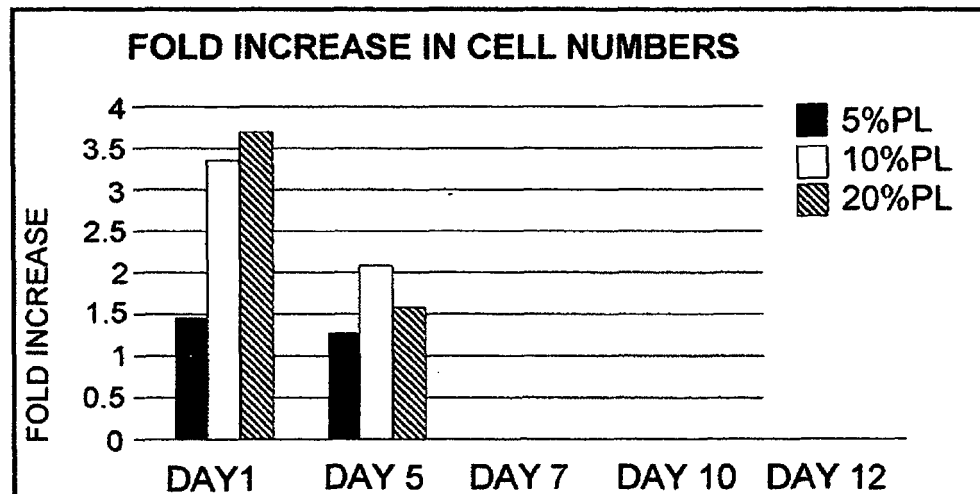
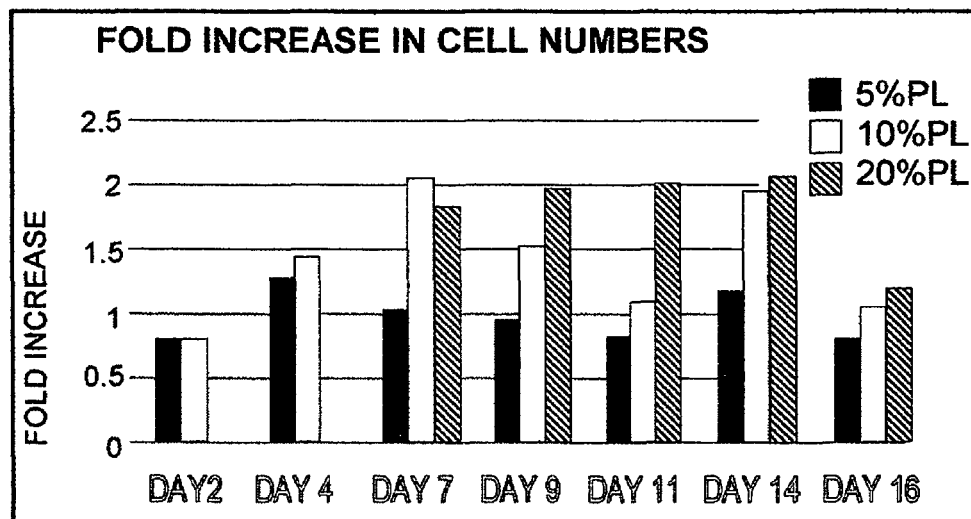
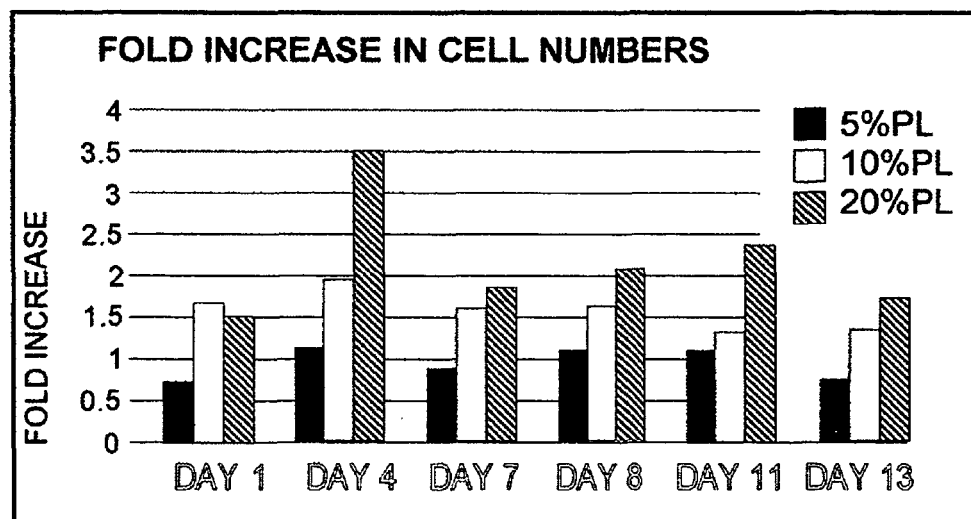


