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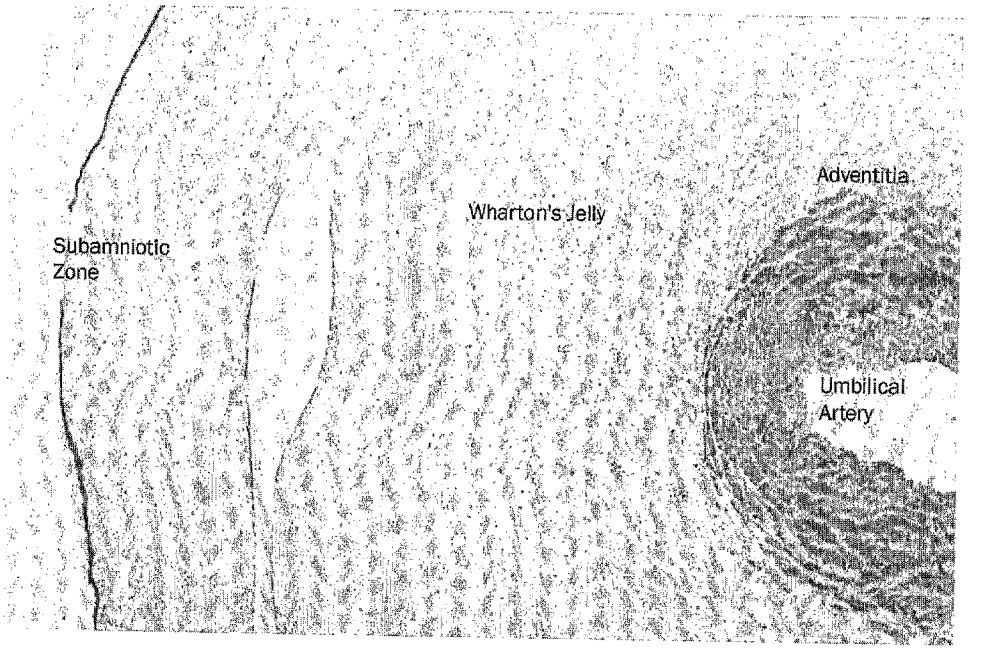

(57) Abrégé/Abstract:
Viable progenitor cells are extracted from frozen umbilical cord tissue. In embodiments, the umbilical cord tissue is a blood vessel bearing perivascular Wharton's jelly, and the extracted progenitor cells are HUCPVCs.

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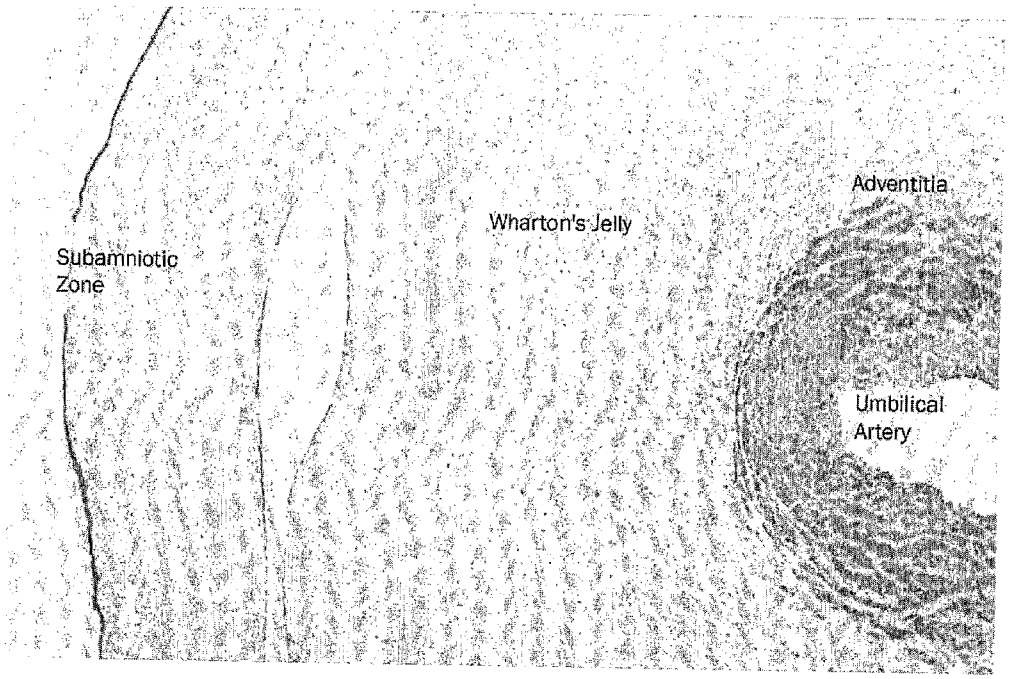
(54) **Title:** VIABLE CELLS FROM FROZEN UMBILICAL CORD TISSUE



(57) **Abstract:** Viable progenitor cells are extracted from frozen umbilical cord tissue. In embodiments, the umbilical cord tissue is a blood vessel bearing perivascular Wharton's jelly, and the extracted progenitor cells are HUCPVCs.

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VIABLE CELLS FROM FROZEN UMBILICAL CORD TISSUE

FIELD OF INVENTION

This invention focuses on the harvesting of a population of rapidly proliferating human cells from the connective tissue of the umbilical cord (UC); the culture of such cells in osteogenic, chondrogenic, adipogenic and myogenic conditions; the demonstration of a high percentage of cells within these populations that are immunologically incompetent, as shown by their lack of cell surface histocompatibility antigens; and the ability of these cells to be used as a source of multipotent progenitor cells for various cell-based therapies. More particularly, the invention relates to the use of frozen tissue as a source for such valuable progenitor cells.

BACKGROUND OF THE INVENTION

The UC is one of the first structures to form following gastrulation (formation of the three embryonic germ layers). As folding is initiated, the embryonic disc becomes connected, by the primitive midgut (embryonic origin) to the primitive yolk sac (extra-embryonic origin) via the vitelline and allantoic vessels which in turn develop to form the umbilical vessels (Haynesworth et al., 1998;Pereda and Motta, 2002;Tuchmann-Duplessis et al., 1972). These vessels are supported in, and surrounded by, what is generally considered a primitive mesenchymal tissue of primarily extra-embryonic derivation called Wharton's Jelly (WJ) (Weiss, 1983). From this early stage, the UC grows, during gestation, to become the 30-50cm cord seen at birth. It can be expected therefore, that WJ contains not only the fibroblast-like, or myo-fibroblast-like cells which have been described in the literature (see below), but also populations of progenitor cells which can give rise to the cells of the expanding volume of WJ necessary to support the growth of the cord during embryonic and fetal development.

WJ was first described by Thomas Wharton, who published his treatise *Adenographia* in (1656) (Wharton TW. Adenographia. Translated by Freer S. (1996). Oxford, U.K.: Oxford University Press, 1656;242-248). It has subsequently been defined as a gelatinous, loose mucous connective tissue composed of cells dispersed in an amorphous ground substance composed of proteoglycans, including hyaluronic acid (Schoenberg et al., 1960), and different types of collagens (Nanaev et al., 1997). The

cells dispersed in the matrix have been described as "fibroblast-like" that are stellate in shape in collapsed cord and elongate in distended cord (Parry, 1970). Smooth muscle cells were initially observed within the matrix (Chacko and Reynolds, 1954), although this was disputed by Parry (1970) who described them as somewhat "unusual fibroblasts" which superficially resemble smooth muscle cells. Thereafter, little work had been done on characterizing these cells until 1993 when Takechi et al. (1993) performed immunohistochemical investigations on these cells. They described the cells as "fibroblast-like" that were "fusiform or stellate in shape with long cytoplasmic processes and a wavy network of collagen fibres in an amorphous ground substance" (Takechi et al., 1993). For the immunohistochemical staining, they used primary antibodies against actin and myosin (cytoplasmic contractile proteins), vimentin (characteristic of fibroblasts of embryonic mesenchyme origin) and desmin (specific to cells of myogenic origin) in order to determine which types of myosin are associated with the WJ fibroblasts. They observed high levels of chemically extractable actomyosin; and although fibroblasts contain cytoplasmic actomyosin, they do not stain for actin or myosin, whereas the WJ fibroblasts stained positively for both. Additionally, positive stains for both vimentin and desmin were observed leading to the conclusion that these modified fibroblasts in WJ were derived from primitive mesenchymal tissue (Takechi et al., 1993). A subsequent, more recent study by Nanaev et al. (1997) demonstrated five steps of differentiation of proliferating mesenchymal progenitor cells in pre-term cords. Their findings supported the suggestion that myofibroblasts exist within the WJ matrix. The immunohistochemical characterization of the cells of WJ, shows remarkable similarities to that of pericytes which are known to be a major source of osteogenic cells in bone morphogenesis and can also form bone nodules referred to as colony forming unit-osteoblasts (CFU-O) (Aubin, 1998) in culture (Canfield et al., 2000).

Recent publications have reported methods to harvest cells from UC, rather than UC blood. Mitchell et al. (Mitchell et al., 2003) describe a method in which they first remove and discard the umbilical vessels to harvest the remaining tissue. The latter, which will include both the remaining WJ (some of which will have been discarded with the vessels, since the umbilical vessels are entirely enveloped in WJ) and the amniotic epithelium, is then diced to produce small tissue fragments that are

transferred to tissue culture plates. These tissue fragments are then used as primary explants from which cells migrate onto the culture substratum.

In another publication, Romanov et al. (2003) indicate they were successful in isolating mesenchymal stem cell-like cells from cord vasculature, although they also indicate their cultures do not contain cells from WJ. Specifically, they employ a single, 15min, collagenase digestion from within the umbilical vein, which yields a mixed population of vascular endothelial and sub-endothelial cells. Romanov et al. show that sparse numbers of fibroblast-like cells appear from this cell harvest after 7 days.

Also, US patent 5,919,702 describes a method of isolating "pre-chondrocytes" from the WJ of human UC, and their use to produce cartilage. Particularly, the method comprises slicing open a one inch section of cord longitudinally, dissecting away the blood vessels and 'casing', which are then discarded, and collecting the WJ into a sterile container where it was cut into 2-3mm^3 sections for culturing. In a preferred method, cells are isolated by placing a 2-3mm^3 section of the WJ on a glass slide on the bottom of a Petri dish, covering it with another slide, and culturing it for 10-12 days in order to allow the 'pre-chondrocytes' to migrate out to the culture dish surface.

In US patent application US2005/0148074 published July 7, 2005, Davies et al describe the isolation of a unique progenitor cell population from a particular region of Wharton's jelly, termed the perivascular region. The Wharton's jelly in this region is on or associated with the external walls of the cord vessels, and remains associated with the vessels when they are excised from the cord. The progenitor cell population has a remarkably short doubling time, and comprises progenitor cells having a wide range of valuable properties, including cells that are multipotent and give rise to various mesenchymal tissues including fat, bone, cartilage, muscle and endothelium; cells that spontaneously differentiate into bone-forming osteoblasts; and cells that lack MHC class I and class II markers. Progenitor cells obtained from the Wharton's jelly in the perivascular region of human umbilical cord vasculature are referred to herein as HUCPVCs.

Cord tissue thus promises to be an important source of progenitor and other cells for use in tissue engineering and other medical procedures. Current practices permit the longer term storage of cord-derived cells under cryogenic conditions, and the recovery of viable cells therefrom. However, techniques have yet to be developed that permit the recovery of viable cells from frozen or cryogenically stored cord tissue, as distinct from cord cells per se. Accordingly, where the recovery of viable cells from cord tissue is required, technicians are required to act promptly to process cord tissue while it is fresh, usually within 24 hours from cord extraction, so that the desired tissue can be extracted, and the desired cells isolated, cultured and stored frozen, while those cells are still viable. Clearly, it would be useful to provide a method permitting the cord tissue to be stored, to serve as an on-demand source of viable cells.

SUMMARY OF THE INVENTION

It has now been determined that viable cells can be recovered from umbilical cord tissues that have been frozen. Accordingly, the present invention contemplates the practice of "banking" such tissues cryogenically, on a post-partum basis, to provide a lasting resource for the on-demand extraction of viable cells from frozen cord tissue.

More particularly, and according to one of its aspects, the present invention comprises the steps of obtaining umbilical cord tissue post-partum, and freezing the post-partum umbilical cord tissue. In a preferred aspect, the present invention is applied to preserve the viability of cells present in umbilical cord tissue, using a method comprising the steps of obtaining umbilical cord tissue comprising viable cells, combining the umbilical cord tissue with a cryopreserving solution comprising serum-containing cell culturing medium and a cryopreservant, and subjecting the combination to a freezing process in which the combination is first refrigerated as a liquid for a period and at a temperature permitting the cryopreservant to penetrate the tissue, then freezing the cooled combination, and then storing the frozen combination under cryogenic conditions.

According to another of its aspects, the present invention provides a method for obtaining viable cells from umbilical cord tissue, comprising the steps of obtaining such tissue in frozen form, thawing the frozen tissue, and extracting viable cells from

the thawed tissue. In a preferred aspect, the present invention is applied to recover viable cells from cord tissue, using a method comprising the steps of obtaining cord tissue that is frozen, and optionally stored cryogenically, particularly by the method of the present invention, thawing the frozen tissue, washing the thawed tissue to remove the cyropreservant, and extracting viable cells from the resulting tissue.

Thus, in another of its aspects, the present invention provides a method for obtaining viable cells from umbilical cord tissue, wherein the cells are extracted from previously frozen umbilical cord tissue.

In a further aspect, the present invention provides umbilical cord tissue, in a frozen state and optionally a cryopreserved state, comprising progenitor cells that are recoverable in a viable state, whenever prepared by the method of the present invention.

In a related aspect of the invention, there is provided umbilical cord tissue in the form of a tissue bank comprising a plurality of containers each comprising a sample of said frozen cord tissue, and a catalog referencing the contents of each container. In a specific embodiment, the containers are containers suitable for cryogenic storage.

In a further aspect, the present invention provides a method comprising the steps of obtaining viable cells from previously frozen umbilical cord tissue, and selecting from within those viable cells, cells that are progenitor cells. In a particular aspect, the progenitors are HUCPVCs and the invention provides a method for recovering viable HUCPVCs from frozen umbilical cord tissue, comprising the steps of:

1) obtaining frozen cord tissue, which frozen cord tissue is optionally cryogenically stored cord tissue, wherein the cord tissue is fresh, isolated human umbilical cord vessel, or a segment thereof, bearing associated perivascular Wharton's jelly, and has been prepared by a method comprising the steps of

a) obtaining said umbilical cord tissue,

b) combining the umbilical cord tissue with a cryopreserving solution comprising serum-containing cell culturing medium and DMSO,

c) subjecting the combination to a cooling process in which the combination is refrigerated as a liquid for a period and at a temperature permitting the DMSO to penetrate the tissue,

d) freezing the cooled combination to provide frozen cord tissue, and, optionally,

e) storing the frozen cord tissue under cryogenic conditions for a period of time; and then

2) thawing the frozen cord tissue;

3) treating the thawed cord tissue to displace the DMSO with water; and

4) digesting the Wharton's jelly associated with the stored vessels to release viable HUCPVCs.

Aspects of the invention will now be described in greater detail with reference being had to the accompanying drawings, in which:

DESCRIPTION OF THE FIGURES

Figure 1 is a light micrograph representing the three distinct zones of tissue represented in the human UC;

Figure 2 is a representative illustration of the looped vessel in the collagenase solution;

Figure 3 is a light micrograph of the cells isolated from the WJ that have attached to the polystyrene tissue culture surface;

Figure 4 is a light micrograph illustrating the initial formation of a CFU-O;

Figure 5 is a light micrograph illustrating a mature CFU-O;

Figure 6 demonstrates tetracycline-labeled CFU-O's under UV fluorescence on a 35mm polystyrene tissue culture dish;

Figure 7 illustrates side by side a phase-contrast light micrograph and a fluorescence micrograph of the same tetracycline-labeled CFU-O;

Figure 8 is a scanning electron micrograph of a mature CFU-O on the tissue culture polystyrene surface;

Figure 9 is a scanning electron micrograph of a cross-section of a CFU-O exposing the underlying matrix;

Figure 10 is a scanning electron micrograph of the lightly mineralized collagen fibres located on the advancing edge of the CFU-O;

Figure 11 is a scanning electron micrograph of the non-collagenous matrix (seen as globules) laid down on the polystyrene interface by differentiating osteogenic cells;

Figure 12 is a scanning electron micrograph of heavily mineralized collagen that comprises the centre of a mature CFU-O;

Figure 13 illustrates the flow cytometry data demonstrating that WJ-derived cells are 77.4% MHC I and MHC II negative;

Figure 14 is a black and white reproduction of a Masson's trichome-stained transverse section of bone nodule showing the distribution of collagen within which cells have become entrapped (osteocytes), and multilayering of peripheral cells some of which are becoming surrounded by the elaborated extracellular matrix;

Figure 15 shows the potential expansion of the adherent perivascular WJ population in relation to the expansion of the committed osteoprogenitor subpopulation and total osteoprogenitor subpopulation;

Figure 16 shows proliferation of the perivascular WJ cells from 0-144 hours illustrating a normal growth curve with a lag phase from 0-24 hrs, log phase from 24-72 hours, and plateau phase from 72-120 hours. The doubling time during the entire culture period is 24 hours, while during log phase it is 16 hours;

Figure 17 shows major histocompatibility complex (MHC) expression of the WJ cells shown over 5 passages, the change in their expression due to free-thawing, and subsequent expression due to reculture;

Figure 18 shows the CFU-F frequency of HUCPVCs;

Figure 19 shows the doubling time of HUCPVCs from P0 through P9. HUCPVCs demonstrate a relatively stable and rapid doubling time of 20 hours from P2 to P8; and

Figure 20 shows the proliferation of HUCPVCs demonstrating that $>10^{14}$ cells can be derived within 30 days of culture. With this rapid expansion, 1,000 therapeutic doses (TDs) can be generated within 24 days of culture;

Figure 21 shows the effects of collagenase concentration and digestion time on cell harvest; and

Figure 22 provides light micrographs showing the presence of cells determined to be viable by Trypan exclusion on day 4 (panel A) and day 9 (panel B) after recovery from cryostorage and culturing on tissue culture treated surfaces in 5% FBS, and alpha-MEM containing penicillin G (167 units/ml), gentamicin (50μg/ml) and amphotericin B (0.3μg/ml). Panel C is a light micrograph showing, for comparison,

results obtained when the cord is frozen after immersion in 10% DMSO without prior cooling.

Detailed Description

The invention relates in one aspect to methods useful for storing umbilical cord tissues. This practice enables the future utilization of cells that are histocompatible with their source subject, should that future need arise for instance for medical reasons such as cellular or tissue repair or regeneration.

