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(54) Title: CELL CULTURE SUPPLEMENTS

(57) Abstract: The invention relates to cell culture supplements based on a platelet rich plasma fraction and a platelet poor plasma fraction. The supplements increase cell proliferation rate, improve selection of clonogenic cells, proliferation of cells from biopsies from elderly patients, maintenance of cell differentiation potential, and in vitro expansion of cell cultures also starting from an extremely low number of initially plated cells. The supplements may be freeze-dried and kept for long period of time as a quality controlled "off the shelf" product.

CELL CULTURE SUPPLEMENTS

BACKGROUND OF THE INVENTION

Fetal bovine serum (FBS) or fetal calf serum (FCS), is the portion of plasma remaining after coagulation of blood, during which process the plasma protein fibrinogen is converted to fibrin and remains in the clot. FBS comes from the blood taken from a bovine fetus via a closed system of collection at the slaughterhouse.

FBS is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells. This is due to the fact that it has a very low level of antibodies and contains much growth factors. FBS is very versatile and can be used in many different cell culture applications. However, FBS as well as other products deriving from a bovine source, are not advisable due to the risk of prion, zoonose and viral contaminations. In particular viral contamination concerns bovine spongiform encephalopathy, commonly known as "mad-cow disease", which is a fatal neurodegenerative disease in cattle that causes a spongy degeneration in the brain and spinal cord and, in humans, is known as new variant Creutzfeldt–Jakob disease. In addition, although FBS can support proliferation of many types of cells, it fails to promote proliferation of some human and animal cells, including articular chondrocytes from elderly subject donors. FBS also does not support certain culture conditions, such as some cell lines (Hela, etc.) or primary culture (skin fibroblasts, osteoblasts, etc.) plated at clonal densities.

Platelet Rich Plasma (PRP) is blood plasma that has been enriched with platelets. PRP contains several different growth factors and other cytokines that stimulate healing of bone and soft tissue.

Platelet Lysate (PL) is a component of PRP. It is obtained from PRP fraction of the blood by a freeze-thaw cycle repeated at least twice or by sonication in order to break the platelet membranes and to release the growth factors content (platelet activation, activated PRP). The activated PRP is then centrifuged at high speed to precipitate the broken platelet membranes. Since PL does not contain red blood cells and other immunogenic cells and related factors (such as blood group-related antibodies), its applications present the benefit to avoid possible immunogenic reactions.

PRP and PL are used to enhance the proliferation of cell cultures, becoming therefore a substitute of FBS. Different articles in literature have already reported that PRP and PL from human or animal (other than bovine) sources are effective and even more beneficial substitutes for fetal bovine serum to support *in vitro* expansion of human or animal cells (i.e. bone marrow stromal cells, mesenchymal stem cells) for different clinical and therapeutic applications (Zaky

SH et al. *J. Tissue Eng. Regen. Med.* 2008, **2**, 472-481; Castegnaro S et al. *Curr. Stem Cell. Res. Ther.* 2011, **6**, 105-114; Schallmoser K et al. *Transfusion* 2007, **47**, 1436-1446; Kocaöemer A et al. *Stem Cells* 2007, **25**, 1270-1278; Bieback K et al. *Stem Cells* 2009, **27**, 2331-2341; Aldén A et al. *Cytotechnology* 2007, **55**, 3-8; Müller I et al. *Cytotherapy* 2006, **8**, 437-444).

US patent application 2011200642 discloses compositions consisting of an agent which induces an inflammatory healing response combined with a platelet lysate from autologous source at a specific concentration which may have demonstrated *in vitro* abilities to expand autologous tissue repair cells.

WO 2011/076414, also US patent application 20110183414, refer to a cell growth medium comprising (a) a human platelet lysate, (b) a human fresh frozen plasma, (c) heparin, (d) L-glutamine and (e) a serum-free, low glucose medium suitable for mammalian cell growth, wherein the cell growth medium permits the expansion of human CD34-stem cells and wherein the resulting expanded CD34-stem cells retain the ability to differentiate.

WO 2010/007502 relates to a method for preparation of a platelet fraction from placental blood, with high concentrations of platelet factors, as well as gels and lysates deriving therefrom and their uses.

US patent application 20110171731 discloses methods and materials for using platelet lysate compositions to grow stem cells, to differentiate stem cells, to grow primary cell cultures, and to identify effective growth factors. The compositions containing platelet lysate are formulated with any appropriate medium to produce a culture medium having enhanced properties, such as DMEM, RPMI, AIMV, X-VIVO15 and other defined serum free or serum requiring media. The compositions containing platelet lysate are also formulated with one or more factors capable of differentiating cells, such as polypeptides, steroids, hormones, dexamethasone, EGF, FGF and BMP4.

US patent application 20100015710 concerns compositions for isolating and expanding human mesenchymal stem/progenitor cells through multiple passages in defined serum-free environments. The complex culture media compositions includes a basal medium supplemented with a nutrient mixture (Ham's F12, glutamine), buffer solutions (sodium bicarbonate), serum albumin, a lipid mixture, insulin, transferrin, putrescine, progesterone, fetuin, hydrocortisone, ascorbic acid or its analogues, growth factors, and platelet lysate as a serum-free substitute.

The international publication WO 2008/110570 discloses medium for culturing endothelial cells which comprises EGM-2, hydrocortisone, VEGF as well as human platelet lysate as supplement.

US 5,198,357 describes platelet lysate used to entirely or partially replace fetal calf serum in cell culture. The platelet lysate is produced from animal plasma and contains an added citrate to

prevent coagulation of the blood during storage. The lysate is prepared by centrifuging plasma to produce a platelet rich paste, adding calcium to the paste to lyse the platelets therein and coagulate fibrinogen to produce a clear liquid containing lysed platelets, sterile filtering the liquid and collecting a liquid filtrate containing the lysed platelets.

The international publication WO 2008/034803 concerns a platelet concentrate, such as platelet-rich plasma, used in a cell culture medium to grow and proliferate cells, which may be from autologous source. The cells grown in the culture medium may be used to treat a patient. The supplement further comprises albumin and/or dextran and/or hydroxyethyl starch.

The international publication WO 2007/149328 discloses a defined serum-free cell culture media useful for culturing fibroblasts, especially dedifferentiated articular cartilage cells. The media avoid problems inherent to the use of serum-containing media. The defined media comprise platelet-derived growth factor (PDGF), chemically defined lipids, oncostatin M, interleukin-6 (IL-6), leukemia inhibitory factor, or combinations of these compounds.

US patent application 20100120144 describes a concentrated blood component, such as platelet-rich plasma, used in a cell culture medium to grow and proliferate cells to treat a patient, possibly with cells from autologous source.

US patent application 20090305401 provides a cell culture medium supplement comprising plasma-free platelet lysate prepared by lysing the platelets from platelet rich plasma. The medium is supplemented with albumin, dextrane, or hydroxyethyl starch.

JP 8308561 discloses the preparation of a medium for culturing cells without adding a serum and without losing functionality of the cells. The medium is composed of a water-soluble selenium added to a mixture of cell stimulating compounds, among them platelet-derived growth factor (PDGF).

All cited documents deal with compositions aimed to improve, enhance, tune the quality and yield for isolation, proliferation, expansion and differentiation of cell cultures.

Nevertheless, experimental data show that platelet derived products used as cell culture supplements may have different effects depending on the starting platelet concentration and on the preparation procedure. Despite the extensive literature about the use of PRP and PL as efficient substitutes of animal serum (Pawitan JA, *Curr Stem Cell Res Ther.* 2012, **7**, 329-35; Schallmoser K et al. *J Vis Exp.* 2009, **30**; pii: 1523, doi: 10.3791/1523; Anitua E et al. *Cell Prolif.* 2009, **42**, 162-70; Kinzebach S et al. *Adv Biochem Eng Biotechnol.* 2012; Blande IS et al. *Transfusion.* 2009, **49**, 2680-5; Ben Azouna N et al. *Stem Cell Res Ther.* 2012, **3**, 6; Lohmann M et al. *PLoS One.* 2012, **7**, e37839; Weibrich G et al. *J Craniomaxillofac Surg.* 2002; **30**, 97-102; Lange C et al. *J Cell Physiol.* 2007, **213**, 18-26; Doucet C et al. *J Cell Physiol* 2005, **205**, 228-

36: Gruber R et al. *Platelets* 2004, **15**, 29-35) there is still a lack of standardized protocols for platelet derived products preparation.

In fact, since the platelet concentration is highly variable from individual to individual and for the same individual it can vary from time to time, the "traditional" platelet derived preparations such as PRP and PL used as culture medium supplements do not allow the reproducibility and the consistency of the experimental results.

The platelet concentration in the platelet derived preparations is wide and, in general, ranges from about $5x10^5$ to about $5x10^6$ platelets/μl (as an example, Bieback K et al. *Stem Cells* 2009, Zaky SH et al. *J. Tissue Eng. Regen. Med.* 2008, **2**, 472-481, Schallmoser K et al. *Transfusion* 2007, **47**, 1436-1446, WO 2010/007502, WO 2011/076414, Lohmann et al. *PlosONE* 2012, **7**, e37839).

As consequence, these PRP and PL contain undefined concentrations of factors and molecules. Moreover, there is still the need for a medium supplement that is practical and of easy handling.

DESCRIPTION OF INVENTION

The authors of the present invention have surprisingly found that cell culture supplement formulations based on two different platelet and plasma derived components, optionally containing defined concentrations of a specific anti-coagulant agent, allow the preparation of cell culture medium additives that may be optimized for any specific cell type.

