(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2012 (19.04.2012)

PCT

(10) International Publication Number
WO 2012/048755 A1

(51) International Patent Classification:
*C07K 7/08* (2006.01)

(21) International Application Number:
PCT/EP2010/065557

(22) International Filing Date:
15 October 2010 (15.10.2010)

(25) Filing Language: English

(26) Publication Language: English

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17**:

— *of inventorship (Rule 4.17(iv))*

**Published**:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/048755 A1

(54) Title: ER-ALPHA-17P PEPTIDE, EFFECTS ON STEM CELL DIFFERENTIATION

(57) Abstract: The present invention provides a new peptide that stimulates mesenchymal stem cell differentiation into other cell types such as chondrocytes, myocytes, osteoblasts, pancreatic beta cells, adipocytes and neurocytes and methods using said peptide.

# ER-ALPHA-17P PEPTIDE, EFFECTS ON STEM CELL DIFFERENTIATION

## FIELD OF THE INVENTION

The present invention is situated in the medical field, more in particular in the field of stem cell differentiation in particular of mesenchymal stem cell (MSC) differentiation into osteoblasts, myocytes, tenocytes, adipocytes, neurocytes etc.

## BACKGROUND OF THE INVENTION

The ER-alpha-p17 is a synthetic peptide corresponding to the amino acids $P_{295}$-$T_{311}$ sequence of the ER-alpha chain protein. This sequence, located between the D- (hinge) and E- (Ligand Binding Domain; LBD) domains, appears to be involved in both the stability and the transcriptional activity of the ER-alpha receptor. This motif, actually situated in the AF-2a (autonomous activation function) domain, can be considered as a platform for various posttranslational modifications such as phosphorylation, acetylation, methylation, SUMOylation and monoubiquitination (see Gallo et al., Mol Cell Endocrinol 2008; 291:20-26). This motif also contains the third nuclear localization signal of the receptor, as well as a proteolysis site and a binding site for calmodulin, a coregulator which enhances both the transactivation and the stabilization of the receptor by impeding its E6-AP (E6-Associated Protein) mediated polyubiquitination (Li et al., J Biol Chem 2006; 281:1978-85). We have described that the ER-alpha-17p synthetic peptide elicits estrogenic responses in ER-alpha-expressing breast carcinoma cells: it stimulates both cell proliferation and ERE-dependent transcriptions (see Gallo et al., Mol Cell Endocrinol 2008; 291:20-26). This is associated with the receptor down regulation, occurring through an increase of ER-alpha degradation rate and a decrease of ER-alpha mRNA level (Gallo et al., Mol Cell Endocrinol 2007; 268:37-49). The present invention provides unexpected applications of the ER-alpha-17p peptide for mesenchymal stem cell differentiation.

The search for new methods and tools of generating new tissues and new cells for regeneration therapies has reached a never seen level today. Using new technologies, multipotent cells can be more easily isolated from subjects, even from human subjects and maintained viable in *ex vivo* cultures, where they are further differentiated for therapeutic use. However, the differentiation step of said cells into the desired mature or differentiated cell-type is still legging behind. The present invention therefore sought new tools and methods for differentiating pluripotent mesenchymal stem cells into their

differentiated cell types being: osteoblasts, adipocytes, myocytes, neurocytes, chondrocytes, tenocytes and ligament cells.

For treating bone-related disorders, grafting or transplantation of mesenchymal stem cells capable of undergoing osteogenic and chondrogenic differentiation, has been shown promising, in particular when the treatment requires production of new bone, especially after grafting. However, although such relatively undifferentiated mesenchymal stem cells can be readily transplanted, they are not yet committed to an osteogenic / chondrogenic lineage and a considerable part of the transplanted stem cells will eventually not contribute to the formation of the desired bone tissue. The use of growth factors to induce this osteogenic or chondrogenic differentiation is currently under investigation, wherein e.g. the FGF growth factor seems to be important (cf. WO 2007/093431). New and improved methods and tools are however strongly needed, especially methods wherein the differentiation to osteoblasts / chondrocytes could be postponed until after grating, whereby the cells are differentiated *in situ* in the subject, i.e. at the spot where they are actually needed, especially whereby said newly formed tissue is fully integrated in the existing structures and tissue.

Myocardial infarction continues to represent a major health problem. Injection of progenitor cells has been proposed as a promising approach to prevent cardiac remodelling or even restore myocardial tissue. Similarly, myocytes can be used to stimulate the formation of new blood vessels and increase organ blood reperfusion e.g. after ischemic events occurred. Several adult stem cell types have shown efficacy in the preclinical setting, including skeletal myoblasts, umbilical cord cells, bone marrow-derived mononuclear cells, and, more recently, adipose tissue-derived stem cells. As for osteoblast differentiation, mesenchymal stem cell (MSCs) are particularly preferred, shown to be capable of homing to infarcted myocardium and inducing angiogenesis. The present invention intends to provide new tools for improving differentiation of MSCs into myocytes.

In plastic and reconstructive surgery, adipocytes are often used. Although this adipose tissue could be recovered from the subject under treatment itself, there is a need for the production of adipose tissue or cells outside the patient. Mesenchymal stem cells are known to be able to differentiate into adipocytes. The present invention intends to provide new tools for improving differentiation of MSCs into adipocytes.

Neurons in the adult brain normally do not proliferate and damage to the brain tissue is therefore not easily repaired. Techniques using stem cells that can be differentiated into neurons are therefore needed.

Many sports or work-related injuries lead to rupture of ligaments or tendons, which often lead to inactivity of the subject for a prolonged time. Using grafting of tenocytes and ligament cells, derived from differentiation of mesenchymal stem cells is an attractive method to accelerate the healing process.

There exists a need for further simple and reliable methods for producing useful phenotype cells from stem cells.

## SUMMARY OF THE INVENTION

The inventors have surprisingly found that the use of a short peptide derived from the Estrogen Receptor alpha (ER-alpha) protein is capable of inducing differentiation of mesenchymal stem cells (MSCs). MSCs are of stromal origin and may differentiate into a variety of tissues such as osteocytes, chondrocytes, myocytes, adipocytes, neurons, tenocytes etc. MSCs can e.g. be isolated from placenta, adipose tissue, lung, bone marrow and blood and umbilical cord.

The present invention unravels the underlying mechanism of action of the ER-alpha-17p peptide and provides new and interesting possibilities for the use of said peptide of the ER-alpha receptor for stem cell differentiation.

The use of the ER-alpha-17p peptide has the advantage of generally inducing differentiation of mesenchymal stem cells, implying that upon using appropriate additional growth factors or signalling agent etc, the differentiation can be additionally steared into the right phenotype of cells i.e. osteoblasts, chondrocytes, myocytes, etc. The invention thus provides a general induction method of differentiating MSCs into its respective cell-types.

The invention provides for the use of the ER-alpha-17p peptide defined by SEQ ID NO:1, optionally carrying one or more post-translational modifications (i.e. lysines / arginines methylation, lysines acéthylation, serines / threonines phosphorylation).

In addition, the invention provides an *in vitro* method for inducing differentiation of mesenchymal stem cells (MSCs) into specific cell types comprising:

a) adding to a culture of MSCs a certain amount of ER-alpha-17p peptide defined by SEQ ID NO: 1,

b) adding to said culture a differentiation factor, selected from the group consisting of: the combination of dexamethasone, ascorbic acid and beta-glycerophosphate (DAG) for osteoblasts, TGF-beta for chondrocytes, MyoD or 5-azacytidine for myocytes, the combination of Insulin, dexamethasone and Indomethacin for adipocytes, the combination of Fibroblast Growth Factor (FGF), Butylated hydroxyanisole and DMSO for neurocytes, the combination of Nicotinamide and beta-mercaptoethanol for pancreatic islet beta-cells. In a preferred embodiment of said method, said ER-alpha-17p peptide is coated on the surface of the culturing support. In a preferred embodiment of said method, said MSCs are or have been recovered from a biological sample of a human subject, selected from bone marrow, adipose tissue, or umbilical cord. Preferably, said MSCs are cultured for a period of between about 7 and about 18 days. The method optionally includes the step of collecting the differentiated cells or cell population obtained by the method. In a further embodiment, said specific cell type is osteoprogenitors, osteoblasts or osteoblast phenotype cells, or a cell population comprising such, and the differentiation factor is the combination of dexamethasone, ascorbic acid and beta-glycerophosphate (DAG).

