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(54) Title: AGENTS HAVING TISSUE GENERATIVE ACTIVITY

(57) Abstract: The invention provides methods for inhibiting, effecting or stimulating the lineage-directed induction of stem cells, the methods comprising contacting the stem cells with a bioactive composition effective to inhibit or induce differentiation thereof into a lineage of choice, wherein the bioactive composition is any one of (a) a fraction of enamel matrix protein (EMP), wherein said fraction is either (i) Fraction C of EMP, wherein Fraction C is the fraction of EMP comprising components less than 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modification, (n) Fraction C-dep of EMP, wherein Fraction C-dep is the fraction of EMP comprising components over 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modifications, (b) an isolated peptide from said fraction, (c) an active portion or fragment of said peptide of (b), (d) a variant of (b) or (c) showing 70% or greater identity therewith, (e) a synthetic analog or derivative of any of the peptides (b)-(d), and wherein the lineage is selected from the group consisting of osteogenic, chondrogenic, angiogenic, and neurogenic. Also provided are particular peptides and fragments having one or more of the relevant activities, and relates methods and compositions.



Agents having tissue generative activity

Technical field

The present invention relates generally to methods and materials having utility in tissue generation or regeneration.

Background art

A number of different types of material have been used to help regenerate new periodontal tissue, including a complex mixture of enamel matrix proteins (EMP; also known as enamel matrix derivative; EMD) obtained from developing teeth.

EMP promote regeneration of the periodontal ligament (PDL) which supports the tooth and gives rise to the new soft connective tissue, cementum and alveolar bone of the periodontium. However, as well as promoting PDL and cementum regeneration in animals and humans (Donos et al, 2003; Sculean et al, 1999), conflicting results on the re-growth of new bone *in vivo* have been reported using the commercially available form of EMP (Emdogain[®]; EMD) (Institute Straumann AG, Basel) (Grestelius et al, 1997; Donos et al, 2005; Rathe et al, 2008). Moreover, *in vitro* studies have also shown that EMP sometimes inhibits bone-associated genes such as alkaline phosphatase (ALP) and osteocalcin (OC) as well as terminal differentiation of bone-forming cells, including PDL (Wada et al, 2008; Hama et al, 2008).

In contrast, EMP appears to promote the formation of mature chondrocytes required for new cartilage (Narukawa et al, 2007) and also the angiogenic differentiation of primary human endothelial cells required for the formation of new blood vessels (Schlueter et al, 2007).

Enamel matrix proteins or derivatives have previously been described in the patent literature to be able to induce hard tissue formation (i.e. enamel formation, U.S. Pat. No. 4,672,032 (Slavkin)), binding between hard tissues (EP-B-0 337 967 and EP-B-0 263 086) and wound healing, such as of skin and mucosa (WO 99/43344).

Definitive studies on the effects of EMP may have been confused by the heterogeneity of this material. One recent study has shown that EMP can be purified into component

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fractions by size-exclusion and reverse-phase HPLC (Mumulidu A et al, 2007). Although some of the constituents of these fractions have been characterised, little is known about the biological functions of these fractions or their constituents, although it has been reported that recombinant amelogenin (a peptide found in EMP) is internalized into target cells (Shapiro et al, 2007).

Building or re-building new healthy tissue is of major medical importance and it can be seen that the characterisation of agents having this activity would be a contribution to the art.

Disclosure of the invention

There is an increasing literature on the role of stem cells in tissue regeneration processes. One recent report is that the adult human PDL also contains stem cells (Singhatanadgit et al, 2009).

However, while EMP have been implicated in the regeneration of certain tissues, the precise effects on progenitor/stem cell functions which are crucial for re-building healthy new tissues have been unclear. Nor was it known whether individual components were responsible for differential effects, and if so what these components and effects were.

The present inventors have now shown that EMP-derived Fraction C, and certain of the individual natural components present in and synthetic components derived therefrom (eg C1 and C2), have been found to have a major impact on a number of essential cell differentiation pathways. These include (A) osteogenesis, (B) chondrogenesis, (C) vasculogenesis and angiogenesis and (D) neurogenesis, leading to the inhibition of new bone but stimulation of cartilage, blood vessels and neuronal tissues. It is thus believed that these materials have differential effects on the progenitor and stem cell populations in PDL and other tissues and can thus be used to control the differentiation of such cells into these different lineages (e.g. osteogenic, chondrogenic, angiogenic, neurogenic respectively.)

Additionally EMP lacking Fraction C ("C-Dep") has been shown to have advantages over EMD e.g. in growing new bone such as alveolar bone and rebuilding PDL. Specifically, since Fraction C has an inhibitory effect on bone formation, its absence from C-Dep is an advantage when used for this purpose.

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Additionally the present inventors have shown that some specific components of EMP and Fraction C are able to bind to the PDL stem cells and thereafter become internalized in specific cellular compartments. Without wishing to be bound by theory, it is believed that the biological effects of the materials on osteogenesis, angiogenesis, chondrogenesis, and neurogenesis (A – D above) may be mediated via these processes. Use of these materials to identify the specific cell receptors responsible, and use of agents to modulate those receptors (e.g. to regulate the formation of new bone, cartilage, blood vessel and nerve cells) form further aspects of the invention.

It will be thus understood that these materials may have significant clinical value in the treatment of diseased and damaged tissues.

Various aspects of the invention will now be discussed in more detail.

Lineage-directed induction of stem cells

In one aspect, the invention provides a method for effecting the lineage-directed induction of stem cells which comprises contacting the stem cells with a bioactive composition effective to induce differentiation thereof into a lineage of choice, wherein the bioactive composition is a fraction of EMP, or a synthetic equivalent or variant thereof, and wherein the lineage is selected from the group consisting of osteogenic, chondrogenic, vasculogenic/angiogenic, and neurogenic.

As used herein the term “stem cells” is intended to encompass also “progenitor cells” and for brevity the term “stem cell” is used in place of “progenitor/stem cell”.

The method may be performed *in vitro*, *ex vivo*, or *in vivo*. Optionally, where the process is *ex vivo*, the cells are contacted with the bioactive composition in a rigid porous vessel which may (by way of non-limiting Example) be a ceramic cube, titanium reactor or implant, or any culture vessel known in the art.

Optionally the cells are contacted with the bioactive composition in an injectable liquid which may be used, for example, during or after surgery e.g. orthopaedic or dental surgery.

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Preferably the bioactive composition comprises one or more factors or fractions derived from or otherwise based on Fractions C and C-Dep, or a synthetic equivalent or variant thereof,

Stem cell therapies

In another aspect, the method of the invention further provides administering to an individual in need thereof stem cells and the bioactive composition effective to induce differentiation of such cells into a lineage of choice. This may in one embodiment comprise administering the bioactive composition to an individual to whom a preparation comprising isolated human stem cells had been administered.

In another aspect, the invention provides a method for inducing the *in vivo* production of bone, blood vessels and neuronal tissues in a patient which comprises administering to the individual isolated stem cells and a bioactive composition effective to induce such cells to differentiate into the respective lineage descendant in such individual.

In this and other aspects, preferably, the stem cells and bioactive composition are administered together or they may alternatively be administered separately.

The stem cells or bioactive composition for use in the above methods, or for use in the preparation of a medicament for the above methods, for further aspects of the invention.

Other therapies

A major contribution of the present invention is a method comprising administering to an individual in need thereof the bioactive composition (e.g. a fraction of EMP, or a synthetic equivalent or variant thereof) to enhance the generation or regeneration of damaged or diseased tissue.

Thus in one embodiment the invention provides a method of treating a mammal in need of tissue generation, said method comprising administering to said mammal a bioactive composition as described herein. Such methods may be used for example for regeneration of a range of diseased and damaged tissues.

As noted above, Fraction C, containing two TRAP isoforms, a 43- and 45-amino acid tyrosine-rich peptide derived from the N-terminal of amelogenin, has been found to inhibit bone gene expression and new bone formation but to strongly up-regulate cartilage, blood vessel and nerve-associated genes and the differentiation of the corresponding chondrocytic, vascular and nerve-like cells *in vitro*.

Thus a fraction of EMP (e.g. Fraction C, or a synthetic equivalent or variant thereof) may preferably be used to promote new cells of the nervous system (e.g. neurons and glial cells). Such may be used in selectively building neurological tissue for the treatment of stroke, Alzheimer's and neuromuscular disorders, and Parkinson's disease.

As shown in the results below, Fraction C (and C1 and C2) was effective at forming endothelial-like tubes. Thus Fraction C (or a fraction thereof, or a synthetic equivalent or variant thereof) may be used to enhance the formation of endothelial-like blood vessel cells. As shown in the results below, Fraction C (and C1 and C2) formed more endothelial-like cells than did EMP. This has utility in cardiac disease. The same bioactive composition has also been found to regulate the function of epithelial cells, and therefore also be clinically useful in treating skin-related and other epithelial disorders.

In view of the inhibitory effect of Fraction C on bone formation (see Examples) it is a particular aspect of the invention to use preparations of EMP lacking the Fraction C bioactive agent (termed "C-Dep") to increase osteogenesis. This may be applied *in vitro* and for the purposes of enhancing the repair and re-building of new bone *in vivo*.