The authors of the present invention have used the different components as above described. The components are combined in different ratios and are used as serum-supplement(s) for *in vitro* cell culture to substitute Fetal Bovine Serum (FBS).

Indeed, the authors' platelet derived products cannot be merely only regarded as additives for cell culture media to replace FBS or other animal sera or to overcome the present limitations of the state of art, as above reported.

In particular, the authors surprisingly found that each type of cell requires a specific ratio between the content of platelet derived components (see below) including bioactive molecules and plasma (or serum). The formulations are based on two components, combined in different ratios and optionally containing a defined concentration of a specific anti-coagulant agent. The first component is a derivative obtained starting from a platelet/plasma fraction containing a well-defined number of platelets per volume and the second component is a derivative from platelet poor plasma.

The first component, or Component A, is a substantially platelet rich plasma fraction containing a well-defined number of platelets per volume. The Component A may be optionally processed in order to remove the fibrinogen part (serum) to avoid the formation of fibrin rich clot once

activated (the fibrinogen-free component A is named Component C). When Component C is used, there is no need to the addition of an anti-coagulant agent.

The second component, or Component B, is a derivative from platelet poor plasma. The Component B may be optionally processed in order to remove the fibrinogen part (the fibrinogen-free component B is named Component D). When component D is used there is no need for the addition of an anti-coagulant agent. The platelet poor plasma is blood plasma with very low number of platelets (e.g. $< 1 \times 10^4/\mu L$).

When using the mixture of Component A and Component B, an anti-coagulant agent is usually used in an optimal ratio. Because fibrinogen may be present in the supplement solution, the anti-coagulant agent prevents the conversion of fibrinogen into fibrin and, therefore allows to carry out an easier and cleaner process. In fact, the conversion of fibrinogen into fibrin involves the formation of agglomerates which can interfere with the cell culture by limiting the cell growth and differentiation and/or by making a more difficult operative handling of the culture steps.

The amount (concentration) of the two components in the mixture and used in cell cultures is, in any case, lower than that of FBS to obtain the same performances. The supplement of the invention shows improved performances.

The mixture of the two components (Component A + Component B or Component C + Component D) at an optimal ratio, with optionally the addition of an anti-coagulant agent at an optimal ratio with the mixture, increased cell proliferation rate (cell doubling time) in both primary cells and cell lines.

Moreover, the same mixtures improve selection of clonogenic cells and/or cell sub-populations. They allow proliferation of cells from biopsies obtained from elderly patients, otherwise not possible with FBS. Further the mixtures maintain cell differentiation potential in human articular chondrocytes and mesenchymal stem cells. Moreover the mixtures allow an *in vitro* expansion of cell cultures starting from an extremely low number of initially plated cells. Finally, the mixtures enhance the cell culture dish coating and cell adhesion, as a further advantageous property.

The reasons of this surprising findings are still unknown and not ascribed solely to an effect of the platelet concentration. The final platelet concentration in the mixture is substantially provided only by the contribution of the Component A, being the contribution in platelet concentration of Component B negligible. Therefore the improved cell adhesiveness observed in the presence of the mixture must be related to element(s) present in Component B and not to a lower platelet concentration. In fact, the PRP fraction (Component A) at the concentration of the present invention (e.g. $10x10^6/\mu l$) does not result in any improvement of the properties of the final product on cell cultures.

The possibility of freeze drying the single components or their mixtures, of sterilizing them and of storing them for long period of time at low temperatures, allows the use of quality controlled "off the shelf" products.

The single component or mixtures thereof are preferably sterilized by gamma radiation or by filtration. The sterilization step may be performed either before or after freeze drying.

In summary, the platelet derived cell culture supplement of the present invention are based on the combination, in different ratios, of two different plasma and platelet derived components processed from blood platelet enriched fractions with a defined and optimized number of platelets per volume. The present invention provides the preparation and use of cell culture supplements specifically optimized for each cell type together with improved reproducibility of the cell culture conditions.

In the present invention, the platelet rich plasma fraction can also be named as Component A and the platelet poor plasma fraction can also be named as Component B.

The platelet rich plasma fraction is preferably further subjected to a lysis step and a centrifugation step and can herein also be named as Component A-Platelet Lysate.

Preferably the platelet rich plasma fraction and/or the platelet poor plasma fraction is processed to remove fibrinogen and can herein also be named as Component C and Component D.

It is therefore an object of the present invention a cell or tissue culture medium supplement consisting of:

a) from 95 % to 0.5 % (volume/volume) of a platelet rich plasma fraction containing at least $2x10^6$ platelets/μL;

b) from 5 % to 99.5 % (volume/volume) of a platelet poor plasma fraction containing less than $5x10^4$ platelets/μL.

Preferably the cell or tissue culture medium supplement consists of:

a) from 70 % to 2.5 % (volume/volume) of the platelet rich plasma fraction and

b) from 30 % to 97.5 % (volume/volume) of the platelet poor plasma fraction.

Still preferably the cell or tissue culture medium supplement consists of:

a) from 60 % to 5 % (volume/volume) of the platelet rich plasma fraction and

b) from 40 % to 95 % (volume/volume) of the platelet poor plasma fraction.

In another preferred embodiment the cell or tissue culture medium supplement consists of:

a) from 90 % to 50 % (volume/volume) of the platelet rich plasma fraction and

b) from 10 % to 50 % (volume/volume) of the platelet poor plasma fraction.

In preferred embodiments, the cell or tissue culture medium supplement according to the invention consists of:

75 % (volume/volume) of the platelet rich plasma fraction and 25% (volume/volume) of the platelet poor plasma fraction, or

50 % (volume/volume) of the platelet rich plasma fraction and 50% (volume/volume) of the platelet poor plasma fraction, or

20 % (volume/volume) of the platelet rich plasma fraction and 80% (volume/volume) of the platelet poor plasma fraction, or

10 % (volume/volume) of the platelet rich plasma fraction and 90% (volume/volume) of the platelet poor plasma fraction.

In another preferred embodiment, the cell or tissue culture medium supplement consists of 95% of the platelet rich plasma fraction and 5% (volume/volume) of the platelet poor plasma fraction.

In a preferred embodiment the platelet rich plasma fraction contains from $5x10^6$ to $15x10^6$ platelets/μL, most preferably from $8x10^6$ to $12x10^6$ platelets/μL.

In a preferred embodiment the platelet poor plasma fraction contains less than $1x10^4$ platelets/μL, most preferably less than $0.4x10^4$ platelets/μL.

The platelet rich plasma fraction is preferably further subjected to a lysis step and a centrifugation step.

In a preferred embodiment the cell or tissue culture medium supplement consists of:

a) from 90 % to 50 % (volume/volume) of Component A-PL and

b) from 10 % to 50 % (volume/volume) of the platelet poor plasma fraction.

Preferably the platelet rich plasma fraction and/or the platelet poor plasma fraction is processed to remove fibrinogen.

Another object of the present invention is a cell or tissue culture medium supplement comprising the cell or tissue culture medium supplement as above disclosed and an anti-coagulant agent.

The anti-coagulant agent is preferably heparin.

Heparin is a well-known and widely used anticoagulant, preventing the formation of clots and extension of existing clots within the blood through a body's natural clot lysis mechanism (Tahir RA, *US Pharm.* 2007, **32**, HS26-HS36; Hirsh J et al. *Chest* 2001, **119**, 64S-94S).

The role of heparin when added to cell cultures is still largely unknown and controversial. Heparin has shown both anti-proliferative action in tumor cell lines, thus evidencing a toxic behavior towards cells (Abu Arab W et al. *Can J Physiol Pharmacol.* 2011, **89**, 705-711; Ichikawa J et al. *Cancer* 2012, **118**, 2494-256) and amplification of growth factors activity and proliferation of stem cells (Hudalla GA et al. *Adv Mater.* 2011, **23**, 5415-5418; Frescaline G et al. *Stem Cell Res.* 2012, **8**, 180-192).

In the proposed range with the cell or tissue culture supplement of the invention, it is pointed out that heparin explicates its well-known action as anti-coagulant agent, thus allowing an easier and cleaner manipulation of the cell culture.

In a still preferred embodiment the concentration of the anti-coagulant agent, e.g. heparin, in the supplement ranges from 20U/ml to 200U/ml.

In another preferred embodiment the concentration of the anti-coagulant agent, e.g. heparin, in the supplement ranges from 2 to 20U/ml or from 4 to 100U/ml.

The final concentration of anti-coagulant agent, e.g. heparin, in the cell culture medium (when 5 % of supplement is used) preferably ranges from 1U/ml to 100U/ml, more preferably from 1U/ml to 50U/ml, even more preferably from 2U/ml to 20U/ml.

In a preferred embodiment the cell or tissue culture medium supplement is frozen and/or freeze-dried and/or sterilized. Preferably the sterilization is performed before or after the freeze-drying step. Still preferably the sterilization is performed by gamma radiation or filtration.

It is a further object of the invention a process for the preparation of the cell or tissue culture medium supplement as above disclosed comprising mixing the platelet rich plasma fraction, the platelet poor plasma fraction and optionally the anti-coagulant agent wherein the platelet rich plasma fraction and/or the platelet poor plasma fraction and/or the anti-coagulant agent are in liquid or powder form.