Fig. 2

Fig. 3A**Fig. 3B****Fig. 3C**

**Fig. 3D****Fig. 3E**

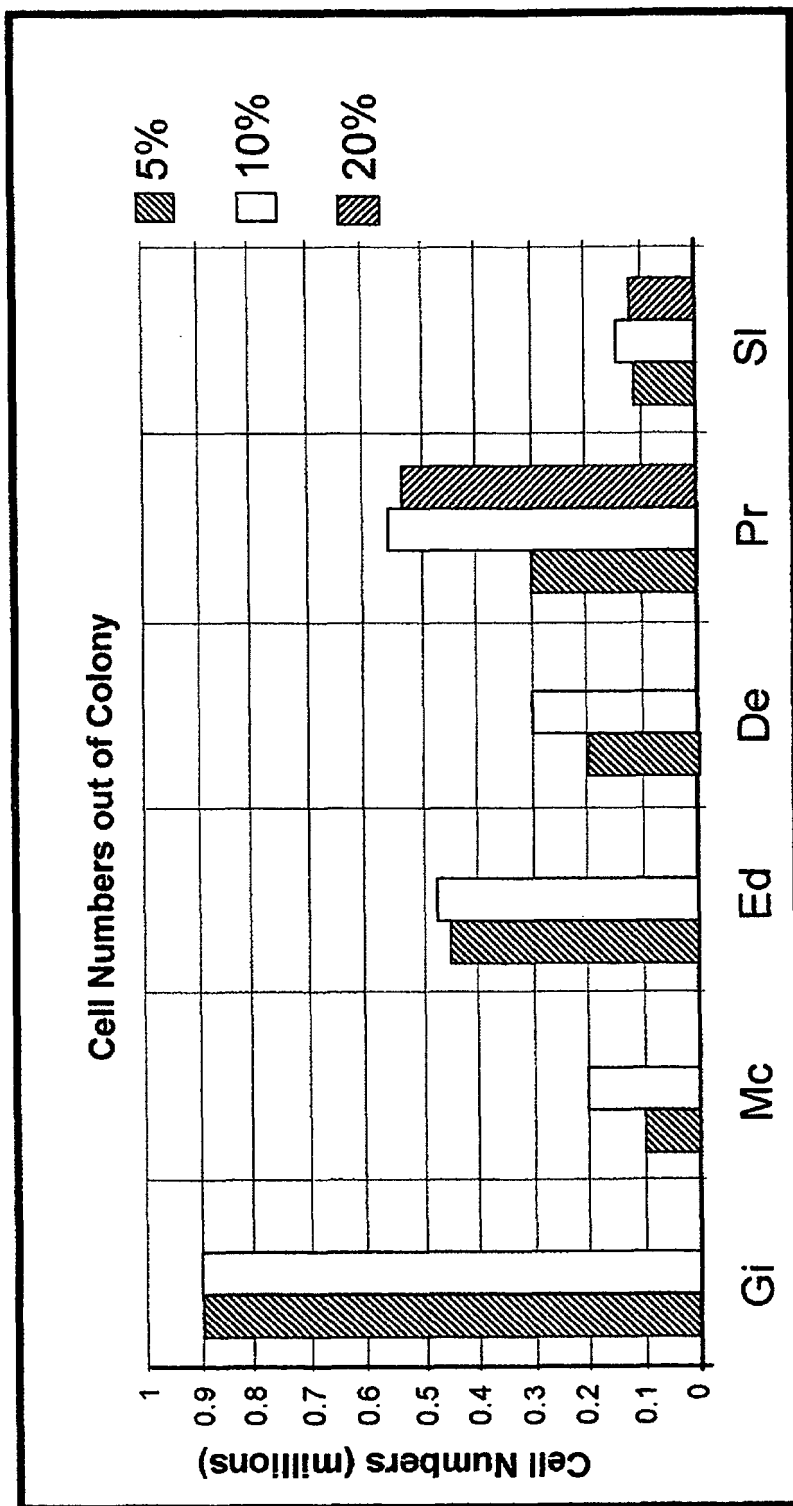


Fig. 4