As explained herein, C-Dep, contains a number of proteins including amelogenin and LRAP, a 56-amino acid leucine-rich peptide derived biologically from amelogenin. C-Dep greatly enhances new bone formation but strongly inhibits chondrogenesis, vasculogenesis/angiogenesis and neurogenesis *in vitro*.

Thus in one embodiment the bioactive composition will comprise C-Dep, or a fraction or factor of C-Dep, or a synthetic equivalent or variant thereof as described herein. Such provides a new treatment for, by way of example only, bone damage and disease and can improve the clinical outcome of orthopaedic and dental surgery.

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In other embodiments, C-Dep (or a fraction of C-Dep, or a synthetic equivalent or variant thereof as described herein) may be used to inhibit chondrogenesis, and/or angiogenesis and/or neurogenesis and/or vasculogenesis.

In preferred embodiments C-Dep (or a fraction of C-Dep, or a synthetic equivalent or variant thereof as described herein) may be used in the regeneration of periodontal (PDL) tissue. As shown herein, such preparations can be superior to EMD e.g. in growing new bone such as alveolar bone and rebuilding PDL.

In the methods described, the composition may (by way of non-limiting example) be administered by continuous injection or bolus injection. Optionally it comprises a pharmaceutically acceptable excipient.

The bioactive composition for use in the above methods, or for use in the preparation of a medicament for the above methods, for further aspects of the invention.

Other aspects

As noted above Fraction C has an inhibitory effect on bone formation. Thus use of Fraction C, or a fraction thereof, or a synthetic equivalent or variant thereof, for this purpose, where that is desired, forms another aspect of the invention.

Another aspect of the invention provides a composition comprising isolated, culture-expanded human stem cells and a bioactive composition as described effective to induce differentiation of such cells into a lineage of choice as described above.

Preferably the composition further comprises a tissue culture medium. Alternatively, the composition can comprise a medium suitable for administration to an animal particularly a human, in need thereof. This aspect of the invention also provides for specific embodiments using the bioactive compositions identified above for lineage induction into the lineages associated therewith as described above.

Another aspect comprises use of EMP, or a synthetic equivalent or variant thereof (more preferably Fraction C, or a fraction of Fraction C, or a synthetic equivalent or variant thereof as described herein, such as C1 or C2) to identify or label PDL stem cell receptors. As shown herein EMP and Fraction C are internalized in specific cellular

compartments in PDL stem cells. Once identified the receptor may be modelled in 3 dimensions to produce EMP fraction mimetics. Alternatively it may be used directly e.g. as a binding partner (optionally in phage display) to screen for compounds. The use of the receptor, and in particular agonists or antagonists thereof, for effecting osteogenesis, chondrogenesis, vasculogenesis/angiogenesis and neurogenesis (e.g. to regulate the formation of new bone, blood vessel and nerve cells) forms a further aspect of the invention.

Preferred embodiments

Stem cells

In the methods of the invention any human stem cells may be employed – for example which are available commercially or via publication to those skilled in the art. In the examples below human PDL stem cells were used (Singhatanadgit et al. 2009). However other stem cells, and stem cell lines, may also be utilised e.g. autologous bone marrow derived mesenchymal stem cells (bm-MSCs) which are often used for the treatment of skeletal defects. Likewise human alveolar bone cells, which form part of the periodontium structure.

Bioactive compositions

As used herein the bioactive compositions are fractions of EMP, or a synthetic equivalent or variant thereof. Sources of EMPs are discussed hereinafter, as are fractionation techniques.

Of particular relevance to the present invention are so called "Fraction C" (or derived or analogous agents) and "C-Dep" (or derived or analogous agents)

Fraction C

"Fraction C" which comprises components less than 6 kDa consisting mainly of a group of hydrophobic peptides derived from the amelogenin gene by alternative splicing and post-translational modifications. These include the 5.1 and 5.3 kDa tyrosine-rich (TRAP) proteins (Fincham et al, 1994, which is specifically incorporated herein by reference), which have previously been isolated and the amino acid sequences fully delineated

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(Fincham et al, 1994). These two components, C1 and C2, have been used in some of the experiments described here, as noted.

In addition, it has been shown by MALDI-TOF analysis, that other proteins are also present in this Fraction C, primarily 2.5 and 4.9 kDa oligopeptides of as yet unknown sequence.

Thus in embodiment utilising Fraction C, the bioactive composition will typically include only proteins of less than 6 kDa present in EMPs, or synthetic equivalents or variants thereof as described herein. Such embodiments may use one or both of C1 and C2 (TRAP proteins as defined) which likewise may be obtained from EMPs, or be synthetic.

As noted below, the present invention specifically embraces peptide variants which are active portions or fragments of the EMP-derived bioactive agent, for example TRAP, or variants of such a portion e.g. showing 75% or greater homology with it. Furthermore, it embraces synthetic analogs of such peptides.

In one embodiment, the agent is a fragment selected from the 43- and 45-amino acid TRAP proteins, derived from the N-terminal of amelogenin:

NH₂ – MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHP YTSYGYEPMG – COOH
(43-amino acids/C1)

NH₂ – MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHP YTSYGYEPMGGW – COOH
(45 amino acids/C2)

In a preferred embodiment the agent comprises or consists of or consists essentially of at least 6, 7, 8, 9, or 10 amino acids of the 10-amino acid C terminal of the 43-amino acid TRAP protein:

NH₂ - YTSYGYEPMG – COOH (which may be termed "TCT1" herein)

In another preferred embodiment the agent comprises or consists of or consists essentially of at least 6, 7, 8, 9, 10, 11, or 12 amino acids of the 12-amino acid C terminal of the 45-amino acid TRAP protein:

NH₂ - YTSYGYEPMGGW – COOH (which may be termed "TCT2" herein)

In a preferred embodiment the agents consist of, or consist essentially of, the TCT1 and/or TCT2 peptides.

In other embodiments the agent is a synthetic analog of such a peptide. The examples herein show that both naturally-occurring and chemically-synthesized TRAP have the same biological activities as Fraction C. For example the TCT1 and TCT2 peptides suppress bone formation and also exhibit the chondrogenic, vasculogenic, angiogenic and neurogenic stimulatory activities of the 'parent' TRAP proteins,

C-dep

"C-Dep" is a preparation of EMP depleted of Fraction C (as above) and comprising components over 6 kDa, mainly 6.9 and 8.1 kDa leucine-rich amelogenin peptides (LRAP), sheathlin proteins (11, 13, 15 and 17 kDa) and full-length amelogenin (>17 kDa protein) (Swanson et al, 2006; Kanazashi et al, 2006; Fincham et al, 1994). These individual proteins have previously been isolated and their amino acid sequences reported (Kanazashi et al, 2006; Maycock et al, 2002; Hu et al, 1997). MALDI-TOF experiments in our laboratory have confirmed the presence of these proteins in C-Dep.

The effect of the 17kDa sheathlin protein on cementogenesis has been reported in experimental models (Swanson et al, 2006) and LRAP has previously been reported to have bone-stimulating activity. However the potential therapeutic value of C-Dep and the individual components therein had not previously been studied.

Compositions consisting of Fraction C, and C-Dep (or synthetic equivalents or variants thereof), and their use in the methods described herein, form further aspects of the invention.

As noted below, the present invention specifically embraces peptide variants which are active portions or fragments of the EMP-derived bioactive agent, for example LRAP, or variants of such a portion e.g. showing 75% or greater homology with it. Furthermore, it embraces synthetic analogs of such peptides.

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In one embodiment, the agent is a fragment of the 56 amino acid LRAP protein derived from the N-terminal of amelogenin:

NH₂ -

MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHPSLLPDLPLEAWPATDKTKREEVD -
COOH

In a preferred embodiment the agent comprises or consists of or consists essentially of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 amino acids of the 23 amino acid C terminal of the 56 amino acid LRAP protein:

NH₂ - SLLPDLPLEAWPATDKTKREEVD - COOH (which may be termed "LCT" herein)

In a preferred embodiment the agent consists of, or consists essentially of, the LCT peptide.

In other embodiments the agent is a synthetic analog of such a peptide. The examples herein show that both naturally-occurring and chemically-synthesized LRAP have the same biological activities as C-dep.

General sources of EMP compositions and variants

Enamel matrix proteins are proteins that normally are present in enamel matrix, i.e. the precursor for enamel (Ten Cate: Oral Histology, 1994; Robinson: Eur. J. Oral Science, January 1998, 106 Suppl. 1:282-91), or proteins which can be obtained by cleavage of such proteins. In general, such proteins have a molecular weight below 120,000 Dalton and include amelogenins, non-amelogenins, proline-rich non-amelogenins and tuftelins. Examples of proteins for use according to the invention are amelogenins, proline-rich non-amelogenins, tuftelins, tuft proteins, serum proteins, salivary proteins, ameloblastin, sheathlin, and derivatives thereof, and mixtures thereof.