The invention further provides for human osteoprogenitors, osteoblasts or osteoblast phenotype cells, or an isolated cell population comprising such, obtainable by the method of the invention.

The invention further provides a method of treating a bone-related disorder using the pharmaceutical composition according to the invention.

The invention further provides a method of producing a bone-forming implantation device comprising:

a) seeding MSCs on an implant and,

b) adding the ER-alpha-17p peptide defined by SEQ ID NO: 1.

Preferably, the addition of the ER-alpha-17p peptide is done through incorporation of said peptide inside the matrix or core of the implant.

The invention further provides for a bone-forming implant obtainable by the method according to the invention.

According to a further embodiment, the invention provides a pharmaceutical composition comprising the cells or cell populations obtained by the methods according to the invention, and suitable for administration to a patient.

The invention further provides a mesenchymal stem cell differentiation kit comprising:
a) an amount of the ER-alpha-17p peptide, and
b) an amount of differentiation-specific component, selected from the group consisting of: DAG for osteoblasts, TGF-beta for chondrocytes, MyoD for myocytes, Insulin - dexamethasone - Indomethacin for adipocytes. In a preferred embodiment of the kit, the ER-alpha-17p peptide is coated onto the surface of culturing supports such as a culture dish, a slide, or a culturing flask.

Preferably, said kit comprises the DAG as the differentiation factor.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1:** Amino acid sequence of the ER-alpha-17p peptide (SEQ ID NO:1)

**Figure 2:** Amino acid sequence of ER-alpha isoform 1 (Genbank Acc. No. NP_000116 (SEQ ID NO: 1)), indicating the identified polypeptide according to the invention.

**Figure 3:** Immunophenotype of MSC. MSC were harvested, labelled with antibodies against CD31, CD34, CD45, HLA-DR, CD29, CD166, CD105 and CD73, and analysed by flow cytometry. Histograms show negative control IgG staining ($10^0$-$10^1$) versus specific antibody staining ($10^1$-$10^2$). Representative examples of MSC phenotype.

**Figure 4:** Induction of osteogenesis. **A.** MSC morphologic changes and formation of an organic matrix induced by ER-alpha-17p at $10^{-5}$M after 7 days of incubation in DMEM + 10% FBS. **B.** Colorization with Alizarin Red-S for calcification visualization. MSCs were cultured in DMEM + 10% FBS (Control) for 10 and 14 days with or without ER-alpha-17p at $10^{-5}$M and / or DAG.

**Figure 5:** Alkaline phosphatase activity and matrix calcification in MSC. **A:** Qualitative analysis. MSCs cultured in osteogenic medium (DAG) were exposed to $10^{-9}$M of estradiol ($E_2$) and $10^{-5}$M of ER-alpha-17p, after 14 days, Cytochemical demonstration of ALP activity was performed by using FAST Violet B as a substrate. After 21 days, Alizarin Red-S staining was used for the demonstration of mineralized bone matrix. Magnification X40. **B:** Quantitative analyses of ALP activity. ALP activity was evaluated in MSC cultured in control (CT) or osteogenic medium (DAG) after 7, 14 and 21 days of treatment with or without $10^{-5}$M of ER-alpha-17p, ALP activity was expressed as enzyme units (U, µmol p-nitrophenol released per min) normalized relative to the total protein content (mg). **C:** Quantitative analyses calcification. Calcium content of the extracellular matrix, of MSCs cultured in DMEM + 10% FBS (CT) or osteogenic medium was determined by colorimetric assay after 21 days of incubation with $10^{-9}$ of $E_2$ and $10^{-5}$M of ER-alpha-17p. Results are presented as mean±SEM (n=6).

**Figure 6:** Expression of bone differentiation markers. **A.** Cytochemical demonstrations. MSCs cultured in DMEM + 10% FBS (CT) with or without osteogenic induction medium (DAG) and or $10^{-5}$M of ER-alpha-17p for 10 days. Histochemical demonstrations of bone related proteins was performed using primary antibodies against osteocalcin (OC) and osteopontin (OPN). **B.** Real-time quantitative PCR analyses. After 4 days of culture, Real-time quantitative PCR were performed using SYBR green dye and sequence-specific primers for OPN and OC. Amplification of ß-actin mRNA as an endogenous control was used to standardize the amount of sample added to the reactions.

**Figure 7:** 3D cultures. MSCs were seeded in Matrigel in 8-well Lab-Tek chambers at high cell density (500.000 cell/well). After matrix solidification, chambers were disassembled and formed cubes were incubated in 6-well plate containing DMEM + 10% FBS (control) with or without DAG and / or ER-alpha-17p at $10^{-5}$M for 14 days. 3D structures were then harvested, fixed in 4% paraformaldehyde, frizzed in OCT and 10µM slices where stained (hematoxylin / eosin).

**Figure 8:** MSC differentiation in chondrocytes. 100.000 MSCs from bone marrow (BM) or adipose tissue (AT) were rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets were incubated in induction medium (insulin, ascorbic acid, and TGF-β) with or without ER-alpha-17p $10^{-5}$ M since formation of a chondrocyts bead (2 weeks).

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, the terms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps.

The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

The term "about" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of and from the specified value, in particular variations of +/-10% or less, preferably +/-5% or less, more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" refers is itself also specifically, and preferably, disclosed.

All documents cited in the present specification are hereby incorporated by reference in their entirety.

Unless otherwise specified, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions may be included to better appreciate the teaching of the present invention.

**Harvesting Techniques of MSCs**

Mesenchymal stromal cells, (also called marrow stromal cells, multipotent stromal cells or shortly “MSCs”) can be isolated from several organs for example including bone marrow, umbilical cord blood and adipose tissue, of which subcutaneous fat appears most easily and repeatedly accessible for high yield MSC isolation. By definition, MSCs are capable of both self-renewal and differentiation into a mature cell type. MSCs divide to form one daughter cell that goes on to differentiate and one daughter cell that retains its MS cell

properties. The classification of cells as stem cells is based on their species of origin, tissue of origin, or differentiation capability of greater than one specific type of the mature cells. MSCs are multipotent, reside in the bone marrow of adult human beings, and can differentiate into bone, fat, muscle, cartilage, and neurons.

Unlike procedures for harvesting autogenous bone, the aspiration of bone marrow or adipose tissues does not require an open surgical site. Using a relatively simple procedure with minimal morbidity, bone marrow or adipose tissue aspirations can be performed in an outpatient setting, as is routinely performed by hematologists and oncologists. The bone marrow aspirant is combined with a matrix which is preferably nonautogenous and/or osteoconductive. This procedure offers a promising alternative to autogenous bone grafts. The large flat bones of the body are rich in red active marrow and are an excellent source of osteoprogenitor cells. Three locations are recommended for harvesting bone marrow from an adult patient: the anterior iliac crest, posterior ilium and sternum.

In a healthy adult human being, aspiration of 2 ml of marrow can provide an average of 36 million ($3.6\times10^7$) marrow cells, of which 360 MSCs would be available for differentiation. An average ratio of 1 MS cell per 100,000 marrow cells is found in healthy individuals, with the highest majority of the marrow cells belonging to the various hematopoietic lineages. In vitro studies have shown that a mean of 2400 osteoblastic bone-forming cells producing alkaline phosphate positive colonies could be calculated. This number diminishes with age and in the presence of systemic disease. After the desired volume of marrow has been harvested, anticoagulation and filtration techniques may be implemented to concentrate the progenitor cells from the rest of the peripheral red blood cells as well as to collect the plasma, which includes cytokines and growth factors, as desired. Alternatively, or additionally, the bone marrow aspirate may be concentrated or separated by centrifuging, for instance for ten minutes.

**Osteoblasts**

Osteoblasts or their precursor cells are needed for bone formation. In one aspect, the present invention provides new methods and tools for bone (re-)generation. The starting product of the methods of the present invention is a pool of mesenchymal stem cells (MSCs), usually obtained from bone marrow aspirate, adipose tissue, or umbillical cord. These MSCs have the ability to differentiate into several specific cell types, e.g. osteoblasts, depending on the growth factors or other initiators or mediators that are supplied to them. Bone tissue is generally composed of cells, insoluble extracellular

matrix, and soluble molecules that serve as regulators for cell function, survival, differentiation and growth. Bone cells can be of three different types: osteocytes, osteoblasts, or osteoclasts. Osteocytes are the mature bone cells found within the dense cortical bone. Cortical bone further contains a significant number of either osteoblasts responsible for bone apposition and osteoclasts involved with remodeling and bone resorption. Cancellous bone is composed of a lattice of large hydroxyapatite plates and rods, known as the trabeculae and lies beneath the cortical bone.