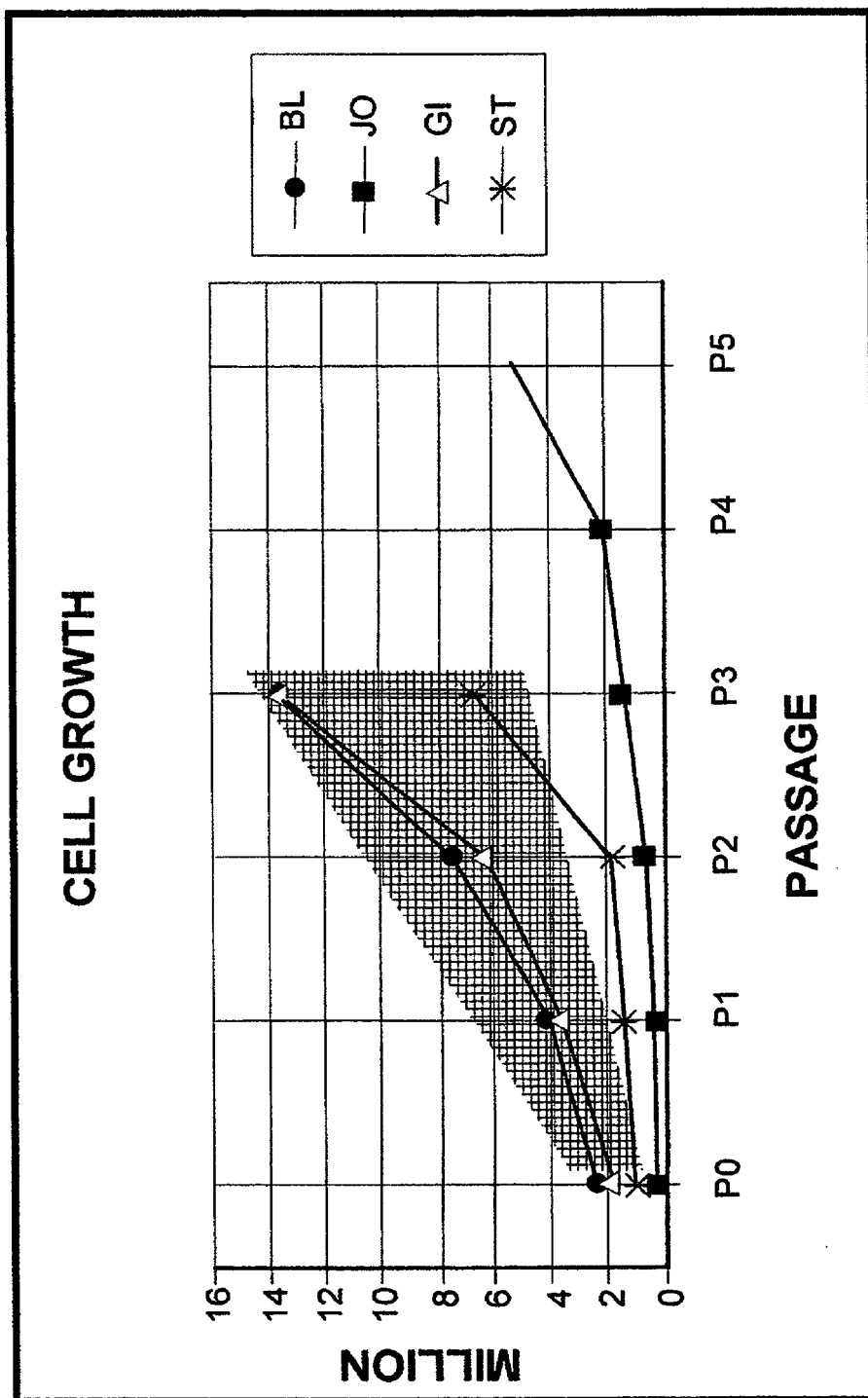


Fig. 5

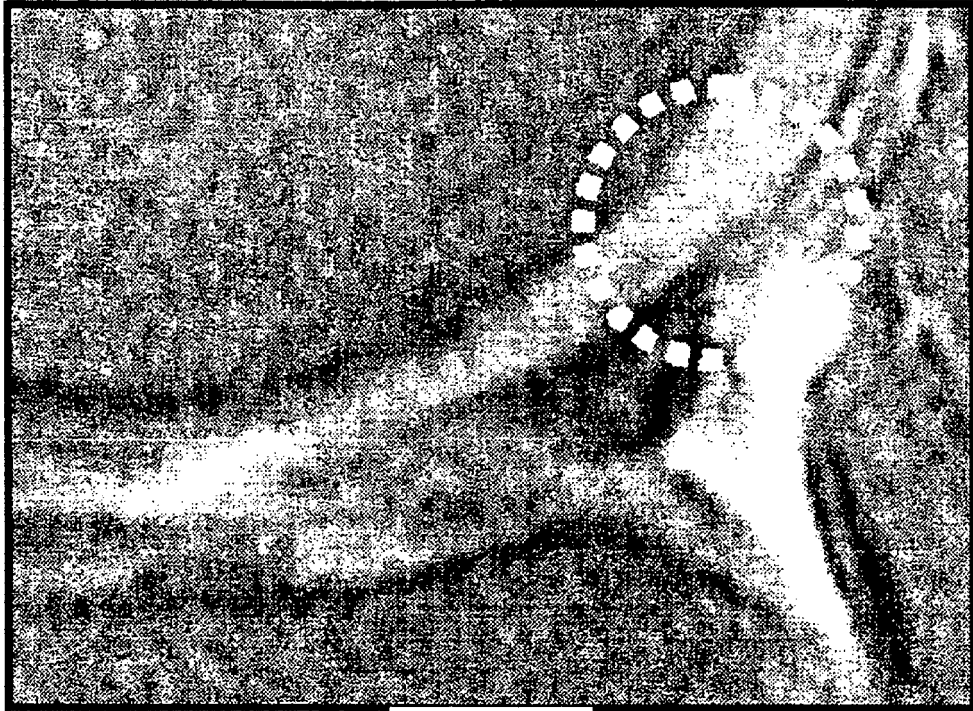


Fig. 6A