In the present invention, umbilical cord tissue is obtained post-partum, and subjected to freezing whereby the frozen umbilical cord tissue is then stored as future source of viable cells. To obtain viable cells from the frozen tissue, the tissue is allowed to thaw and is then extracted to provide cells that, when cultured, exhibit viability.

The present method can be applied to various cord tissues, such as the vasculature including vessel walls and endothelium, the Wharton's jelly, the amniotic epithelium and the like. The cord from which such tissues are obtained can be cord from any mammal, and is preferably obtained from human umbilical cord. In one embodiment of the invention, the umbilical cord tissue is Wharton's jelly. In a preferred embodiment, the tissue is Wharton's jelly associated with the perivascular region of umbilical cord vasculature, desirably human umbilical cord vasculature. In another embodiment of the invention, the umbilical cord tissue is vascular tissue. In a preferred embodiment, the tissue is vascular tissue having Wharton's jelly associated with the perivascular region bound thereto. In a particularly preferred embodiment, the umbilical cord tissue to be frozen is the vasculature (i.e., vessels) and associated Wharton's jelly that remains associated therewith when the vasculature is removed from within the resected cord. Such cord tissue includes the entire length of the intact vasculature, individual vessels, longitudinally sectioned forms thereof from which blood has been optionally removed, and transverse sections of such tissues.

Thus, in all aspects of the present invention, the preferred cord tissues are the vessels within human cord tissue that have Wharton's jelly either on or associated with the external walls thereof, i.e., Wharton's jelly that lies within the perivascular region of

the cord. The Wharton's jelly that lies within this particular region is a rich source of the progenitor cells that are described above, and in further detail below. This Wharton's jelly is associated so intimately with the vessel walls that its isolation from the walls is procedurally difficult. Nevertheless, recovery of this perivascular Wharton's jelly per se, and without associated vessels is technically possible, and the present invention thus further embraces the use of such isolated perivascular Wharton's jelly as cord tissue useful as source material to be frozen and, optionally, cryogenically stored, for subsequent harvest of viable progenitor cells.

The cord tissue desirably is obtained fresh, as post-partum tissue, and following optional dissection to provide tissue of the nature just described above, is then prepared for freezing. Desirably, the cord tissue is processed within about 24 hours from harvest, and the tissues thus extracted are frozen, and desirably enter cryogenic storage, within at least about 72 hours from harvest, and more desirably within 48 hours and particularly 24 hours from harvest. The fresh tissue can be cooled during this period, and is desirably washed and optionally disinfected, in accordance with standard practice, but should not be frozen during this period expect as noted herein, so that cell viability is not adversely affected.

In the preferred embodiment, where the cord tissues to be preserved are the cord vessels with associated, perivascular Wharton's jelly, the vessels can be extracted as described in greater detail hereinbelow, and as described by Davies et al, in US2005/0148074. In brief, intact cord vessels with associated Wharton's jelly are obtained by gently pulling the vessels from cord that has been opened longitudinally and sectioned transversely to yield vessel segments that are 1-3 inches in length, e.g., about 4cm. This process sheds the bulk of Wharton's jelly within the cord, but leaves Wharton's jelly in the perivascular region associated with the extracted vessels. The ends of the vessels are tied off for instance using clamps, suturing or the like to prevent the escape of any blood remaining within the vessels. In addition or in the alternative, blood within the vessels can be removed by repeating rinsing in suitable vehicle such as saline or buffered saline, such as PBS. The absence of blood in the specimen ensures that progenitors of hematopoietic origin are not present as contaminants in the preparation. In the case where blood is removed, it will be appreciated that cord vessel segments useful for storage include not only segments

that have been generated by sectioning of the vessels transversely but also segments that have been generated by longitudinal sectioning, particularly of the transverse segments, to generate segments that are strips of the otherwise tubular segments. All forms of cord vessel segments that bear perivascular Wharton's jelly are useful in the present method.

In order to preserve the tissue under conditions that permit the recovery of viable cells, the cord tissue is placed in a vehicle suited to the freezing process, such as any vehicle suitable for cryogenic storage. Optionally, the vehicle further comprises a supplement suitable for cell culturing. In one embodiment, the vehicle comprises dimethylsulfoxide (DMSO), e.g., from 10-30% DMSO, such as 15-25% DMSO, including 20% DMSO. In another embodiment, wherein the vehicle comprises a cell culturing supplement, the DMSO is present as from 1 to 25% of the vehicle by volume, e.g., 5-20%, such as 8-15%, i.e, about 10%. In another embodiment, the supplement is a serum-based supplement, such as fetal bovine serum. In a specific embodiment, the fetal bovine serum medium, itself comprising from 5-20% (e.g., 10% or 15%) by volume of FBS, is present as from 75 to 99% of the vehicle by volume, such as 80-95%, i.e, about 90%. In a further specific embodiment, the vehicle comprises 90% by volume of a 10% FBS solution, and 10% DMSO.

In a particularly preferred aspect of the invention, preparation of the tissue for freezing and optional cryogenic storage proceeds through a graduated, transitional cooling process that is designed particularly to permit the cyropreservant to penetrate the tissue sufficiently both to protect the tissue and resident cells during storage and to displace water that upon freezing will damage the tissue and cells. Particularly, the preferred freezing/cryopreservation method makes use of a cryopreserving solution that, as noted above, comprises a cyropreservant and a cell nutrient medium such as a cell culturing medium that is supplement with serum. The cyropreservant can be any liquid agent, or an agent solution, that protects the tissue and resident cells from the damaging effect of freezing and ice formation. Most preferably, the cyropreservant is dimethylsulfoxide (DMSO). In the alternative, the cyropreservant can be glycerol, or mixtures of glycerol and DMSO. The glycerol can be used in the same manner and at the same concentrations set out herein with reference to DMSO. The properties of DMSO make it particularly well suited use in the present cryopreservation procedure.

The preferred serum-supplemented culturing medium present in the cryopreserving solution is fetal bovine serum (FBS). Alternatively, the culturing medium may comprise any nutrient medium suitable for cell culturing, such as DMEM, M 199, RPMI 1640 or the like, to which is added a serum supplement, such as FBS, human serum and the like. Generally, but depending on the type of nutrient medium selected, the serum component of the culturing medium will comprise from about 5-30% serum, e.g., 10-20% serum. As noted above, the preferred cryopreserving solution comprises, by volume, about 10% DMSO, and about 90% culturing medium, such as FBS.

To prepare the tissue for freezing and subsequent cryogenic storage, the tissue is combined with the cryopreserving solution and refrigerated as a liquid for a period and at a temperature effective for the cyropreservant to permeate the tissue thus, desirably, displacing associated water. For this purpose, where the tissue is cord vessel segments bearing perivascular Wharton's jelly, the tissue suitably is held at a temperature of about 4C for a period of about 15-60 minutes, such as 20-40 minutes and preferably 30 minutes. Lower temperatures are less desirable, given that the DMSO solidifies below this temperature and has reduced tissue permeation. Higher temperatures are suitable, but it is desirable to use the lower temperatures in order to slow the metabolic processes within the cord during handling. The term of refrigeration can vary. It is desirable to strike a balance so that the cyropreservant is given time sufficient to permeate the tissue, while reducing the time during which the cord is handled. In general, the refrigeration period should not be less than about 10 minutes and can approach up to 60 minutes or more.

During the refrigeration step, the tissue can be incubated within any suitable container, according to standard practice, such as a centrifuge tube e.g. 50mL in volume. When the tissues are cord vessels bearing perivascular Wharton's jelly, each container may receive about 5-10 vessel segments. It is useful not to crowd the container, so that DMSO permeation is not hindered.

After refrigeration, the vessels are subjected to a freezing process. This process is desirably performed after transferring the refrigerated tissue to a cryocontainer, so that the frozen tissue can readily then be transferred into cryostorage. In one embodiment,

the vessels are made of a non-brittle polymer that can be sterilized such as polypropylene or the like, and is provided in the form of a tube or other receptacle adapted to receive a removable closure to retain the specimen with the vessel during storage and allow its release thereafter. Most desirably, each cryocontainer comprises one tissue sample, e.g., one vessel segment per container, in the case where the tissue is cord vessel bearing the perivascular Wharton's jelly. So-called cryotubes are suitable, as are polyethylene bags. The refrigerated vessels thus are transferred to a cryocontainer, and combined with fresh cryopreserving solution, in a volume sufficient preferably to immerse the tissue fully.

The prepared tissue then is frozen by placing the specimen into a freezing unit where temperatures can be controlled at the low level required to freeze the tissue. In one embodiment, the containers comprising the tissue and solution are placed into a freezer to reduce the temperature to about -70C, e.g., below about -40C, for a period of at least about 6 hours, e.g., for at least about 8-12 hours. Most preferably, the freezing is performed using a controlled rate -70C freezer.

It will be appreciated that the frozen tissue can be used directly as starting material from which viable cells can be recovered. Such frozen material, when maintained at -70C, can exhibit at least some cellular activity, and should therefore be used relatively promptly so that the cell death that can occur over time at this temperature is avoided. More preferably, according to the method of the present invention, the frozen tissue is placed into cryogenic storage, i.e., is stored at a temperature at which cell metabolism is in stasis.

Thus, after the tissue is frozen, the containers comprising the frozen tissue is preferably transferred to cryogenic storage, in the manner already developed for the storage of similar cells and tissues. Suitably, the containers are stored in the vapour phase of liquid nitrogen, at about -180C to -200C. In the alternative, the frozen cells can be stored cryogenically in mediums other than liquid nitrogen, such as liquid carbon dioxide or liquid halocarbon.

The tissue specimen can remain in this cryogenic state for long periods of days, week, months or years, for retrieval when a source of progenitor cells is required.

When required, viable cells resident within the stored tissue can be obtained using a recovery process according to the present invention. More particular, tissue within the cryogenically stored container is first obtained and thawed to permit subsequent removal of the cryopreservant. The tissue can be thawed for instance in a bath of warm water, at a temperature generally not exceeding 40C. A temperature of 10C-40C is suitable, and a temperature of 37C is desirable. Once thawed, which at 37C takes about 5-15 minutes, e.g., 10 minutes, the tissue can be transferred to a container such as a 50mL tube, and the tissue washed to dilute or remove the DMSO. The washing is desirably performed using cool (e.g., refrigerated, such as 4C) liquid, such as water or buffered saline, e.g., PBS, by immersing the tissue in the cool liquid. Vigorous washing of the tissue is desirably avoided, so that shock or damage to the cells is minimized. The immersed tissue can be retained in a refrigerator for another period to permit further dilution and replacement of the cyropreservant by water, and then still further diluted by addition of further cooled liquid.

The resulting restored tissue can then be used to recover viable cells resident within the tissue, using the same practices established for recovering viable cells from fresh cord tissue. The present method thus enables the recovery of cells that are viable from cord tissue that has been frozen and stored cryogenically. The viability of such cells can be confirmed using any of the techniques established for this purpose. Conveniently, the viability of the recovered cells can be confirmed simply by Trypan blue exclusion (a procedure that identifies dead cells as those which are unable to exclude the dye), or in a more sophisticated manner, by detecting incorporation of BrdU, to confirm DNA synthesis is occurring. Thus, the term "viable" is used herein with reference to cells that exhibit an active metabolism, and which further desirably display, for cells cultured based on adherence, the characteristics of adherence to culture substrate and subsequent spreading and proliferation. An example of such viability is shown in Figure 22, particularly panels A and B, which demonstrate the spreading and proliferation phenomena characteristic of viable HUCPVCs.

In particular embodiments of the invention, the viable cells recovered from the stored umbilical cord tissue are progenitor cells. In other particular embodiments, the viable

progenitor cells are recovered from the perivascular Wharton's jelly associated with stored umbilical cord vessels or segments thereof, as exemplified herein.

It will be appreciated that the storage and recovery processes of the present invention make possible the organization of cord tissues in storage collections or "banks", which comprise a plurality of containers each comprising an umbilical cord tissue sample. Such a collection or bank of umbilical cord tissue samples constitutes another embodiment of the present invention. Each bank of tissues will be accompanied by a catalog denoting, with respect to each sample, any information that is usefully associated with each sample, such as sample origin, with reference for instance to the donor individual and perhaps a genetic or medical history thereof, the date of storage, the identity of the agency or person who produced the sample, etc. With this approach, the tissue bank can serve as repository for future use of the tissue and its inherent cells, and the catalog can be used to select particular tissues and cells for use with particular patients or to treat particular medical conditions.

To recover viable progenitor cells in the case where the stored tissue is cord vessels or vessel segments bearing perivascular Wharton's jelly, reference may be made to the procedures described in our co-pending international patent application WO04/072273 published August 26, 2004, and US 2005/0148074. More particularly, the isolation of such progenitors from fresh umbilical cord tissue, and their properties and end-uses are described below. These progenitors are referred to as human umbilical cord perivascular cells or "HUCPVCs".

The noted references teach a procedure for extracting cells from Wharton's jelly of human umbilical cord, which yields a unique cell population characterized by rapid proliferation, the presence of osteoprogenitor and other human progenitor cells, including cells which display neither of the major histocompatibility markers (human leukocyte antigen (HLA) double negative). The cell population is a useful source of progenitor cells from which to grow bone and other connective tissues including cartilage, fat and muscle, and for autogenic and allogeneic transfer of progenitor cells to patients, for therapeutic purposes.

More particularly the procedure provides a Wharton's jelly extract, wherein the extract comprises human progenitor cells and is obtained by enzymatic digestion of the Wharton's jelly proximal to the vasculature of human umbilical cord, in a region usefully termed the perivascular zone of Wharton's jelly. The tissue within this perivascular zone, and from which the progenitor cells are extracted, can also be referred to as perivascular tissue. The extraction procedure suitably results in an extract that is essentially free from cells of umbilical cord blood, epithelial cells or endothelial cells of the UC and cells derived from the vascular structure of the cord, where vascular structure is defined as the tunicae intima, media and adventia of arteriolar or venous vessels. The resultant extract is also distinct from other Wharton's jelly extracts isolated from the bulk Wharton's jelly tissue that has been separated from the vascular structures.

The procedure thus provides a method for obtaining a human progenitor cell, comprising the step of isolating the cell from the Wharton's jelly extract obtained in accordance with the invention.

The procedure provides a cell population obtained by culturing of the cells present in the Wharton's jelly extract, including a population of osteoprogenitor cells and a population of MHC -/- progenitor cells.

The extracted progenitor cell population is characterized as an adherent cell population obtained following culturing of the extracted cells under adherent conditions. In another embodiment, the extracted progenitor cell population is characterized as a non-adherent (or "post-adherent") (PA) cell population present within the supernatant fraction of extracted cells grown under adherent conditions. This PA fraction is derived by transferring the supernatant of the initially plated HUCPVCs into a new T-75 flask to allow the as yet non-adhered cells to attach to the culture surface. This process is repeated with this new T-75 flask, transferring its media into another new T-75 flask in order to harvest any remaining PA cells. This PA cell population comprises a subpopulation of progenitor cells that, when cultured under adherent conditions, proliferates rapidly and forms bone nodules and fat cells spontaneously. This technique provides a means to increase the yield of adherent cells isolated from the enzymatic digest cell population.

The procedure also provides a population of committed osteoprogenitor cells characterized by the property of differentiating into bone cells when cultured in the absence of supplements otherwise required for such differentiation.

There is also provided a method for producing connective tissue, including bone tissue, cartilage tissue, adipose tissue and muscle tissue, which comprises the step of subjecting cells obtained from the Wharton's jelly extract to conditions conducive to differentiation of those cells into the desired connective tissue phenotype. In this respect, the invention further provides for the use of such cells in cell-based therapies including cell transplantation-mediated treatment of medical conditions, diseases and disorders.

There is also provided a composition and the use thereof in tissue engineering, comprising progenitor cells in accordance with the invention or their differentiated progeny, and a carrier suitable for delivering such cells to the chosen tissue site.

There is provided an extract of Wharton's jelly (WJ), as a source of a rapidly proliferating cell population comprising human progenitor cells including osteoprogenitor cells, as well as immuno-incompetent cells.

The extracted cell population can be referred to as human umbilical cord perivascular (HUCPV) cells. The HUCPV cell population constitutes a rich source of multipotent progenitor cells that are unique in their phenotype, particularly as revealed by the variety of cell subpopulations contained therein. The perivascular zone of the Wharton's jelly from which the cells are extracted can be referred to as perivascular tissue.

As used herein, the term "progenitor cells" refers to cells that will differentiate under controlled and/or defined conditions into cells of a given phenotype. Thus, an osteoprogenitor cell is a progenitor cell that will commit to the osteoblast lineage, and ultimately form bone tissue when cultured under conditions established for such commitment and differentiation. A progenitor cell that is "immuno-incompetent" or "non-immunogenic" is a cell having a phenotype that is negative for surface antigens

associated with class I and class II major histocompatibility complexes (MHC). Such a progenitor cell is also referred to herein as an HLA double negative, or as MHC -/-..

The HUCPV cell population extracted from WJ is also characterized by "rapid proliferation", which refers to the rate at which the extracted cells will grow relative to other known progenitor cell populations, under conditions that are standard for progenitor cell expansion. As will be appreciated from the experimental results presented herein, and as shown in Figure 16, the progenitor cell population can double within at least about 25 hours and as quickly as 7-15 hours, and thus expands far more rapidly than other known osteoprogenitor cell populations and other progenitor cell populations extracted from WJ.

The cells and cell populations can be obtained by extraction from WJ of human umbilical cord. Unlike the prior art such cells are extracted from the WJ that is associated with, i.e., proximal to, the exterior wall of the umbilical vasculature. The Wharton's jelly that is associated with or very near to the external surface of the cord vasculature lies within a region termed the perivascular zone, and typically remains associated with the vasculature when the vessels are excised from the cord, as is done for instance either to extract Wharton's jelly from the cord, or to extract the vessels from the cord and associated Wharton's jelly. It has remarkably been found that the Wharton's jelly within this perivascular zone, and which has typically been discarded in prior art practice, is a rich source of progenitor cells having the characteristics herein described. Accordingly, one exploits the tissue from this perivascular zone of the Wharton's jelly as a source for useful human progenitor cells, termed HUCPVCs.

In embodiments, the HUCPV cell population is characterized by the presence of progenitor cells having many markers indicative of a functional mesenchymal (non-hematopoietic) phenotype, i.e., CD45-, CD34-, SH2+, SH3+, Thy-1+ and CD44+. Of particular significance, the population is characterized generally as harbouring cells that are positive for 3G5 antibody, which is a marker indicative of pericytes. The extracted cell population generally is a morphologically homogeneous fibroblastic cell population, which expresses alpha-actin, desmin, and vimentin, and provides a very useful source from which desired cell subpopulations can be obtained through

manipulation of culturing conditions and selection based for instance on cell sorting principles and techniques.