It is a further object of the invention a method to *in vitro* expand a cell and /or to promote proliferation and/or differentiation of a cell and/or select of clonogenic cell and/or a cell sub-population and/or to maintain the differentiation potential of a cell and/or to enhance the cell culture dish coating and/or cell adhesion comprising culturing said cell in a medium supplemented with 0.1 to 30 % of the cell culture medium supplement as defined above.

Preferably, the cell is cultured in a medium supplemented with 0.5 to 20 % of the cell culture medium supplement as defined above. Most preferably the cell is cultured in a medium supplemented with from 1% to 15% of the cell culture medium supplement as defined above.

Even more preferably, the cell is cultured in a medium supplemented with 5% of the cell culture medium supplement as defined above.

E.g. when a medium is supplemented with 5% of the cell culture medium supplement according to the invention, the cell culture medium supplement may be a combination of platelet rich plasma fraction and platelet poor plasma fraction as above defined, respectively in the following percentages: 2.5% and 2.5%, 1% and 4% or 0.5% and 4.5%.

In another preferred embodiment the cell is cultured in a medium supplemented with 5% of the cell culture medium supplement according to the invention and the cell culture medium

supplement is a combination of platelet rich plasma fraction and platelet poor plasma fraction as above defined in a 75%/25% relative volume ratio. In a yet preferred embodiment the cell is cultured in a medium supplemented with 5% of the cell culture medium supplement according to the invention and the cell culture medium supplement is a combination of Component A - platelet lysate (PL) and component B in a 75%/25% relative volume ratio.

In a further preferred embodiment the cell is cultured in a medium supplemented with 5% of the cell culture medium supplement according to the invention and the cell culture medium supplement is a combination of Component A - platelet lysate (PL) and component B in a 50%/50% relative volume ratio.

In a another preferred embodiment the cell is cultured in a medium supplemented with 5% of the cell culture medium supplement according to the invention and the cell culture medium supplement is a combination of Component C and component D in a 75%/25% relative volume ratio.

According to the invention, the cell is preferably selected from the group consisting of: a primary cell, a cell line, a cell obtained from a biopsy of an elderly patient, an articular chondrocyte, a stem cell and an iPS cell.

More preferably, the cell is a bone marrow mesenchymal stem cell or bone marrow stromal cell, preferably human (hBMSC), osteoblast, preferably human (hOB), skin fibroblast, preferably human (hSF), umbilical cord derived MSC, preferably human (hUC-MSC), articular chondrocytes, preferably human (hAC).

In a preferred embodiment the cell is plated at density below 3×10^3 per cm^2.

According to the invention, the first component which contains a well-defined and optimized number of platelets per volume, is, as a not limitative example, platelet rich plasma, alone or suitably pre-diluted and platelet lysate, obtained by lysis of platelet rich plasma, alone or suitably pre-diluted to get the desired concentration range.

According to the invention, the first component can be derived from plasma or is suitably derived after processing to eliminate the fraction which can trigger coagulation.

According to the invention, the first component is mixed with the second component, preferably by combining Component A (fraction with higher platelet concentration) and Component B (platelet poor fraction), both with fibrinogen and agents triggering coagulation, or by combining Component C (fraction with higher platelet concentration) and Component D (platelet poor fraction), both without fibrinogen and agents triggering coagulation.

According to the invention, the mixture containing the first and second component, optionally with the addition of anti-coagulant agent, is used for primary cell cultures and cell lines.

According to the invention, the mixture containing the first and second component, optionally with the addition of anti-coagulant agent is used, as a not limitative example, for cell proliferation and differentiation, selection of clonogenic cells and/or cell sub-populations, proliferation of cells from biopsies obtained from elderly patients, maintaining the cell differentiation potential as, for example, in human articular chondrocytes and mesenchymal stem cells, in vitro expansion of cell cultures also starting from an extremely low number of initially plated cells, enhancement of cell culture dish coating and cell adhesion.

According to the invention, the components and/or their mixtures can be frozen and freeze-dried for a long term storage at low temperature.

The possibility of freeze drying the mixture preparations, sterilizing (either before or after freeze drying) and storing them for long time at low temperatures, allows therefore the usage of quality controlled "off the shelf" and "ready to use" products.

According to the invention, the components and/or their mixtures are sterilized preferably by gamma radiation, before or after freeze-drying process, or by filtration.

The present invention will be described by means of non limiting examples referring to the following figures:

Fig. 1 Proliferation rate of human bone marrow mesenchymal stem cells cultured with platelet derivatives at different percentages.

Bone marrow derived MSC were initially plated in 10% FCS medium at low density (5000 cells/well) and after 24 hours were transferred in medium containing Component A-PL at different percentages (2.5%, 1%, 0.5%) or combination of Component A-PL and Component B (Component A 2.5% / Component B 2.5%; Component A-PL 1% / Component B 4%; Component A-PL 0.5 % / Component B 4.5%). The proliferation rate was tested at different time points in the culture with the MTT assay. Cells cultured with the combination of Component A-PL and Component B show a higher proliferation rate in comparison with the cells grown in the presence of the Component A-PL alone.

Fig. 2 Clonogenic efficiency of human bone marrow mesenchymal stem cells cultured with platelet derivative supplemented medium.

Bone marrow nucleated cells were plated at low density in the culture conditions 10% FCS, 10% FCS+bFGF, 5% of the mixture Component A-PL (75%) / Component B (25%). After 14 days from plating, colonies were stained for alkaline phosphatase expression (ALP) and methylene blue (MB). The condition Component A-PL and Component B in a 75%/25% relative volume ratio increases the total number of colonies and the number of ALP positive colonies (90-100%) when compared to both 10% FCS and 10% FCS+ bFGF.

Fig. 3 Growth curve of human articular chondrocytes cultured with platelet derivative supplemented medium.

Human articular chondrocytes were cultured in 10%FCS (control) or 5% of the mixture Component A-PL (75%) / Component B (25%) supplemented medium and the cell population doublings were evaluated at different time points. Platelet derivative expanded cells showed a higher proliferation potential compared to the FCS expanded cells.

Values represent the number of doublings obtained at 80% confluence in the culture time period range (n = 3 primary cultures).

Fig. 4 In vitro chondrogenic differentiation of human articular chondrocytes expanded in platelet derivative supplemented medium.

Human articular chondrocytes isolated and expanded in medium supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio were tested for in vitro differentiation in the micromass culture assay at different passages in culture.

Cells derived from the expansion in the platelet derivative supplemented medium undergo chondrogenic differentiation as well as the control culture condition (FCS) expanded cells producing a methacromatic cartilagineous matrix (toluidine blue staining).

Fig. 5 In vitro osteogenic differentiation of human bone marrow mesenchymal stem cells cultured with platelet derivatives supplemented medium.

Cells expanded in the medium supplemented with 5% of a mixture of Component A-PL and Component B (3:1 volume:volume) or with Component B or 10% FCS were stimulated with an osteo-inductive medium. Platelet derivatives expanded cells were able to produce a marked osteogenic mineralized matrix than cells expanded with FCS (weak staining). Further those cells differentiated earlier (10 days compared to 15-20 days for FCS).

Fig. 6 Platelet derivatives stability.

Different aliquots of lyophilized Component A preparations were stored at different temperatures (RT, 4°C, -20°C). The frozen not freeze-dried Component A preparation stored at -80°C (Component A frozen -80°C) was used as internal control of the experiments. Preparations derived from each storage condition were evaluated in the clonogenic assay with hBMSC immediately after preparation (T0), 1 month (T1), 3 months (T3), 6 months (T6), 15 months (T15) after storage. The colony number of cells cultured in medium supplemented with preparations stored at RT and 4°C significantly decreased after 3 and 6 months of storage, respectively. After 15 months of storage at -20°C the platelet derivative is still able to support colony formation with an efficiency close to that at T0.

Fig. 7 Component A-PL and Component B characterization.

Component A-PL and Component B were analyzed for several parameters as listed in tables 6 and 7. Comparative morphology of cells in bone marrow plating derived colonies (A, B).

Colonies derived from bone marrow plating with 5% Component A-PL supplemented medium without (panel A) or with (panel B) Component B were analyzed for their phenotype. A higher number of fibroblastic spindle-shaped cells is evident in the culture maintained in the presence of Component B (panel B) while very few rounded cells are present in the culture condition without Component B (panel A).

EXAMPLES

Example 1

Preparation of Component A - platelet rich plasma fraction (PRP)

Component A is the fraction with an optimal concentration of platelet.

Component A is prepared starting from human blood buffy coats (Hospital Transfusional Center of Genova). Buffy coat samples from 10 to 20 healthy donors are pooled together in a single sterile blood bag connected to a satellite blood bag. The pooled buffy coat containing bag is centrifuged at low speed (1,100 RPM x 10 minutes, centrifuge ROTOSILENTA 630 RS, Hettich) and the upper phase represented by the platelet rich plasma fraction (PRP) is collected in the satellite bag, while the red blood cells containing phase is discarded. The platelet concentration in the PRP is measured by means of an automatic hemocytometer.

The PRP containing bag is connected in a sterile way to a satellite bag and subjected to a second high speed (2,600 RPM X 20 minutes, centrifuge ROTOSILENTA 630 RS, Hettich) centrifugation. After this second centrifugation, the platelets are concentrated at the bottom of the bag and the upper phase, represented by the platelet poor plasma fraction (PPP, Component B) is transferred to the satellite empty bag.

After measuring the platelet concentration of PRP, PRP can optionally be diluted in order to obtain a final platelet concentration from $8x10^6$ to $12x10^6$ platelets/µl.