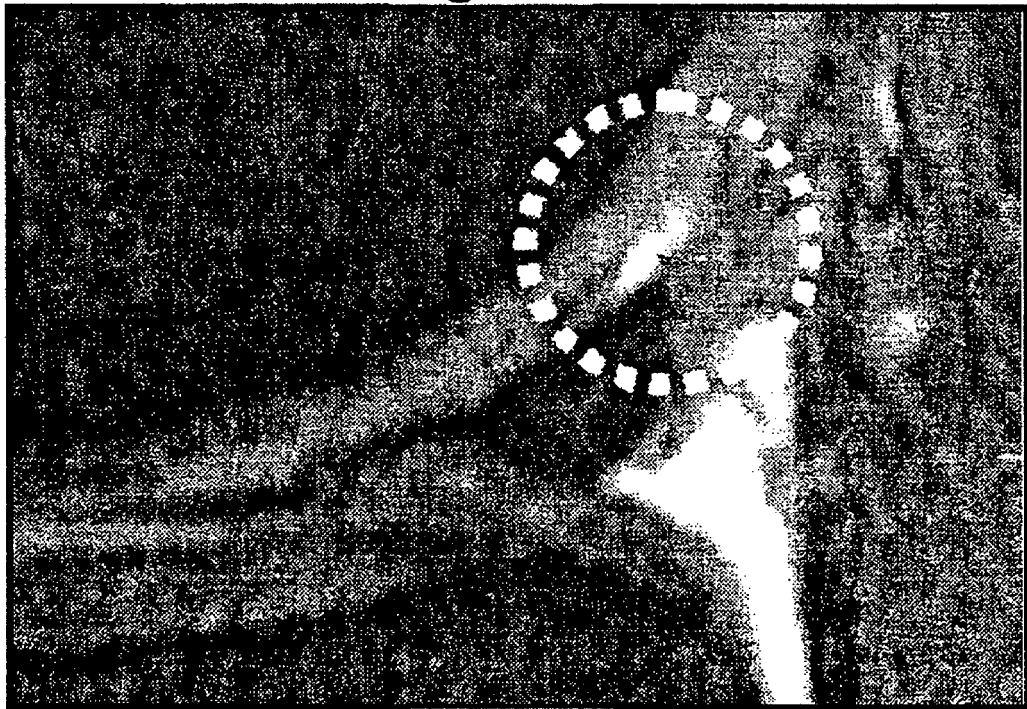


Fig. 6B

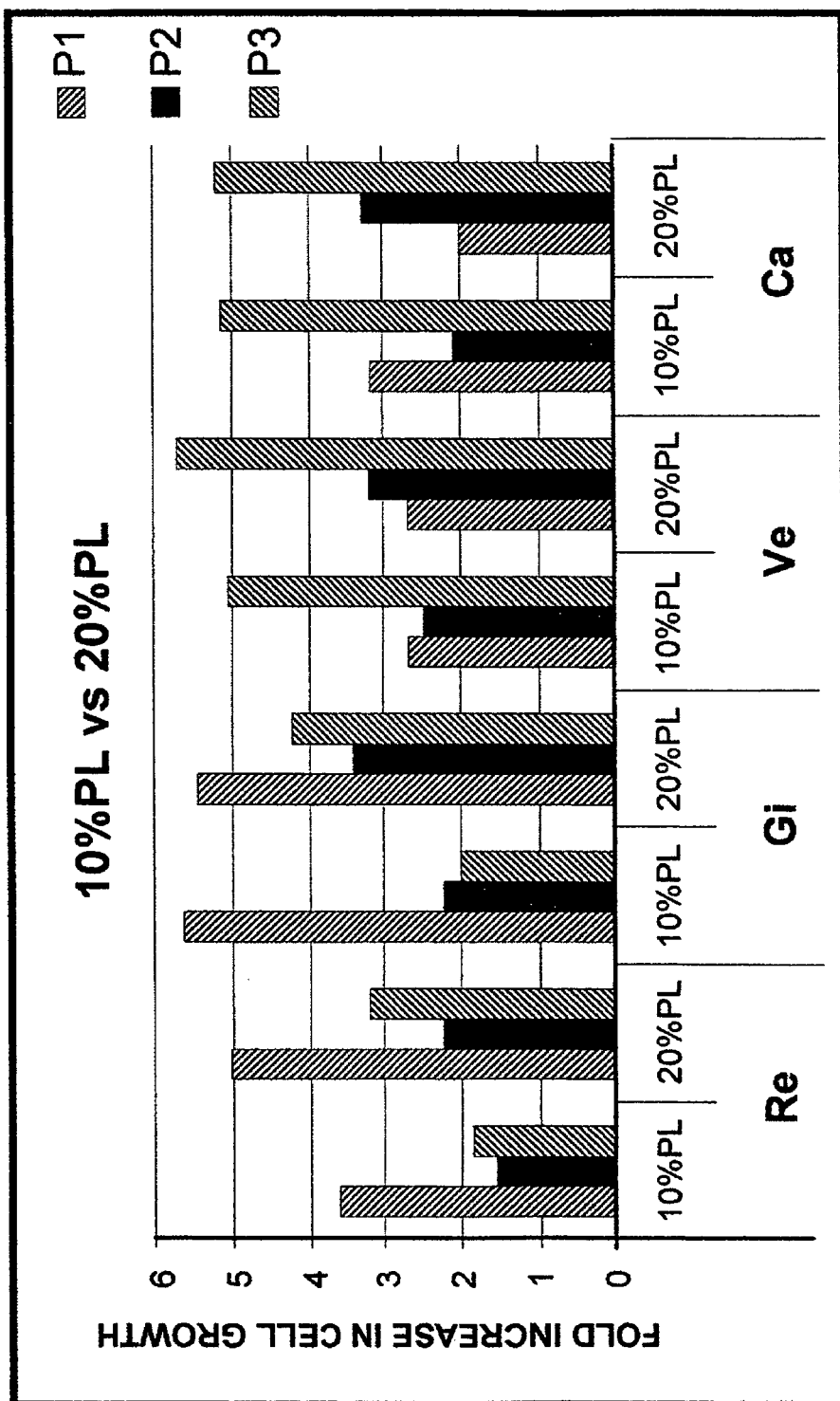
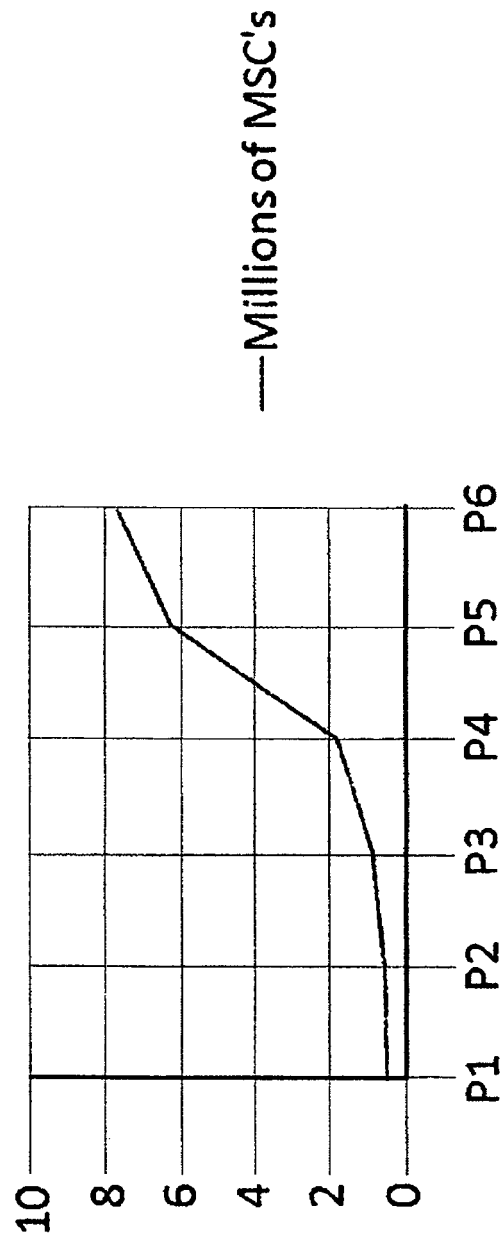