Osteoblasts create new bone. For a successful graft, the graft matrix must be populated by osteoblasts or primitive mesenchymal cells that can be transformed into osteoblasts. If osteoblasts are not present in the recipient bed, they must be harvested and brought to the site. The absence of a sufficient population of osteoblasts will likely cause the graft to fail.

**Matrix, implants, grafting**

The ideal scaffold should mimic the extra-cellular matrix of autogenous bone. Preferably it is non-toxic, biocompatible, biodegradable at a rate that is compatible with bone remodeling without lowering the pH of surrounding tissues, has a porous structure of a geometry that permits cell ingrowth, and can be easily integrated into new bone. Further, the scaffold should preferably be a gel or have a microporosity that supports and promotes angiogenesis and capillary in-growth.

A variety of tissue-engineered bone substitute materials are commercially available. Preferably, an osteoconductive scaffold is provided to serve as a delivery vehicle for the aspirated cells. This scaffold contains the cells within the graft. Because fast resorbing matrices may not remain at the recipient site for a long enough period to permit the full process of osteogenesis to unfold, a slowly resorbable, biodegradable, and biocompatible scaffold material may be provided. Porous, resorbable hydroxylapatite matrix, is an excellent material and produces satisfactory results. Beta-tricalcium phosphate is another material of choice for these procedures.

Preferably, the scaffold should possess both micro-porosity and macro-porosity with open-pore architecture and inter-connecting geometry to allow a large surface-to-area volume ratio. This will facilitate cell ingrowth and vascularization of the graft from the surrounding tissues. As is the case for all bone grafts, the graft material must be stabilized at the

recipient site with at least one of screws, guided bone regenerative membranes, a titanium mesh and/or other suitable materials.

Cell transfer strategies of bone marrow aspirate stem cells to the scaffold delivers living cells to allografts, alloplasts, and xenografts or combinations thereof. Suitable allograft material includes demineralized bone an undemineralized bone. Suitable xenograft material includes bovine resorbable hydroxyapatie. Suitable alloplast material includes β-tricalcium phosphate, calcium sulfate and β-tricalcium sulfate, as well as combinations thereof. After the transplanted cells from the bone marrow differentiate, they provide the osteoblasts and osteoclasts that are needed for bone regeneration. When properly handled and administered, the transplanted cells lay down an initial unmineralized bone matrix, an osteoid, and initiate the mineralization process of laying the mineral component of bone, hydroxylapatite. When suspended into a suitable graft matrix the transplanted cells lay down the initial unmineralized bone, the osteoid, and initiate the mineralization process of proliferating the mineral component of bone, hydroxylapatite.

For bone reconstruction, diverse types of ceramics or natural products or synthetic products (hydroxyapatite / tricalcic phosphate / alpha-hydroxy-acids) are widely used. Unfortunately these materials rarely stimulate bone regeneration as such. By stimulating osteogenesis, the ER-alpha-p17, in combination with strategies using mesenchymal stem cells obtained from bone marrow or adipose tissue could clearly augment the regeneration of bone tissue.

Therefore, in order to create more rapidly bone tissue in vitro, coating the materials currently used for bone regeneration with said ER-alpha-p17 peptide could be used to create bone tissue in vitro.

Gels such as Matrigel, gelatin or other biocompatible gel-forming polymers may also be used to create bone implants. MSCs seeded in such gels and treated with ER-alpha-p17 peptide to favor osteoblastic differentiation could therefore be used to create three-dimensional customized bone implants.

In another aspect, analogous of the peptides of the invention, derived from ER-alpha, such as ER-alpha-p17, could be developed that have lost their ability to act on breast cancer cells, but maintaining their effect on the differentiation of mesenchymal cells into osteoblasts could be used to for in vivo applications e.g. to treat osteoporosis in vivo.

Compositions comprising bioactive compositions or extracts thereof which include the ER-alpha-17p peptide according to the invention in an appropriate concentration and ratio that enhance osteoblast production, and methods of preparation and use thereof, are envisaged by the present invention. The compositions can be included in implantable devices that are capable of inducing tissue formation in autogeneic, allogeneic and xenogeneic implants, for example as coatings and/or matrix materials. Examples of such devices include prosthetic implants, sutures, stents, screws, plates, tubes, and the like. Aqueous extracts of the bioactive glass compositions, which extracts are capable of stimulating osteoblast production, are also disclosed. The compositions can be used, for example, to induce local tissue formation from a progenitor cell in a mammal, for accelerating allograft repair in a mammal, for promoting in vivo integration of an implantable prosthetic device to enhance the bond strength between the prosthesis and the existing target tissue at the joining site, and for treating tissue degenerative conditions.

The combination of bone marrow aspirate graft techniques and an appropriate graft scaffold matrix of either an allograft, xenograft, or alloplast, or a combination thereof presents a methodology yielding a bone-graft material that is most likely to be transformed into viable new bone, and ensures that bone regeneration results, as opposed to fibrous repair.

The term “bone-related disorder” as used herein refers to any type of bone disease, the treatment of which may benefit from the administration of osteogenic lineage cells, e.g., osteoprogenitors, osteoblasts or osteoblast phenotype cells to a subject having the disorder. In particular, such disorders may be characterised, e.g., by decreased bone formation or excessive bone resorption, by decreased number, viability or function of osteoblasts or osteocytes present in the bone, decreased bone mass in a subject, thinning of bone, compromised bone strength or elasticity, etc. By way of example, but not limitation, bone-related disorders which can benefit from administration of osteoblasts or osteoblast phenotype cells of the present invention may include local or systemic disorders, such as, any type of osteoporosis or osteopenia, e.g., primary, postmenopausal, senile, corticoid-induced, any secondary, mono- or multisite osteonecrosis, any type of fracture, e.g., non-union, mal-union, delayed union fractures or compression, conditions requiring bone fusion (e.g., spinal fusions and rebuilding), maxillo-facial fractures, bone reconstruction, e.g., after traumatic injury or cancer surgery, cranio-facial bone reconstruction, osteogenesis imperfecta, osteolytic bone cancer, Paget's Disease, endocrinological disorders, hypophsophatemia, hypocalcemia, renal

osteodystrophy, osteomalacia, adynamic bone disease, rheumatoid arthritis, hyperparathyroidism, primary hyperparathyroidism, secondary hyperparathyroidism, periodontal disease, Gorham-Stout disease and McCune-Albright syndrome.

As used herein in the context of the methods of the invention, the term "osteoprogenitors", "osteoblasts" or "osteoblast phenotype cells" generally encompass cells which can contribute to, or are capable of developing to cells which can contribute to, the formation of bone material or bone matrix. It is to be understood that this aspect of the invention provides methods resulting in cells and cell populations which are useful for restoring bone formation in therapeutic settings. Consequently, the recitation osteoprogenitors, osteoblasts or osteoblast phenotype cells should be construed as wishing to encompass any such useful cells of the osteogenic lineage resulting from the methods of the invention.

In an aspect, osteoprogenitors, osteoblasts or osteoblast phenotype cells obtainable by or directly obtained by methods of the present invention, or an isolated cell population comprising osteoprogenitors, osteoblast or osteoblast phenotype cells, said population obtainable or directly obtained using the methods of the invention as described above, may be administered at a site of bone lesion, e.g., surgery or fracture.

In another aspect, the invention provides a method for preventing and/or treating bone disease, comprising administration of osteoprogenitors, osteoblasts or osteoblast phenotype cells obtainable by or directly obtained by methods of the present invention, or of an isolated cell population comprising osteoprogenitors, osteoblast or osteoblast phenotype cells, said population obtainable or directly obtained using the methods of the invention as described above, to a subject in need of such treatment.

Implants can for example be artificial bone graft implant such as the ones described in US patent 6,607,557; 6,149,688; 4,863,472 and any other implant known in the art.

A bone grafting method according to the present invention typically comprises the steps of:

a) harvesting a bone marrow or adipose tissues aspirate from a patient, said aspirate containing at least one type of cells selected from the group consisting of adult stem cells and primitive mesenchymal cells;

b) combining said cells from aspirate with a suitable graft material;

c) adding a suitable amount of ER-alpha-17p peptide according to the invention,

c) adding a suitable amount of DAG, and

d) securing the graft material at a site in a patient to receive a graft.