In the present context "enamel matrix proteins" (EMP) includes enamel matrix derivatives, or enamel matrix protein derivatives. These are derivatives of enamel matrix which include one or several enamel matrix proteins or parts of such proteins, produced naturally by alternate splicing or processing, or by either enzymatic or chemical cleavage of a natural length protein, or by synthesis of polypeptides in vitro or in vivo (recombinant

DNA methods or cultivation of diploid cells, either plant or animal cells). Enamel matrix protein derivatives also include enamel matrix related polypeptides or proteins. The polypeptides or proteins may be bound to a suitable biodegradable carrier molecule, such as polyamine acids or polysaccharides, or combinations thereof. Furthermore, the term enamel matrix derivatives also encompasses synthetic analogous substances.

EMDOGAIN® (BIORA AB, S-205 12 Malmö, Sweden) contains 30 mg enamel matrix protein, heated for 3 hours at about 80°C in order to inactivate residual proteases, and 1 ml Vehicle Solution (Propylene Glycol Alginate), which are mixed prior to application, unless the protein and the Vehicle are tested separately. The weight ratio is about 80/8/12 between the main protein peaks at 20, 14 and 5 kDa, respectively.

Enamel matrix is a precursor to enamel and may be obtained from any relevant natural source, i.e. a mammal in which teeth are under development. A suitable source is developing teeth from slaughtered animals such as, e.g., calves, pigs or lambs. Another source is e.g. fish skin. Enamel matrix can be prepared from developing teeth as described previously (EP-B-0 337 967 and EP-B-0 263 086). The enamel matrix is scraped off and enamel matrix derivatives (EMD) are prepared, e.g. by extraction with aqueous solution such as a buffer, a dilute acid or base or a water/solvent mixture. Methods of purifying peptides from heterogenous mixtures are well known in the art (e.g. selective precipitation, proteolysis, ultrafiltration with known molecular weight cut-off filters, ion-exchange chromatography, gel filtration, etc.) Typical protocols are set out "Protein Purification" – principles and practice" Pub. Springer-Verlag, New York Inc (1982), and by Harris & Angal (1989) "Protein purification methods – a practical approach" Pub. O.U.P. UK, or references therein. Further methods which are known to be suitable for protein purification are disclosed in "Methods in Enzymology Vol 182 – Guide to Protein Purification" Ed. M P Deutscher, Pub. Academic Press Inc.

As an alternative source of the enamel matrix derivatives or proteins one may also use generally applicable synthetic routes, well known to a person skilled in the art, or use cultivated eukaryotic and/or prokaryotic cells, alternatively modified by DNA-techniques. In order to obtain expression of the bioactive agent peptide-encoding nucleic acid sequences, the sequences can be incorporated into a vector having control sequences operably linked to the bioactive agent nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the bioactive agent peptide is

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produced as a fusion and/or nucleic acid encoding secretion signals so that the peptide produced in the host cell is secreted from the cell. bioactive agent peptide can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the peptide is produced and recovering the peptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of *E. coli*, yeast, and eukaryotic cells such as COS or CHO cells.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, "Molecular Cloning: a Laboratory Manual": 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press.

Cells and techniques may be selected such as to permit or enhance the folding and/or formation of disulphide bridges (see e.g. "Protein Folding" by R. Hermann, Pub. 1993, European Patent Office, The Hague, Netherlands, ISBN 90-9006173-8).

Chemical synthesis may also be employed. Peptides may be synthesized by any suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution couplings. In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence.

The use of variants of any of the recited proteins, provided those variants share the requisite activity, is expressly embraced by the present invention.

Variants can be produced by a mixture of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as

the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptides conformation. Also included are variants having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure. In regions which are critical in determining the peptides conformation or activity such changes may confer advantageous properties on the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e.g. altered stability or specificity.

The present invention also includes variants which are active portions or fragments of the EMP-derived bioactive agents employed in the invention.

An "active portion" of bioactive agent peptide means a peptide which is less than said full length bioactive agent peptide, but which retains at least some (say, 50%, 60%, 70%, 80%, 90% or more) of its activity as assayed above – e.g. in respect of stem cell differentiation.

Examples of active portions of the LRAP and TRAP proteins are described herein.

Included within the definition of a "variant" protein of the invention are amino acid variants of the naturally occurring proteins and peptides as described above or in the References referred to herein (including 5.1 and 5.3 kDa tyrosine-rich proteins; 6.9 and 8.1 kDa leucine-rich amelogenin peptides; sheathlin proteins (11, 13, 15 and 17 kDa); full-length amelogenin (>17 kDa protein) etc.) and which share the relevant activity of those proteins and peptides as assayed above – e.g. in respect of stem cell differentiation. Preferably, variant sequences are at least 75% homologous to the wild-type sequence, more preferably at least 80% homologous, even more preferably at least 85% homologous, yet more preferably at least 90% homologous or most preferably at least 95% homologous to at least a portion of the reference protein. In some embodiments the homology will be as high as 94, 95, 96, 97, 98, or 99%. Homology in this context means sequence similarity or identity, with identity being preferred. To determine whether a candidate peptide region has the requisite percentage similarity or identity to a reference polypeptide or peptide oligomer, the candidate amino acid sequence and the reference amino acid sequence are first aligned using a standard computer programme such as are commercially available and widely used by those skilled in the art. In a preferred

embodiment the NCBI BLAST method is used (<http://www.ncbi.nlm.nih.gov/BLAST/>). Once the two sequences have been aligned, a percent similarity score may be calculated.

Alternatively the proteins may share at least about 50%, 60%, 70%, 80%, 90% or more sequence identity with an authentic sequence. In this connection, "sequence identity" means strict amino acid identity between the sequences being compared.

Derivatives of peptides

Proteins and peptide agents according to the present invention may be subject to degradation by a number of means (such as protease activity at a target site). Such degradation may limit their bioavailability and hence therapeutic utility. There are a number of well-established techniques by which peptide derivatives that have enhanced stability in biological contexts can be designed and produced. Such peptide derivatives may have improved bioavailability as a result of increased resistance to protease-mediated degradation. Preferably, a derivative suitable for use according to the invention is more protease-resistant than the protein or peptide from which it is derived. Protease-resistance of a peptide derivative and the protein or peptide from which it is derived may be evaluated by means of well-known protein degradation assays. The relative values of protease resistance for the peptide derivative and peptide may then be compared.

Peptoid derivatives of proteins and peptides according to the invention may be readily designed from knowledge of the primary sequences given herein. Commercially available software may be used to develop peptoid derivatives according to well-established protocols.

Retropeptoids, (in which all amino acids are replaced by peptoid residues in reversed order) are also able to mimic proteins or peptides according to the invention. A retropeptoid is expected to bind in the opposite direction in the ligand-binding groove of a receptor or other binding partner, as compared to a peptide or peptoid-peptide hybrid containing one peptoid residue. As a result, the side chains of the peptoid residues are able to point in the same direction as the side chains in the original peptide.

A further embodiment of a modified form of peptides or proteins according to the invention comprises D-amino acid forms. In this case, the order of the amino acid residues is reversed. The preparation of peptides using D-amino acids rather than L-

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amino acids greatly decreases any unwanted breakdown of such derivative by normal metabolic processes, decreasing the amounts of the derivative which needs to be administered, along with the frequency of its administration.

Derivatives of peptide agents used according to the invention include derivatives that increase the half-life of the agent *in vivo*. Examples of derivatives capable of increasing the half-life of polypeptides according to the invention include peptoid derivatives, D-amino acid derivatives and peptide-peptoid hybrids.

Thus the term "amino acid," as herein, comprises not only the residues of the natural amino acids (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Hyl, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in D or L form but also unnatural amino acids (e.g., phosphoserine, phosphothreonine, phosphotyrosine, hydroxyproline, gamma-carboxyglutamate; hippuric acid, octahydroindole-2-carboxylic acid, statine, 1,2,3,4,-tetrahydroisoquinoline-3-carboxylic acid, penicillamine, ornithine, citruline, α -methyl-alanine, para-benzoylphenylalanine, phenylglycine, propargylglycine, sarcosine, and tert-butylglycine). The term also includes natural and unnatural amino acids bearing a conventional amino protecting group (e.g., acetyl or benzyloxycarbonyl), as well as natural and unnatural amino acids protected at the carboxy terminus (e.g. as a (C₁-C₆)alkyl, phenyl or benzyl ester or amide). Other suitable amino and carboxy protecting groups are known to those skilled in the art (see for example, Greene, *Protecting Groups In Organic Synthesis*; Wiley: New York, 1981, and references cited therein). An amino acid can be linked to another molecule through the carboxy terminus, the amino terminus, or through any other convenient point of attachment, such as, for example, through the sulfur of cysteine.

The term "peptide" when used herein, describes a sequence of usually 5 to 25 amino acids (e.g. as defined hereinabove) or peptidyl residues. The sequence may be linear or cyclic. For example, a cyclic peptide can be prepared or may result from the formation of disulfide bridges between two cysteine residues in a sequence. A peptide can be linked to another molecule through the carboxy terminus, the amino terminus, or through any other convenient point of attachment, such as, for example, through the sulfur of a cysteine. Preferably a peptide comprises 10 to 25 amino acids. Peptide derivatives can be prepared as disclosed in U.S. Patent Numbers 4,612,302; 4,853,371; and 4,684,620.

Mimetics of peptides

Also embraced by the present invention are uses of agents which are functional mimetics of the peptides described herein, and which retain the essential biological activity of the peptides.

Examples of such mimetics include chemical compounds which are modeled to resemble the three dimensional structure of the peptides described herein.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptide are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR.