The bag containing the plasma derivative at a desired platelet concentration is then transferred to a -80°C freezer or freeze-dried and stored at -20°C.

Example 2

Preparation of Component A - Platelet Lysate (PL)

The PRP fraction, prepared as in Example 1 and kept at -80°C at least for 15 hours, is gently thawed at 37°C and the platelet extract transferred to a bag which is frozen down to liquid nitrogen temperature (-196°C). Then the bag is thawed at 37°C. This freeze-thaw cycle is repeated for three times in order to fully break the platelets membranes and to obtain a Platelet Lysate (PL). After the third cycle is completed the bag is centrifuged at high speed (4,500 RPM

X 20 minutes, centrifuge ROTANTA 460R, Hettich) to remove platelet membranes and cell debris. The clear liquid fraction within the bag is carefully separated from the settled membranes and debris and collected in a sterile container under a laminar flow hood.

The liquid is then frozen at -80°C, freeze-dried and stored at -20°C. In some applications the freeze-dried product can be sterilized by gamma radiation (1 KGy) and stored at -20°C.

Example 3

Preparation of Component B, platelet poor plasma (PPP) fraction

Component B is obtained during Component A preparation in the procedure described in Example 1 (number of platelets < 5 x $10^4/\mu L$). Component B is freeze-dried and then stored at -20°C. In some applications the freeze-dried product can be sterilized by gamma radiation (1 KGy) and stored at -20°C. .

Before the freeze-drying process, different concentrations 4 to 100 U/ml of heparin can be added to the Component B.

Example 4

Preparation of Component C

Component C is Component A-Platelet Lysate (PL) without fibrinogen and fraction of coagulating agents.

Component C is prepared starting from pooled buffy coats samples. Up to the 3 freeze-thaw cycles the preparation protocol followed is the same described for Component A-PL (as described in Example 2). At the end of the last freeze-thaw cycle, the platelet extract is mixed with 0.1 M $CaCl_2$ (9:1 volume:volume) to trigger a clot formation. Alternatively at the end of the last freeze-thaw cycle, the bag containing the platelet extract is centrifuged at 4,500 RPM X 20 minutes (centrifuge ROTANTA 460R, Hettich) to remove membranes and cell debris and the supernatant collected in another bag prior the mixing with the 0.1M $CaCl_2$ solution. Bags are then left for 12-20 hours at room temperature in constant linear agitation to facilitate the clot formation. The bag is then centrifuged at high speed (4,500 RPM for 20 minutes, centrifuge ROTANTA 460R, Hettich) to sediment the formed clot. The liquid fraction is collected in a tube and further centrifuged at 3150 RPM for 10 minutes (centrifuge 5810R, Eppendorf) and the liquid clear phase is collected in sterile tubes under a laminar flow hood. The tubes are then frozen at -80°C, freeze-dried and sterilized by gamma radiation (1 KGy). Alternatively to the gamma irradiation, the final liquid preparation could be sterilized by filtration (0.45 – 0.22 microns) prior to freeze-drying.

Example 5

Preparation of Component D

Component D is a Component B without fibrinogen and fraction of coagulating agents.

The PPP residual from the Component A PRP preparations (Example 1) contained within the blood bag is mixed with 0.1M $CaCl_2$ (9:1 volume:volume) and the bag is left for about 4 h hours at room temperature in constant linear agitation to obtain the maximum of the clot formation. $CaCl_2$ causes coagulation of the plasma. The bag is then centrifuged at high speed (4,500 RPM X 20 minutes, centrifuge ROTANTA 460R, Hettich) to remove the clot. The liquid fraction is collected in a tube and further centrifuged at 3150 RPM for 10 min (centrifuge 5810R, Eppendorf) and the liquid clear phase collected in sterile tubes under a laminar flow hood. The product is freeze-dried, sterilized by gamma radiation and stored as described above for Component C. Alternatively to gamma irradiation (1 KGy), the final liquid preparation may be sterilized by filtration (0.45 – 0.22 microns) prior to freeze-drying.

Example 6

Cell proliferation rate with culture media containing different percentages of Component A-PL and different combinations of Component A-PL and Component B

Human bone marrow stromal cells (hBMSC) were plated in medium containing Component A-PL at different percentages (0.5%, 1%, 2.5%) or combinations of Component A-PL and Component B at different percentages

Component A-PL 2.5% / Component B 2.5%, therefore 50%/50% relative volume ratio,

Component A-PL 1% / Component B 4%, therefore 20%/80% relative volume ratio, and

Component A-PL 0.5% / Component B 4.5%, therefore 10%/90% relative volume ratio.

The proliferation rate was tested at different time points in the culture with the MTT assay.

Cells cultured with the combination of Component A-PL / Component B in all the different percentages tested showed a higher proliferation rate in comparison with the cells grown in the presence of the Component A-PL alone (Fig. 1).

Example 7

Proliferation rate of different cell types

Primary cultures of human osteoblasts (hOB), hBMSC, human skin fibroblasts (hSF) and human umbilical cord derived MSC (hUC-MSC) were established from tissue obtained during surgery and otherwise destroyed. All procedures were performed after obtaining informed consent from patients and Ethical Committee approval. Cells have been initially plated at 4 to 8 x 10^3 per 1 cm^2 and cultured with basal tissue culture medium (Iscove's modifies Dulbecco's medium Euroclone ECM0192L for hOB; Coon's modified Ham's F12, Biochrom FZ0855 for hBMSC; D-MEM Euroclone ECB7501L for hSF; alpha-MEM Glutamax Gibco 32561 for hUC-MSC) supplemented with the mixture Component A-PL (50% volume) / Component B (50% volume)

and incubated at 37°C in humidified atmosphere containing 95% air and 5% CO_2. At confluence cells were detached and replated for the evaluation of the doubling number performed during the culture. Cells grown in 10% FCS were used as control condition. The obtained results are indicated in Table 1.

Table 1. Doubling number after 22 days of cultures with mixtures containing Component A-PL and Component B.

Primary culture	10% FCS	Component A-PL (50%) / Component B (50%)
hOB	2	4.3
hBMSC	7.7	10
hSF	5	10.2
hUC-MSC	3	6

The results from Table 1 show that the mixture Component A-PL (50% volume) / Component B (50% volume) has a higher mitogenic effect on the analyzed cell culture populations when compared to the FCS control condition.

Example 8

Proliferation rate of human articular chondrocytes with Component A and Component A-PL

Primary cultures of articular chondrocytes (hAC) have been plated at 1 X 10^5 per 6 cm diameter dish in a serum-free medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of a mixture of Component A-PL and Component B at a 75%/25% relative volume ratio. The cultures were tested for proliferation rate with the determination of doubling number that occurs per unit of time (after 30 days). A 5% mixture of Component A and Component B at a 75%/25% relative volume ratio was used also used. The results are indicated in Table 2.

Table 2. Doubling number after 30 days of cultures with mixtures containing Component A and Component A-PL.

5% mixture of Component A (75%) and Component B (25%)	5% mixture of Component A-PL (75%) and Component B (25%)
23	22

No significant difference between the two used mixtures (Component A and Component A-PL) was observed.

Example 9

Proliferation rate (doubling number) of human articular chondrocytes with mixtures of Component A + Component B and Component C + Component D

Primary cultures of articular chondrocytes from articular cartilage biopsy have been cultured with basal tissue culture medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of Component A-PL and Component B in a 75%/25% relative volume ratio or supplemented with a 5% of a mixture of Component C and Component D in a 75%/25% relative volume ratio. Cells were incubated at 37°C in humidified atmosphere containing 95% air and 5% CO_2. At first confluence, cells were detached and re-plated at low density 1 x 10^5 in 6 cm diameter dish and tested for proliferation rate with the determination of doubling number that occurs per unit of time (after 30 days). Cells grown in 10% FCS were used as control condition. The results are presented in Table 3.

Table 3. Doubling number after 30 days of cultures containing different concentrations of mixture (10% FCS, doubling number = 2).

% in cell culture	Component A-PL (75%) and Component B (25%)	Component C (75%) and Component D (25%)
0,5	5	6
1	10	10
2,5	19	18
5	23	22
10	18	18
20	3	4

The results show that both mixtures enhance cell population growth rate when compared with 10% FCS control (doubling number after 30 days = 2). This effect is observed even at a very low concentration of the mixtures. No significant differences were observed between the two mixtures.

Example 10

Addition of heparin

Human umbilical cord derived MSC from donned cord have been cultured with basal tissue culture medium (alpha-MEM Glutamax, GIBCO 32561) supplemented with 5% of a mixture of Component A-PL and Component B in a 50%/50% relative volume ratio, further containing different concentrations of heparin. Cell proliferation was evaluated via MTT assay (Sigma M5655). The results are reported in Table 4.

Table 4. MTT assay (expressed as an optical density between 570 and 670 nm) after 120 hours of cultures containing increasing concentration of heparin in a 5% of Component A-PL and Component B in a 50%/50% relative volume ratio.

Heparin concentration (U/mL)	OD (570-670 nm)
No heparin	-
0,5	-
1	-
2	1.25
10	1.32
20	1.20
50	0.83

The results show that an optimal range (2-20 U/mL) of heparin enhances the proliferation rate when compared to other heparin concentrations.