The methods and tools of the invention can be used for making all kinds of gels, pastes, and bioactive compositions known in the art comprising bone cells. The bioactive composition typically comprises mesenchymal stem cells and a certain amount of the active ER-alpha-17p peptide as identified herein, capable of inducing differentiation of said stem cells into osteoblasts, even after administration to the subject.

The invention particularly envisages the provision of or coating with osteoblasts onto a bone grafting matrix, scaffold, gel or implant known in the art. The incorporation of the active ER-alpha polypeptide as identified herein into said matrix, scaffold, gel or implant gives it the ability to induce or maintain differentiation of mesenchymal stem cells into osteoblasts, even after implantation.

**Myocardiocytes**

The most common cause of loss of cardiac muscle tissue with heart failure is coronary artery disease. Myocardial infarctions (heart attacks) kill cells. The longer the period of no blood flow (from coronary thrombosis) the greater the heart muscle cell destruction, and the poorer the resultant heart function. Opening heart arteries soon after the heart attack restores blood flow and potentially limits the amount of heart muscle damage. The sooner the heart artery is opened, the more the muscle can recover, and the smaller the damaged area is. Heart muscle damage shows itself in falling heart function, which may lead to heart failure, a syndrome of breathlessness and leg swelling. This heart failure can be treated with medical therapy, with good effect, especially if the heart failure is mild to moderate (reflecting a mild to moderate area of heart muscle damage). In severe heart failure (large, extensive heart muscle damage) medical therapy may not be adequate, and the patient may benefit from certain devices and ultimately may require cardiac transplantation. Although cardiac transplantation is a good and effective way to treat patients with severe heart failure from extensive heart muscle failure, there is a severe lack of donor hearts. An alternative approach is to use myocardiocytes (heart muscle cells) for regeneration of the damaged heart. Direct injection of cardiomycytes is the preferred mode of administration. Myocardiocytes are inherently self excitable. This is a

property of all cardiac cells. In our heart, this self triggering potential is channeled through a proper channeling pathway, so that they are trigger in synchrony with other cells, and so the heart contracts in-synchrony like a group of marching soldiers. The most convenient way of monitoring the treatment is the use of echocardiogram, MRI scans or MSCT scans.

**Adipocytes**

Adipocytes, also called lipocytes or fat cells, are the cells that primarily compose adipose tissue, specialized in storing energy as fat. Producing adipocytes e.g. finds it use in reconstruction of the body in case of amputations or surgical removal of certain body parts, for example in case of cancerectomy, or even skin reconstruction after an accident, or in case of burns or wounds etc… Also in cosmetics, the use of adipocytes is well known for reduction of wrinkles of aged skin, correction and reconstruction of lip line, face line and hairline, and rhinoplasty, otoplasty, blepharoplasty, labiaplasty, mammoplasty, abdominoplasty, buttock augmentation, chin augmentation, cheek augmentation, etc. Adipocytes yielded according to the method of the present invention can be reintroduced in the patient directly or in combination with a biodegradable scaffold, wherein the adipocytes are seeded and first grown in vitro.

**Chondrocytes**

Chondrocytes form cartilage, which is another expectative target for tissue engineering, because of its limited capacity for self-repair. The difficulty in the self-repair of cartilage seems to be due to the lack of a sufficient supply of healthy chondrocytes to the defective sites or to the low productivity of matrices in regenerated chondrocytes. Cartilage tissue engineering, i.e. administering chondrocytes at the place of need, could overcome such limitations. In principle, chondrocyte activities are maintained when they are placed in the proper 3D environment. During the development and growth of cartilage, the chondrocytes produce abundant matrices, encase themselves within cavities, and are eventually separated from each other. In contrast, when chondrocytes are isolated from their 3D environment, they rapidly lose the typical phenotype and protein synthesis (dedifferentiation). The chondrocytes produced by the method of the invention can be conditioned in a 3D environment, mimicking the physiological situation with favorable scaffolds so as to reproduce their functions and enhance and maintain chondrocyte specific protein synthesis. Two types of scaffolds for cartilage tissue engineering are generally used. The first type is a solid type of scaffold including a honeycomb, porous body, mesh, sponge, and unwoven fabric, and the second type is a hydrogel, using materials from animals or plants, for example, collagen type I gel, atelopeptides of

collagen, fibrin glue, gelatin, agarose, or alginate. The hydrogel type of scaffold could be used to reconstruct the 3D environment for the chondrocytes in cartilage tissue engineering (cf. e.g. Hisayo Yamaoka et al., J Biomed Mater Res 78A: 1–11, 2006).

**Neurocytes**

The production of neurocytes offers a new treatment strategy for previously incurable diseases such as Alzheimer's and Pick's disease having a main lesion at the cerebral cortex, Parkinson disease and Huntington chorea having a main lesion at the cerebral basal nuclei, spino-cerebellar degeneration having a main lesion at the cerebellum, amyotrophic lateral sclerosis having a main lesion at the spinal cord or Multiple Sclerosis, creating lesions on the myelin sheets. Here the defined aim is to replace the damaged or death neurocytes with MSC derived neurocytes or neuroblasts.

**Pancreatic beta cells**

Pancreatic beta-cells are responsible for insulin synthesis, storage, and release. Lack or defect of insulin produces diabetes mellitus, a devastating disease suffered by 150 million people in the world. Transplantation of insulin-producing cells could be a cure for type 1 and some cases of type 2 diabetes, however this procedure is limited by the scarcity of material. Obtaining pancreatic beta-cells from mesenchymal stem cells according to the method of the present invention overcomes this problem.

**Culturing of mesenchymal stem cells and inducing differentiation**

The majority of modern culture techniques still take a CFU-f approach, where raw unpurified bone marrow or ficoll-purified bone marrow monocytes are plated directly into cell culture plates or flasks. Mesenchymal stem cells, but not red blood cells or haematopoetic progenitors, are adherent to tissue culture plastic within 24 to 48 hours. The present invention additionally provides for culture dishes, flasks or slides that are coated with the ER-alpha-p17peptide according to the invention. Such culture dishes, flasks or slides have the advantage that they will induce differentiation of the stem cells immediately when used in culturing, i.e. without the need of administrating said peptide to the culturing medium. Mesenchymal stem cells adhering to such culture dishes, flasks or slides would then be immediately triggered for differentiation, without the need of supplying the peptide of the invention to the culturing medium. The choice of differentiation into e.g. osteoblasts, adipocytes, myocytes/cardiocytes, chondrocytes, neurocytes etc. can then be done by adding a cell-type-specific differentiation factor according to the invention to the medium.

Said cell-type-specific differentiation factor can be one of the following components, depending on the desired cell-type to be obtained (reviewed e.g. in Cao and Feng, Chinese Medical Journal 2009; 122(2):225-231):

- DAG (dexamethasone / ascorbic acid / beta-Glycerophosphate) for obtaining osteoblasts for use e.g. in regenerating or forming bone tissue, which can be identified by osteopontin or osteocalcin expression, alkaline phosphatase activity and calcification and bone nodule formation (cf. e.g. Wexler et al., Br J heamatol 2003; 121:368-374; Lu et al., Haematologica 2006; 91:1017-1026; Wang et al., Stem Cells 2004; 22:1330-1337),

- TGF-beta, optionally combined with insulin transferrin selenium, for obtaining chondrocytes for e.g. forming cartilage, tendons, etc, identified by type-II collagen and/or aggrecan expression (cf. e.g. Lu et al., Haematologica 2006; 91:1017-1026; Wang et al., Stem Cells 2004; 22:1330-1337),

- MyoD or 5-azacytidine for obtaining cardiomyocytes e.g. or use in regenerating heart tissue, which can be identified e.g. based on troponin I and N-cadherin expression (cf. e.g. Lu et al., Haematologica 2006; 91:1017-1026; Wang et al., Stem Cells 2004; 22:1330-1337),

- A combination of Insulin, Dexamethasone (DXM) and Indomethacin and optionally isobutylmethylxanthine for obtaining adipocytes e.g. for forming adipose tissue, identified based on peroxisome proliferation activation receptor gamma-2, and/or lipoprotein lipase activity (cf. e.g. Frank and Sayegh Lancet 2004; 363:1411-1412; Wexler et al., Br J heamatol 2003; 121:368-374; McElreavey et al., Biochem Soc Trans 1991; 19:29S; Wang et al., Stem Cells 2004; 22:1330-1337),

- A combination of Fibroblast Growth Factor (FGF), Butylated hydroxyanisole, DMSO and optionally composite salvia injection for neurocytes or a combination of insulin, cAMP and Nerve Growth Factor (NGF) for neurocytes, forming neuronal cells, which can be identified based on expression of NSE, III beta tubulin, Neurofilament M, tyrosine hydroxylase and/or GAP43 (cf. e.g. Lu et al., Haematologica 2006; 91:1017-1026; Weiss et al., Stem Cells 2006;

24:781-792; Fu et al., Stem Cells 2006; 24:115-124; Ma et al., Clin Med J 2005; 118:1987-1993; Mitschell et al., Stem Cells 2003; 21:50-60; Tontreau et al., Stem Cells 2005; 23:1105-1112),

- Nicotinamide and beta-mercaptoethanol for pancreatic islet beta-cells, identified by insulin production (cf. e.g. Li-Bo Chen et al., WJG 2004; 10(20):3016-3020).