The three dimensional structure may be determined. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modeling process.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound.

Alternatively, where the mimetic is peptide based, further stability can be achieved by cyclising the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Therapeutic compositions

The bioactive agents and nucleic acids encoding them can be formulated into pharmaceutical compositions. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Furthermore, a composition may be adapted to administration in connection with surgery, e.g. as a systemic administration by infusion into the blood, lymph, ascites, or spinal fluids, or by inhalation.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution (PBS is preferred), dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

For delayed release, the modulators may be included in a pharmaceutical composition for formulated for slow release, such as in microcapsules formed from biocompatible polymers or in liposomal carrier systems according to methods known in the art.

For continuous release of proteins or peptides, the protein or peptide may be covalently conjugated to a water soluble polymer, such as a polylactide or biodegradable hydrogel derived from an amphipathic block copolymer, as described in U.S. Pat. No. 5,320,840. Collagen-based matrix implants, such as described in U.S. Pat. No. 5,024,841, are also useful for sustained delivery of peptide therapeutics. Also useful, particularly for subdermal slow-release delivery to perineural regions, is a composition that includes a biodegradable polymer that is self-curing and that forms an implant in situ, after delivery in liquid form. Such a composition is described, for example in U.S. Pat. No. 5,278,202.

Compositions may be formulated according to conventional pharmaceutical practice, see, e.g., "Remington: The science and practice of pharmacy" 20th ed. Mack Publishing, Easton Pa., 2000 ISBN 0-912734-04-3 and "Encyclopaedia of Pharmaceutical Technology", edited by Swarbrick, J. & J. C. Boylan, Marcel Dekker, Inc., New York, 1988 ISBN 0-8247-2800-9.

Thus in a further aspect, the present invention provides a pharmaceutical composition comprising a bioactive agent peptide-encoding nucleic acid molecule and its use in methods of therapy or diagnosis.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more bioactive agents as defined above and its use in methods of therapy or diagnosis.

In further aspects, the present invention provides the above bioactive agents and nucleic acid molecules for use in the use of bioactive agent peptides in the preparation of medicaments for therapy.

Compositions, particularly those based on peptides, may include anti-oxidant effective to prevent methionine oxidation.

Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

The disclosure of all references cited herein, inasmuch as it may be used by those skilled in the art to carry out the invention, is hereby specifically incorporated herein by cross-reference.

Figures

Figure 1a) shows the effects of Fraction C on terminal osteogenic differentiation *in vitro*. The numbers are the alizarin red staining intensity. The number in () is the % inhibition by Fraction C compared with EMP.

Figure 1b) shows the effects of Fraction C and C-Dep on chondrogenic gene expression and terminal differentiation *in vitro*. The Figure is a representative RT-PCR gel showing chondrogenic gene expression of PDL cells cultured in CM with EMD/Fractions for 2 weeks.

Figure 1c) shows the dose effects of EMD and EMD Fractions on PDL chondrogenesis using Alcian blue staining at 2 weeks. Terminal chondrogenic differentiation was examined by Alcian blue staining of paraffin sections of PDL cell pellets. Red arrows show proteoglycans stained by Alcian blue; Nuclei stained purple with hematoxylin.

Figure 2 shows *in vitro* endothelial-like tube formation assay in the presence of EMP, and Fraction C. The numbers are the angiogenic scores. Number in () is the % stimulation by Fraction C, compared with control cultures in growth medium (GM) and endothelial growth medium (EGM-2) alone.

Figure 3 shows the effects of Fraction C on blood vessel development *in vitro*.

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Figure 4 shows the effects of EMP and Fraction C on terminal neural differentiation. The numbers are the % of neuron-specific β III tubulin positive cells. The number in () is the % stimulation by Fraction C, compared with EMP.

Figure 5 shows the effects of Fraction C on nerve cell differentiation *in vitro*.

Figure 6 shows the effects of C-Dep on terminal osteogenic differentiation of bone-forming cells *in vitro*.

Figure 7 shows that EMP and Fraction C bind to and are internalized into PDL stem cells.

Figure 8 shows that Fraction C binds to the cells (green-fluorescence) at 4°C for 4 h and is then internalized at 37°C (red-fluorescence). Black arrows show the binding and white arrows show internalization of Fraction C into cellular vesicles. Cell nuclei are stained blue using Hoechst dye.

Figure 9 shows fractionation of EMD by low pressure SEC (BioGel P-30 Fine, 100 cm×5.0 cm) (from Mumulidu 2007).

Figure 10 shows RP HPLC analysis of the Figure 9 "5 kDa component" using different buffer concentrations in the mobile phase (YMC-Pack ODS-A, 250mm×4.6 mm). Top: 100 mM, middle: 50 mM, and bottom: 10 mM (from Mumulidu 2007).

Examples

Experimental Protocols

Isolation of primary human PDL cells

Human PDL cells were used as they have previously been shown to be highly heterogeneous, possibly including stem cells, and to have the ability to give rise to several different types of tissue (Singhatanadgit et al, Tissue Eng Part A. 2009 Sep;15(9):2625-36; this is specifically incorporated herein by reference). They were obtained from periodontally healthy patients undergoing routine extractions at the Eastman Dental Hospital, as previously described (Singhatanadgit et al, 2009). Briefly, tissue was separated from the surface of the middle portion of the root and digested with

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3 mg/ml of collagenase type I and 4 mg/ml of dispase for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer and cultured in α -Modified Eagle's Medium (α -MEM), containing 10% fetal calf serum (FCS) supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO₂ in air. Three separate PDL cell populations from three different healthy donors (all male, aged between 18 to 25) were used between passages 3 and 6.

Preparation of EMP and EMP Fractions

EMP (in the form of the commercially available product Emdogain®; Institut Straumann) was prepared at a concentration of 10 mg/ml in 0.1% acetic acid. Fraction C and C-Dep, obtained following fractionation of EMP as described previously (Mumulidu A et al, *Journal of Chromatography B*, 857 (2007) 210–218 this is specifically incorporated herein by reference) were prepared at 1 and 10 mg/ml in 0.1% acetic acid, respectively.

Briefly, in Mumulidu 2007, High performance liquid chromatography (HPLC) methods were used to analyse a 5 kDa component purified from EMD, After initial purification by size-exclusion chromatography (SEC) on a 100 cm×5 cm column (Bio-Gel P-30 Fine, 280 nm), collected fractions were analysed by size-exclusion HPLC (SE HPLC; TSK-Gel Super SW2000, 220 nm). The fractions containing only the 5 kDa component were analysed by reversed-phase high-pressure chromatography (RP HPLC; YMC-Pack ODS-A, 200 nm), revealing four peaks of the 5 kDa component.

The SEC used a 5 cm×100 cm column including BioGel P-30 Fine, with 125mM formic acid, measurement at 280 nm, and ambient temperature. The following fractions and molecular weights were obtained:

<u>Fraction</u>	<u>MW by SDS of most abundant band</u>
P1	12–80 (20)
P2	12–30 (20)
P3	9–20 (20)
P4	4–9
P5	5

This fractionation of EMD by low pressure SEC (BioGel P-30 Fine, 100 cm×5.0 cm) is shown in Figure 9 (from Mumulidu 2007).

Further analysis was performed using SEHPLC and RPHPLC as follows (from Mumulidu 2007):

Table 1
Summary of parameters for the quality control steps based on SE HPLC and RP HPLC

Parameter	SE HPLC	RP HPLC
Column	TSK-Gel, SuperSW2000, 300 mm × 4.6 mm	YMC-Pack ODS-A, S-5 μm, 30 nm, particle AP-303, 250 mm × 4.6 mm
Elution type	Isocratic	Gradient; $t = 0$ min, 100% A; $t = 1$ min, 100% A; $t = 30$ min, 100% B; $t = 32$ min, 100% B; $t = 35$ min, 100% A; $t = 45$ min 100% A
Mobile phase	30% ACN in 0.9% NaCl	A = 65% KHFP/35% ACN, B = 35% KHFP/65% ACN
Injection volume	5 μl	20 μl
Flow rate	0.3 ml/min	1.5 ml/min
Run time	15 min	45 min
Autosampler temperature	Ambient	4 °C
Column oven temperature	30 °C	40 °C
Wavelength	220 nm	200 nm

Figure 10 shows RP HPLC analysis of the 5 kDa component using different buffer concentrations in the mobile phase (YMC-Pack ODS-A, 250mm×4.6 mm). Top: 100 mM, middle: 50 mM, and bottom: 10 mM (from Mumulidu 2007).

As used in the invention herein herein "Fraction C" includes only proteins up to 6 kDa (based on Maldi TOF analysis). This preparation thus includes the "TRAP" proteins or peptides referred to in Mumulidu 2007, and other proteins or peptides in this size range, but will exclude LRAPs between 6 and 9 kDa for example.

Two purified fractions, each containing only one of these two TRAP proteins, are referred to herein as Fractions C1 and C2. Results have also been obtained with synthetic TRAP proteins with equivalent or improved effect (results not shown).

"C Dep" as used herein is prepared from EMD by subtraction of "Fraction C". It includes proteins between 6 and 20 kDa, *inter alia*, the LRAPs between 6 and 9 kDa (usually 6.9 and 8.1) referred to by Mumulida.