Example 11

Cell clonogenicity (hBMSC)

The clonogenic potential of hBMSC was evaluated with the colony forming unit-fibroblast assay (CFU-f). Bone marrow nucleated cells were plated at 7.5 to 12.5 X 10^3 per cm^2 in basal tissue culture medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio or in the same medium supplemented with 10% FCS or 10% FCS + bFGF (1 ng/mL, Peprotech, 100-18B) (control conditions).

After 14 days of culture, colonies were stained for alkaline phosphatase expression (ALP) and methylene blue (MB). The results are presented in Figure 2. Figure 2 shows that the 5% mixture of Component A-PL and Component B in a 75%/25% relative volume ratio increases the total number of colonies and the number of ALP positive colonies (90-100%) when compared to both 10% FCS and 10% FCS+ bFGF .

Example 12

Proliferation of cells derived from elderly patients

Human articular chondrocytes (hAC) from articular cartilage biopsy have been cultured with basal tissue culture medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume

ratio or in the same medium supplemented with 10% FCS (FCS, control). The cell population doublings were evaluated at different time points as shown in Figure 2. Values represent the number of doublings obtained at 80% confluence in the culture time period range (n = 3 primary cultures).

The results demonstrate that hAC cells expanded in a medium supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio showed a higher proliferation potential when compared to hAC cells expanded in 10% FCS (Figure 3).

Example 13

hAC micromass assay

Human articular chondrocytes from articular cartilage biopsy have been expanded in basal tissue culture medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio or in the same medium supplemented with 10% FCS. Cells were tested for *in vitro* differentiation in the micromass culture assay as described by Johnstone B. et al. 1998, *Exp. Cell Res.* **238**, 265-272. The results are shown in Figure 4.

Cells expanded in the medium supplemented with 5% of a mixture of Component A-PL and Component B in a 75%/25% relative ratio undergo chondrogenic differentiation, comparable to FCS expanded cells, and produce a metachromatic cartilaginous matrix (toluidine blue staining) incorporating chondrocytes within lacunae (Figure 4).

In Figure 4, human articular chondrocytes were expanded in 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio or 10% FCS and tested for in vitro differentiation in the micromass culture assay at different passages in culture (p0, p1, p2, etc.). Each passage in culture corresponds to a different doubling number. Cells expanded in 5% of a mixture of Component A-PL and Component B in a 75%/25% relative volume ratio undergo a higher doubling number (dbs) in comparison with 10% FCS expanded cells which do not proliferate beyond the first passage (p1).

Cells derived from the expansion in of mixture of Component A-PL and Component B in a 75%/25% relative volume ratio supplemented medium undergo chondrogenic differentiation as well as FCS expanded cells producing a methacromatic cartilagineous matrix (toluidine blue staining) with chondrocytes in lacunae.

This finding provides evidence of the maintenance of the chondrogenic potential by the chondrocytes expanded in the presence of the platelet product. It is to note that the chondrogenic differentiation is maintained also after a number of doublings never reached by chondrocytes expanded in the presence of FCS.

Example 14

hBMSC *in vitro* osteogenic differentiation

hBMSC from iliac crest aspirates have been expanded in a basal tissue culture medium (Coon's modified Ham's F12, Biochrom FZ0855) supplemented with 5% of a mixture of Component A-PL and Component B (3:1 volume:volume) or with 5% of only Component B or with 10% FCS containing medium (control condition). At confluence, cells have been induced with an osteogenic medium according to the work of Muraglia A. et al., *J. Cell Sci.* 2000, **113**, 1161-1166, containing ascorbic acid (50 μg/ml), dexamethasone (10^{-7}M) and β glycerophosphate (10 mM) every other day (STIM). The *in vitro* osteogenic differentiation was assessed by means of histochemical staining with Alizarin Red S which stains in red the mineralized matrix and by Alkaline Phosphatase (AP) staining which stains in violet cells positive for the AP osteogenic marker. The results are presented in Figure 5.

Figure 5 shows that cells expanded in the medium supplemented with 5% of a mixture of Component A-PL and Component B (3:1 volume:volume) and cells expanded in the medium supplemented with Component B were able to produce a marked osteogenic mineralized matrix than cells expanded with FCS (weak staining). Further those cells differentiated earlier (10 days compared to 15-20 days for FCS).

Example 15

Cell density plating

Hela cells were obtained from the cell bank of the authors' institute (www.iclc.it) and their proliferation in the different media was evaluated by means of the xCELLIgence technology (RTCA DP Instrument, Roche Applied Science) which evaluates the "cell index" parameter. Cells were cultured with 5% of a mixture of Component A-PL and Component B in different relative volume ratios and 5% FCS (control). The cells were plated at different densities (0.5 to 4 x 10^3) per well and, as indicated in Table 5.

Table 5. Cell index after 120 hours of culture in presence of 5% of a mixture of Component A-PL and Component B, at different relative volume ratios between the Components, or 5% FCS in plates at different cell densities.

5% supplemented medium		Cell density (cells/well)		
		500	2000	4000
FCS		0,22	1,4	1,5
Component A-PL (%)	Component B (%)			
100	0	0,51	0,98	1,33
90	10	1,66	1,82	2,23

80	20	1,22	2,03	2,21
50	50	1,08	1,70	2,00
30	70	1,00	1,31	1,60

The results show that an optimal range of 50 to 90% of Component A-PL and of 10 to 50% of Component B induces the best cell proliferation. Moreover, the proliferation was observed also when the cells were plated at very low concentration at variance with the behavior of the same cells in control cultures when the supplement was 5-10% FCS (no cell growth at 500 cells per well (not shown).

Example 16

Stability

Different aliquots of freeze-dried Component A preparations have been stored at Room Temperature (Component A freeze-dried RT), 4°C (Component A freeze-dried 4°C), -20°C (Component A freeze-dried -20°C). The frozen not freeze-dried Component A preparation stored at -80°C (Component A frozen -80°C) was used as internal control of the experiments. For each of these conditions, preparations have been evaluated in the clonogenic assay with hBMSC as decribed in Example 11 immediately after preparation (T0), 1 month (T1), 3 months (T3), 6 months (T6), 15 months (T15) after storage. The results are shown in Figure 6. The colony number of cells cultivated in the presence of preparations stored at RT and 4°C significantly decreased after 3 and 6 months of storage, respectively (Figure 6). After 15 months of storage, the preparations stored at -20°C are still able to support colonies formation with an efficiency close to that at T0.

Example 17

Platelet derivatives characterization

Component A-PL and Component B were analyzed for the presence of mycoplasma (PCR analysis) and endotoxin (quantitative chromogenic LAL method). The content of several factors, insulin, PDGF-BB, VEGF were determined by Elisa assay; haemoglobin was quantified by emogasanalysis. Cell cloning and cell proliferation were used as biological test; fibrinogen content was determined by Fibrinogen Clauss assay. pH analysis was performed with a traditional pHmeter. Results are shown in tables 6 and 7:

Table 6

COMPONENT A-PL		
Test	**Detection Method**	**Result**
Mycoplasma	PCR analysis	Negative
Endotoxin	Quantitative Chromogenic LAL method	< 3 EU/ml
Insulin	Elisa	< 10 mU/L
Haemoglobin	Emogasanalysis	< 5 g/dl
PDGF-BB	Elisa	At least 100 ng/ml
VEGF	Elisa	At least 2 ng/ml
Quality and performance testing	Cell cloning (MSC); cell growth	Enhancing of cell proliferation and cloning efficiency of MSC
pH	Ph meter	6-6.5
Fibrinogen	Fibrinogen Clauss assay	not detectable

Table 7

COMPONENT B		
Test	**Detection Method**	**Result**
Mycoplasma	PCR analysis	Negative
Endotoxin	Quantitative Chromogenic LAL method	< 3EU/ml
Insulin	Elisa	< 20 mU/L
Haemoglobin	Emogasanalysis	< 5 g/dl
PDGF-BB	Elisa	18 ng/ml
VEGF	Elisa	0.450 ng/ml
Quality and performance testing	Cell cloning (MSC); cell growth	Enhancing of MSC proliferation
pH	Ph meter	7.5-8
Fibrinogen	Fibrinogen Clauss assay	2 g/L

The effect of the Component B presence in the medium was observed in parallel MSC cultures cultured with Component A-PL with or without Component B. As shown in figure 7 (panels A, B) the presence of Component B in the culture allows the attachment of a higher number of cells in comparison with the culture condition without Component B (panel A) where only very few rounded like cells are present.

CLAIMS

1. A cell or tissue culture medium supplement consisting of:

a) from 95 % to 0.5 % (volume/volume) of a platelet rich plasma fraction containing at least $2x10^6$ platelets/μL;

b) from 5 % to 99.5 % (volume/volume) of a platelet poor plasma fraction containing less than $5x10^4$ platelets/μL.

2. The cell or tissue culture medium supplement according to claim 1 consisting of:

a) from 70 % to 2.5 % (volume/volume) of the platelet rich plasma fraction and

b) from 30 % to 97.5 % (volume/volume) of the platelet poor plasma fraction.

3. The cell or tissue culture medium supplement according to claim 1 consisting of:

75 % (volume/volume) of the platelet rich plasma fraction and 25% (volume/volume) of the platelet poor plasma fraction, or

50 % (volume/volume) of the platelet rich plasma fraction and 50% (volume/volume) of the platelet poor plasma fraction, or

20 % (volume/volume) of the platelet rich plasma fraction and 80% (volume/volume) of the platelet poor plasma fraction, or

10 % (volume/volume) of the platelet rich plasma fraction and 90% (volume/volume) of the platelet poor plasma fraction.