The invention thus provides a method for inducing differentiation of mesenchymal stem cells (MSCs) into specific cell types comprising:
a) adding to a culture of MSCs a certain amount of ER-alpha-17p peptide defined by SEQ ID NO: 1,
b) adding to said culture an amount of a cell-type specific differentiation factor, as defined above,
c) allowing the cells to differentiate *in vitro*, and
d) optionally sorting and harvesting said cells according to the cell-type specific markers expressed or activities performed.

Said ER-alpha-17p peptide can be coated on the surface of the culturing support or on the surface or even inside the structure of an implant or scaffold.

The MSCs can be or can have been recovered from a biological sample of a human subject, selected from bone marrow, adipose tissue, or umbilical cord blood.

The cells are generally cultured for a period of between about 7 and about 18 days and can be collecting afterwards to obtain a population of differentiated cells for use in e.g. therapy.

The invention further provides a mesenchymal stem cell differentiation kit comprising:
a) an amount of the ER-alpha-p17 peptide, and
b) an amount of cell-type specific differentiation factor as defined herein.
In a preferred embodiment, the kit according to the invention could comprise:
a) culturing supports such as a culture dish, a slide, or a culturing flask, coated with the ER-alpha-p17 peptide of the invention, and
b) amount of cell-type specific differentiation factor as defined herein.

## EXAMPLES

The embodiments of the present invention are further illustrated by the following non-limiting examples.

### Example 1: Influence of the ER-alpha-17p peptide on the osteogenic differentiation of Mesenchymal Stem Cells.

The influence of the ER-alpha-17p peptide on the osteogenic differentiation of Mesenchymal Stem Cells was studied. The Mesenchymal Stem Cells were obtained from bone marrow and adipose tissue of healthy individuals. The cells are selected based on their adhesion preference to plastic and on their high proliferation capacity. These cells are expanded in complete medium, whereto fetal calf serum was added and they were identified using flow cytometry. MSCs were labeled with antibodies against CD31, CD34, CD45, HLA-DR, CD29, CD166, CD105 and CD73, and analysed by flow cytometry (Figure 3). Histograms show negative control IgG staining ($10^0$-$10^1$) versus specific antibody staining ($10^1$-$10^2$). Representative examples of MSC phenotype.

The complete differentiation into osteoblasts up to the phase of mineralisation of the extracellular matrix is generally induced by culturing the MSCs in the presence of Dexamethasone, Ascorbic Acid and Beta-Glycerophosphate (DAG) for 14 to 21 days.

Our results indicate that the ER-alpha-17p peptide stimulates the differentiation of MSCs into osteoblasts. Indeed, after 7 days ER-alpha-17p induces a change in the MSCs morphology as well as the formation of dense extracellular matrix (Figure 4A). Moreover, the calcification of the matrix as such is accelerated by the treatment of with the ER-alpha-17p peptide in the presence of DAG (Figure 4B). Although a very faint calcification is visible in the control samples or in the presence of estradiol, the colorisation with Alizarin Red enabled us to observe a clear and profound massive calcification after 14 days when the cells were treated with the ER-alpha-17p peptide. After 21 days, we have equally observed a more profound calcification in the samples treated with the ER-alpha-17p peptide.

ER-alpha-17p exhibiting pseudo-estrogenic activities, the potential effect of estradiol ($E_2$ was assessed (Figure 5) to ensure that the ER-alpha-17p effect is not relevant to a trivial estrogenic-like activity. Thus, MSCs were cultured in the presence of DAG in the presence

or absence of $E_2$ or ER-alpha-17p during 14 days for the visualization of the alkaline phosphatase (ALP) activity or 21 days for calcification (Figure 5A). Cytochemical demonstration of ALP activity was performed using FAST Violet B as a substrate and Alizarin Red-S staining was used for the demonstration of mineralized bone matrix. Results indicate that ER-alpha-17p increases both ALP activity and calcification (*vs* DAG alone; control) whereas $E_2$ only slightly increases ALP activity and has no effect on calcification indicating that the mode of action of ER-alpha-17p is not relevant to an estrogenic effect. ALP activity was also quantified in MSC cultured in control CT or osteogenic medium (DAG) after 7, 14 and 21 days of treatment with $10^{-5}$M ER-alpha-17p, ALP activity was expressed as enzyme units (U, µmol p-nitrophenol released per min) normalized relative to the total protein content (mg) (Figure 5B). Results show that ER-alpha-17p significantly increases the effect of DAG especially at 14 days. Subsequently, qualitative analysis of matrix calcification in MSCs culture was performed (Figure 5C). The calcium content of the extracellular matrix, of MSCs cultured in DMEM (CT) or osteogenic medium (DAG) was determined by colorimetric assay after 21 days of incubation with $10^{-9}$ of $E_2$ or $10^{-5}$M ER$\alpha$17p. The data indicates that, while $E_2$ has no significant effect, ER-alpha-17p increases the effect of DAG. Hence, the chronical treatment of MSCs with the ER-alpha-17p peptide accelerates and increases both the alkaline phosphatase activity and calcification of the bone matrix.

Cytochemical demonstration of bone related proteins was also performed using primary antibodies raised against osteocalcin (OC) and osteopontin (OPN) (Figure 6A). MSCs were cultured in DMEM or osteogenic medium (DAG) and exposed or not to $10^{-5}$M of ER-alpha-17p for 10 days. Results show that ER-alpha-17p increases expression of these markers especially for OC the expression of which is enhanced even in the absence of DAG. In addition, after 4 days of culture, Real-time quantitative PCR were performed (Figure 6B) using SYBR green dye (SYBR green PCR master mix; Applied Biosystems) and sequence-specific primers for OPN and OC. The amplification was performed in an ABI prism 7900 sequence detector system (Applied Biosystems) using 40 cycles of a two-step PCR (15 s at 95°C and 60 s at 60°C) after an initial activation step (95°C for 10 min). Melting curves from 60°C to 99°C were assessed to evaluate specificity. Serial dilutions of purified amplicons served to generate standard melting curves. Amplification of beta-actin mRNA as an endogenous control was used to standardize the amount of sample added to the reactions. Results show that ER-alpha-17p significantly potentialize the effect of DAG on the expression of both markers.

In a next step, MSCs were seeded at high density (500.000 cell/well) in Matrigel in 8-well Lab-Tek chambers in order to produce an artificial bone structure. After matrix solidification, chambers were disassembled and formed cubes were incubated in 6-well plate containing DMEM with or without DAG and / or ER-alpha-17p at $10^{-5}$M for 14 days. 3D structures were then harvested, fixed in 4% paraformaldehyde, frizzed in OCT and 10µM slices where stained (hematoxylin / eosin) (Figure 7). As depicted here, the combination of ER-alpha-17p with DAG produce an optimal differentiation of MSCs into osteocytes, leading to the formation of long and rectilinear bone fibres organized in a 3 dimensional network.

In conclusion, the provision of DAG in combination with ER-alpha-p17 to MSCs induces an optimal osteoblast differentiation; a property that can be exploited to rapidly generate quality bone implant.

**Example 2: Influence of the ER-alpha-17p peptide on the differentiation of Mesenchymal Stem Cells into Chondrocytes.**

In analogy to the MSC differentiation into osteoblasts, the inventors show that the ER-alpha-17p peptide, as an activator or promoter of stem cell differentiation, can be used to improve differentiation of stem cells towards other, non-osteoblast cell types, such as myocytes/cardiocytes, adipocytes, neurons, etc. using other factors or stimuli than DAG. For instance we provide, here, evidence that this peptide favors the differentiation of MSCs into chondrocyte cell type (Figure 8).