To extract such perivascular cells from human umbilical cord care is taken during the extraction process to avoid extracting cells of the umbilical cord blood, epithelial cells or endothelial cells of the UC, and cells derived from the vascular structure of the cord, where vascular structure is defined as the tunicae intima, media and adventia of arterial or venous vessels. Obtaining an extract that is essentially free of these unwanted cells can be achieved by careful flushing and washing of the umbilical cord prior to dissection, followed by careful dissection of the vessels from within the cord. The vessels can also be carefully pulled away from the surrounding cord tissue in which case the perivascular tissue is excised with the vessels. It will be appreciated that, with care being taken to avoid extracting these unwanted cells, they may still be present to a small extent in the resulting extract. This is acceptable provided they occur at a frequency too low to interfere with the observed results presented herein, i.e., observation of cell colonies derived from mesenchymal and specifically mesodermal origin, frequency and rapidity of formation of CFU-F, CFU-O and CFU-A, and characterization of HLA phenotypes observed in the cultured population.

The tissue that lies within the perivascular zone is the Wharton's jelly proximal to the external wall of the umbilical vasculature, and lies typically within a zone extending to about 3mm from the external wall of the vessels. Suitably, the target extraction zone can lie within about 2mm, e.g., about 1mm from the external wall of any one of the three vessels. The extraction of WJ from this region can be readily achieved using the technique described in the examples. In this technique the vessels are used as a carrier for the WJ, and the vessels per se are used as the substrate from which the progenitor cells are extracted. Thus cord vessels bearing a thin coating of perivascular tissue are excised either surgically or manually from fresh umbilical cord that has been washed thoroughly to remove essentially all cord blood contaminants. The vessels bearing the proximal perivascular tissue, or sections thereof, are then incubated at about 37°C in an extraction medium such as phosphate buffered saline (PBS) containing an enzyme suitable for digesting the collagen matrix of the perivascular tissue in which the desired cells reside. For this purpose, digestion with a collagenase is suitable, at a concentration within the range from about 0.1mg/mL to

10.0mg/mL or more, e.g., 0.5mg/mL. The enzyme type, concentration and incubation time can vary, and alternative extraction conditions can be determined readily simply by monitoring yield of cell phenotype and population under the chosen conditions. For instance, a higher collagenase concentration of 4mg/mL (e.g., 1-4mg/mL) is also suitable over a shorter digestion period of about 3 hours (e.g., 1-5 hours). During the extraction, the ends of the vessels are tied, or clipped, off and can be suspended above the extraction medium to avoid contamination by agents contained within the vessel. It will thus be appreciated that the Wharton's jelly extract is essentially free from cord blood cells, umbilical cord epithelial cells, vessel endothelial cells and vessel smooth muscle cells.

According to the present invention, such tissue is frozen and preferably stored cryogenically, and restored from the frozen or cryogenic state in a manner permitting the extraction of viable HUCPV progenitor cells from the stored tissue.

Other digestive enzymes that can be used in the isolation procedure are 0.1 to 10 mg/ml hyaluronidase, 0.05 to 10 mg/ml trypsin as well as EDTA. The optimum collagenase concentration is 4mg/ml for a digestion period of 3 hours, although a less expensive alternative is to use 0.5mg/ml for 18-24 hours. Still other alternatives to collagenase concentrations are illustrated in Figure 21. Desirably, digestion is halted at or before the vessels begin to degrade which, as shown in Figure 21, occurs at different time points depending on the collagenase concentration.

After about 24 hours in the 0.5mg/mL collagenase extraction medium, e.g., 12- 36 hours, such as 18-24 hours, or after about 3 hours in the 4.0mg/mL collagenase extraction medium, the vessels are removed, leaving a perivascular tissue extract that contains human progenitor cells. These cells are expanded under conditions standard for expansion of progenitor cells. The cells can, for instance, be selected on polystyrene to select for adherent cells, such as in polystyrene dishes or flasks and then maintained in a suitable culturing medium. The extracted cells can be cultured for expansion, with or without prior selection for adherent cells, under conditions of stirred suspension, as described for instance by Baksh et al in WO02/086104.

The extracted population of HUCPVCs can be cultured under adherent conditions, and non-adherent cells resident in the supernatant are recovered for further culturing. These "post-adherent" cells are characterized as a subpopulation by a propensity to form bone nodules and fat cells spontaneously. Thus, the procedure provides an isolated population of progenitor cells extracted from perivascular tissue, the cells having the propensity to form at least one of several differentiated cell types including bone cells, cartilage cells, fat cells and muscle cells, wherein such progenitor cells constitute the non-adherent fraction of the HUCPVCs cultured under adherent conditions. Such cells are obtained by culturing the perivascular tissue-extracted HUCPVCs under adherent conditions, selecting the non-adherent cell population, and then culturing the non-adherent cell population under conditions useful to (1) expand said population or (2) to cause differentiation thereof into a desired cell phenotype. Culturing conditions useful therein are those already established for such expansion and differentiation, as exemplified herein.

The HUCPV subpopulations can be cultured and expanded under standard adherent culturing conditions. As is revealed herein, such adherent cell populations are known to comprise the immunoincompetent or non-immunogenic, progenitors, and mesenchymal progenitors.

The cells present in the extract can, either directly or after their expansion, be sorted using established techniques to provide expandable subpopulations enriched for cells of a given phenotype. Thus, there is further provided perivascular tissue extracted cell populations that are enriched for multipotent mesenchymal progenitor cells, osteoprogenitor cells, cell populations that are enriched for immuno-incompetent progenitor cells, and cell populations that are enriched for multipotent and osteoprogenitor cells that are immuno-incompetent. Further, the cells can be enriched to select for only those that are positive for the pericyte marker 3G5, using antibody thereto, and to select only for those that are negative for either one or both of the MHC class I and class II markers. The cell population can also be enriched by selection against other surface markers, such as by depletion of those bearing CD45 to remove hematopoietic cells, for instance.

As is revealed in Figure 17, the distribution of MHC markers within the progenitor cell population is altered by freeze-thawing. Upon passaging of fresh cells, the frequency of MHC double negative cells is relatively constant/marginally increased. However, the frequency of MHC double negative cells in the progenitor population is increased significantly in cells plated following freezing. Thus, in the progenitor cell population, cells of the MHC double negative phenotype are further characterized by the propensity to increase in frequency following freezing. Such freezing is performed in the usual manner, by first preparing a cell aliquot, and then storing the cell preparation for the desired period. It will be appreciated that such cells can be stored for many years if desired.

The method is useful for producing MHC double negative progenitor cells, by obtaining a perivascular tissue extract as herein described, or an MHC double negative-enriched fraction thereof, subjecting the extract or fraction thereof to freezing, and then culturing the frozen cells. The resulting cells as noted are potentially useful to induce tissue formation or repair in human subjects.

The cell populations obtained from the extract or from a suitably enriched fraction thereof, are useful either directly or following their expansion to provide differentiated cell populations. All of the procedures suitable for their fractionation and enrichment, and for their expansion are established in the prior art. Expansion can proceed, for instance, in the presence of factors such as IL-3 and Stem Cell Factor, and similar agents known in the art. The cell population, and particularly the osteoprogenitor cells therein, can be subjected to differentiation using conditions established for the growth of bone tissue therefrom. Remarkably, a subpopulation of osteoprogenitor cells that arise from the culturing of the progenitor cell population, referred to as committed osteoprogenitors, have shown the ability to differentiate in the absence of osteogenic supplements. Alternatively, the osteoprogenitor cells are cultured in a medium supplemented with one or more agents that stimulate osteogenesis, such as dexamethasone. In addition, the progenitor cells can also be cultured with supplements suitable for stimulating differentiation into other mesenchymally-derived connective tissues (Caplan, 1991), including cartilage, muscle, tendon, adipose etc., all in accordance with standard practice in the art.

As a practical alternative to *in vitro* culturing of cells in the cell population, it will be appreciated that the cells can be transplanted *in vivo* to induce the formation of a desired tissue directly within a patient. By this route, the *in situ* formation of bone is provided by implanting osteoprogenitor, for the benefit of patients suffering from various bone conditions, diseases and disorders, particularly including bone fracture and osteoporosis. Such therapies can also be applied to attenuating maladies of other connective tissues such as, but not limited to, cartilage, fat and muscle. The immuno-incompetent progenitor cells present in the cell population are particularly valuable in this respect, given the substantially reduced rejection response that can be expected following their implantation.

For use in transplantation, the cells can be provided as a composition, further comprising a carrier useful for their delivery to the tissue site selected for engineering. The cells are presented in a dose effective for the intended effect. It is expected that an effective cell dose will lie in the range from 10^3 to 10^7 cells, e.g., 10^4-10^6 such as 2 x 10^5 cells, per dose. The carrier selected for delivery of those cells can vary in composition, in accordance with procedures established for delivery of viable cells. In embodiments, the cells are exploited for purposes of bone tissue engineering. In one embodiment, the cells are presented with a carrier in the form of a scaffold material that serves to localize the cells as an implant at a bone site that is defective or fractured, or is surgically prepared to receive the implant. A variety of materials are suitable as carriers for this purpose. In a particular embodiment, the carrier is formed of resorbable material such as calcium phosphate, PLGA or mixtures thereof. Equivalent materials can be used, provided they allow for the cells to remain viable during formation and delivery of the composition, and are otherwise physiologically compatible at the implantation site.

Still other carriers suitable for delivery of the progenitor cells will include vehicles such as PBS and gels including hyaluronic acid, gelatin and the like with equivalents being useful provided they possess the pH and other properties required for cell viability.

It will also be appreciated that the cells are useful as hosts for delivering gene expression products to the desired tissue site. That is, the cells can be engineered

genetically to receive and express genes that upon expression yield products useful in the tissue repair process, such as the various growth factors which, in the case of bone tissue, can usefully include PTH, the BMPs, calcitonin, and the like. The cells can also be developed as transgenics for other purposes, such as by introduction of genes that alter the cell phenotype, to make it more robust, or more suitable to a given end-use.

Embodiments of the invention are described in the following examples.

Harvest of Progenitor Cells from Human Wharton's Jelly

The UCs were collected from full-term caesarian section infants immediately upon delivery at Sunnybrook & Women's College Hospital, Toronto, Canada. The UC was transferred by the surgeon into a sterile vessel containing medium (80% α-MEM, 20% antibiotics), and immediately transported to our laboratories at the Institute of Biomaterials & Biomedical Engineering, University of Toronto.

All procedures from this point on were performed aseptically in a biological safety cabinet. The UC was washed in Phosphate Buffered Saline (PBS) (-Mg^{2+}, -Ca^{2+}) three times to remove as much of the UC blood as possible, and transferred back into a container with medium. A length of approximately 6 cm of cord was cut with sterile scissors and placed onto a sterile cork dissection board. The remaining cord (30-45 cm) was returned to the medium-filled container and placed into an incubator at 37°C. The 6 cm section of cord was 'twisted' against its helix, and pinned at both ends to reveal a smooth and straight surface of the UC epithelium. Using fine scissors, the UC was cut approximately 1-2 mm deep along its length to reveal the WJ. Starting with each 'flap' of cut epithelium, the WJ was teased from its inner surface using the blunt edge of a scalpel, and the teased away epithelium (approximately 0.5mm thick) was pinned down. This procedure resulted in the WJ being exposed, and with its three vessels embedded in it running straight from end to end rather than helically along its longitudinal axis. Care was taken to constantly bathe the section with 37°C PBS. Isolating one of the ends of a vessel with forceps, it was teased away from the WJ along its length until it was free of the bulk of the WJ matrix. Alternatively, the middle of the vessel could be dissected from the matrix,

held with tweezers, and teased from the matrix in each direction toward its ends. Once freed by either method, the vessel was surrounded with approximately 1-2mm of the cell-bearing WJ matrix. The dissected vessel was then clipped at both ends with either a surgical clamp, mosquito clip or sutured to create a 'loop,' blocking the passage of fluid either into or out of the vessel. The 'loop' was immediately placed along with the scissors into a 50ml tube containing a 0.5mg/ml collagenase solution with PBS (-Mg^{2+}, -Ca^{2+}), and placed into an incubator at 37°C. The remaining two vessels were dissected in a similar fashion, looped, and also placed in the collagenase solution in the incubator. Subsequent to the removal of the vessels, strips of WJ, constituting perivascular tissue, could easily be dissected off the epithelium and placed into 50 ml tubes with the collagenase solution. The remaining epithelial layer was then disposed of in a biohazard waste container. The same protocol was used with the remaining 30-45 cm of UC, producing 15 to 25 tubes with either 'loops' or perivascular tissue strips.

Initiation of Wharton's Jelly Progenitor Cell Cultures

After 18-24 hours, the 'loops' were removed with the aid of their attached suspension clamp or suture and a pipette, and the remaining suspensions were then diluted 2-5 times with PBS and centrifuged at 1150rpm for 5 minutes to obtain the cell fraction as a pellet at the bottom of the tube/s. After removal of the supernatant, the cells were resuspended in eight times volume of 4% NH_4Cl for 5 minutes at room temperature in order to lyse any contaminating red blood cells. The suspensions were then centrifuged again at 1150rpm for 5 minutes to isolate the cell fraction as a pellet, and the supernatant was removed. After counting the cells with the use of hemocytometer, they were plated directly onto T-75 cm^2 tissue culture polystyrene dishes, and allowed to incubate at 37°C for 24-72 hours in order to allow the cells to attach to the polystyrene surface. The medium was then changed every two days.

The results detailed below have been reproduced using the procedure described above, but in which collagenase-based digestion proceeded either at 4mg/mL for 3 hours, 2mg/ml for six hours and at 1mg/mL for 12 hours.

The attached cells were passaged using 0.1% trypsin solution after 7 days, at which point they exhibited 80-90% confluency, as observed by light microscopy, and there

was evidence of 'mineralized' aggregate formation, as revealed under phase microscopy and indicated by expected changes in optical properties. Upon passage, cells were plated either in 35mm tissue culture polystyrene dishes or 6 well plates at $4x10^3$ cells/cm^2 in supplemented media (SM) (75% α-MEM or D-MEM, 15% FBS, 10% antibiotics) and treated with 10^{-8}M Dex, 5mM β-GP and 50 μg/ml ascorbic acid to test the osteogenic capacity of these cells. These plates were observed on days 2, 3, 4 and 5 of culture for CFU-O otherwise referred to as 'bone nodule' formation.

In order to test the chondrogenic capacity of these cells, $2x10^5$ cells were centrifuged at 1150 rpm for 5 minutes in order to obtain the cells as a pellet. Once the supernatant was removed, the cells were maintained in SM supplemented with 10ng/ml transforming growth factor-beta (TGF-β) (and optionally with 10^{-7}M dexamethasone). The supplemented medium was replaced every two days, maintaining the cultures for 3-5 weeks, at which point they were harvested for histology (by fixation with 10% neutral formalin buffer (NFB)), embedded in paraffin, cut into 6μm section, and stained for the presence of collagen II (antibody staining) and the presence of glycosaminoglycans (alcian blue staining). To assess the adipogenic differentiation capacity of the cells, they were initially cultured in 6-well plates in SM (with D-MEM), which was replaced every 2 days, until they reached 60% confluence. At that point the medium was replaced with the adipogenic induction medium (AIM) (88% D-MEM, 3% FBS, 33μM Biotin, 17μM Pantothenate, 5μM PPAR-gamma, 100nM Bovine insulin, 1μM Dexamethasone, 200μM Isobutyl methylxanthine and 10% antibiotics). The AIM was replaced every 2 days for 10 days at which point the cells were fixed in 10% NFB and stained with Oil Red O which stains the lipid vacuoles of adipocytes red. Finally, in order to assess the myogenic capacity of the cells, they were initially cultured in T-75 cm^2 tissue culture flasks in SM (with D-MEM) until they reached 80-90% confluence, at which point the medium was replaced with myogenic medium (MM) (75% D-MEM, 10% FBS, 10% Horse serum, 50μM hydrocortisone and 10% antibiotics). The MM was replaced every 2 days. After 3-5 weeks in culture, the cells were removed from the culture surface (see subculture protocol), lysed in order to obtain their mRNA, and assessed by rtPCR for the presence of several myogenic genes, including: MyoG, MyoD1, Myf5, Myosin heavy chain, myogenin and desmin.

Another useful approach to obtaining the perivascular tissue-derived progenitor cell cultures has been adopted, using the following protocol:

1. Obtain sterile umbilical cord (UC) from caesarian-section patient and transport to biological safety cabinet in media (80% α-MEM, 20% antibiotics)
2. Wash the UC 3x in sterile 37°C phosphate buffered saline (PBS)
3. Cut the UC into approximately 1-2 inch sections with a sharp pair of scissors
4. Wash each section of UC 2x in sterile 37°C PBS to remove as much residual umbilical cord blood (UCB) as possible.
5. Isolate one of the UC sections on a dry sterile dish
6. Using two sets of forceps, grasp the epithelium approximately 2mm apart, and pull away from each other, tearing the epithelium.
7. Grasping the epithelium along the length of the UC section, continue to tear the epithelium away, exposing the WJ underneath
8. Similarly to step 6, continue tearing the epithelium away in 'strips' until approximately half of the epithelium has been torn away.
9. The umbilical vessels should be clearly visible through the WJ, and the ends loose on the cut edges of the UC section.
10. By grasping a remaining part of the epithelium with one set of forceps, and the end of a vessel with the other, the vessel can be 'pulled' from the bulk WJ with its surrounding perivascular tissue (PVT).
11. This process is repeated with each vessel, until all three are free of the underlying WJ matrix.
12. Once released, each vessel is placed into 37°C PBS.
13. Steps 5-12 are repeated with each section of UC until all the vessels have been isolated in a sterile 37°C PBS-filled beaker.
14. Then, by placing each vessel individually on a clean, sterile surface, the ends can be ligated together with a suture using a double knot into a 'loop'
15. Once all of the vessels have been ligated into loops, the loops are placed into a 0.5mg/ml collagenase solution in a sterile 50ml tube. In the alternative, and according to the present invention, the vessels can be frozen, stored cryogenically and then restored, maintaining viability of the resident

progenitor (HUCPVCs) using the present method, and the viable progenitors resident in the thawed and treated cord tissue can be recovered using the method commencing at step 15.

16. The 50ml tube is placed into a rotator in a 37°C, 5%CO_2 incubator overnight.
17. The following day, the collagenase is inactivated with 1ml fetal bovine serum (FBS), and the loops removed from the suspension.
18. The remaining suspension is diluted with PBS, centrifuged at 1150rpm for 5 minutes, and the supernatant removed.
19. The pellet is then resuspended in 8 times volume of 4% NH_4Cl for 5 minutes at room temperature to lyse all contaminating red blood cells, then centrifuged at 1150rpm for 5 minutes, and the supernatant removed.
20. The cells remaining in the pellet are resuspended in supplemented media (SM) (75% α-MEM, 15% FBS, 20% antibiotics), and aliquot is counted on a hemocytometer.
21. The cell suspension is then plated onto a T-75 tissue-culture polystyrene flask, and allowed to proliferate.
22. After 2 days, the supernatant from the flask is transferred to a new T-75 flask in order to harvest the post adherent"" (PA) cells.
23. After 2 days, the supernatant from the first PA flask is transferred to a new T-75 flask in order to harvest any remaining PA cells.
24. The SM is replaced in all three T-75 flasks every 2 days until the cells reach sub-confluence (1-2 weeks), at which point they are sub-cultured (passaged).