4. The cell or tissue culture medium supplement according to any one of preceding claims wherein the platelet rich plasma fraction contains from $5x10^6$ to $15x10^6$ platelets/μL.

5. The cell or tissue culture medium supplement according to any one of the preceding claims wherein the platelet poor plasma fraction contains less than $1x10^4$ platelets/μL.

6. The cell or tissue culture medium supplement according to any one of the preceding claims wherein the platelet rich plasma fraction is further subjected to a lysis step and a centrifugation step.

7. The cell or tissue culture medium supplement according to any one of the preceding claims wherein the platelet rich plasma fraction and/or the platelet poor plasma fraction is processed to remove fibrinogen.

8. A cell or tissue culture medium supplement comprising the cell or tissue culture medium supplement according to any one of the preceding claims and an anti-coagulant agent.

9. The cell or tissue culture medium supplement according claim 8 wherein the anti-coagulant agent is heparin.

10. The cell or tissue culture medium supplement according to any of claims 8-9 wherein the concentration of the anti-coagulant agent in the supplement ranges from 20U/ml to 200U/ml.

11. The cell or tissue culture medium supplement according to any one of previous claim being frozen and/or freeze-dried and/or sterilized.

12. A process for the preparation of the cell or tissue culture medium supplement according to any one of previous claims comprising mixing the platelet rich plasma fraction, the platelet poor plasma fraction and optionally the anti-coagulant agent wherein the platelet rich plasma fraction and/or the platelet poor plasma fraction and/or the anti-coagulant agent are in liquid or powder form.

13. A method to *in vitro* expand a cell and /or to promote proliferation and/or differentiation of a cell and/or select of clonogenic cell and/or a cell sub-population and/or to maintain the differentiation potential of a cell and/or to enhance the cell culture dish coating and/or cell adhesion comprising culturing said cell in a medium supplemented with 0.1 to 30 % of the cell culture medium supplement as defined in claims 1 to 11.

14. The method of claim 13 wherein the cell is cultured in a medium supplemented with 0.5% to 20 % of the cell culture medium supplement as defined in claims 1 to 11.

15. The method according to claim 14 wherein the cell is cultured in a medium supplemented with 5 % of the cell culture medium supplement as defined in claims 1 to 11.

16. The method according to any one of claims 13-15 wherein the cell is selected from the group consisting of: a primary cell, a cell line, a cell obtained from a biopsy of an elderly patient, an articular chondrocyte, a stem cell and an iPS cell.

17. The method according to any one of claims 13 to 16 wherein the cell is plated at density below 3×10^3 per cm^2.

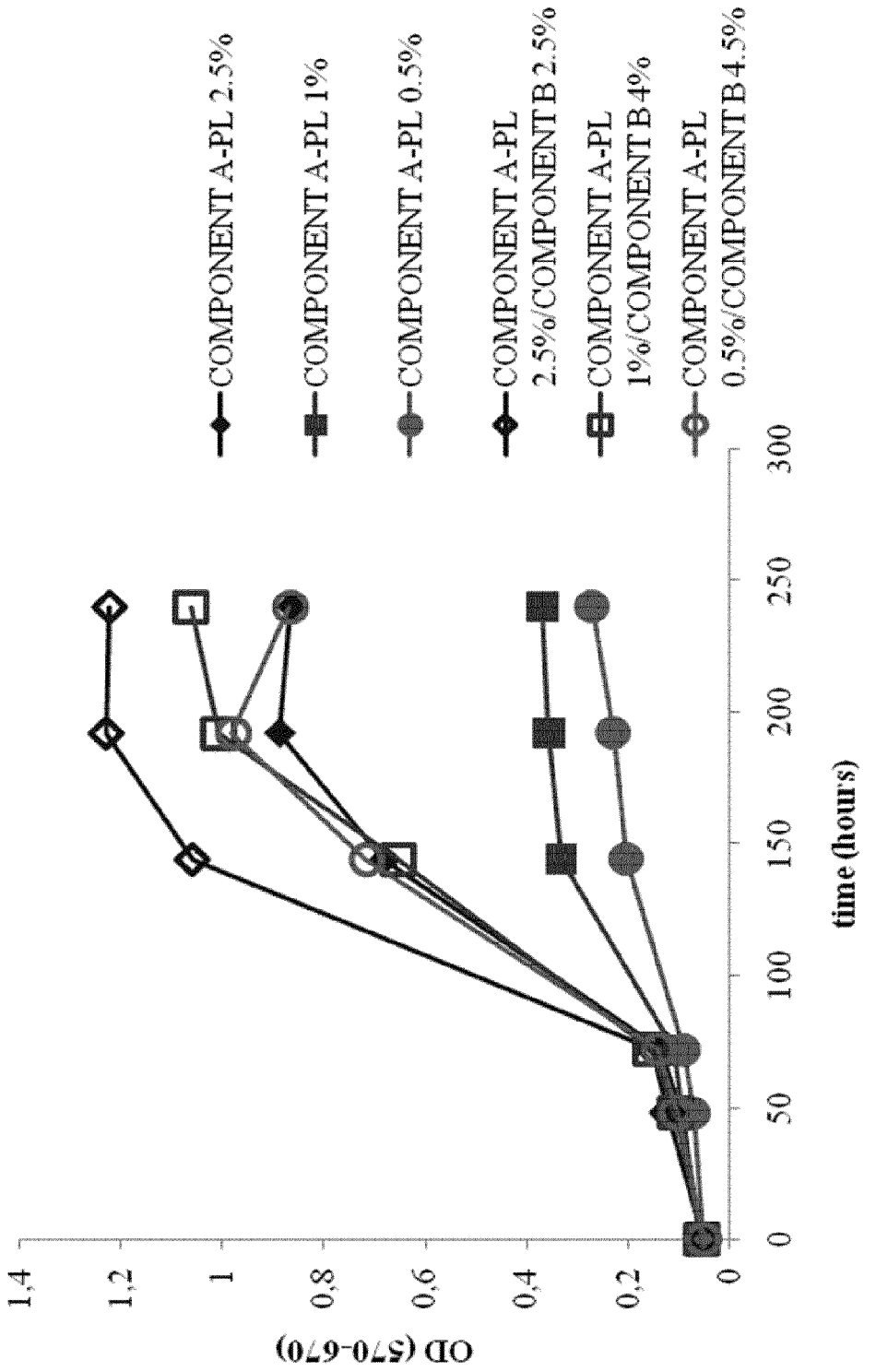
COMPONENT A-PL 2.5%
COMPONENT A-PL 1%
COMPONENT A-PL 0.5%
COMPONENT A-PL 2.5%/COMPONENT B 2.5%
COMPONENT A-PL 1%/COMPONENT B 4%
COMPONENT A-PL 0.5%/COMPONENT B 4.5%
OD (570-670)
time (hours)
0
0,2
0,4
0,6
0,8
1
1,2
1,4
0
50
100
150
200
250
300