MSCs from bone marrow (BM) or adipose tissue (AT) were rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets were incubated in induction medium (combination of insulin, ascorbic acid, and TGF-β) with or without ER-alpha-17p $10^{-5}$ M for 14 days (Figure 8). Results show ER-alpha-17p induces a increase of the size of chondrocyte beads as well as the development of a large extracellular matrix.

In conclusion, the provision of TGF-β in combination with ER-alpha-p17 to MSCs increases differentiation into chondrocytes.

**Example 3: Influence of the ER-alpha-17p peptide on the differentiation of Mesenchymal Stem Cells into cardiomyocytes.**

In analogy to the MSC differentiation into osteoblasts and chondrocytes, the ER-alpha-17p peptide can be used as an activator or promoter of stem cell differentiation towards myocytes/cardiocytes using MyoD or 5-azacytidine.

MSCs from bone marrow (BM) or adipose tissue (AT) are rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets are incubated in induction medium (comprising MyoD or 5-azacytidine) with or without ER-alpha-17p $10^{-5}$ M for 14 days.

The formation of myocyte cells can be evaluated by means of detecting e.g. troponin I and N-cadherin expression.

**Example 4: Influence of the ER-alpha-17p peptide on the differentiation of Mesenchymal Stem Cells into adipocytes.**

In analogy to the MSC differentiation into osteoblasts and chondrocytes, the ER-alpha-17p peptide can be used as an activator or promoter of stem cell differentiation towards adipocytes using a combination of Insulin, Dexamethasone (DXM) and Indomethacin and optionally isobutylmethylxanthine.

MSCs from bone marrow (BM) or adipose tissue (AT) are rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets are incubated in induction medium (comprising a combination of Insulin, Dexamethasone (DXM) and Indomethacin and optionally isobutylmethylxanthine) with or without ER-alpha-17p $10^{-5}$ M for 14 days.

Adipose cells can be identified based on peroxisome proliferation activation receptor gamma-2, and/or lipoprotein lipase activity.

**Example 5: Influence of the ER-alpha-17p peptide on the differentiation of Mesenchymal Stem Cells into neurocytes.**

In analogy to the MSC differentiation into osteoblasts and chondrocytes, the ER-alpha-17p peptide can be used as an activator or promoter of stem cell differentiation towards

neurocytes using a combination of Fibroblast Growth Factor (FGF), Butylated hydroxyanisole, DMSO and optionally composite salvia injection; or using a combination of insulin, cAMP and Nerve Growth Factor (NGF).

MSCs from bone marrow (BM) or adipose tissue (AT) are rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets are incubated in induction medium (comprising a combination of either Fibroblast Growth Factor (FGF), Butylated hydroxyanisole, DMSO and optionally composite salvia injection; or insulin, cAMP and Nerve Growth Factor (NGF)) with or without ER-alpha-17p $10^{-5}$ M for 14 days.

Neurocytes can be identified based on expression of NSE, III beta tubulin, Neurofilament M, tyrosine hydroxylase and/or GAP43.

**Example 6: Influence of the ER-alpha-17p peptide on the differentiation of Mesenchymal Stem Cells into pancreatic beta cells.**

In analogy to the MSC differentiation into osteoblasts and chondrocytes, the ER-alpha-17p peptide can be used as an activator or promoter of stem cell differentiation towards pancreatic beta cells using a combination of Nicotinamide and beta-mercaptoethanol.

MSCs from bone marrow (BM) or adipose tissue (AT) are rinsed in PBS and then centrifugated in a 15 ml tube in order to form cell pellets. Pellets are incubated in induction medium (comprising a combination of Nicotinamide and beta-mercaptoethanol) with or without ER-alpha-17p $10^{-5}$ M for 14 days.

Pancreatic beta cells can be identified based on insulin secretion.

**CLAIMS**

1. The use of the estrogen receptor-alpha (ER-alpha) 17p peptide (ER-alpha-17p) defined by SEQ ID NO:1, optionally carrying one or more of the following post-translational modifications: methylation, acetylation and/or phosphorylation for inducing differentiation of mesenchymal stem cells.

2. An *in vitro* method for inducing differentiation of mesenchymal stem cells (MSCs) into specific cell types comprising:
a) adding to a culture of MSCs a certain amount of ER-alpha-17p peptide defined by SEQ ID NO: 1,
b) adding to said culture a differentiation factor, selected from the group consisting of: a mixture of Dexamethasone, Ascorbic acid and beta-Glycerophosphate (DAG) for osteoblasts, TGF-beta for chondrocytes, MyoD or 5-azacytidine for myocytes, the combination of Insulin, DXM and Indomethacin for adipocytes, the combination of Fibroblast Growth Factor (FGF), Butylated hydroxyanisole and DMSO or a combination of insulin, cAMP and Nerve Growth Factor (NGF) for neurocytes, the combination of Nicotinamide and beta-mercaptoethanol for pancreatic islet beta-cells.

3. The method according to claim 2, wherein said ER-alpha-17p peptide is coated on the surface of the culturing support or added to a gel or a paste to make a three dimensional cell structure.

4. The method according to claims 2 or 3, wherein said MSCs are or have been recovered from a biological sample of a human subject, selected from bone marrow, adipose tissue, or umbilical cord.

5. The method according to any one of claims 2 to 4, wherein the cells are cultured for a period of between about 7 and about 18 days.

6. The method according to any of claims 2 to 5 further comprising collecting the cells or cell population obtained by the method.

7. The method according to any one of claims 2 to 6, wherein the specific cell type is osteoblasts.

8. The method according to any one of claims 2 to 6, wherein the specific cell type is chondrocytes.

9. The method according to any one of claims 2 to 6, wherein the specific cell type is myocardiocytes.

10. The method according to any one of claims 2 to 6, wherein the specific cell type is adipocytes.

11. The method according to any one of claims 2 to 6, wherein the specific cell type is neurocytes.

12. The method according to any one of claims 2 to 6, wherein the specific cell type is pancreatic beta cells.

13. A pharmaceutical composition comprising the cells or cell populations according to any one of claims 6 to 12, and suitable for administration to a patient.

14. A mesenchymal stem cell differentiation kit comprising:
a) an amount of the ER-alpha-17p peptide, and
b) an amount of differentiation-specific component, selected from the group consisting of:
a mixture of Dexamethasone, Ascorbic acid and beta-Glycerophosphate (DAG) for osteoblasts, TGF-beta for chondrocytes, MyoD or 5-azacytidine for myocytes, the combination of Insulin, DXM and Indomethacin for adipocytes, the combination of Fibroblast Growth Factor (FGF), Butylated hydroxyanisole and DMSO or a combination of insulin, cAMP and Nerve Growth Factor (NGF) for neurocytes, the combination of Nicotinamide and beta-mercaptoethanol for pancreatic islet beta-cells.

15. The mesenchymal stem cell differentiation kit according to claim 14, wherein the ER-alpha-17p peptide is coated onto the surface of culturing supports such as a culture dish, a slide, a culturing flask or a gel or a paste to make a three dimentional cell structure.