Treatment of cells with EMP and EMP Fractions

EMP and EMP Fractions were added directly to the cells when they reached approximately 90% confluence. EMP and C-Dep were used at a final concentration of 100 μg/ml, and Fraction C was used at 10 μg/ml for osteogenesis and chondrogenesis-

related experiments and 30 µg/ml for the vasculogenesis/angiogenesis- and neurogenesis-related experiments, unless otherwise noted. These concentrations were optimised from the dose effects of EMP, C-Dep and Fraction C on terminal PDL osteogenesis, chondrogenesis, vasculogenesis/angiogenesis and neurogenesis *in vitro*.

Quantitative polymerase chain reaction (Q-PCR) analysis of lineage associated genes

Total RNA was isolated from PDL cells using the RNeasy Mini Kit, after culture in GM and in several differentiation media each specific for a different cell lineage, as described below. For reverse transcription first strand cDNA was synthesized using 1 µg of total RNA, as previously described (Singhatanadgit et al, 2009), with primers obtained from Applied Biosystems. Q-PCR analysis was carried out using the ABI Prism® 7300 sequence detector, the Taqman® Gene Expression Assay consisting of the unlabelled specific PCR primers and Taqman® MGB probes with FAM™ dye labelling in a 96-well plate format. Thermal cycler conditions were used as recommended by the manufacturer and the data were collected and analyzed by the SDS 1.2 software. All PCR reactions were performed in triplicate and each of the gene cycle threshold (ct) values were normalized to the GAPDH ct value detected simultaneously on the same plate.

Differentiation of PDL cells

(A) Osteogenic differentiation

PDL cells were plated into 24-well plates at a density of 2.5×10^4 cells/well and cultured in GM for 2-3 days, then osteogenic medium (OM) added, consisting of GM supplemented with 0.1 mM L-ascorbic acid 2-phosphate, 10 mM β- glycerophosphate and 10 nM dexamethasone. EMP, C-Dep and Fraction C were added to half of the cultures and the OM and EMD changed every 3-4 days. On several days between 1-14, and total RNA was extracted and Q-PCR carried out to measure osteopontin (OP), osteocalcin (OC) and bone sialoprotein (BSP) gene expression. At the same time points, the ALP activity of these cells was measured using previously described method (Singhatanadgit et al, 2006). Briefly, triplicate wells for each condition were rinsed twice with PBS and the cells were incubated with 200 µl of 5 mM *p*-nitrophenyl phosphate in 50 mM glycine, 1 mM MgCl₂ and 150 mM 2-amino-2-methyl-1-propanol buffer, pH 10.5 at 37°C for 1 h. A 50 µl aliquot of 3 M NaOH was added to stop the enzymatic reaction and the absorbance was measured at 405 nm (A_{405}).

The formation of mineralized nodules, a measure of terminal osteogenic differentiation, was determined by alizarin red S staining of calcium-containing deposits, as follows. Cells were cultured in OM with and without EMP, C-Dep and Fraction C for 21 days, fixed with 10% formaldehyde for 15 min and washed with distilled water. The samples were incubated with 2% Alizarin Red S (pH 4.2) for 15 min at room temperature, then washed, air-dried and photographed. The level of alizarin red staining was quantified by absorbance at 562 nm (A_{562}), after eluting the stain for 2 h with 10% cetylpyridinium chloride in 10 mM sodium phosphate buffer, pH 7.0.

(B) Chondrogenic differentiation

Cells were trypsinized and 2.5×10^5 cells suspended in 0.5 ml of serum-free chondrogenic medium (CM) containing low glucose-DMEM, transforming growth factor- β 3 (TGF- β 3), dexamethasone, L-ascorbate2-phosphate, sodium pyruvate, L-proline and insulin/transferrin/selenium and placed in 15 ml conical polypropylene centrifuge tubes (Nunc). After centrifugation at 200g for 5 min, the pellets were incubated at 37°C for 2 weeks in the presence of EMD, with caps loosened to allow gas exchange, then total RNA extracted for RT-PCR analysis of the late markers aggrecan, Col2a1 and COMP (after 2 weeks), as previously described.

Alcian blue staining for acid mucopolysaccharides and glycosaminoglycans was also carried out, after 3 weeks, to assess terminal chondrogenic differentiation as follows. Cell pellets were fixed in 10% formalin at 4°C for 24 h, dehydrated in an ascending series of ethanol and embedded in paraffin. Sections (3 μ m) were cut, stained with 1% alcian blue (pH 2.5) (Sigma) for 5 min. The deposition of mucopolysaccharides and glycosaminoglycans was visualized as blue staining of the extracellular matrix (ECM). Nuclei were stained purple using Harris Hematoxylin.

(C) Vasculogenic/Angiogenic differentiation

PDL cells were cultured in GM as in a. above and endothelial cell growth medium-2 (EGM-2) then added. The EGM-2, EMP and Fraction C were changed every 3-4 days. Total RNA was extracted to measure the early angiogenic marker gene Ang-1 (at week 1) and the late marker gene vWF (at week 2), as described previously (Gang et al, 2006). Angiogenic differentiation of PDL cells was performed using an *in vitro* angiogenesis assay kit. Briefly, 10^4 cells were plated on ECMatrix gel coated 96-well plates and cultured in the presence of EGM-2 with and without EMP and Fraction C. After 6 h, digital images were obtained using bright-field microscopy and angiogenic tube formation

scored from 0 to 5, as previously described (Cochran et al, 2007), based on the progressive appearance of morphological features associated with angiogenesis: individual cells, (0); aligned cells, (1); capillary tubes without sprouting, (2); sprouting of new capillaries, (3); the formation of closed polygonal structures, (4); and complex-mesh-like structures, (5). Each condition for each culture was scored using 5 separate fields.

(D) Neural differentiation

2×10^4 Cells/well were cultured in poly-L-lysine/laminin coated 8-well chamber slides in GM for 2 days and neural differentiation medium (NM) then added, consisting of 20 ng/ml EGF, 20 ng/ml bFGF, 10 ng/ml heregulin β -2, 4 μ M forskolin and 10 μ M retinoic acid, with and without EMP and Fraction C. After 2 weeks, total RNA was extracted and the late markers MAP-2 and GFAP measured as previously described (Coura et al, 2008). Immunostaining was also carried out as previously described (Singhatanadgit et al, 2008). Briefly, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT) and permeabilised using 0.1% Triton X for 15 min at RT. They were then treated with a blocking solution containing 10% normal donkey serum (NDS) in PBS for 1 h and incubated for 1 h at RT with primary mouse monoclonal anti- β III tubulin antibody diluted 1:1000 in PBS containing 1% NDS. Incubation was then carried out with donkey anti-mouse Alexa Fluor secondary antibody diluted 1:200 in PBS containing 2% NDS for 1 h at RT. The neuron-like cells were visualized as green fluorescent stained cells with long axonal projections. Nuclei were stained blue using Hoechst dye. The proportion of neuron-specific β III tubulin-positive cells was determined by manual counting of 5 separate fields of each culture.

EMP and Fraction C binding and internalization

10^4 Cells/well PDL cells were cultured in 24-well plates on sterilized glass coverslips in GM for 2 days. The medium was replaced with cold GM containing 30 μ g/ml of biotin-labeled EMP and Fraction C and the cells were incubated at 4°C on ice for 4 h. The cells were washed with PBS and fixed in 4% PFA for 15 min for subsequent analysis of EMP binding to the cells. To examine internalization, after the initial binding at 4°C as above, the cells were washed 3 x, incubated with warm GM at 37°C for 5 h, fixed and permeabilised using 0.1% Triton-X for 10 min. The samples were then reacted with FITC-streptavidin for 20 min and nuclei stained blue using Hoechst dye.

Example 1- Fraction C regulates cell differentiation *in vitro***(A) Fraction C inhibits osteogenesis by bone-forming PDL cells****1. Effects of Fraction C on osteogenesis-associated markers**

<u>Osteogenesis-associated markers</u>	<u>Relative Gene Expression</u>		
	<u>EMP</u>	<u>Fraction C</u>	<u>% Inhibition by Fraction C</u>
OP gene expression (day 7)	3.8	1.0	73
OC gene expression (day 10)	2.3	1.2	48
BSP gene expression (day 14)	5.4	3.6	34
ALP activity (day 14)	1.5	1.1	25

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OP, OC and BSP gene expression, and the ALP activity of bone-forming cells, were significantly down-regulated when cultured in the presence of Fraction C, compared to EMP.

2. Effects of Fraction C on terminal osteogenic differentiation *in vitro*

The results are shown in Figure 1.

These results show that terminal osteogenic differentiation of the PDL cells was severely inhibited when cultured in OM in the presence of Fraction C, compared with EMP.

Very similar results to those above were obtained using C1 and C2 (data not shown here).

(B) Fraction C stimulates chondrogenesis *in vitro***1. Effects of Fraction C on chondrogenesis-associated genes**

As shown in Figure 1b, 10 µg/ml Fraction C appeared to strongly stimulate chondrogenic genes compared with CM alone and CM + EMD or C-Dep .