Fig. 1

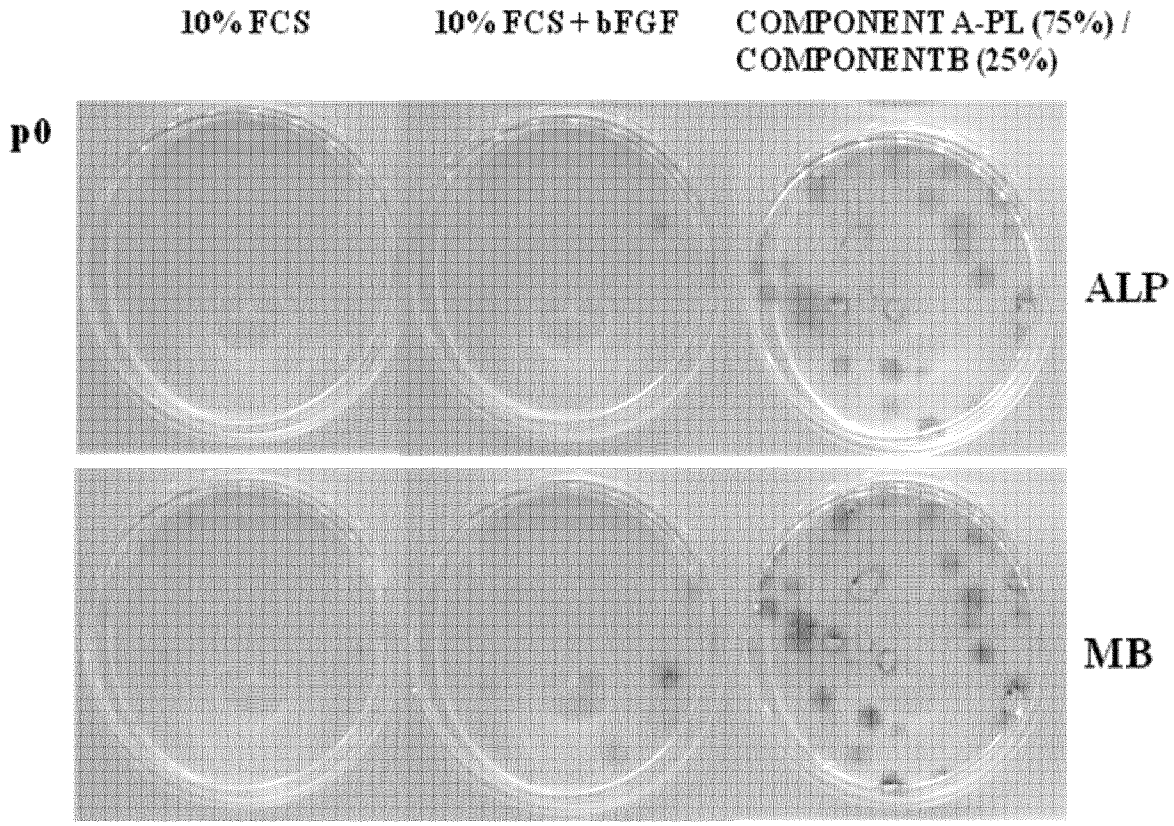

Fig. 2

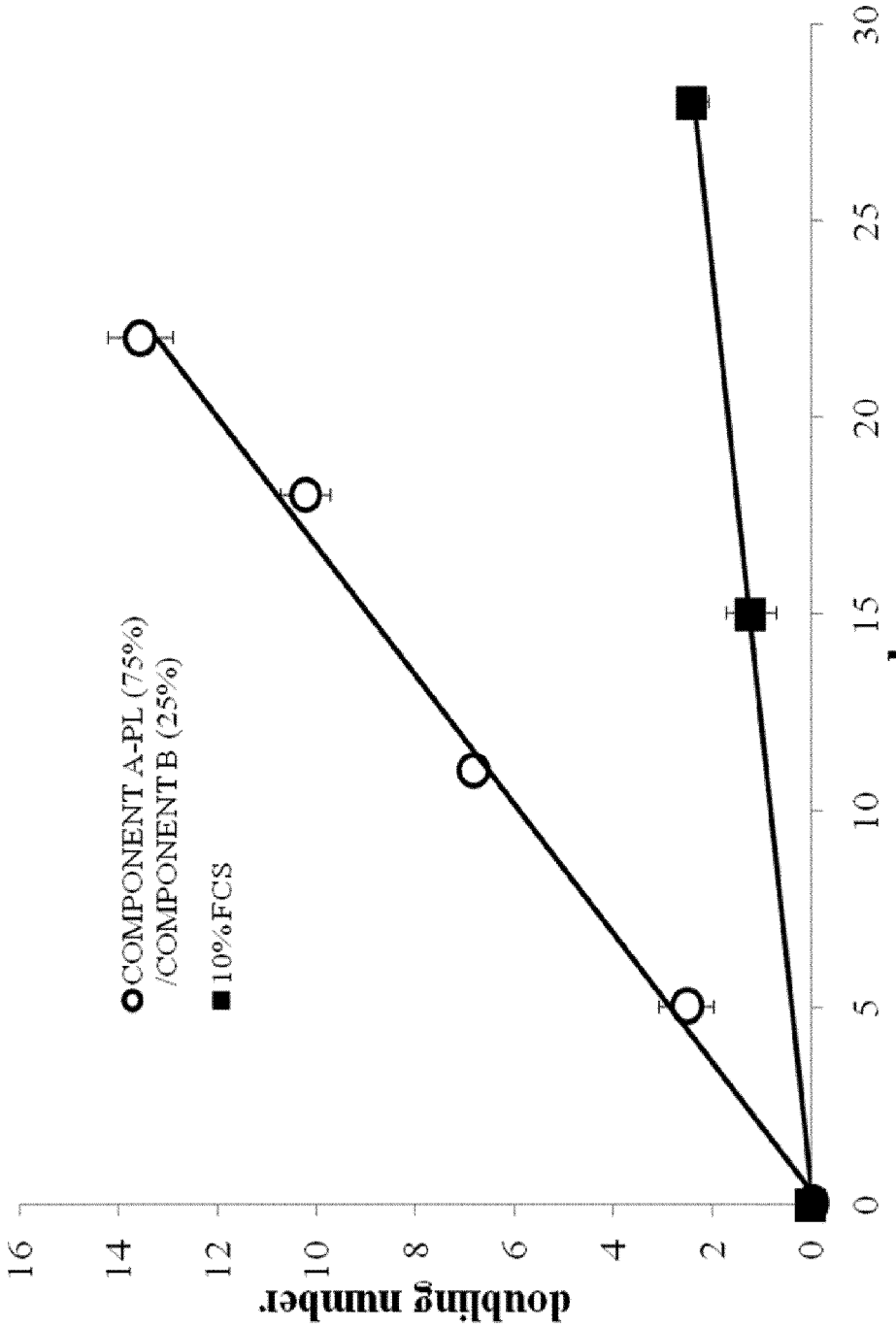
doubling number
days
COMPONENT A-PL (75%)
/COMPONENT B (25%)
10%FCS
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30

Fig. 3

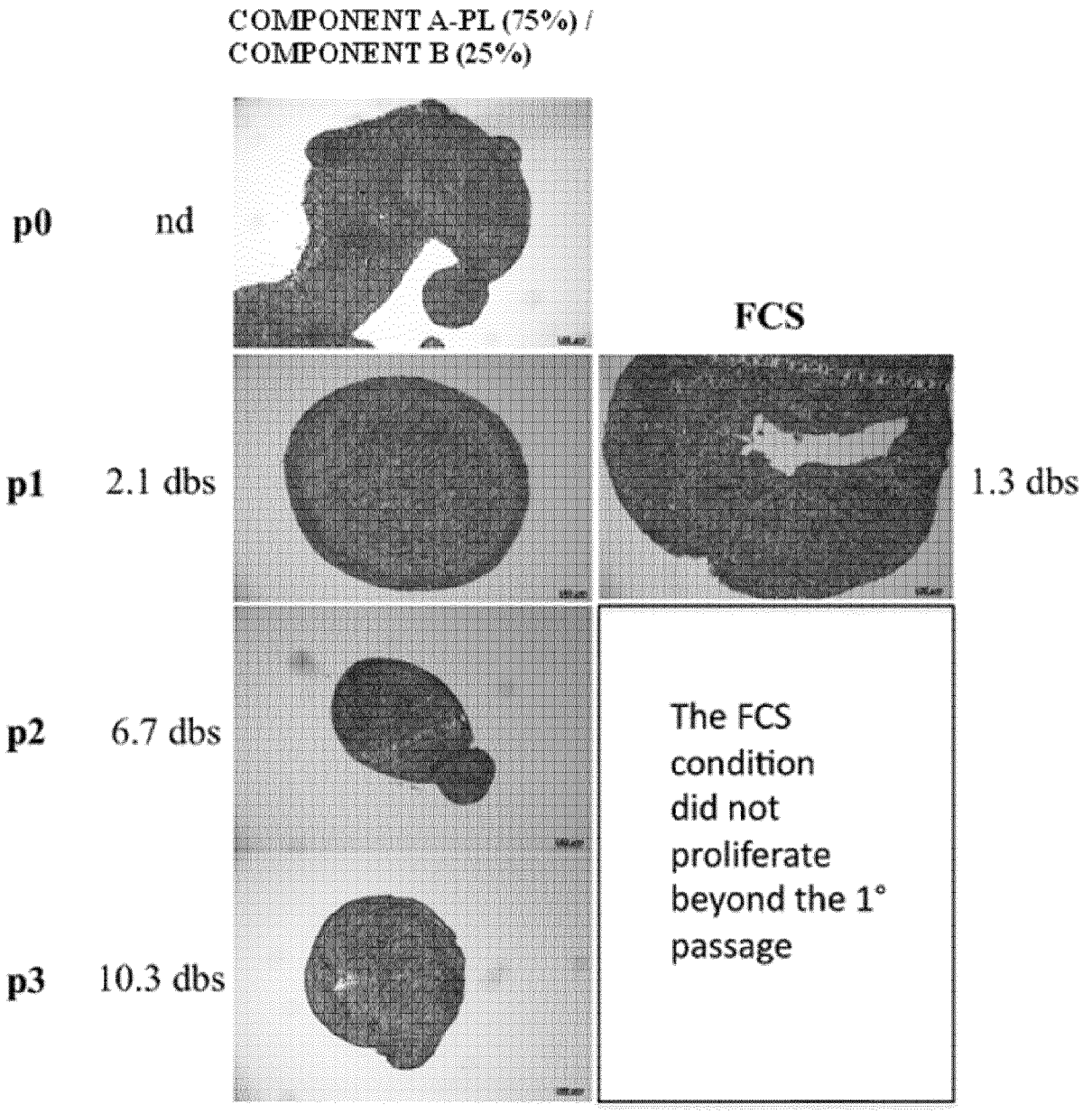
COMPONENT A-PL (75%) /
COMPONENT B (25%)
FCS
p0
nd
p1
2.1 dbs
1.3 dbs
p2
6.7 dbs
p3
10.3 dbs
The FCS condition did not proliferate beyond the 1° passage