CRYOPRESERVATION OF WHOLE UMBILICAL CORD VESSELS

The present invention allows for the retrieval of viable cells from cord tissue stored cryogenically, using the following exemplified procedure:

Freezing

Individual cord vessels were isolated as described above, and the ends were tied off with sutures forming loops as described above. The vessels were placed into 15 mL polypropylene tubes each containing 5 mLs of cryo-preservation media, consisting of 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO). The prepared

tubes were then placed into a -70C freezer overnight, and were transferred the following day to liquid nitrogen for longer term storage.

Thawing

A tube containing the prepared specimen was removed from liquid nitrogen after 3 days in storage, and placed immediately into a 37C water bath. Once fully thawed, approximately five minutes, the specimen loops were placed into a collagenase digest medium as describe above and placed into a rotisserie in a 37C incubator. After 18-24 hours of incubation, the digested vessels were removed, and progenitor cells were isolated using the procedure described above. After plating on tissue culture plates and culturing in 5-15% FBS supplemented media, also as described above, viable cells were identified by Trypan exclusion

An improved method, was also developed, as follows. The vessels with associated Wharton's jelly were removed from the cord and sectioned into segments about 1-2 inches, or about 4cm, long_, using the removal technique as described above. The removed vessel segments (about 5-10 segments per tube, to avoid crowding) were then placed in 10% DMSO and 90% FBS (cooled @ 4°C) in a 50mL centrifuge tube, 1mL per vessel. In place of the 90% FBS, different serum-containing mixtures or blends can be used, such as 10% serum (FBS or other) and 80% DMEM or other media suitable for cell culturing. The tubes containing the vessel segments in the DMSO/serum solution were then cooled transitionally by first incubating for 30 minutes in a fridge at 4°C. The tubes were then removed, and the vessels were transferred to cryopreservation containers, known as cryotubes. One vessel segment was added to each tube. To the cryotubes containing the vessels was added 1mL of cryoprotectant solution (90% Serum, 10% DMSO as above) per 4cm section of vessel, or enough to fully submerge the vessel.

The cryotubed vessel segments were then placed in a controlled rate freezer overnight (about 6-12 hours), reducing and holding the temperature to about -70°C. Thereafter, the tubes were transferred to liquid nitrogen storage (vapour phase), labeled for identification, and maintained at -196°C, for about one week.

To thaw the vessels, the cryotubes were removed from the liquid nitrogen storage. The vessels were removed from the cryotubes, and allowed to thaw for 10 minutes in a 37°C waterbath. The vessel segments were then transferred to 50mL tubes, and washed in cooled (4C) PBS, using about 1mL of the PBS per vessel segment. The tubes with vessel segments in the PBS were then allowed to incubate in the fridge at 4°C, to remove DMSO. The amount of cool PBS in each tube was then doubled, and the tubes were incubated for an additional 5 minutes in the fridge.

Following this procedure, the vessel segments are ready to serve as a source of viable progenitor cells, including HUCPVCs, particularly according to the progenitor cell extraction, culturing and differentiation techniques described further herein.

To confirm that viable cells are recoverable from the tissue so preserved, the vessel segments were subjected to the digestion procedure noted above, the cells released were plated as described with reference to Figure 22, and the cells were counted in a ViCell-XR™ machine using trypan blue exclusion. Following this, cells determined to be viable were plated in standard culture media as mentioned herein, and demonstrated their ability to form colonies and proliferate, as shown in panels A and B.

This method resulted in cells which are bioequivalent to the cells which are removed from the standard digestion procedure.

Other methods have been attempted, including immersion of the dissected tissues in 10% DMSO then step frozen at -70C followed by cryopreservation. These methods resulted in very few viable cells, as shown in panel C of Figure 22.

PROGENITOR ASSAYS

Cell Proliferation Assay

During the weekly passage procedure (occurring every 6 days), aliquots of 3 x 10^4 cells were plated into each well of 24 6-well tissue culture polystyrene plates. On days 1,2,3,4,5 and 6 days of culture, four of the 6-well plates were passaged and the cells were counted. The exponential expansion of these cells was plotted, and the mean doubling time for the cells in these cultures was calculated. Results are shown

in Figure 16. It will be noted that the doubling time for the PVWJ cell culture is about 24 hours across the entire culturing period. During the log phase, the doubling time is a remarkable 16 hours. This compares with literature reported doubling times of about 33-36 hours for bone marrow mesenchymal cells (Conget and Minguell, 1999), and about 3.2 days for mesenchymal stem cells derived from adipose tissue (Sen et al., 2001). For observation of proliferation with successive passaging, $3x10^5$ cells were plated into 4 T-75 flasks (n=4) and fed with SM which was replaced every 2 days. After 6 days of culture the cells were subcultured (see subculture protocol above), and counted with the use of a hemocytometer. Aliquot of $3x10^5$ cells were seeded into 4 new T-75 flasks, cultured for 6 days, and the process of counting was repeated. This process was repeated from P0 through P9 for 4 cord samples.

Figure 18 illustrates the CFU-F frequency of HUCPVCs. The frequency of 1:300 is significantly higher than that observed for other mesenchymal progenitor sources including neonatal BM ($1:10^4$) (Caplan, 1991), and umbilical cord blood-derived "unrestricted somatic stem cells" (USSCs) (Kogler et al., 2004) which occur at a frequency of $1:2x10^8$. Figure 19 illustrates the proliferation rate of HUCPVCs with successive passaging. The initial doubling time of 60 hours at P0 drops to 38 hours at P1, which drops and maintains itself at 20 hours from P2-P8. The cells begin to enter senescence thereafter and their proliferation rate begins to drop rapidly. Interestingly, when observed during the first 30 days of culture (Figure 20), HUCPVCs derive $2x10^{10}$ cells within 30 days. As one therapeutic dose (TD) is defined as $2x10^5$ cells (Horwitz et al, 1999) (Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999; 5:309-313.), HUCPVCs can derive 1 TD within 10 days of culture, and 1,000 TDs within 24 days of culture.

As show in Figure 15, the perivascular tissue-derived progenitors comprise different sub-populations of progenitor cells. Within this population, there are the so-called "committed" osteoprogenitor cells characterized by an ability to form bone nodules, as shown herein, in the absence of the culturing supplements normally required to induce such differentiation, such as culturing in the presence of dexamethasone. These committed osteoprogenitors thus differentiate spontaneously to form bone

nodules. Also within the progenitor population are osteogenic cells that can be induced to form bone nodules, when cultured in the presence of the required factors, such as dexamethasone, β-glycerophosphate and ascorbic acid. Thus, the "total osteogenic" sub population graphed in Figure 15 includes a "committed osteogenic progenitor" population, as well as an "uncommitted osteogenic progenitor" population, and reveals the total number of cells that can be induced to differentiate along the osteogenic differentiation pathway. The actual ratio between the "committed" and "uncommitted" population is approximately 1:1, and so the ratio between the "total osteogenic progenitor" population and the "committed osteogenic progenitor" population is about 2:1. Analysis of the bone forming properties of the progenitors was performed as noted below.

Chondrogenic, adipogenic and myogenic differentiation of the cells has also been observed. Although osteogenic and occasional adipogenic differentiation has been observed spontaneously, the other phenotypes were only observed when cultured under specific induction conditions for the respective phenotype.

Serial dilution and CFU-F assays

Dilutions of 1 x 10^5, 5 x 10^4, 2.5 x 10^4, 1 x 10^4, 5 x 10^3, 1 x 10^3, HUCPVCs were seeded onto 6-well tissue culture plates (Falcon# 353046) and fed every two days with SM. The number of colonies, comprising >16 cells, were counted in each well on day 10 of culture, and confirmed on day 14. CFU-F frequency, the average number of cells required to produce one colony, was consequently determined to be 1 CFU-F/300 HUCPVCs plated. Based on this frequency, the unit volume required to provide 300 HUCPVCs (done in triplicate from each of 3 cords) was calculated, and 8 incremental unit volumes of HUCPVCs were seeded into individual wells on 6-well plates. Again, colonies comprising >16 cells (CFU-Fs) were counted on day 10 of culture to assay CFU-F frequency with incremental seeding.

CFU-O Assay

During the weekly passage procedure, aliquots of test cell populations were directly plated on tissue-culture polystyrene in bone forming medium containing 75% α-MEM, 15% FBS (StemCell Batch #: S13E40), 10% antibiotic stock solution containing penicillin G (167 units/ml), gentamicin (50μg/ml) and amphotericin B

(0.3 µg/ml), and Dex (10-8M), (3-glycerophosphate (5mM) and L-ascorbic acid (50ug/ml), at a cell seeding density of 1 x 10^4 cells/cm^2. Cultures were re- fed every two days for a period of 12 days. The cultures were maintained until mineralized nodular areas, detected as bone nodules, were observed (usually 3 days) at which point the cultures were re-fed with tetracycline containing medium at the last culture re-feed, then fixed in Karnovsky's fixative and prepared for analysis. A Leitz Aristoplan™ microscope (Esselte Leitz GmbH & Co KG, Stuttgart, Germany) was used to visualize the tetracycline labelled cultures under phase contrast as well as UV fluorescence and a Hitachi S-2000™ scanning electron microscope at an accelerating voltage of 15 kV was used to generate images to demonstrate the presence of morphologically identifiable bone matrix.

CFU-C Assay

In order to test the chondrogenic capacity of these cells, $2x10^5$ cells were centrifuged at 1150 rpm for 5 minutes in order to obtain the cells as a pellet. Once the supernatant was removed, the cells were maintained in SM supplemented with 10ng/ml transforming growth factor-beta (TGF-β) (and optionally with 10^{-7} M dexamethasone). The supplemented medium was replaced every two days, maintaining the cultures for 3-5 weeks, at which point they were harvested for histology (by fixation with 10% neutral formalin buffer (NFB)), embedded in paraffin, cut into 6 µm section, and stained for the presence of collagen II (antibody staining) and the presence of glycosaminoglycans (alcian blue staining). Staining confirmed the formation of chondrocytes under induction conditions.

CFU-A Assay

To assess the adipogenic differentiation capacity of the cells, they were initially cultured in 6-well plates in SM (with D-MEM), which was replaced every 2 days, until they reached 60% confluence. At that point the medium was replaced with the adipogenic induction medium (AIM) (88% D-MEM, 3% FBS, 33 µM Biotin, 17 µM Pantothenate, 5 µM PPAR-gamma, 100nM Bovine insulin, 1 µM Dexamethasone, 200 µM Isobutyl methylxanthine and 10% antibiotics). The AIM was replaced every 2 days for 10 days at which point the cells were fixed in 10% NFB and stained with Oil Red O which stains the lipid vacuoles of adipocytes red. Staining confirmed the

formation of adipocytes, not only under induction conditions, but also in their absence, i.e., spontaneously

CFU-M Assay

In order to assess the myogenic capacity of the cells, they were initially cultured in T- 75 cm^2 tissue culture flasks in SM (with D-MEM) until they reached 80-90% confluence, at which point the medium was replaced with myogenic medium (MM) (75% D-MEM, 10% FBS, 10% Horse serum, 50 µM hydrocortisone and 10% antibiotics). The MM was replaced every 2 days. After 3-5 weeks in culture, the cells were removed from the culture surface (see subculture protocol), lysed in order to obtain their mRNA, and assessed by rtPCR for the presence of several myogenic genes, including: MyoG, MyoDl, Myf5, Myosin heavy chain, myogenin and desmin. Results confirmed the presence of myocytes under these induction conditions.

DATA ANALYSIS

Tetracycline Stain

Tetracycline (9 µg/ml) was added to the cultures prior to termination. At termination, the cells were fixed in Karnovsky's fixative overnight and then viewed by UV- excited fluorescence imaging for tetracycline labeling of the mineral component of the nodular areas.

Scanning Electron Microscopy (SEM)

Representative samples of CFU-O cultures were prepared for SEM by first placing them in 70%, 80%, 90% and 95% ethanol for 1 hour, followed by immersion in 100% ethanol for 3 hours. They were then critical point dried. A layer of gold approximately 3 nm layer was sputter coated with a Polaron SC515 SEM Coating System™ onto the specimens, which were then examined at various magnifications in a Hitachi S- 2000 scanning electron microscope at an accelerating voltage of 15 kV. The images generated are used to demonstrate the presence of morphologically identifiable bone matrix.

Flow cytometry for HLA-typing

Test cell populations of >1 x 10^5 cells were washed in PBS containing 2% FBS (StemCell Batch #: S 13E40) and re-suspended in PBS + 2% FBS with saturating

concentrations (1:100 dilution) of the following conjugated mouse IgGI HLA- A,B,C- PE and HLA-DR,DP,DQ-FITC for 30 minutes at 4°C. The cell suspension was washed twice with PBS + 2% FBS, stained with 1 g/ml 7-AAD (BD Biosciences) and re-suspended in PBS + 2% FBS for analysis on a flow cytometer (XL™, Beckman-Coulter, Miami, FL) using the ExpoADCXL4™ software (Beckman-Coulter). Positive staining was defined as the emission of a fluorescence signal that exceeded levels obtained by >99% of cells from the control population stained with matched isotype antibodies (FITC- and PE-conjugated mouse IgGl,x monoclonal isotype standards, BD Biosciences). For each sample, at least 10,000 list mode events were collected. All plots were generated in EXPO 32 ADC™ Analysis software.

In addition to HLA typing, the HUCPV cell population was also assessed for other markers, with the following results:

Marker	Expression
CD105 (SH2)	+ +
CD73 (SH3)	+ +
CD90 (Thy1)	+ +
CD44	+ +
CD117 (c-kit)	15% +
MHC I	75% +
MHC II	-
CD106 (VCAM1)	-
STRO1	-
CD123 (IL-3)	-
SSEA-4	-
Oct-4	-
HLA-G	-
CD34	-
CD235a (Glycophorin A)	-
CD45	-

RESULTS

Light micrographs of bone nodule Colonies

Figures 3, 4 and 5 illustrate CFU-O's that were present in the cultures on day 3 and day 5. They demonstrated the confluent layer of "fibroblast-like" cells surrounding a nodular area represented by an 'aggregation' of polygonal cells that were producing the bone-matrix. These CFU-O's were observed in both the Dex (+) and Dex (-) cultures, and displayed similar morphology over successive passages.

Tetracycline Labeling of CFU-O Cultures

Tetracycline labeling of cultures was used for labeling newly formed calcium phosphate associated with the biological mineral phase of bone. The tetracycline labeling of the cultures coincide with the mineralized nodular areas, which is visualized by exposing the cultures to UV light. Figures 6 and 7 depict tetracycline labeled CFU-O cultures of Day 3 and Day 5 cultures of progenitor cells. These images were generated by UV-excited fluorescence imaging, and photographed.

Scanning Electron Microscopy

The CFU-O's were observed under SEM for formation of mineralized collagen matrix which demonstrates the formation of the CFU-O's from the initial stages of collagen formation through to the densely mineralized matrix in the mature CFU-O. Figures 8, 9, 10, 11, 12 and 14 represent scanning electron micrographs of the CFU-Os.

Flow Cytometry & HLA-typing

The flow cytometry, identifying cell-surface antigens representing both Major Histocompatibility Complexes (MHCs) demonstrated 77.4% of the population of isolated cells as MHC $^{-/-}$. Figure 13 illustrates the flow cytometry results in relation to the negative control. Figure 17 shows the impact of freeze-thawing on the frequency of MHC -/- cells in the progenitor population. The effect of freeze-thawing was studied as follows:

Test cell populations of >1 x 10^5 cells were washed in PBS containing 2% FBS and re-suspended in PBS + 2% FBS with saturating concentrations (1:100 dilution) of the following conjugated mouse IgG1 HLA-A,B,C-PE (BD Biosciences #555553 , Lot M076246) (MHC I), HLA-DR,DP,DQ-FITC (BD Biosciences #555558 , Lot M074842) (MHC II) and CD45-Cy-Cychrome (BD Biosciences # 555484, Lot 0000035746) for 30 minutes at 4°C. The cell suspension was washed twice with PBS + 2% FBS and re-suspended in PBS + 2% FBS for analysis on a flow cytometer (XL, Beckman-Coulter, Miami, FL) using the ExpoADCXL4 software (Beckman-Coulter). Positive staining was defined as the emission of a fluorescence signal that exceeded levels obtained by >99% of cells from the control population stained with matched isotype antibodies (FITC-, PE-, and Cy-cychrome-conjugated mouse IgG1,κ

monoclonal isotype standards, BD Biosciences), which was confirmed by positive fluorescence of human BM samples. For each sample, at least 10,000 list mode events were collected. All plots were generated in EXPO 32 ADC Analysis software.

Sub-culture & cell seeding

The attached cells were sub-cultured (passaged) using 0.1% trypsin solution after 7 days, at which point they exhibited 80-90% confluency as observed by light microscopy. Upon passage, the cells were observed by flow cytometry for expression of MHC-A,B,C, MHC-DR,DP,DQ, and CD45. They were then plated in T-75 tissue culture polystyrene flasks at $4x10^3$ cells/cm^2 in SM, and treated with 10^{-8}M Dex, 5mM β-GP and 50 μg/ml ascorbic acid to test the osteogenic capacity of these cells. These flasks were observed on days 2, 3, 4, 5 and 6 of culture for CFU-O or bone nodule, formation. Any residual cells from the passaging procedure also were cryopreserved for future use.

Cryopreservation of cells

Aliquots of $1x10^6$ PVT cells were prepared in 1ml total volume consisting of 90% FBS, 10% dimethyl sulphoxide (DMSO) (Sigma D-2650, Lot# 11K2320), and pipetted into 1ml polypropylene cryo-vials. The vials were placed into a -70°C freezer overnight, and transferred the following day to a -150°C freezer for long-term storage. After one week of cryo-preservation, the PVT cells were thawed and observed by flow cytometry for expression of MHC-A,B,C, MHC-DR,DP,DQ, and CD45. A second protocol was used in which the PVT cells were thawed after one week of cryopreservation, recultured for one week, sub-cultured then reanalyzed by flow cytometry for expression of MHC-A,B,C, MHC-DR,DP,DQ, and CD45.

The results are presented in Figure 17. It will be noted that the frequency of MHC-/- within the fresh cell population is maintained through several passages. When fresh cells are frozen after passaging, at -150°C for one week and then immediately analyzed for MHC phenotype, this analyzed population displays a remarkably enhanced frequency of cells of the MHC -/- phenotype. Thus, cells of the MHC -/- phenotype can usefully be enriched from a population of PVT cells by freezing. Still further enrichment is realized upon passaging the cultures of the previously frozen cells. In particular, and as seen in Figure 17, first passage of cryopreserved cells

increases the relative population of MHC -/- cells to greater than 50% and subsequent freezing and passaging of those cells yields an MHC -/- population of greater than 80%, 85%, 90% and 95%.The frozen PVT cells per se are potentially very useful in human therapy, given their non immunogenic nature.