2. Effects of Fraction C on terminal differentiation

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As shown in Figure 1c, 10 µg/ml Fraction C appeared to strongly stimulate chondrogenesis of PDL cells compared with EMD and C-Dep

(C) Fraction C stimulates angiogenic differentiation in vitro

1. Effects of EMD and Fraction C on endothelial-associated marker genes

<u>Endothelial-associated marker genes</u>	<u>Relative Gene Expression</u>		
	<u>EMP</u>	<u>Fraction C</u>	<u>% Increase by Fraction C</u>
Ang-1 gene expression (day 7)	2.5	5.6	217
vWF gene expression (day 14)	4.0	7.5	186

This Table shows that Ang-1 and vWF gene expression of PDL cells were significantly up-regulated when cultured in EGM-2 in the presence of EMP and, most notably, Fraction C.

2. *In vitro* endothelial-like tube formation assay in the presence of EMP, and Fraction C

Figure 2 shows that EMP and, most notably, Fraction C stimulated PDL cells to form complex tube-like structures when cultured in EGM-2 for 5 h, compared to control cultures GM and EGM-2 alone.

Very similar results as above were obtained using C1 and C2 (data not shown here).

3. Effects of Fraction C on blood vessel development *in vitro*

Figure 3 shows the formation of elongated blood vessel-like structures after culture in the presence of Fraction C, compared to EGM-2 alone, which contained less elongated and smaller structures.

(D) Fraction C stimulates neurogenic differentiation in vitro

1. Effects of EMP and Fraction C on neural-associated marker genes

<u>Relative Gene Expression</u>			
<u>Neural-associated marker genes</u>	<u>EMP</u>	<u>Fraction C</u>	<u>% Stimulation by Fraction C</u>
MAP-2 gene expression (day 7)	0.06	3.8	6333
GFAP gene expression (day 7)	0.33	5.3	1606

These data show that Fraction C up-regulates the neural-associated genes MAP-2 and GFAP when PDL cells are cultured in NM, compared with control cultures in NM alone and with EMP.

2. Effects of EMP and Fraction C on terminal neural differentiation

Figure 4 shows that Fraction C stimulated PDL cells to form more green-fluorescent stained neuronal-like cells than in control cultures NM alone and EMP.

Very similar results as above were obtained using C1 and C2 (data not shown here).

3. Effects of Fraction C on nerve cell differentiation *in vitro*

Figure 5 shows the morphology of nerve-like cells which are induced in cultures incubated with Fraction C.

Example 2 - C-Dep enhances bone-forming cell differentiation and osteogenesis *in vitro*

1. Effects of C-Dep on osteogenesis-associated markers

<u>Osteogenesis-associated markers</u>	<u>Relative Gene Expression</u>		
	<u>EMP</u>	<u>C-Dep</u>	<u>% Stimulation by C-Dep</u>
OP gene expression (day 7)	3.8	5.1	135
OC gene expression (day 10)	2.3	3.1	132
BSP gene expression (day 14)	5.4	22.9	421
ALP activity (day 14)	1.5	1.9	13

This Table shows that OP, OC and BSP gene expression, and ALP activity of bone-forming cells, were significantly up-regulated when cultured in the presence of C-Dep, compared to EMP.

2. Effects of C-Dep on terminal osteogenic differentiation of bone-forming cells *in vitro*

The numbers are the alizarin red staining intensity. The number in () is the % stimulation by C-Dep compared with EMP.

Figure 6 shows that terminal osteogenic differentiation of bone-forming cells was significantly stimulated in the presence of C-Dep, compared with EMP.

Example 3 - EMP and Fraction C bind to and are internalized into PDL stem cells

The biological function of many exogenous mediators are mediated by their binding to specific cell surface receptors, and followed by a sequence of intracellular processes that can elicit marked changes in cell differentiation and activity.

Figure 7 shows that EMP (visualized by green fluorescent staining) binds to the PDL cell surface at 4°C, and is then transported to intra-cellular vesicles, possibly lysosomes, after 5 h of incubation at 37°C. Cell nuclei are stained blue using Hoechst dye.

Figure 8 shows that Fraction C binds to the cells (green-fluorescence) at 4°C for 4 h and is then internalized at 37°C (red-fluorescence). Black arrows show the binding and white

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arrows show internalization of Fraction C into cellular vesicles. Cell nuclei are stained blue using Hoechst dye.

This Example shows for the first time that at least some specific components of EMP and Fraction C are able to bind to the PDL stem cells and thereafter become internalized in specific cellular compartments. The biological effects of the materials on osteogenesis, angiogenesis and neurogenesis (A – C above) are highly likely to be mediated via these processes.

Thus the present findings provide for the characterisation (identification and isolation) of the specific receptors involved in EMP 'uptake' and determining the ultimate fate of these EMP proteins in the 'target' stem cells, especially in relation to their specific biological effects on cell differentiation and tissue regeneration.

Based on this information the EMP derived product(s) and their intracellular transport can be adapted, thereby improving their activity and potential clinical efficacy. Likewise the receptors may be modified, with consequent change in the activity of the EMP products, and improved efficacy of materials e.g. to regulate the formation of new bone, blood vessel and nerve cells.

Example 4 – Synthetic peptides derived from TRAP and LRAP proteins

The present inventors have compared the amino acid sequences of the amelogenin-derived TRAP and LRAP peptides and probed their active domains, as follows.

1. Despite having apparently completely opposite biological activities, TRAP and LRAP have the identical 33 N-terminal amino acid sequence as in the parent amelogenin protein. Without wishing to be bound by theory, it is proposed that this common overlapping N-terminal peptide (ONT) does not contribute specifically to the biological activities of the parent proteins examined herein.

This was supported by obtaining the ONT peptide by chemical synthesis. Preliminary results indicated that ONT did not appear to exert the biological effects (results not shown).

Amelogenin-derived peptide sequences	
TRAP (43 amino acids)	NH ₂ - <u>MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHP YTSYGYEPMG</u> - COOH
TRAP (45 amino acids)	NH ₂ - <u>MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHP YTSYGYEPMGGW</u> - COOH
TCT1 (10 amino acids)	NH ₂ - <u>YTSYGYEPMG</u> - COOH
TCT2 (12 amino acids)	NH ₂ - <u>YTSYGYEPMGGW</u> - COOH
LRAP (56 amino acids)	NH ₂ - <u>MPLPPHPGHPGYINFSYEVLTPWKYQNMIRHP SLLPDLPLEAWPATDKTKREEVD</u> - COOH
LCT (23 amino acids)	NH ₂ - <u>SLLPDLPLEAWPATDKTKREEVD</u> - COOH

2. The present inventors then obtained the synthetic 10- and 12-amino acid C-terminal (TCT1 and TCT2; PepC1 and PepC2) peptide that is unique to TRAP. Data indicated that this small peptide suppressed bone and also exhibited the chondrogenic, vasculogenic, angiogenic and neurogenic stimulatory activities of the 'parent' TRAP protein, possibly at an even lower dosage. These data thus indicate that the TCT1 and TCT2 peptides, which are chemically produced and does not itself occur as a natural product, is the critically important sequence of TRAP responsible for the range of tissue developmental activities we have studied.

3. The present inventors then obtained the synthetic 23-amino acid C-terminal (LCT; PepDep) peptide that is unique to LRAP. Results obtained indicated that this new LCT peptide is likely to be responsible for both bone-forming activity as well as for suppression of cartilage, blood vessel and nerve development.

Thus these 10-, 12- and 23-amino acid peptide sequences (TCT1, TCT2 and LCT, respectively) and related analogs or variants have potential as therapeutics for wound healing and regeneration of damaged and diseased tissues.

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Claims

1 A method for inhibiting, effecting or stimulating the lineage-directed induction of stem cells,

which method comprises contacting the stem cells with a bioactive composition effective to inhibit or induce differentiation thereof into a lineage of choice,

wherein the bioactive composition is any one of:

(a) a fraction of enamel matrix protein (EMP), wherein said fraction is either:

(i) Fraction C of EMP, wherein Fraction C is the fraction of EMP comprising components less than 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modification,

(ii) Fraction C-dep of EMP, wherein Fraction C-dep is the fraction of EMP comprising components over 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modifications,

(b) an isolated peptide from said fraction;

(c) an active portion or fragment of said peptide of (b);

(d) a variant of (b) or (c) showing 70% or greater identity therewith;

(e) a synthetic analog or derivative of any of the peptides (b)-(d),

and wherein the lineage is selected from the group consisting of osteogenic, chondrogenic, angiogenic, and neurogenic.