Fig. 4

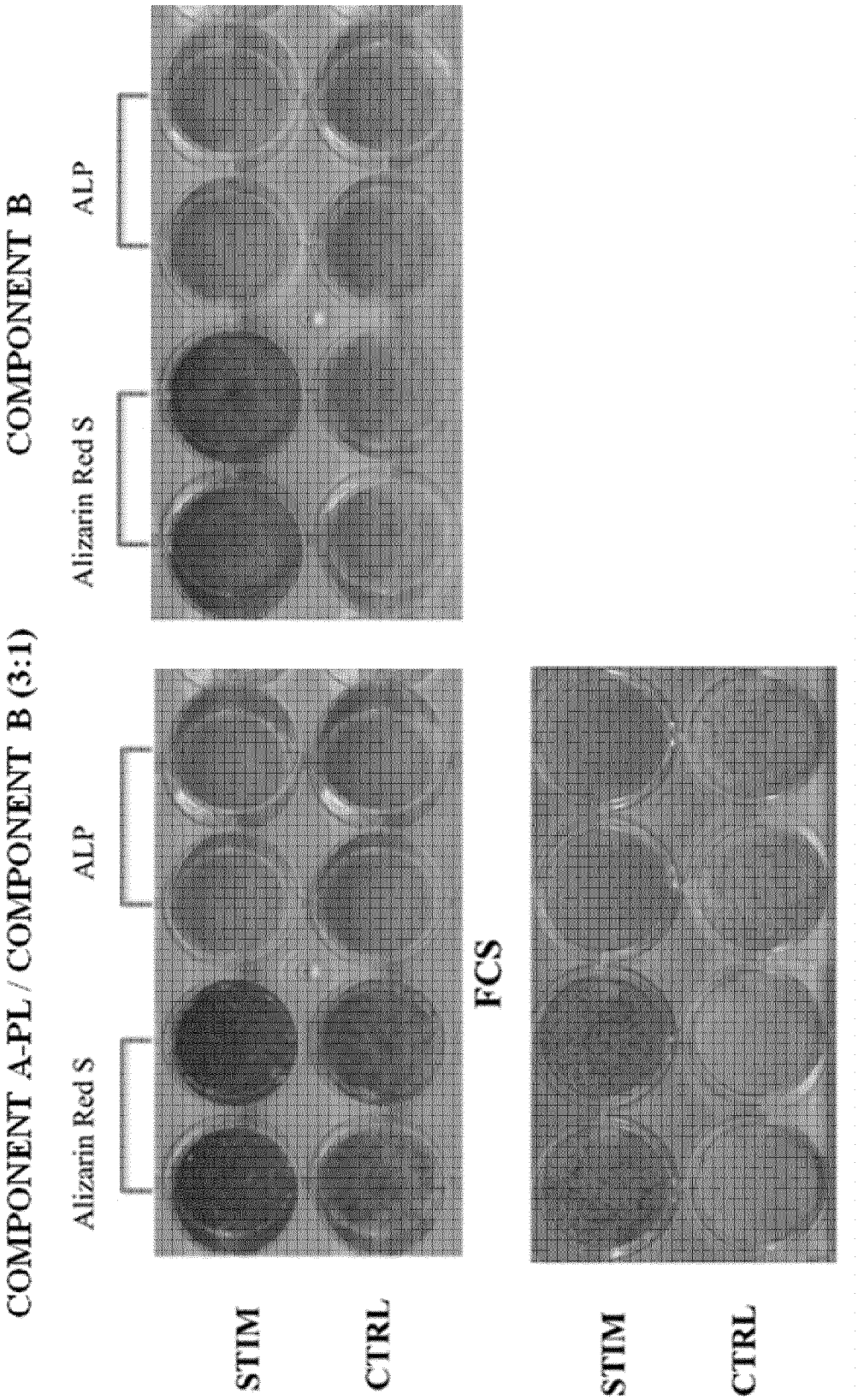
COMPONENT A-PL / COMPONENT B (3:1)
COMPONENT B
Alizarin Red S
ALP
Alizarin Red S
ALP
STIM
CTRL
FCS
STIM
CTRL

Fig. 5

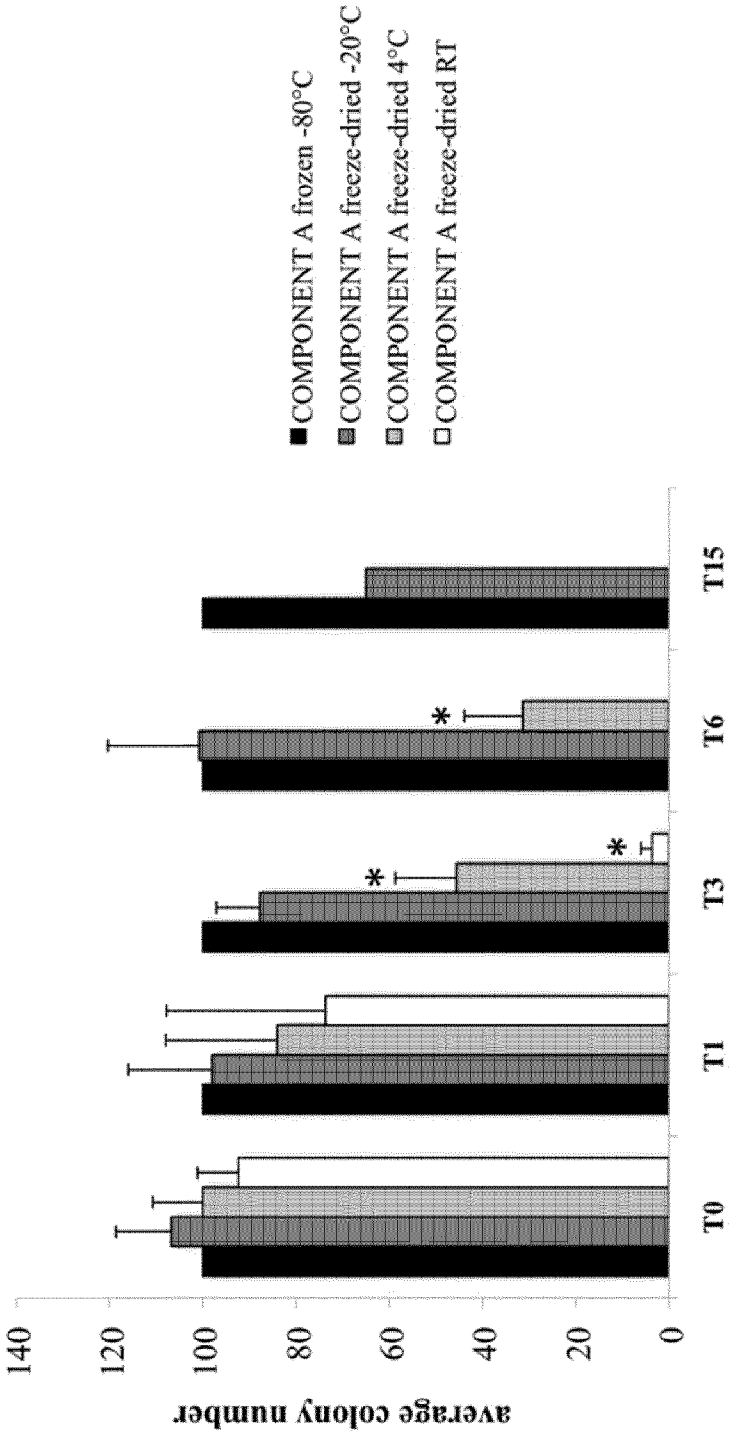
COMPONENT A frozen -80°C
COMPONENT A freeze-dried -20°C
COMPONENT A freeze-dried 4°C
COMPONENT A freeze-dried RT
average colony number
0
20
40
60
80
100
120
140
T0
T1
T3
T6
T15
*
*
*

Fig. 6

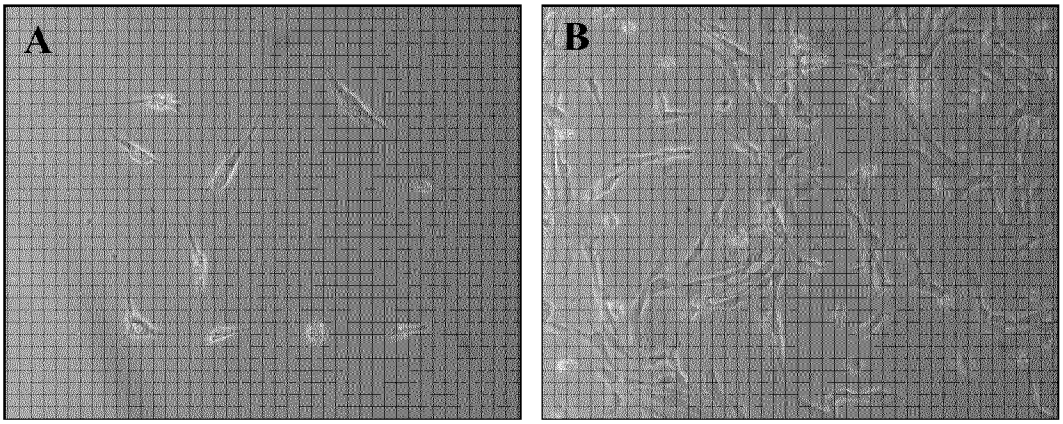

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/073865

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIEBACK KAREN ET AL: "Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow", STEM CELLS, ALPHAMED PRESS, INC, UNITED STATES, vol. 27, no. 9, 1 September 2009 (2009-09-01), pages 2331-2341, XP001525902, ISSN: 1549-4918, DOI: 10.1002/STEM.139 [retrieved on 2009-06-04] cited in the application the whole document ----- -/--	1-7, 11-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 December 2013	17/12/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mauhin, Viviane

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHALLMOSER KATHARINA ET AL: "Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells", TRANSFUSION, AMERICAN ASSOCIATION OF BLOOD BANKS, BETHESDA, MD, US, vol. 47, no. 8, 1 August 2007 (2007-08-01) , pages 1436-1446, XP002454868, ISSN: 0041-1132, DOI: 10.1111/J.1537-2995.2007.01220.X cited in the application the whole document	1-7, 11-17
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