Harvest of Post Adherent HUCPV cell fraction

The yield of progenitors recovered from the perivascular tissue can be enhanced in the following manner. In order to harvest the "post adherent" (PA) fraction of HUCPVCs, the supernatant of the initially seeded HUCPV harvest was replated onto a new T-75 flask, and incubated at 37°C, 5% CO_2 for 2 days. The initially seeded HUCPV flask was then fed with fresh SM. After 2 days this supernatant was again transferred to a new T-75 flask, and the attached cells fed with fresh SM. Finally, the supernatant of the third seeded flask was aspirated, and this flask fed with fresh SM. (Consequently, for each cord, 3 flasks are generated: the initially seeded flask, the first PA fraction and the second PA fraction.) Similar to identical characteristics of these cells are seen compared to the initially seeded cells, confirming that higher cell yields are obtained by isolating these PA fractions. Similar to the initially seeded HUCPVCs, these PA cells have a rapid proliferation rate, spontaneously produce bone nodules in culture, and can be induced to differentiate into the other three lineages: cartilage, fat and muscle.

Tissue Engineering Compositions comprising the progenitor cells

In this example, the cells are combined with a carrier in the form of CAP/PLGA used commonly in the bone engineering field. In order to seed the CAP/PLGA scaffolds, they were cut into 5mm by 5mm cylinders. Then, a 200µl suspension of $2x10^5$ HUCPVCs was placed into a sterile 1.5ml eppendorf tube, and the scaffold placed into the suspension. Using a modified pipette tip (with a suction diameter of 5mm), the suspension of cells was suctioned and washed through the scaffold several times by pipetting up and down. The scaffolds were then incubated in this suspension for 4 hours at 37°C, 5%CO_2, to allow for attachment of the cells to the scaffold. After the 4 hours, the scaffolds were removed from the suspensions and placed into individual wells of a non-tissue culture treated 24-well plate, fed with 1ml of SM and incubated at 37°C, 5%CO_2 for 14 days, the SM being replaced every 2 days. The scaffolds were

then fixed in Karnovsky's fixative and prepared for SEM analysis (see above). After 14 days of culture, the HUCPVCs completely covered the scaffold.

As noted, the PVT progenitor cell population may also be exploited to give rise to mesenchymal cells and tissues other than bone, by culturing under conditions appropriate for such differentiation. To generate adipocytes, for instance, the progenitors are prepared at a concentration of 10^4 cells/cm^2 and plated in 35 mm tissue culture dishes. The cells are maintained in Preadipocyte Medium (PM) (DMEM/Ham's F-10 (1:1, vol/vol), 10% fetal calf serum, 15 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B) for 3 days. After 3 days, the PM is removed, and the cells are fed with Adipogenic medium (DMEM/ Ham's F-10 nutrient broth, 1:1, v/v; HEPES buffer (15 mM); Fetal Bovine Serum (3%); Biotin (33 µM), Pantothenate (17 µM), human insulin (100 nM), dexamethasone (0.5 µM), PPARY agonist (1 µM) and antibiotics), and cultured for 3 days. After the 3 day induction, the Adipogenic medium is removed, and the cultures are maintained in Adipocyte Medium (AM) (DMEM/Ham's F-10 (1:1, vol/vol), 3% fetal calf serum, 1 µM dexamethasone, 100 nM human insulin, 33 µM D-biotin, 17 µM Na-pantothenate, 15 mM HEPES, 100 U/ml penicillin , 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B), with regular feeding every 3 days, ensuring to only remove half the medium, replenishing with an equal volume of AM since adipocytes will float if all the media is removed. After four feedings (12 days), cells appear rounded with lipid droplets. Positive identification of differentiated mesenchymal cells into adipocytes can be confirmed by staining with Oil Red O and Nile Red.

Similarly, chondrocytes may be generated using cell suspensions prepared at a concentration of 10^4 cells/cm^2 and plated in 35 mm tissue culture dishes. To promote chondrogenic cells are cultured without serum and with transforming growth factor-β3. The cell pellets develop a multilayered matrix-rich morphology and histologically show an increased proteoglycan-rich extracellular matrix during culture.

To generate myoblasts, cell suspensions are prepared at a concentration of 10^4 cells/cm^2 and plated in 35 mm tissue culture dishes. The cells are maintained in MCDB 120 medium completed with 15% fetal bovine serum (FBS) for 1 week (myoblast proliferation medium, MPM). At 1 week, the serum level in the basal

medium (MPM) is dropped to 2% (myoblast differentiation medium, MDM) and the cultures are terminated after 7 days. The cultures are re-fed 3-times a week with appropriate culture medium.

It will thus be appreciated that the present invention provides cryogenically preserved umbilical cord tissue that can be a useful source of viable human progenitor cells having properties useful in the production of various connective tissues including bone, and progenitor cells that are immune incompetent and ideal for transplantation into human patients to treat connective tissue conditions including bone diseases and disorders. The human progenitor cells are generated from extracts of a particular zone of human umbilical cord Wharton's jelly, termed the perivascular zone, extending proximally from the external wall of the cord vessels. The cell population extracted from this zone displays remarkable properties, including rapid proliferation, changes in cell morphology, as witnessed by the formation of cell colonies occurring before day 7 in all subcultured flasks (approximately 7-10 doublings) and the appearance of bone nodule formation without the addition of osteogenic supplements to the culture medium, as well as relatively high frequency of MHC double negative cells, the frequency of which is increased upon culturing of cells that have been frozen.

When used herein, the term "about" refers to a value that is +/- 10% of the value qualified by this term. Thus, a temperature of "about -70C" can refer to a temperature that is within the range from 63C to 77C.

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WE CLAIM:

1. A method for recovering viable cells from cryogenically frozen umbilical cord tissue, comprising the steps of:

1) obtaining cryogenically stored cord tissue, wherein the cord tissue is (1) an umbilical cord blood vessel bearing perivascular Wharton's jelly that comprises progenitor cells, (2) a segment of said blood vessel, or (3) Wharton's jelly tissue obtained from said vessel, and is prepared by a method comprising the steps of

a) obtaining said umbilical cord tissue comprising viable cells,

b) combining the umbilical cord tissue by immersion in a cryopreserving solution comprising serum-containing cell culturing medium and a cryopreservant, and

c) subjecting the combination to a cooling process in which the combination is refrigerated as a liquid for a period and at a temperature permitting the cryopreservant to penetrate the tissue,

d) freezing the cooled combination, to provide frozen tissue, and

e) storing the frozen tissue under cryogenic conditions;

2) thawing the frozen cord tissue;

3) treating the thawed cord tissue to displace the cryopreservant with water or buffer; and

4) recovering viable progenitor cells from the cord tissue so treated.

2. A method for preserving the viability of progenitor cells present in umbilical cord tissue, comprising the steps of

a) obtaining umbilical cord tissue comprising viable progenitor cells, wherein said umbilical cord tissue is (1) an umbilical cord blood vessel bearing perivascular Wharton's jelly that comprises said progenitor cells, (2) a segment of said blood vessel, or (3) Wharton's jelly tissue obtained from said vessel,

b) combining the umbilical cord tissue by immersion with a cryopreserving solution comprising serum-containing cell culturing medium and a cryopreservant;

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c) subjecting the combination to a cooling process in which the combination is refrigerated as a liquid for a period and at a temperature permitting the cryopreservant to penetrate the tissue,

d) freezing the cooled combination, and optionally, then

e) storing the frozen combination under cryogenic conditions.

3. The method according to claim 1 or claim 2, wherein the cryopreservant is DMSO.

4. The method according to claim 1 or 2, wherein the cell culturing medium comprises fetal bovine serum.

5. The method according to claim 1 or claim 2, wherein the cryopreserving solution comprises, by volume, from 5-25% DMSO, and from 75-95% cell culturing medium supplemented with fetal bovine serum.

6. The method according to claim 5, wherein the cryopreserving solution is 10% DMSO, and 90% cell culturing medium supplemented with fetal bovine serum.

7. The method according to any one of claims 1-6, wherein the umbilical cord tissue is human umbilical cord tissue.

8. The method according to claim 6 or claim 7 wherein the umbilical cord tissue is essentially free from blood.

9. The method according to any one of claims 1 to 8, wherein the cooling process is performed at about 4°C for a period of at least 20 minutes.

10. The method according to any one of claims 1 to 8, wherein the freezing process is conducted at about -70°C.

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11. The method according to any one of claims 1 to 8, wherein the cryogenic storage is conducted at about -196°C.

12. The method according to claim 1, wherein the thawing process is performed at about 37°C.

13. The method according to claim 1 wherein the thawed cord is treated by immersion in water or buffer at about 4°C.

14. The method according to claim 1 wherein the viable cells recovered from the stored cord are human umbilical cord perivascular cells.

15. The method according to claim 14, wherein the viable cells are recovered by digestion of the Wharton's jelly.

16. A method for recovering viable HUCPVCs from frozen umbilical cord tissue, comprising the steps of:

1) obtaining cryogenically stored cord tissue, wherein the cord tissue is (1) fresh, isolated umbilical cord blood vessel bearing perivascular Wharton's jelly that comprises said HUCPVC progenitor cells, (2) a segment of said blood vessel, or (3) Wharton's jelly tissue obtained from said vessel, and is prepared by a method comprising the steps of

a) obtaining said umbilical cord tissue,

b) combining the umbilical cord tissue by immersion with a cryopreserving solution comprising serum-containing cell culturing medium and DMSO,

c) subjecting the combination to a cooling process in which the combination is refrigerated as a liquid for a period and at a temperature permitting the DMSO to penetrate the tissue,

d) freezing the cooled combination, and

e) storing the frozen combination under cryogenic conditions for a period of time; and then

2) thawing the stored cord tissue;

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3) treating the thawed cord tissue to displace the DMSO with water or buffer; and

4) digesting the Wharton's jelly associated with the stored tissue to release viable HUCPVCs.

17. The method according to claim 16 wherein said cord tissue is a segment of an umbilical cord blood vessel bearing perivascular Wharton's jelly that comprises said HUCPVC progenitor cells.

18. The method according to any one of claims 1-15 wherein said cord tissue is a segment of an umbilical cord blood vessel bearing perivascular Wharton's jelly that comprises HUCPVC progenitor cells.

19. The method according to any one of claims 1-15 wherein said cord tissue is Wharton's jelly comprising HUCPVC progenitor cells, wherein the Wharton's jelly is obtained from the perivascular region of an umbilical cord blood vessel.

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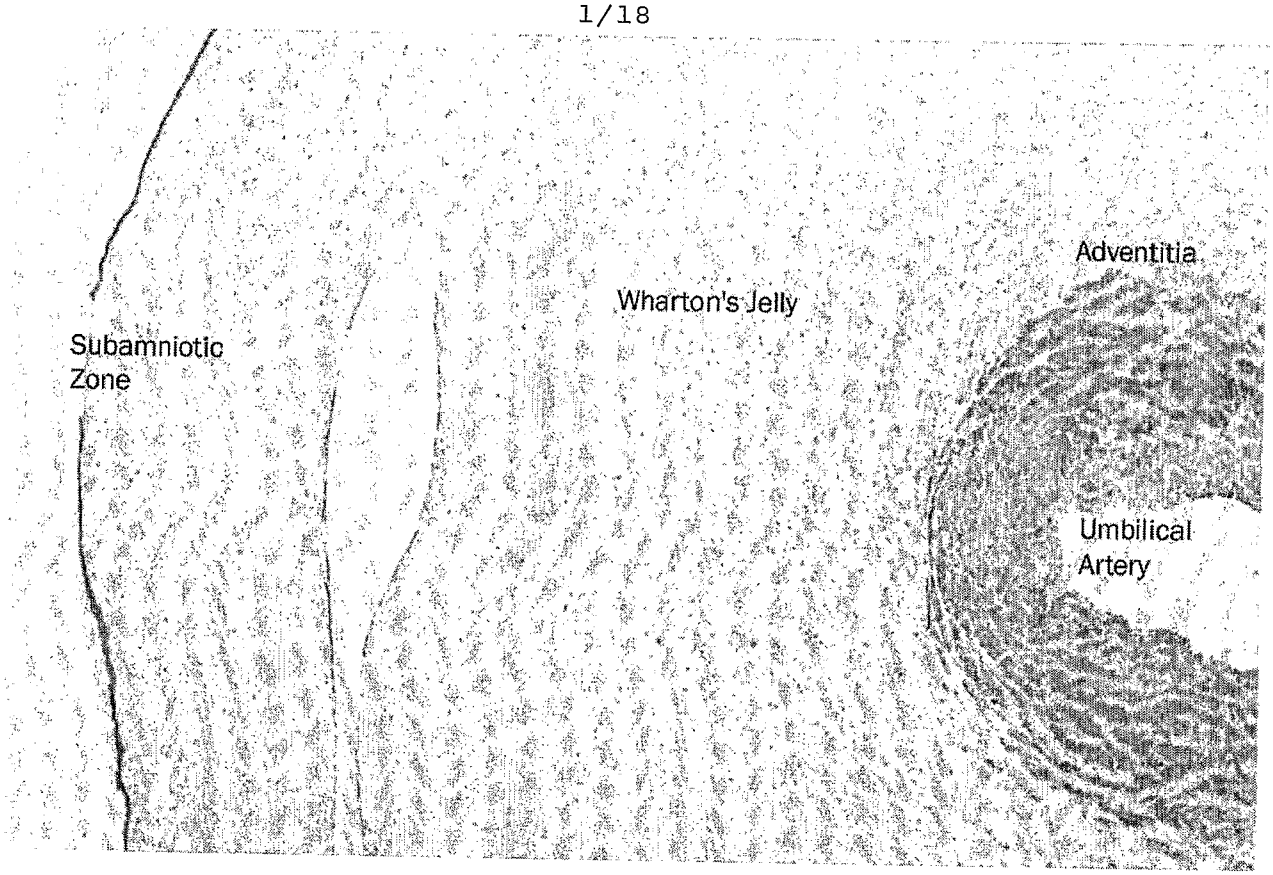