Fig. 7

Expansion of MSC's-AVN Patient



Expansion of MSC's Isolated from patient
with Avascular Necrosis of the Hip

Fig. 8

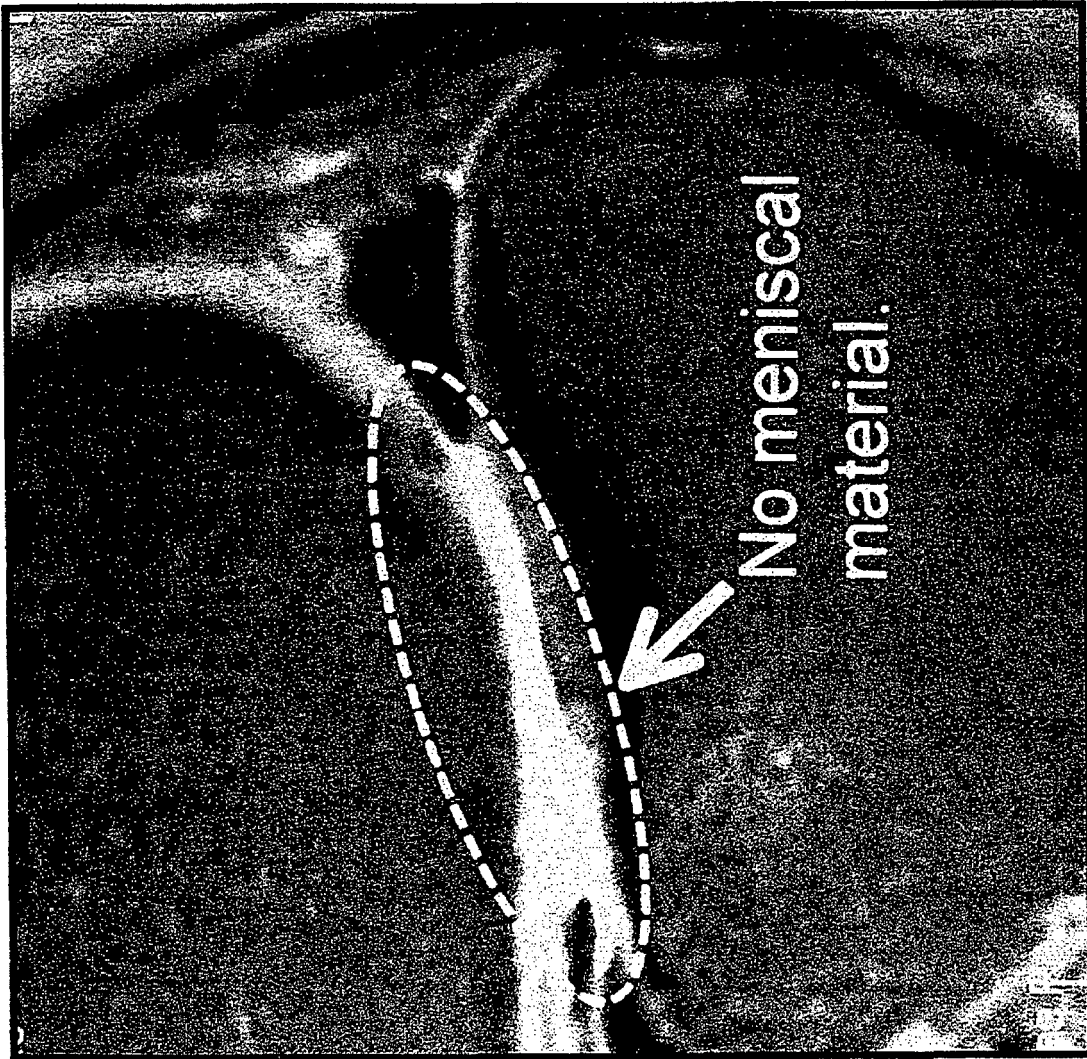


Fig. 9A

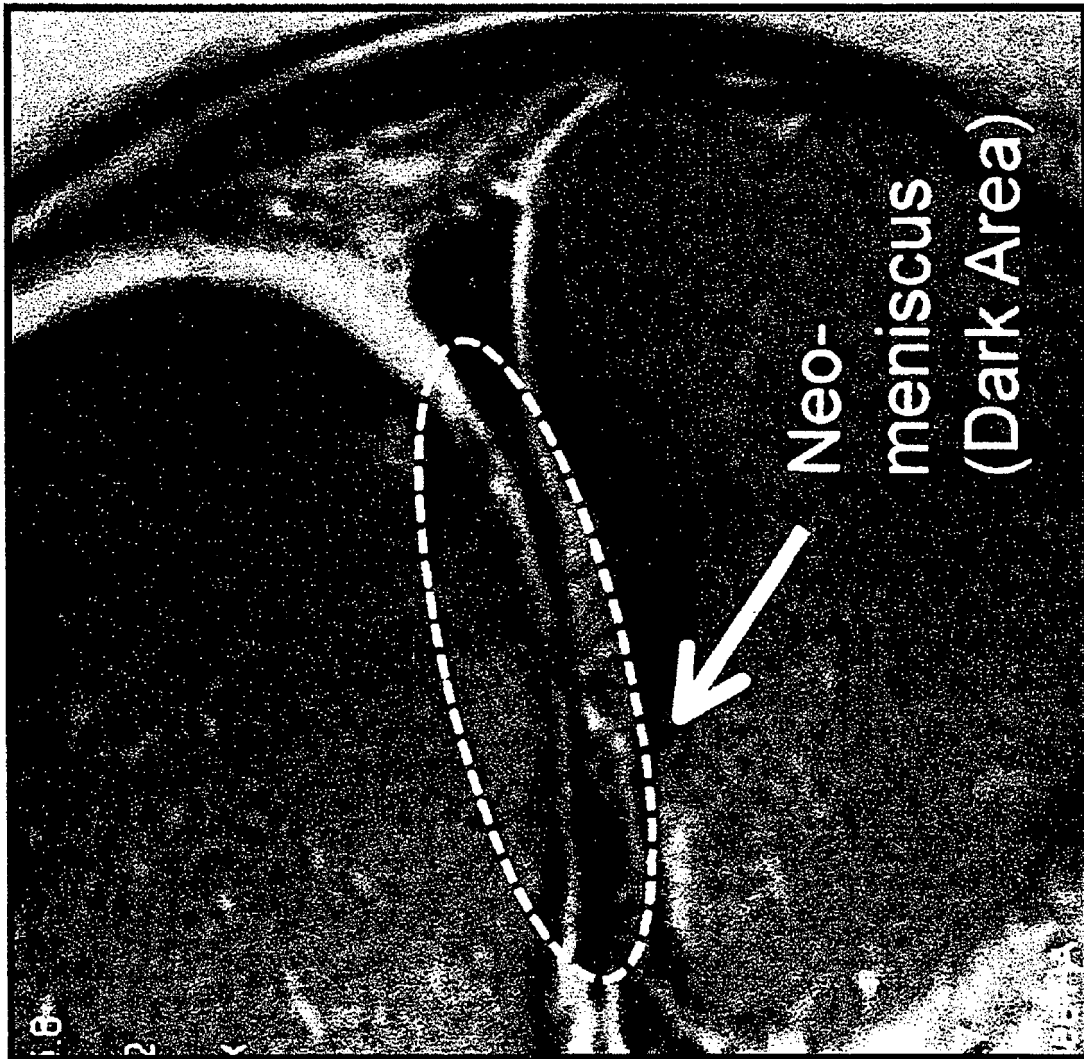


Fig. 9B

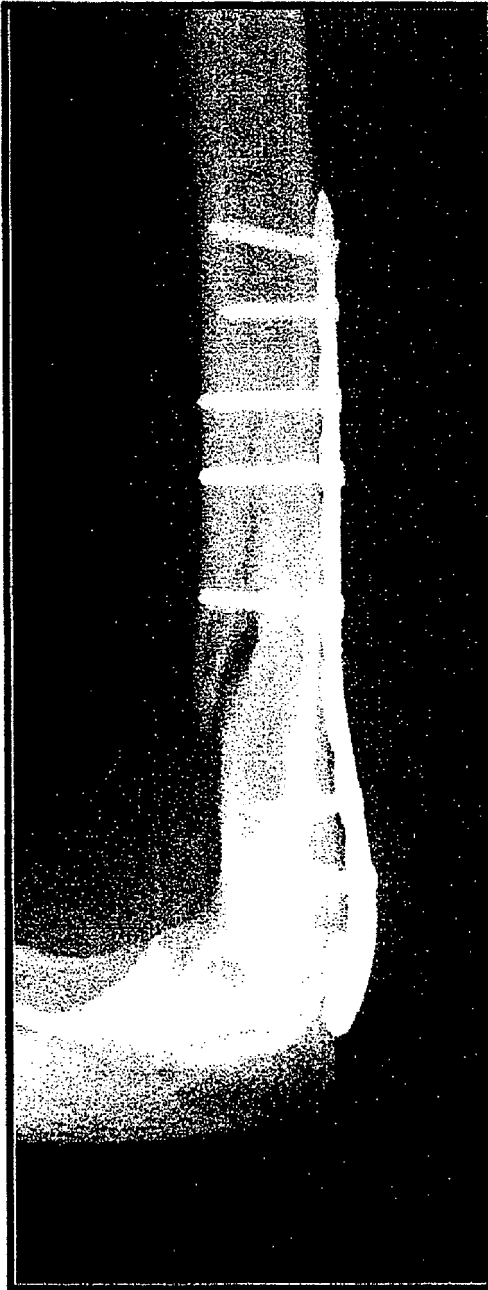