2 A method as claimed in claim 1 for wound healing or regeneration of damaged and diseased tissues.

3 A method as claimed in claim 1 or claim 2 wherein the bioactive composition consists of or consists essentially of:

(a) Fraction C of EMP, wherein Fraction C is the fraction of EMP comprising components less than 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modification;

(b) an isolated peptide from said fraction;

(c) an active portion or fragment of said peptide of (b);

(d) a variant of (b) or (c) showing 70% or greater identity therewith;

(e) a synthetic analog or derivative of any of the peptides (b)-(d),

4 A method as claimed in claim 3 wherein the bioactive composition consists of or consists essentially of:

(i) a peptide which has a sequence selected from:

MPLPPHPGHPGYINFSYEVLTPWKWYQNMIRHP YTSYGYEPMG;

MPLPPHPGHPGYINFSYEVLTPWKWYQNMIRHP YTSYGYEPMGGW;

(ii) an active portion of the peptide of (i) being equal to or up to 40, 30, 20 amino acids in length, and having 6, 7, 8, 9, 10, 11, or 12 contiguous amino acids of the C terminal of the peptide;

(iii) a variant of such a peptide of (i) or (ii) showing 70% or greater identity therewith,

(iv) a synthetic analog or derivative of any of the peptides (i)-(iii)

5 A method as claimed in claim 3 wherein the bioactive compositions consist of or consist essentially of:

(i) YTSYGYEPMG or YTSYGYEPMGGW

(ii) a variant of (i) showing 70% or greater identity therewith,

(iii) a synthetic analog or derivative of any of the peptides (i)-(ii)

6 A method as claimed in any one of claims 3 to 5 wherein the bioactive composition:

(i) inhibits bone gene expression and/or new bone formation and up-regulates cartilage-associated genes and the differentiation or promotion of chondrocytic cells and/or

(ii) up-regulates blood vessel and/or nerve-associated genes and the differentiation or promotion of the corresponding vascular and nerve-like cells.

7 A method as claimed in any one of claims 3 to 6 for promoting angiogenesis or neurogenesis, optionally for the treatment of stroke, Alzheimer's Disease, neuromuscular disorders, cardiac disease, or epithelial disorders.

8 A method as claimed in claim 1 or claim 2 wherein the bioactive composition consists of or consists essentially of:

- 37 -

- (a) Fraction C-dep of EMP, wherein Fraction C-dep is the fraction of EMP comprising components over 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modifications;
- (b) an isolated peptide from said fraction;
- (c) an active portion or fragment of said peptide of (b);
- (d) a variant of (b) or (c) showing 70% or greater identity therewith;
- (e) a synthetic analog or derivative of any of the peptides (b)-(d),
and wherein the lineage is selected from the group consisting of osteogenic, chondrogenic, angiogenic, vasculogenic and neurogenic.

9 A method as claimed in claim 8 wherein the bioactive composition consists of or consists essentially of:

- (i) a peptide which has the following sequence:

PLPPHPGHPGYINFSYEVLTPLKQYQNMIRHPSLLPDLPLEAWPATDKTKREEVD

- (ii) an active portion or fragment of the peptide of (i) having equal to or up to 50, 40, 30, 25 amino acids in length, and having 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 contiguous amino acids of the 23 amino acid C terminal of the peptide;
- (iii) a variant of such a peptide of (i) or (ii) showing 70% or greater identity therewith;
- (iv) a synthetic analog or derivative of any of the peptides (i)-(iii)

10 A method as claimed in claim 9 wherein the bioactive composition consists of or consists essentially of:

- (i) SLLPDLPLEAWPATDKTKREEVD,
- (ii) a variant of (i) showing 70% or greater identity therewith;
- (iii) a synthetic analog or derivative of any of the peptides (i)-(ii)

11 A method as claimed in any one of claims 8 to 10 wherein the bioactive composition:

- (i) up-regulates bone gene expression and/or new bone formation and/or
- (ii) inhibits chondrogenesis, vasculogenesis, angiogenesis and/or neurogenesis

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12 A method as claimed in any one of claims 3 to 6 for promoting of osteogenesis in periodontal or orthopedic treatments and/or for stimulating bone formation in or for a patient in need of the same.

13 A method as any one of the preceding claims wherein:

- (i) the method is *ex vivo*, and the cells are contacted with the bioactive composition in a rigid porous vessel which may optionally be a ceramic cube, titanium reactor or implant;
- (ii) the cells are contacted with the bioactive composition in injectable liquid form which may optionally be during or after surgery;

14 A bioactive composition as defined in the method of any one of claims 1 to 14 for use in a method for inhibiting, effecting or stimulating the lineage-directed induction of stem cells,

which method comprises contacting the stem cells with the bioactive composition effective to inhibit or induce differentiation thereof into a lineage of choice,

wherein the lineage is selected from the group consisting of osteogenic, angiogenic, and neurogenic.

15 A bioactive composition as claimed in claim 14 further comprising isolated, culture-expanded human stem cells.

16 A method for wound healing or regeneration of damaged and diseased tissues, which method comprises administering to an individual in need thereof the bioactive composition of claim 14 or claim 15.

17 A method as claimed in claim 16 for inducing the *in vivo* production of bone, cartilage, blood vessels and/or neuronal tissues in the individual.

18 An agent which is:

- (i) a peptide consisting of or consisting essentially of the sequence YTSYGYEPMG
- (ii) a variant of (i) showing 70% or greater identity therewith;
- (iii) a synthetic analog or derivative of any of the peptides (i)-(ii)

19 An agent which is:

- 39 -

- (i) a peptide consisting of or consisting essentially of the sequence YTSYGYEPMGGW
- (ii) a variant of (i) showing 70% or greater identity therewith;
- (iii) a synthetic analog or derivative of any of the peptides (i)-(ii)

20 An agent which is:

- (i) a peptide consisting of or consisting essentially of the sequence
SLLPDLPLEAWPATDKTKREEVD
- (ii) a variant of (i) showing 70% or greater identity therewith;
- (iii) a synthetic analog or derivative of any of the peptides (i)-(ii)

21 A pharmaceutical composition comprising an agent of any one of claims 18 to 20 for use in a method of therapy or diagnosis.

22 Use of any of:

- (a) Fraction C of EMP, wherein Fraction C is the fraction of EMP comprising components less than 6 kDa in size and being derived from the amelogenin gene by alternative splicing and post-translational modification;
 - (b) an isolated peptide from said fraction;
 - (c) an active portion or fragment of said peptide of (b);
 - (d) a variant of (b) or (c) showing 70% or greater identity therewith;
- to identify or label PDL stem cell EMP receptors by internalization therein.