Subamniotic
Zone
Wharton's Jelly
Adventitia
Umbilical
Artery

Figure 1

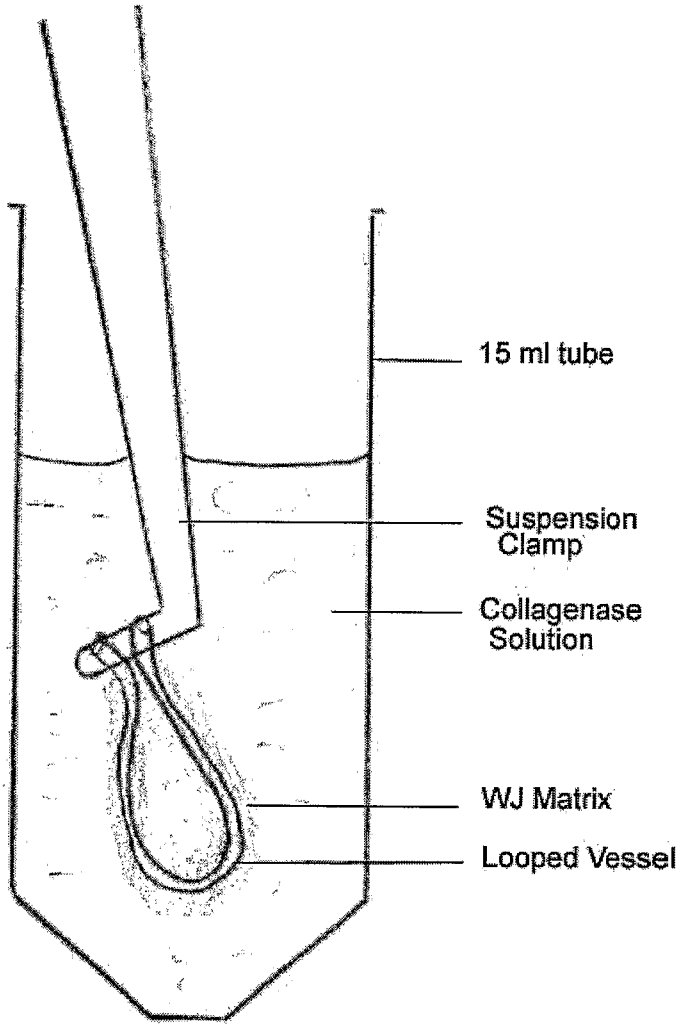

15 ml tube
Suspension Clamp
Collagenase Solution
WJ Matrix
Looped Vessel

Figure 2

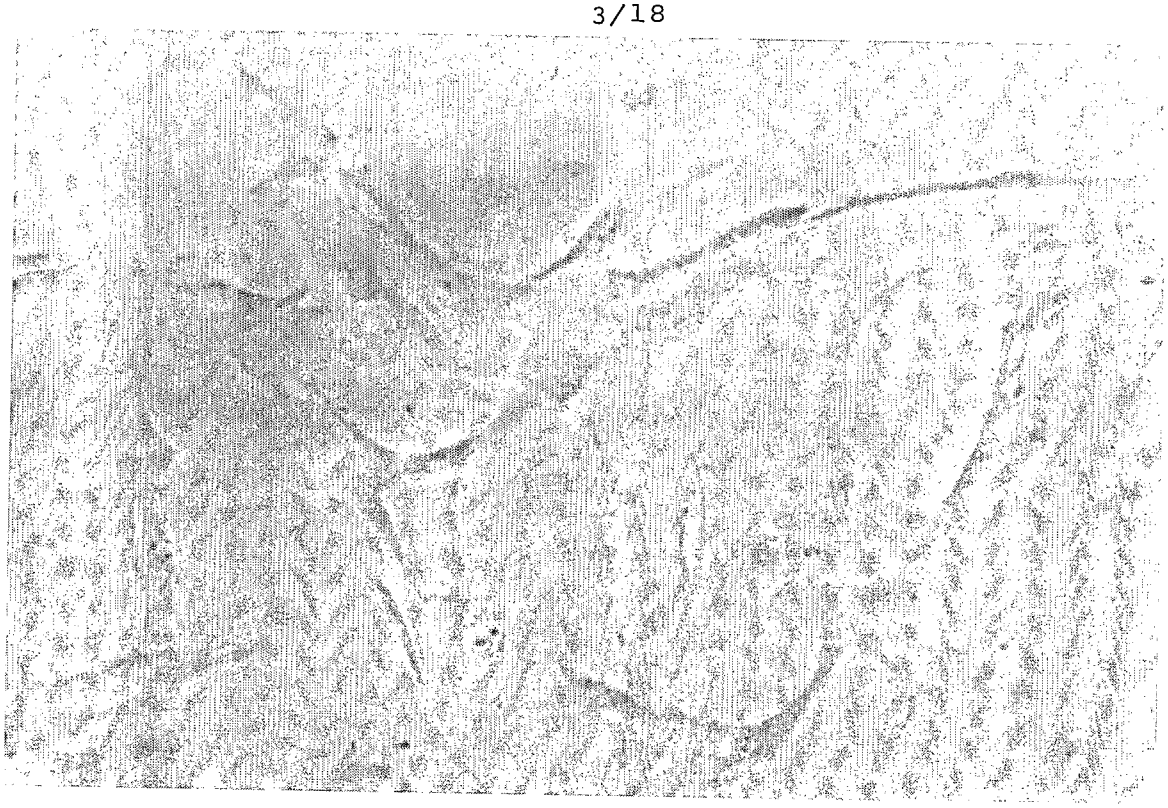


Figure 3

Figure 4

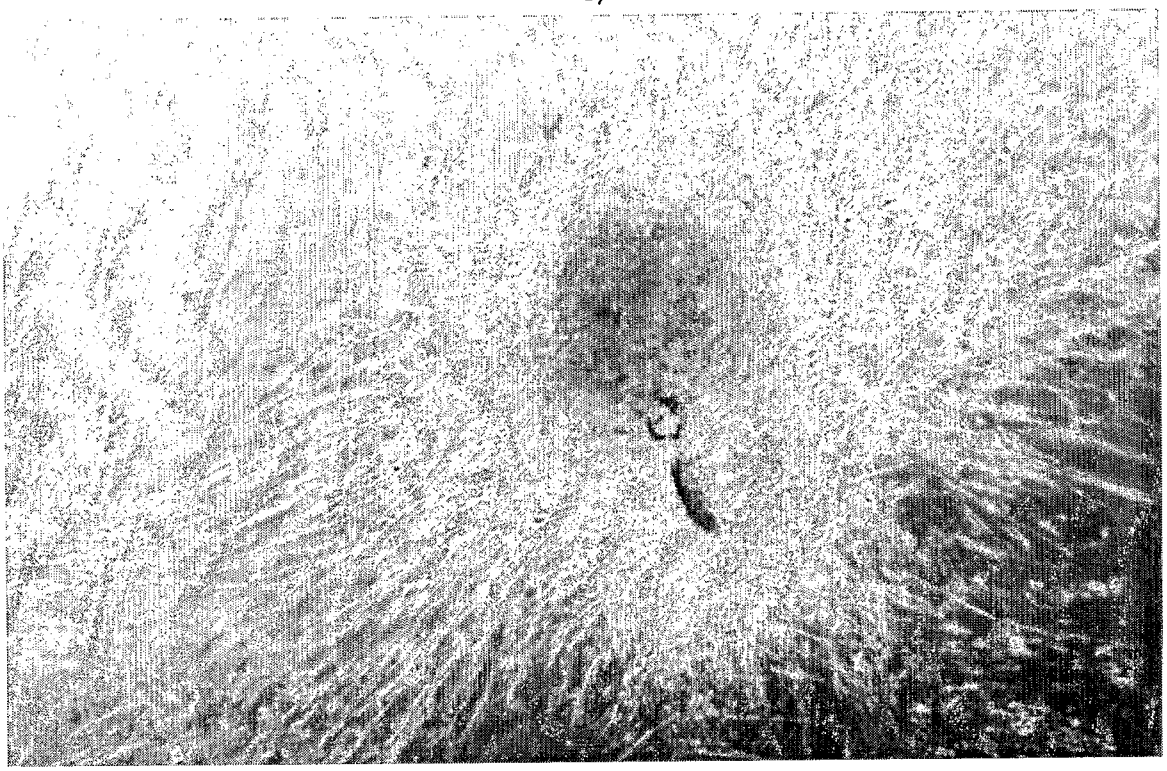


Figure 5

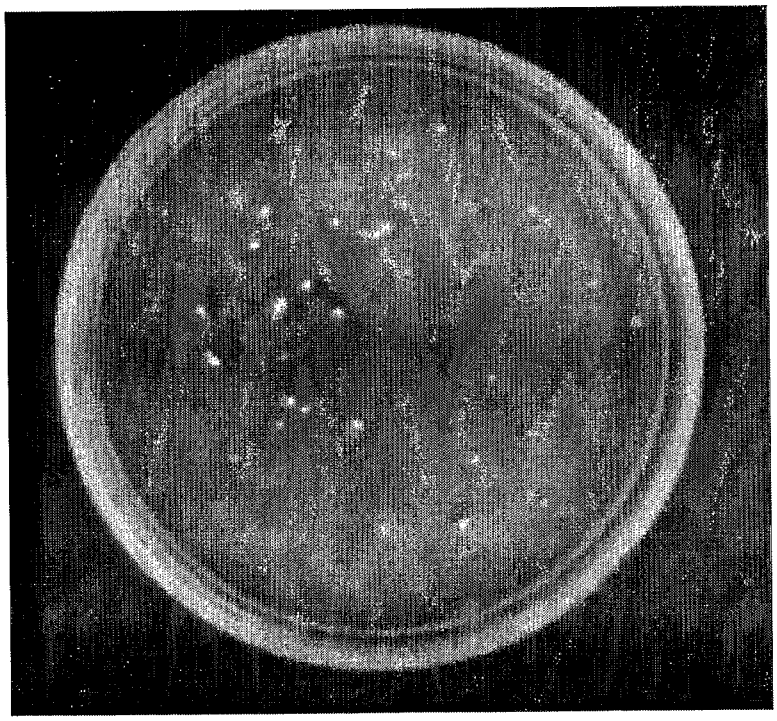


Figure 6

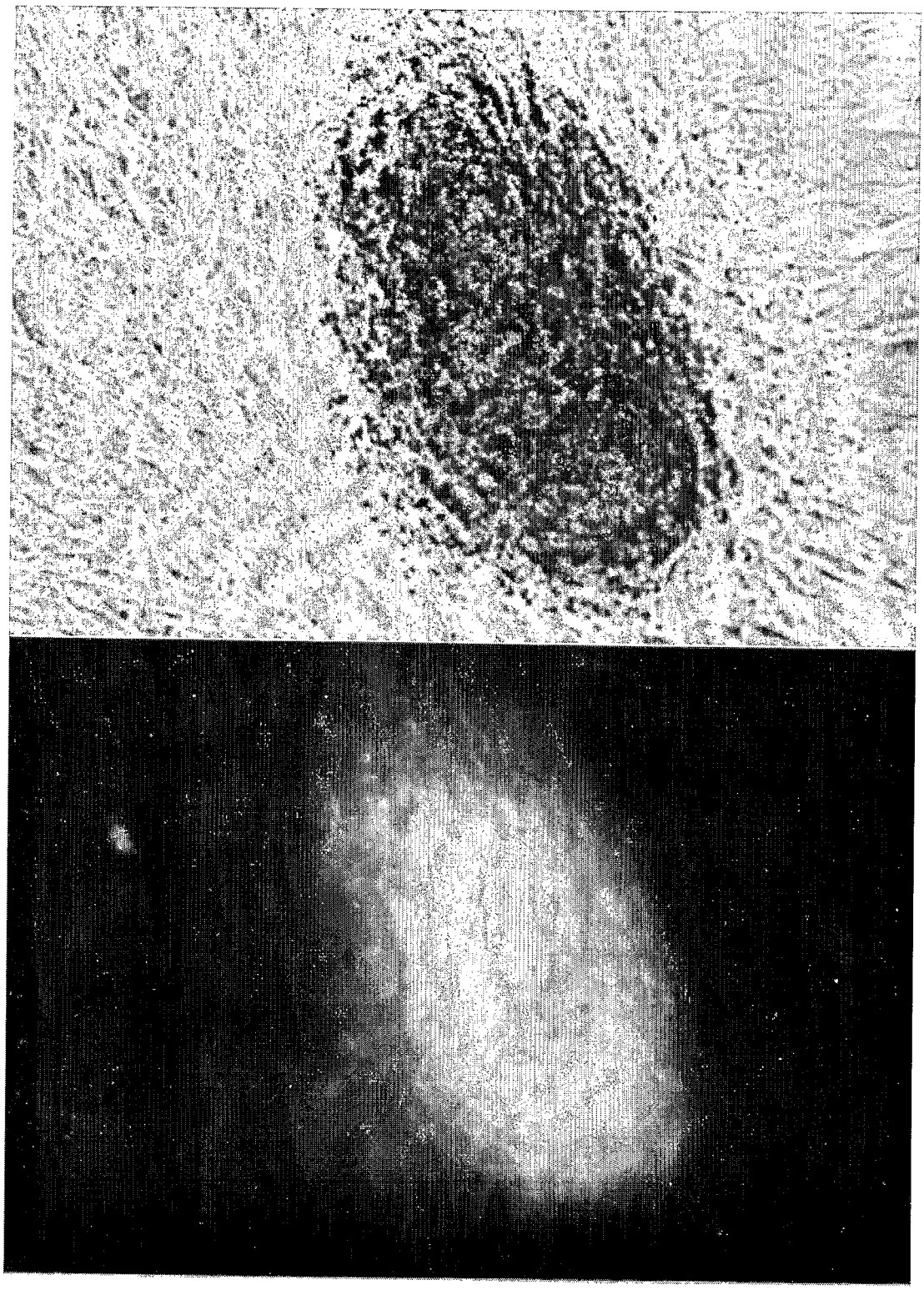


Figure 7

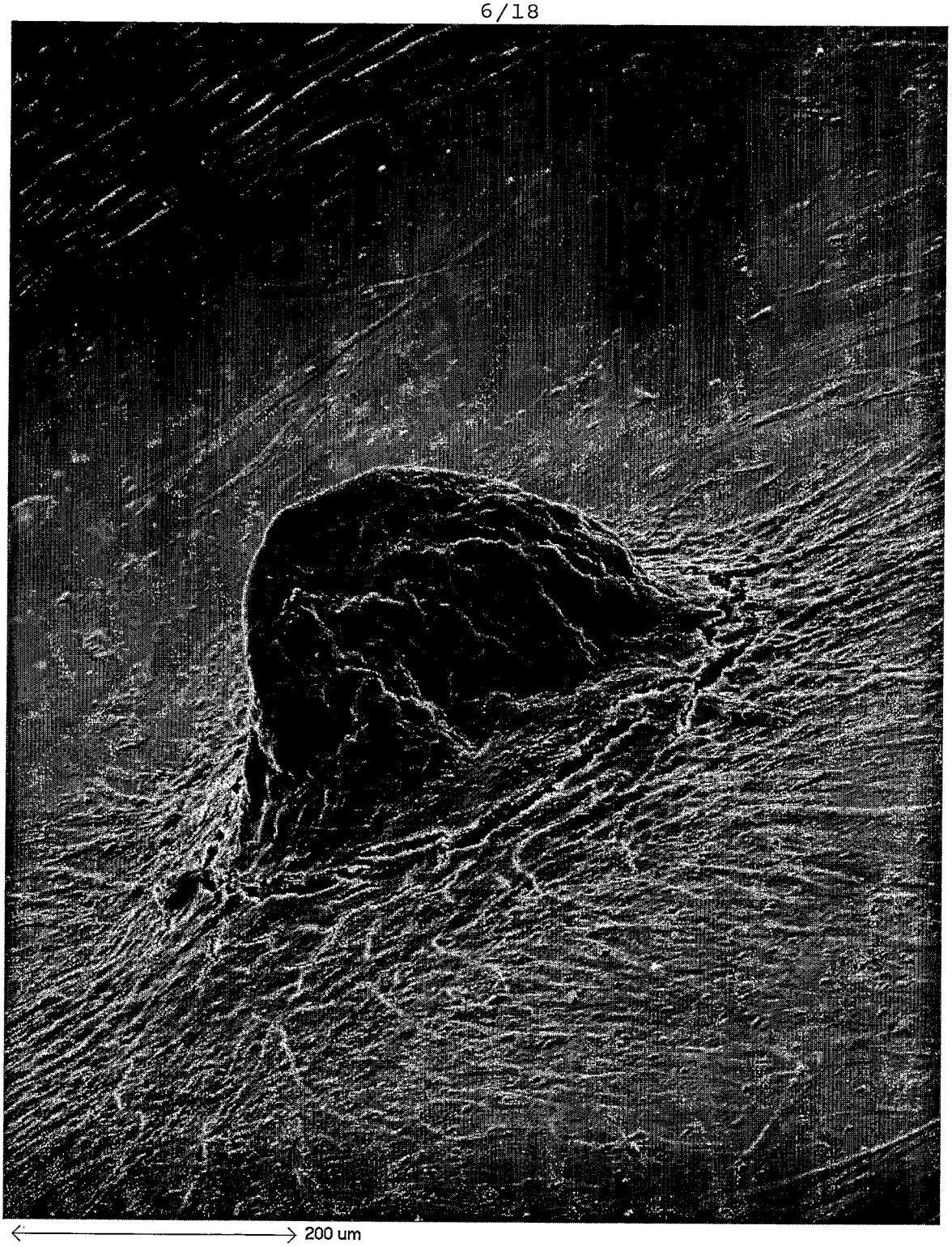


Figure 8

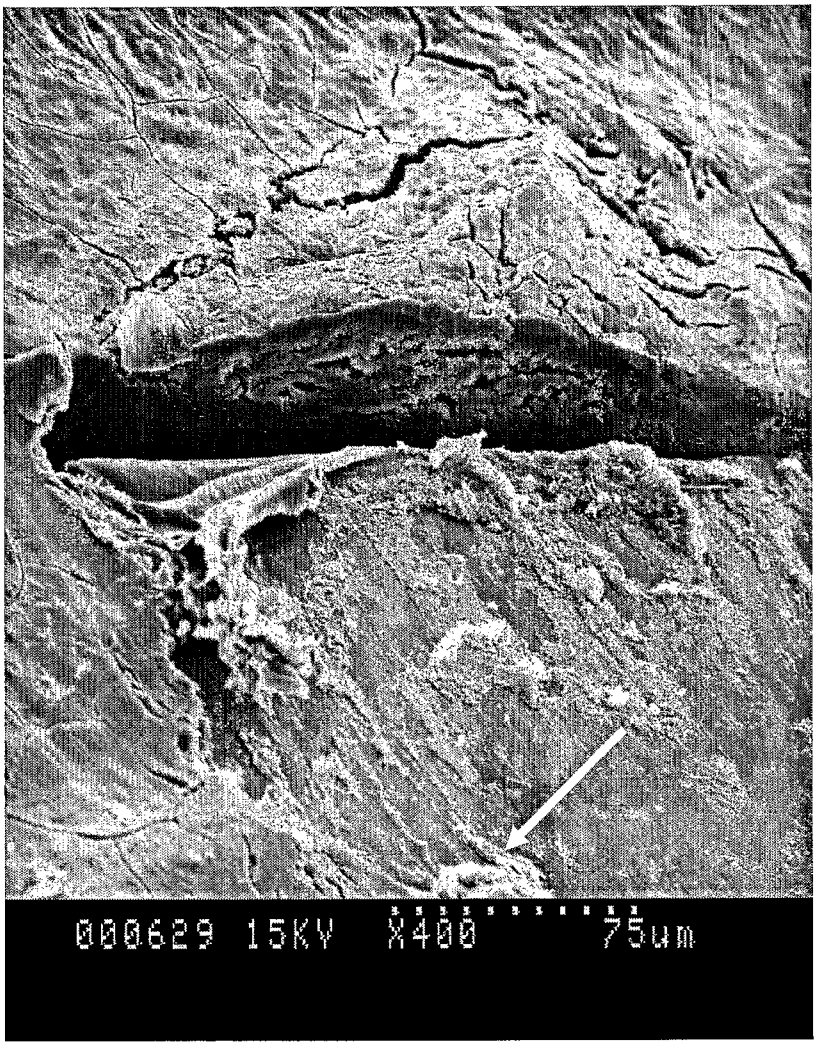


Figure 9

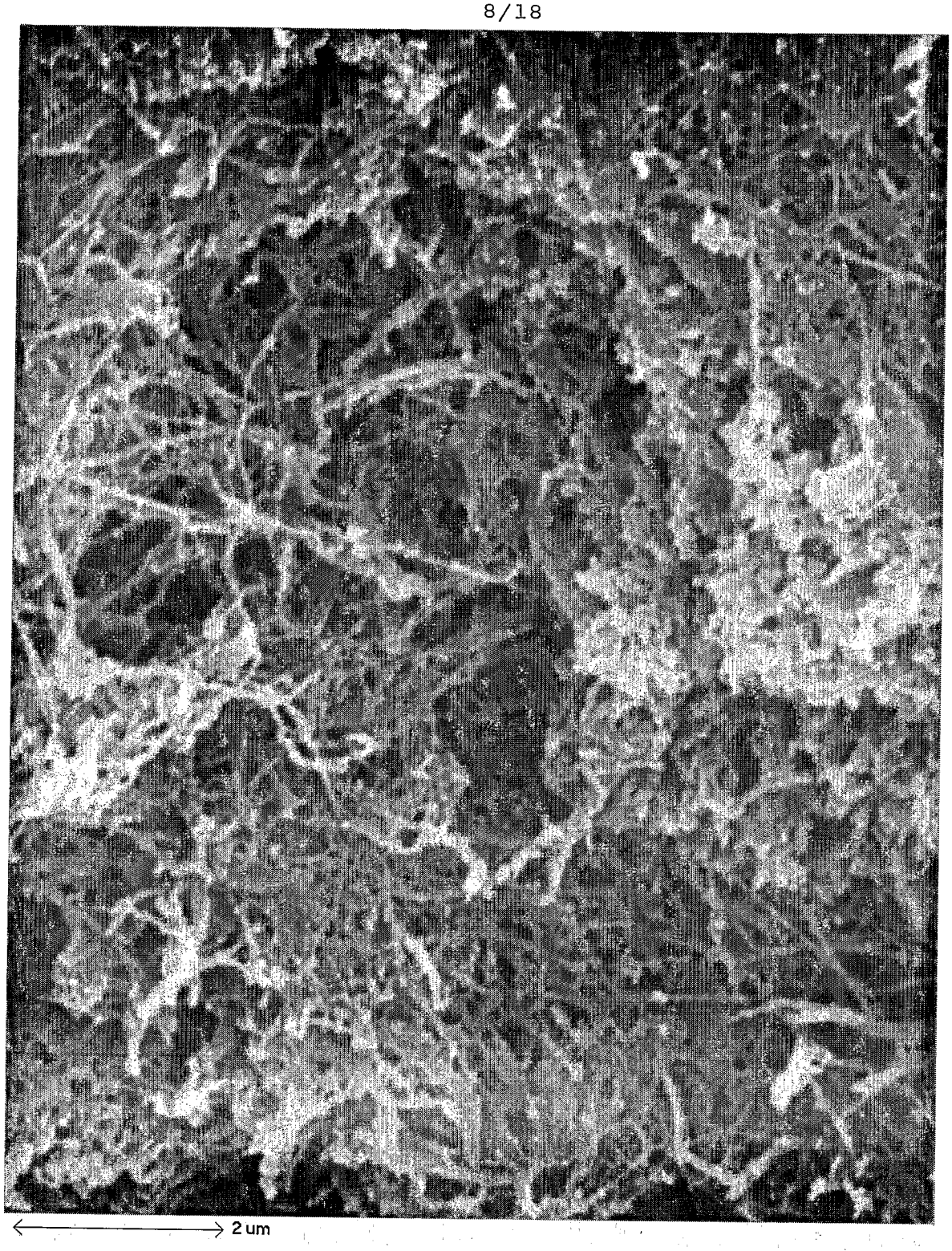


Figure 10

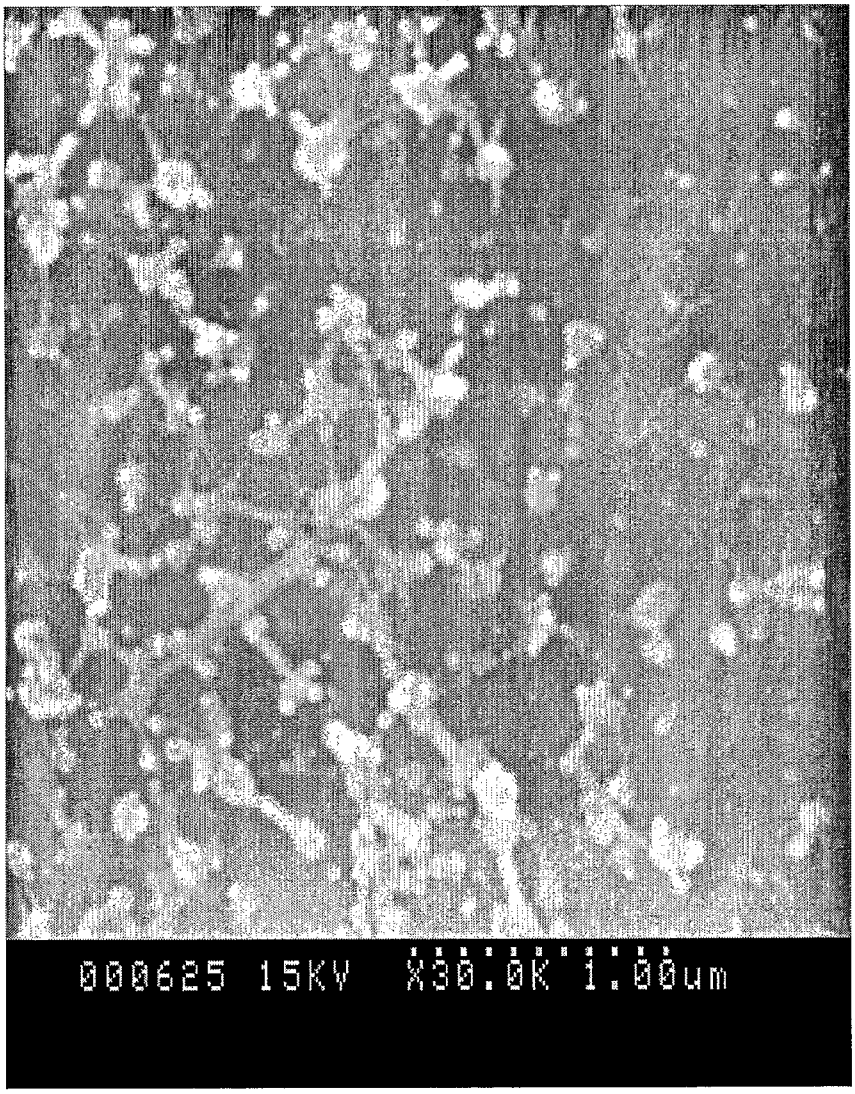


Figure 11

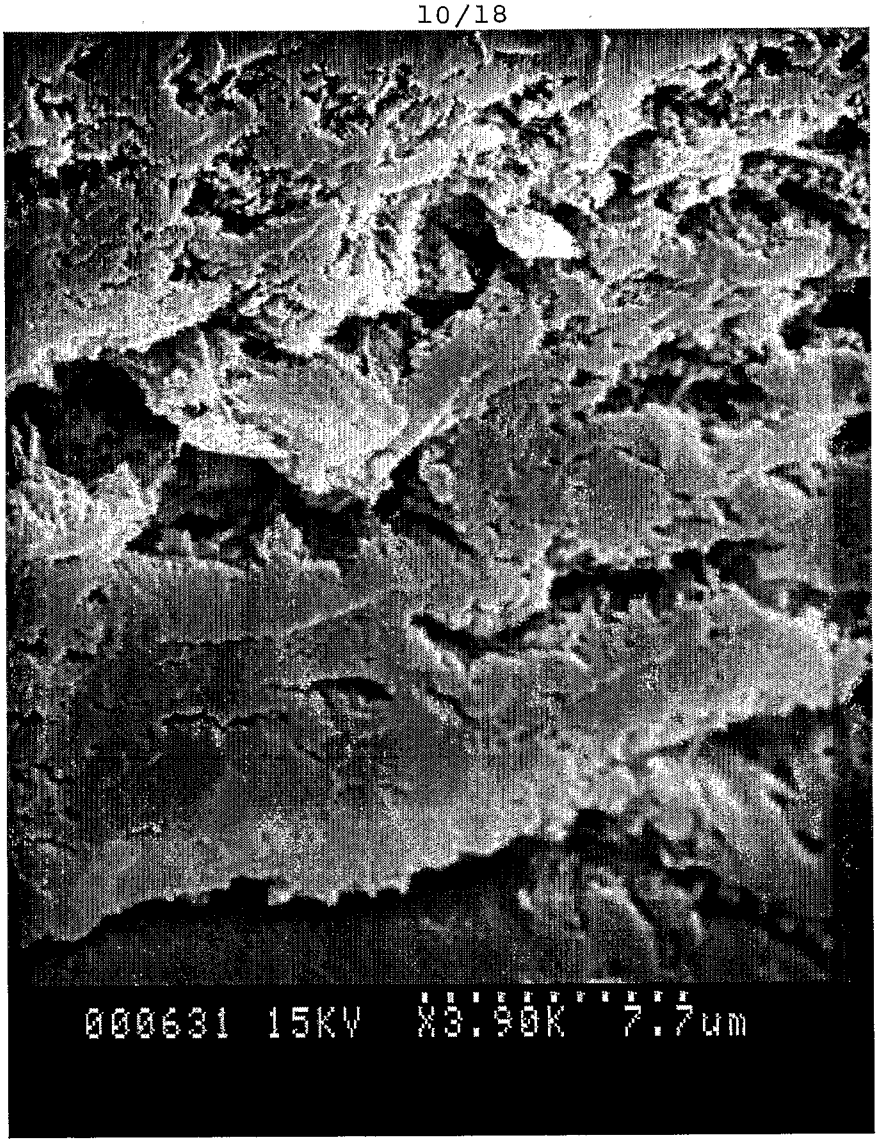