Fig. 10A

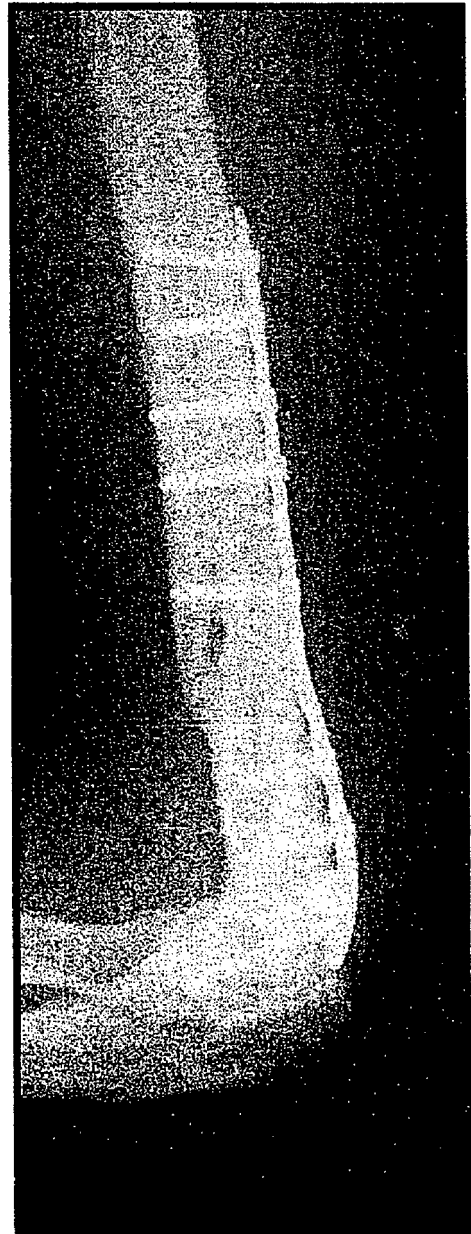


Fig. 10B

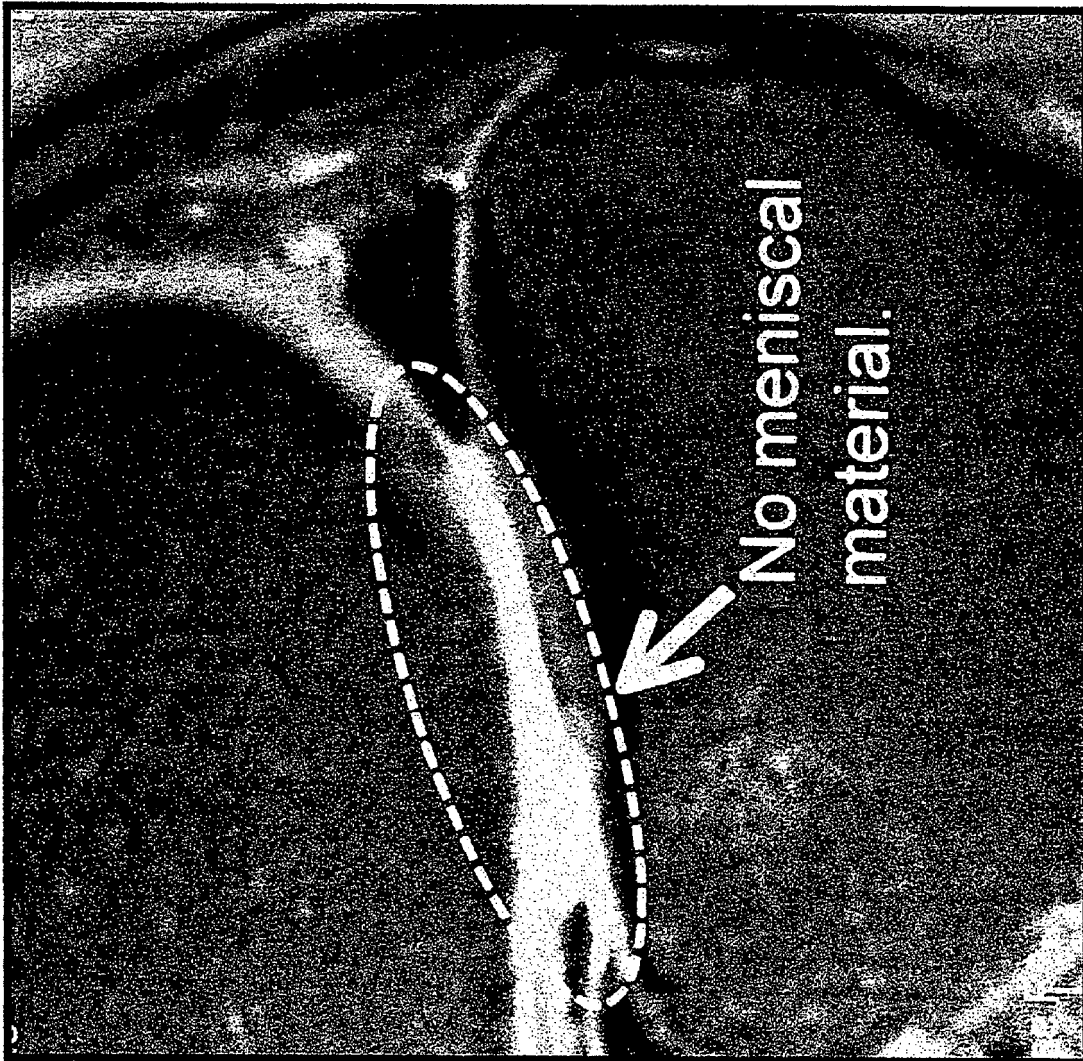


Fig. 11A