Figure 1

$P_{295}$LMIKRSKKNSLALSL$T_{311}$

Figure 2

```
  1 mtmtlhtkas gmallhqiqg neleplnrpq lkiplerplg evyldsskpa vynypegaay
 61 efnaaaaana qvygqtglpy gpgseaaafg snglggfppl nsvspsplml lhpppqlspf
121 lqphgqqvpy ylenepsgyt vreagppafy rpnsdnrrqg grerlastnd kgsmamesak
181 etrycavcnd yasgyhygvw scegckaffk rsiqghndym cpatnqctid knrrkscqac
241 rlrkcyevgm mkggirkdrr ggrmlkhkrq rddgegrgev gsagdmraan lwpsplmikr
                                                          p295
301 skknslalsl tadqmvsall daeppilyse ydptrpfsea smmglltnla drelvhminw
              t311
361 akrvpgfvdl tlhdqvhlle cawleilmig lvwrsmehpg kllfapnlll drnqgkcveg
421 mveifdmlla tssrfrmmnl qgeefvclks iillnsgvyt flsstlksle ekdhihrvld
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Figure 3

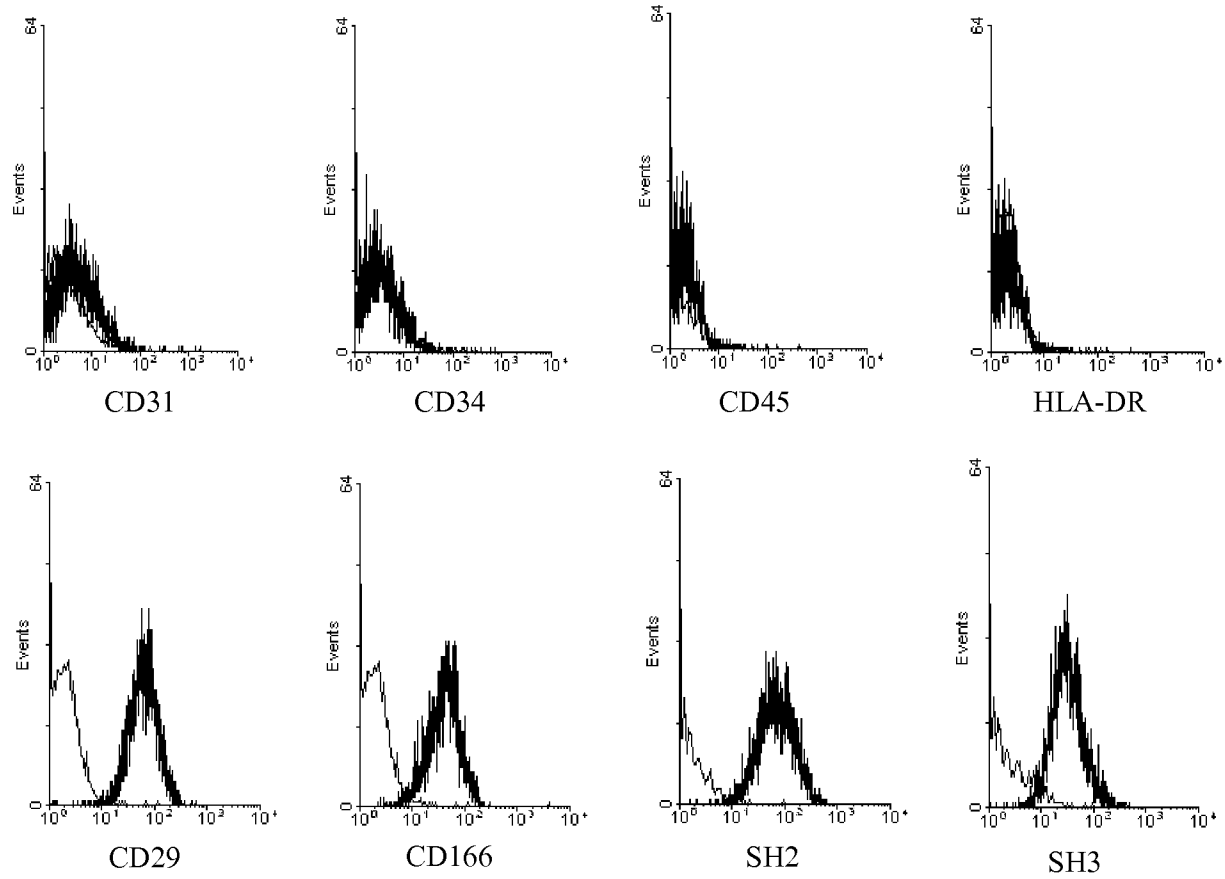
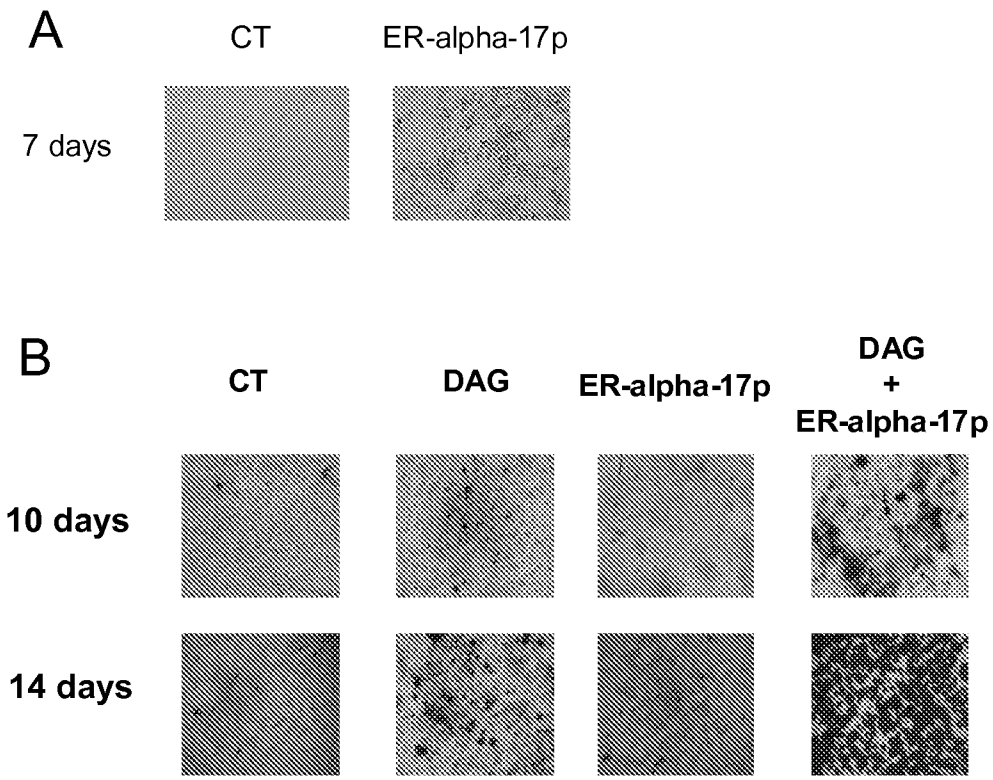


Figure 4


A
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7 days
B
CT
DAG
ER-alpha-17p
DAG + ER-alpha-17p
10 days
14 days

Figure 5

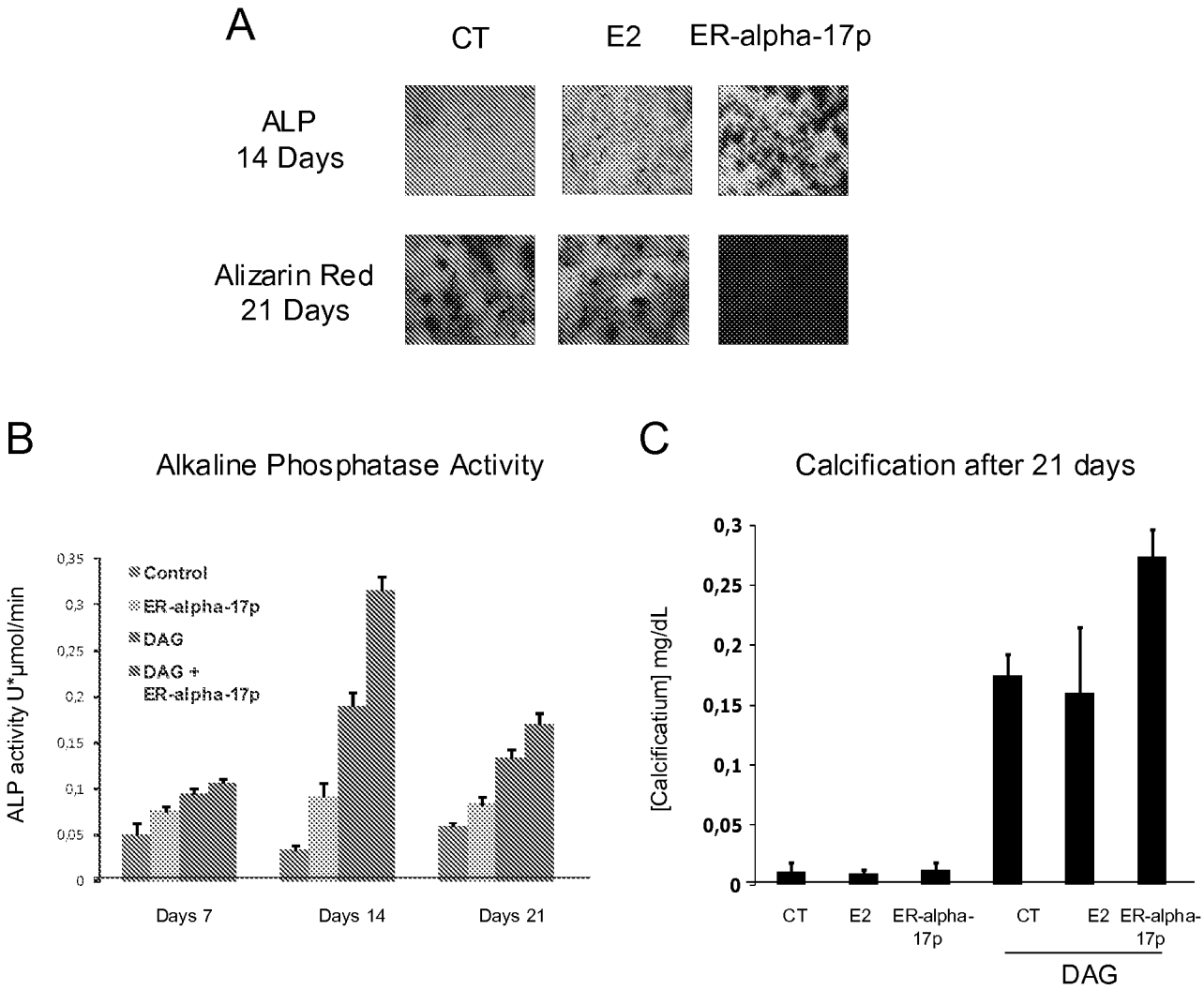
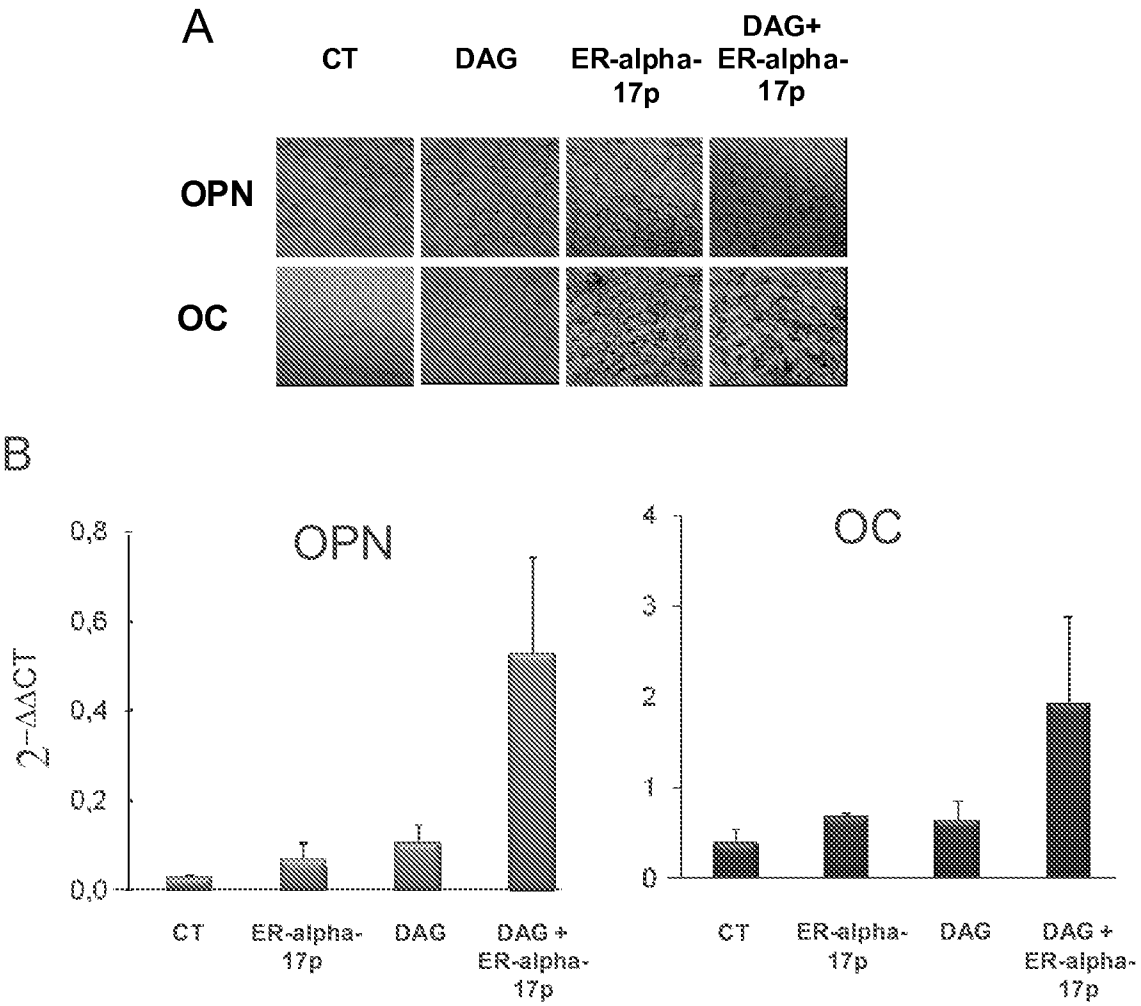

A
CT
E2
ER-alpha-17p
ALP
14 Days
Alizarin Red
21 Days
B
Alkaline Phosphatase Activity
ALP activity U*µmol/min
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Control
ER-alpha-17p
DAG
DAG +
ER-alpha-17p
Days 7
Days 14
Days 21
C
Calcification after 21 days
[Calcificatium] mg/dL
0,3
0,25
0,2
0,15
0,1
0,05
0
CT
E2
ER-alpha-17p
CT
E2
ER-alpha-17p
DAG

Figure 6


A
CT
DAG
ER-alpha-17p
DAG+ ER-alpha-17p
OPN
OC
B