Figure 12

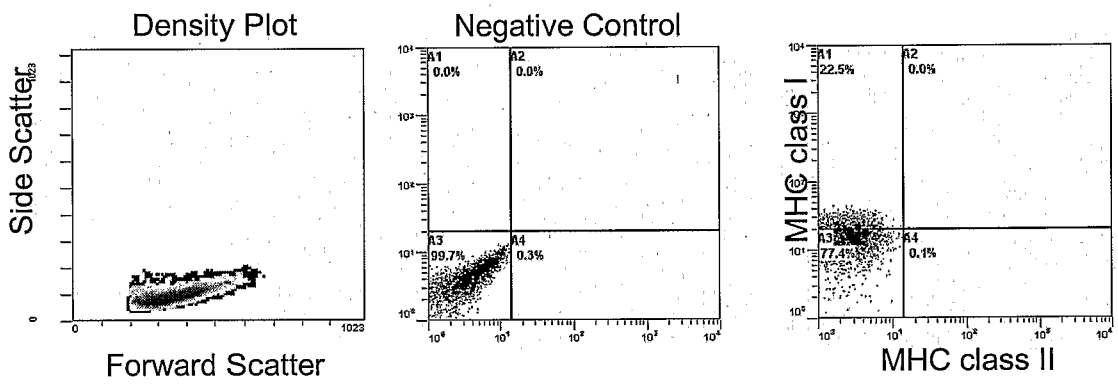

Density Plot
Side Scatter
Forward Scatter
Negative Control
A1 0.0%
A2 0.0%
A3 99.7%
A4 0.3%
MHC class I
MHC class II
A1 22.5%
A2 0.0%
A3 77.4%
A4 0.1%

Figure 13

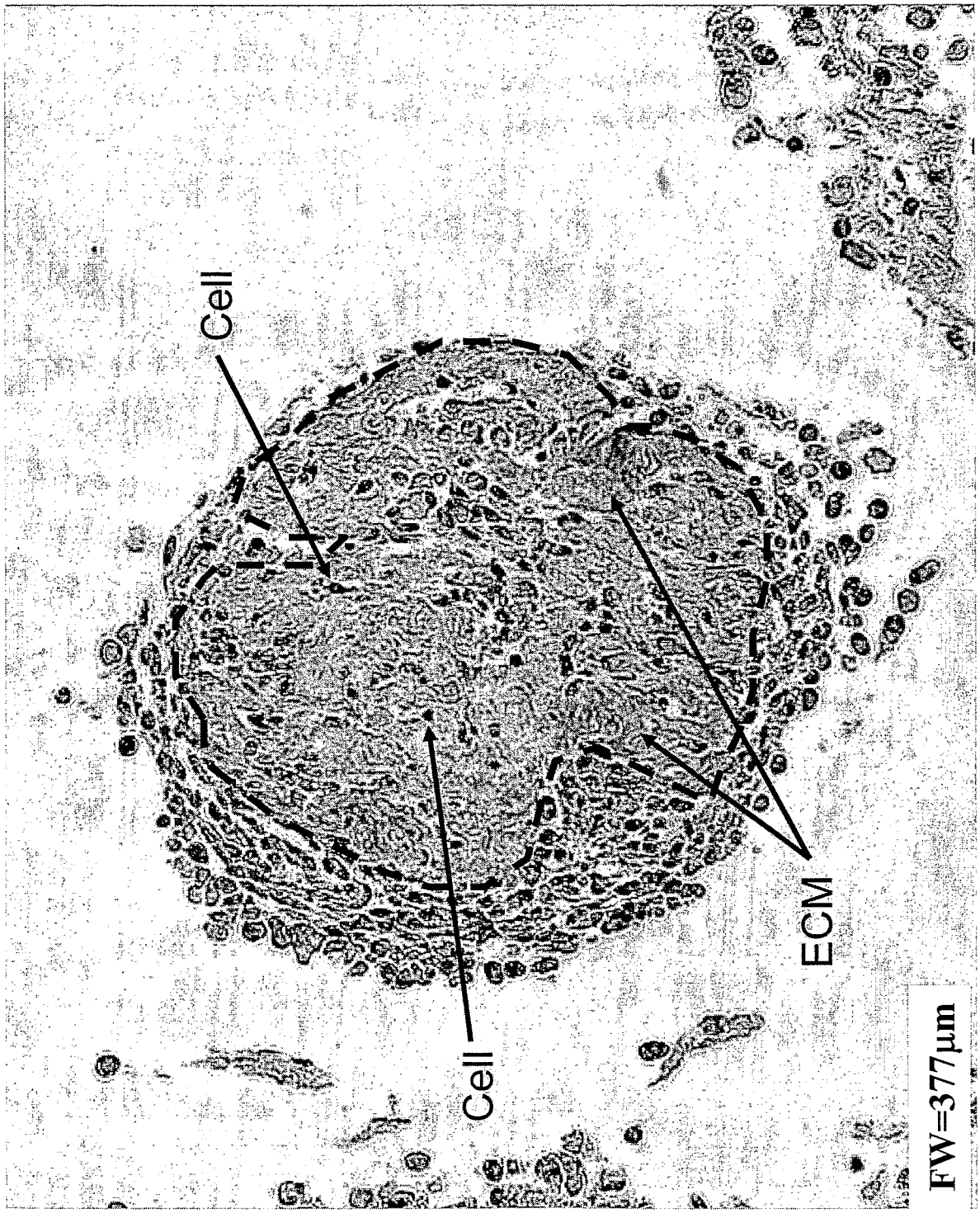


Figure 14

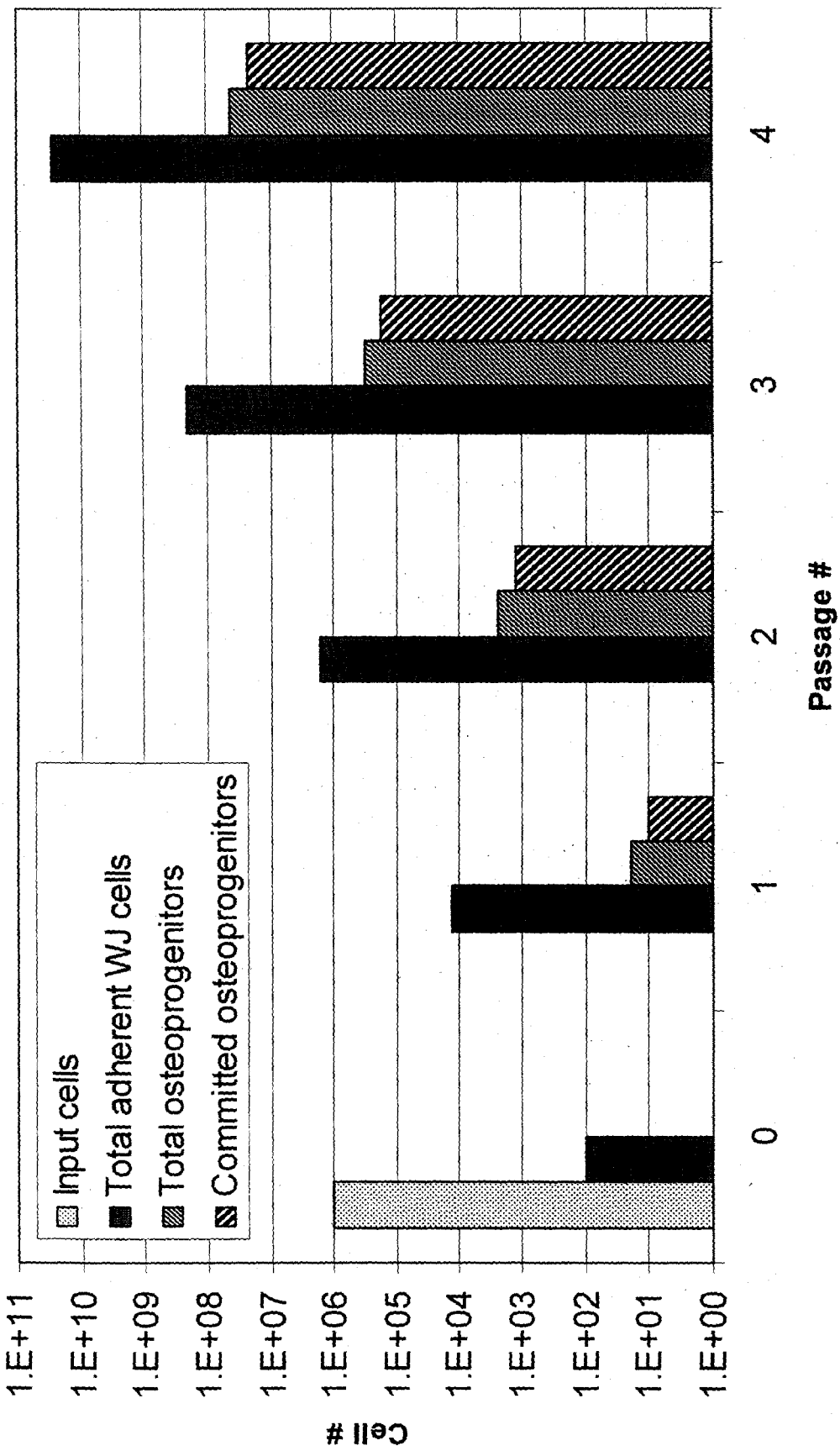

Cell #
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Input cells
Total adherent WJ cells
Total osteoprogenitors
Committed osteoprogenitors
0
1
2
3
4
Passage #

Figure 15

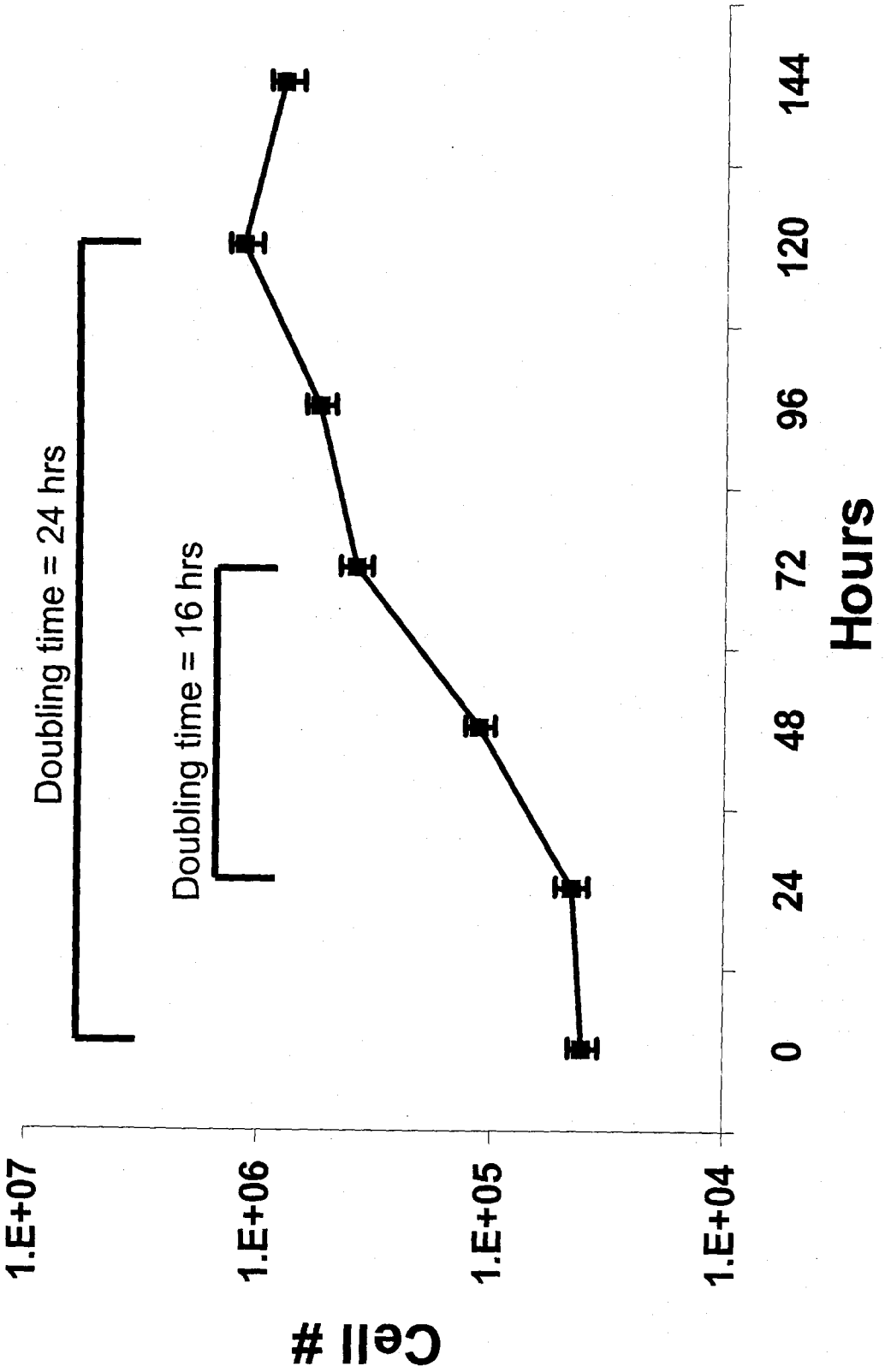

Doubling time = 24 hrs
Doubling time = 16 hrs
1.E+07
1.E+06
1.E+05
1.E+04
Cell #
0
24
48
72
96
120
144
Hours