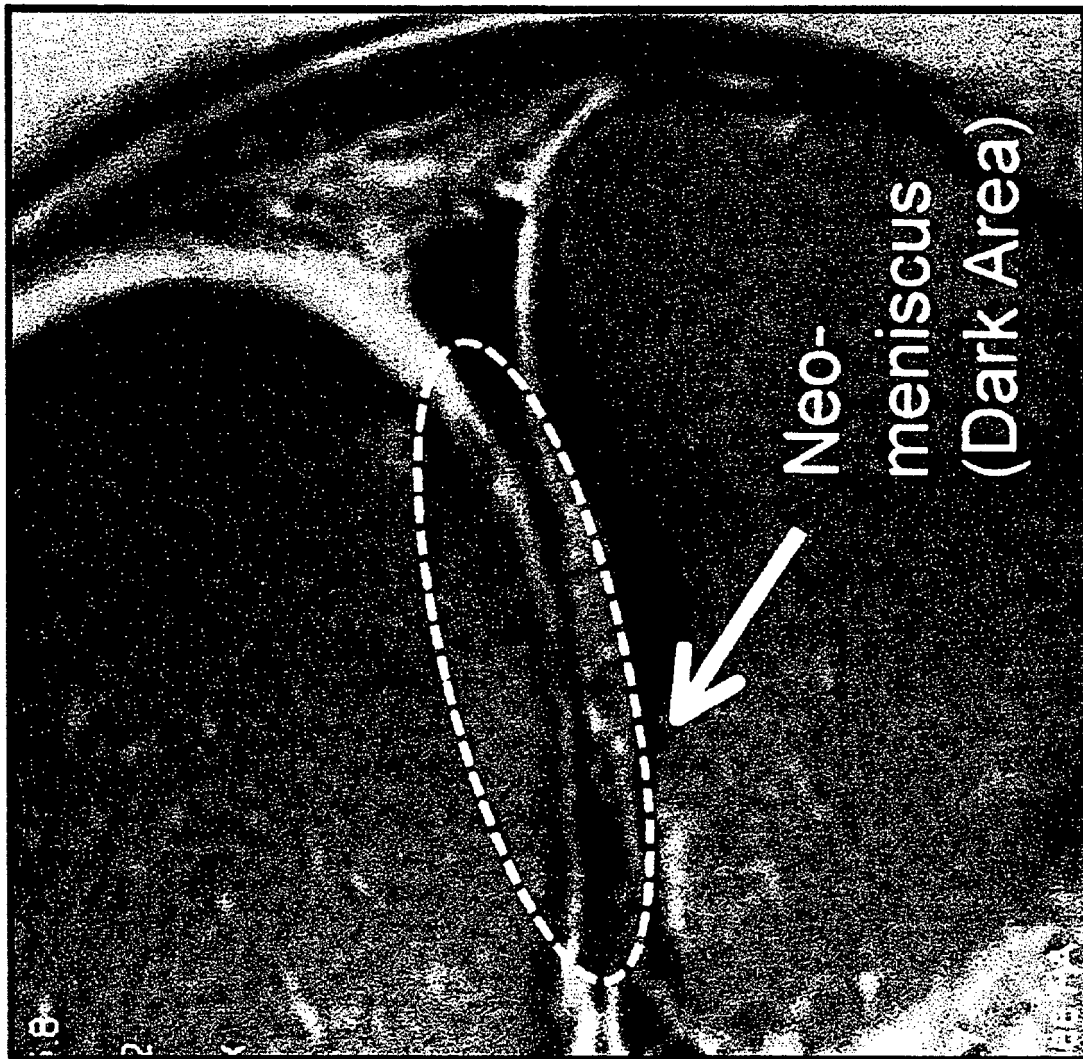


Fig. 11B

REFERENCES CITED IN THE DESCRIPTION

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