OPN
OC
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0,6
0,4
0,2
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4
3
2
1
0
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ER-alpha-17p
DAG
DAG + ER-alpha-17p

Figure 7

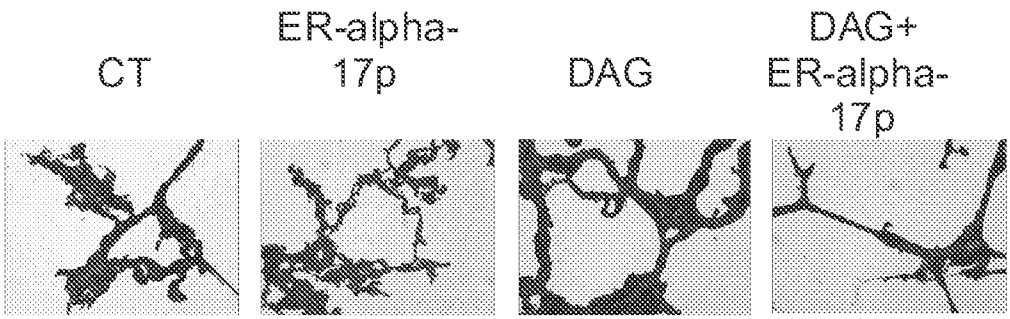
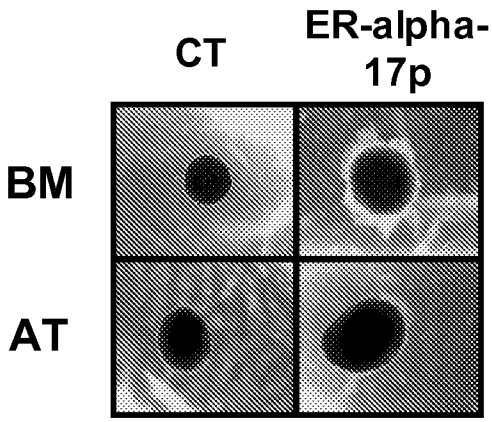


Figure 8



# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/065557

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/08
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)
C07K 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 practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, 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 | Dominique Gallo: "Role de la sequence P295-T311 dans l'activation du recepteur d'oestrogenes alpha", , 31 December 2009 (2009-12-31), XP002647445, Retrieved from the Internet: URL:http://theses.ulb.ac.be/ETD-db/collection/available/ULBetd-06252009-145141/unrestricted/These_Dominique_GALLO.pdf [retrieved on 2011-07-05] the whole document page vi - page vii<br>-----<br>-/-- | 1-15 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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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 |
|---|---|
| 5 July 2011 | 25/07/2011 |

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

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/065557

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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