Figure 16

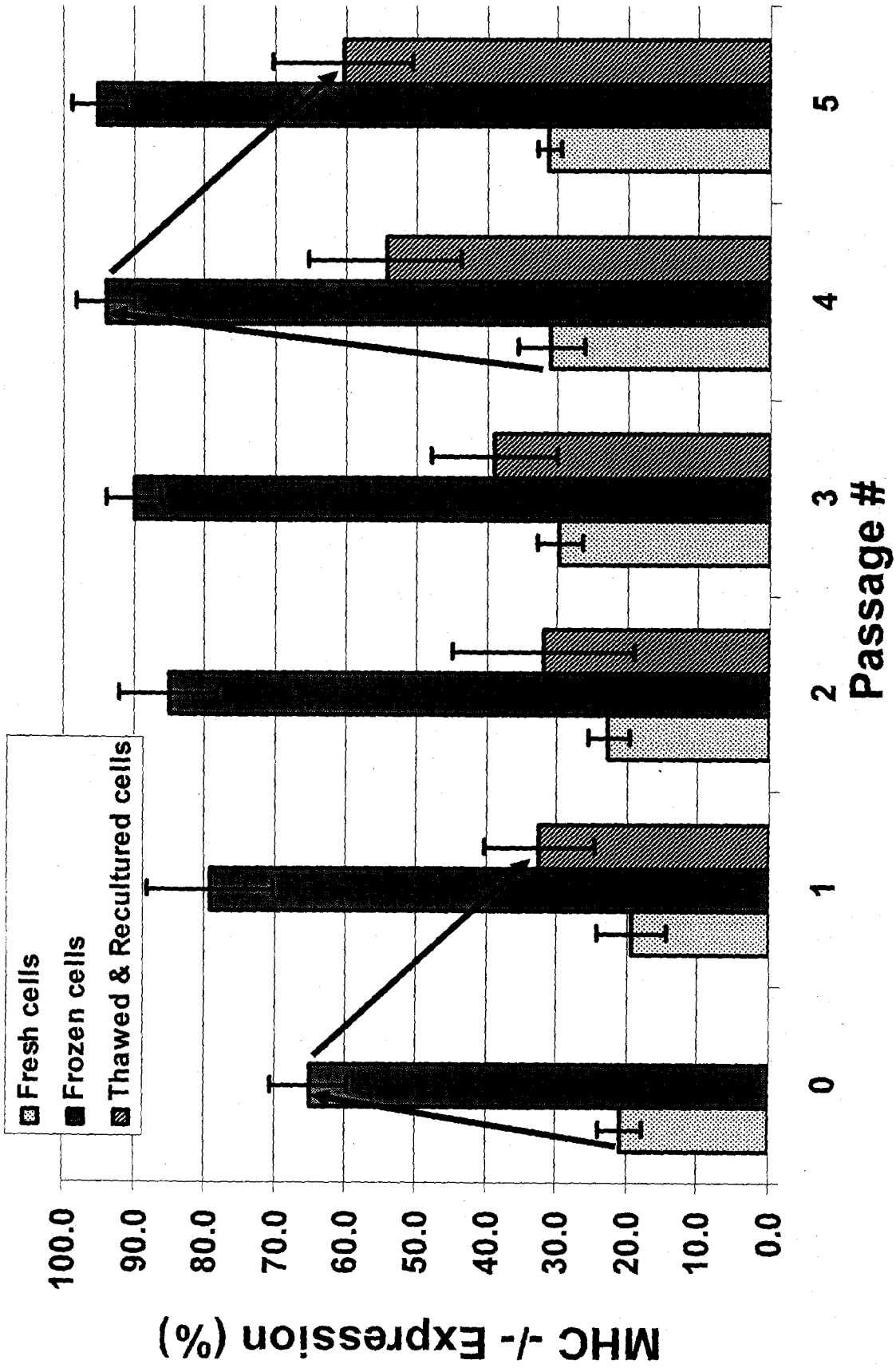

MHC -/- Expression (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Passage #
0
1
2
3
4
5
Fresh cells
Frozen cells
Thawed & Recultured cells

Figure 17

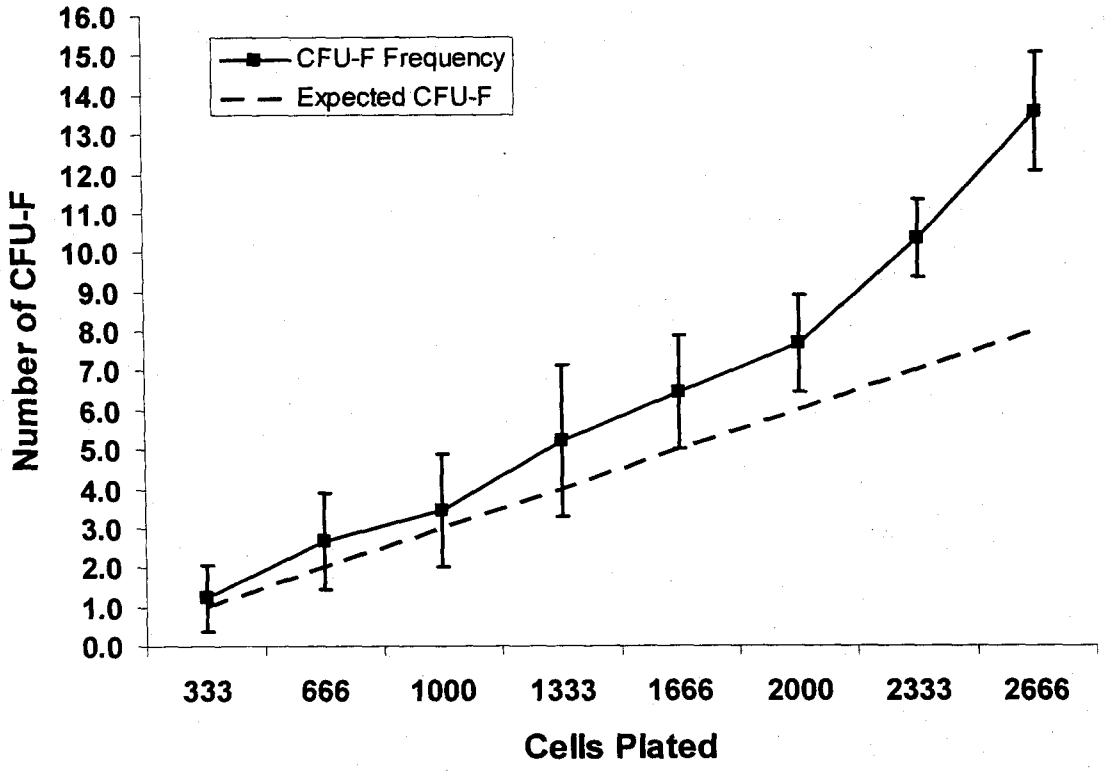


Figure 18

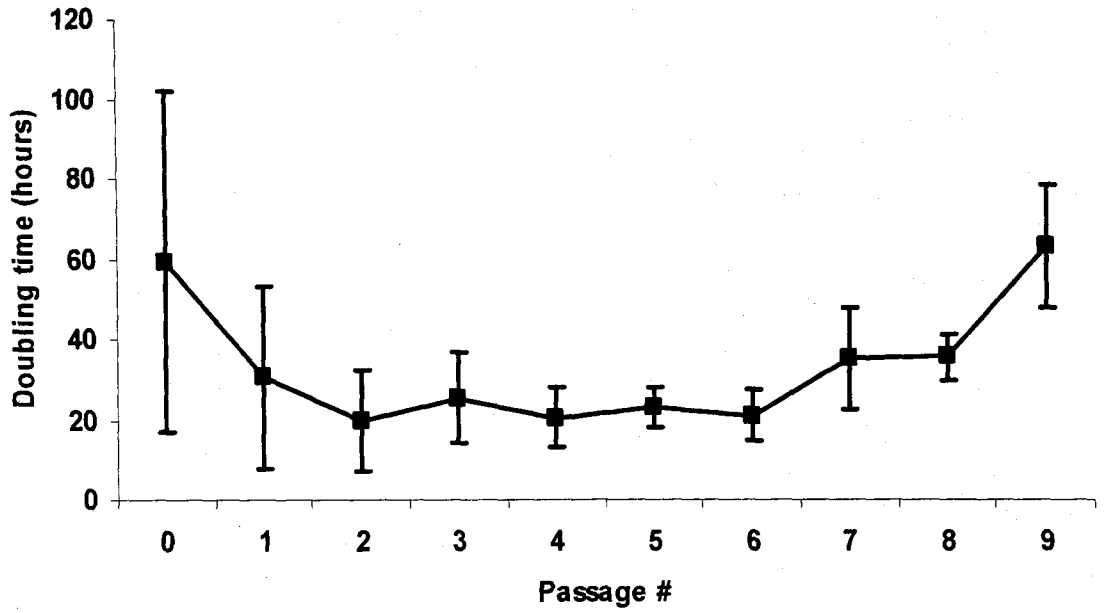


Figure 19

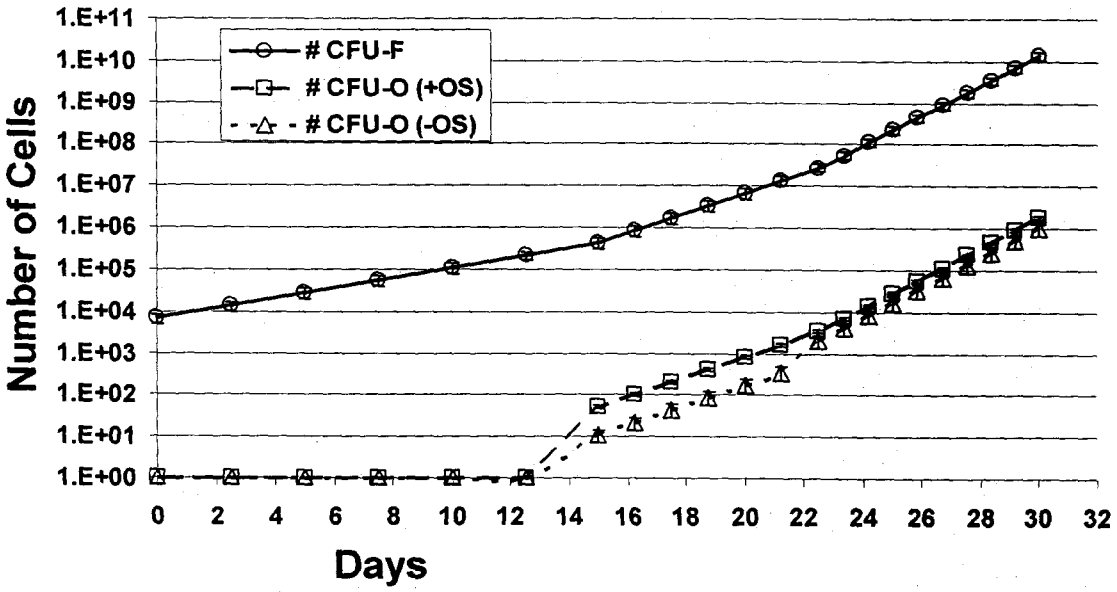


Figure 20

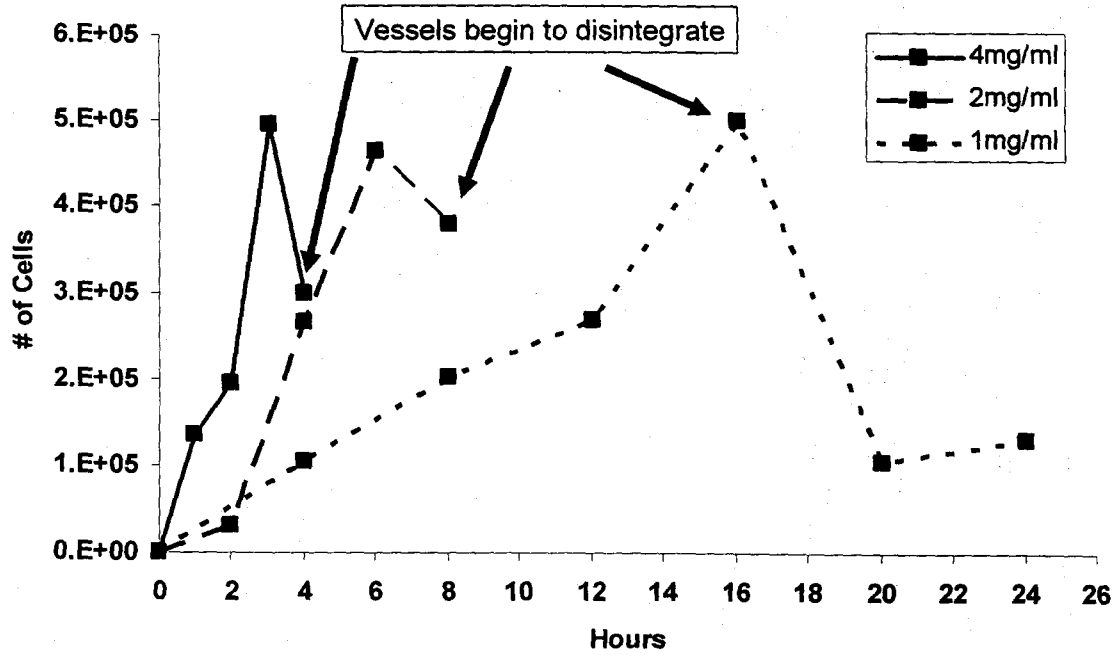
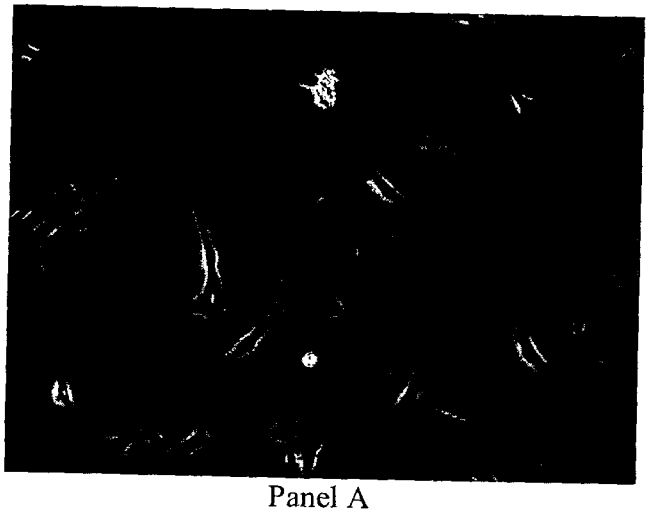
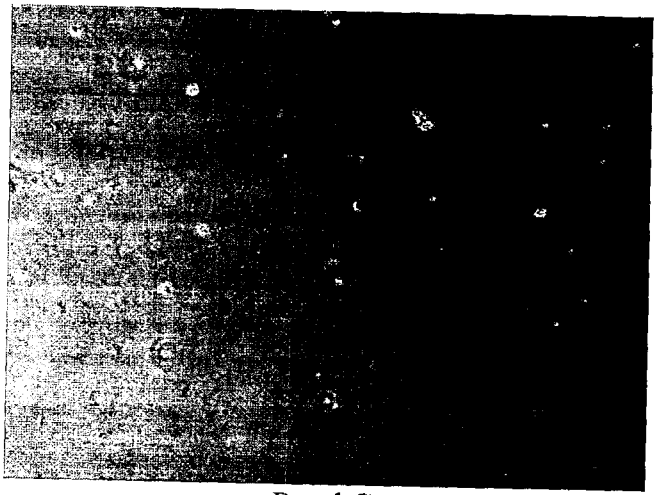


Figure 21



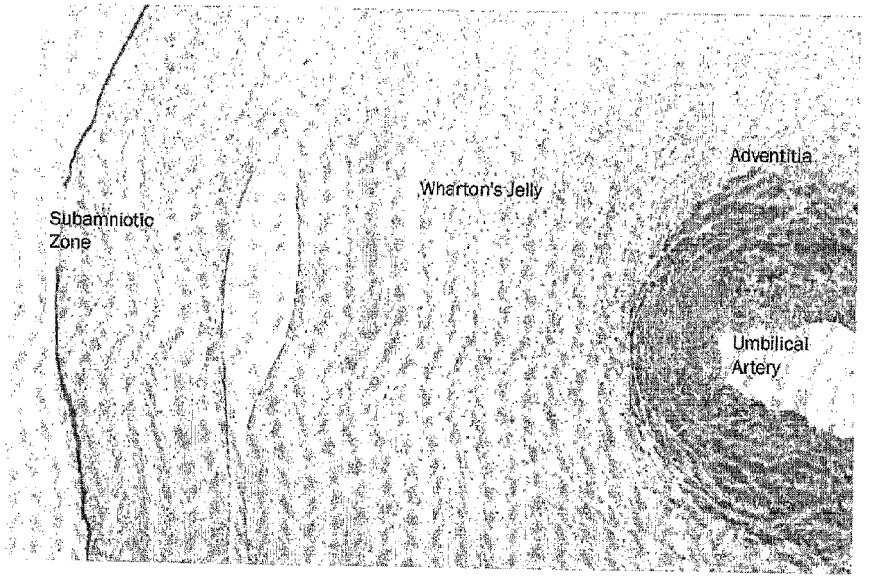
Panel A

Panel B



Panel C

Figure 22


Adventitia
Wharton's Jelly
Subamniotic
Zone
Umbilical
Artery