Figure 1a

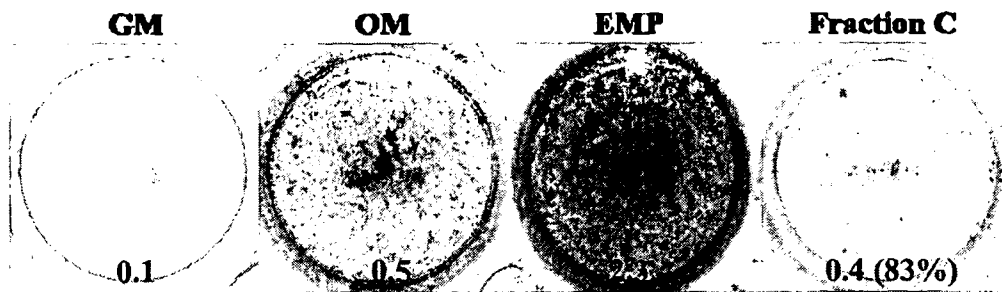


Figure 1b

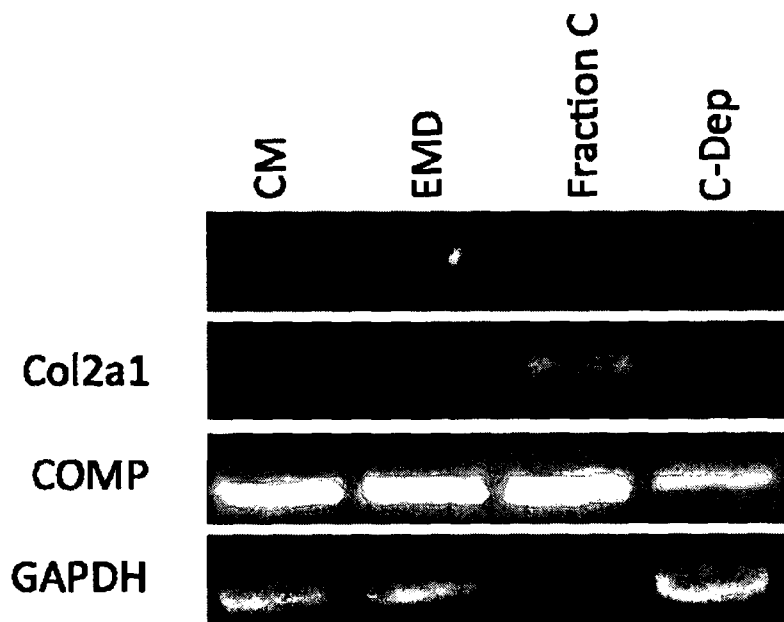


Figure 1c

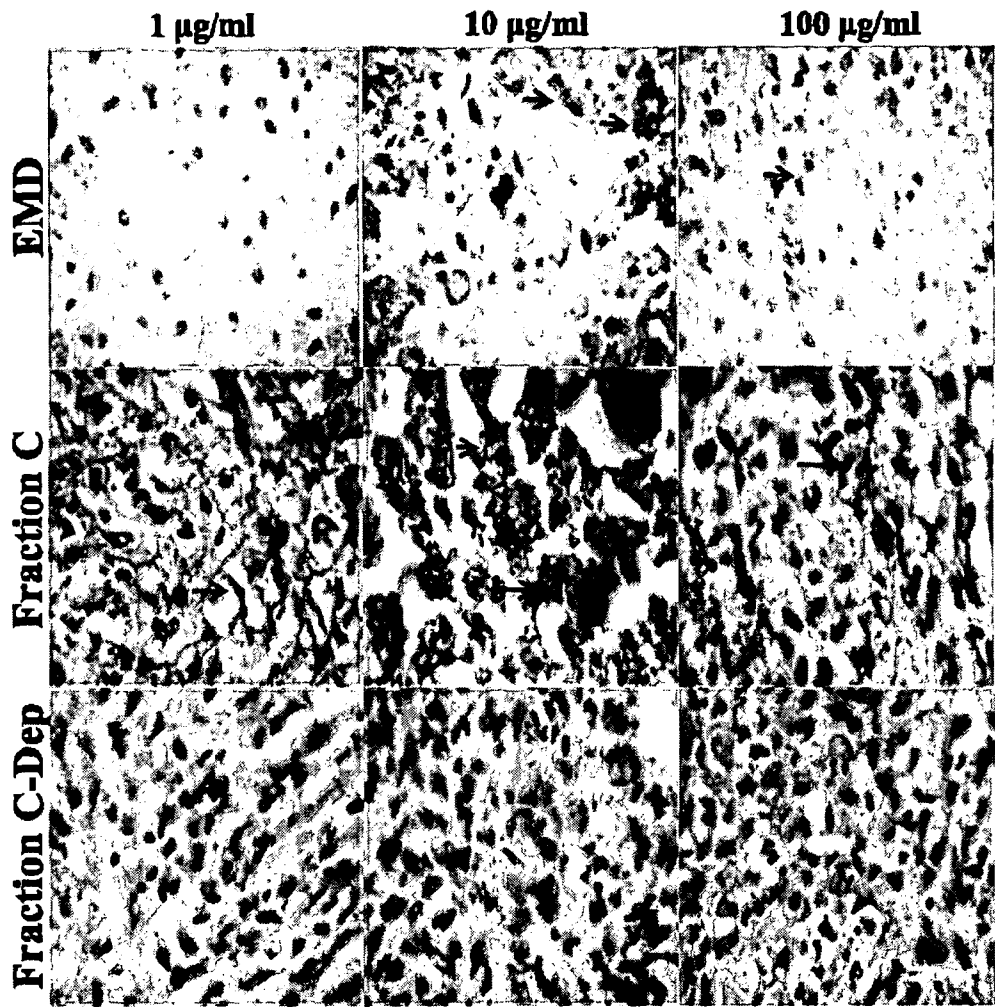


Figure 2

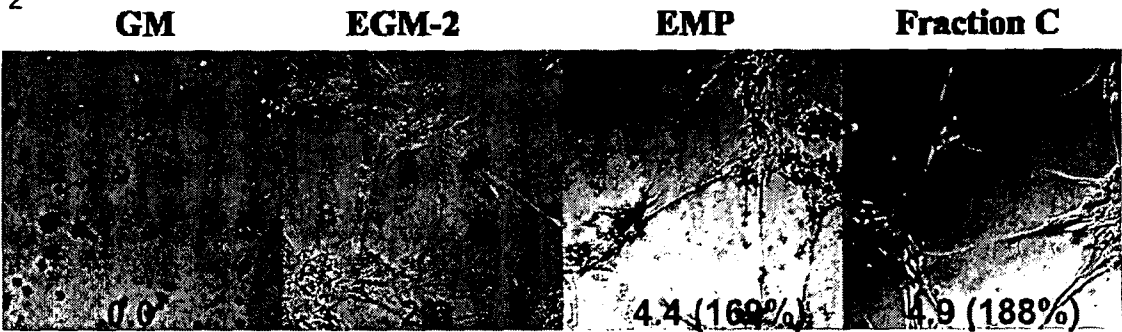


Figure 3

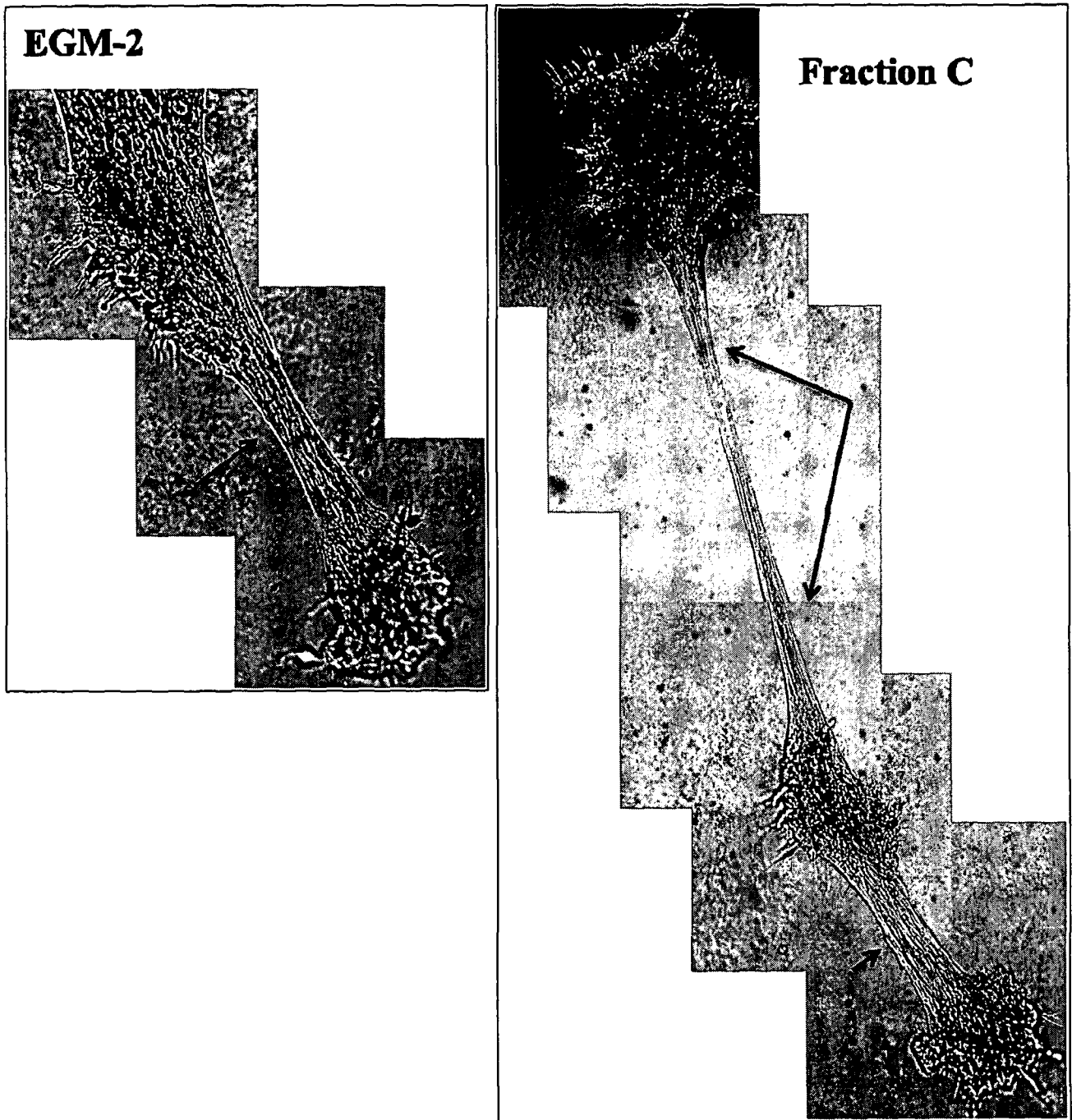


Figure 4

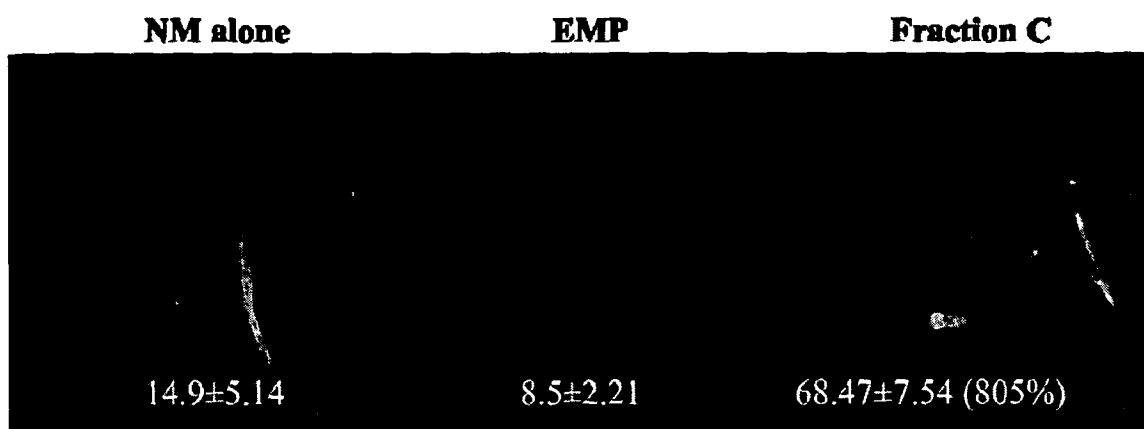


Figure 5

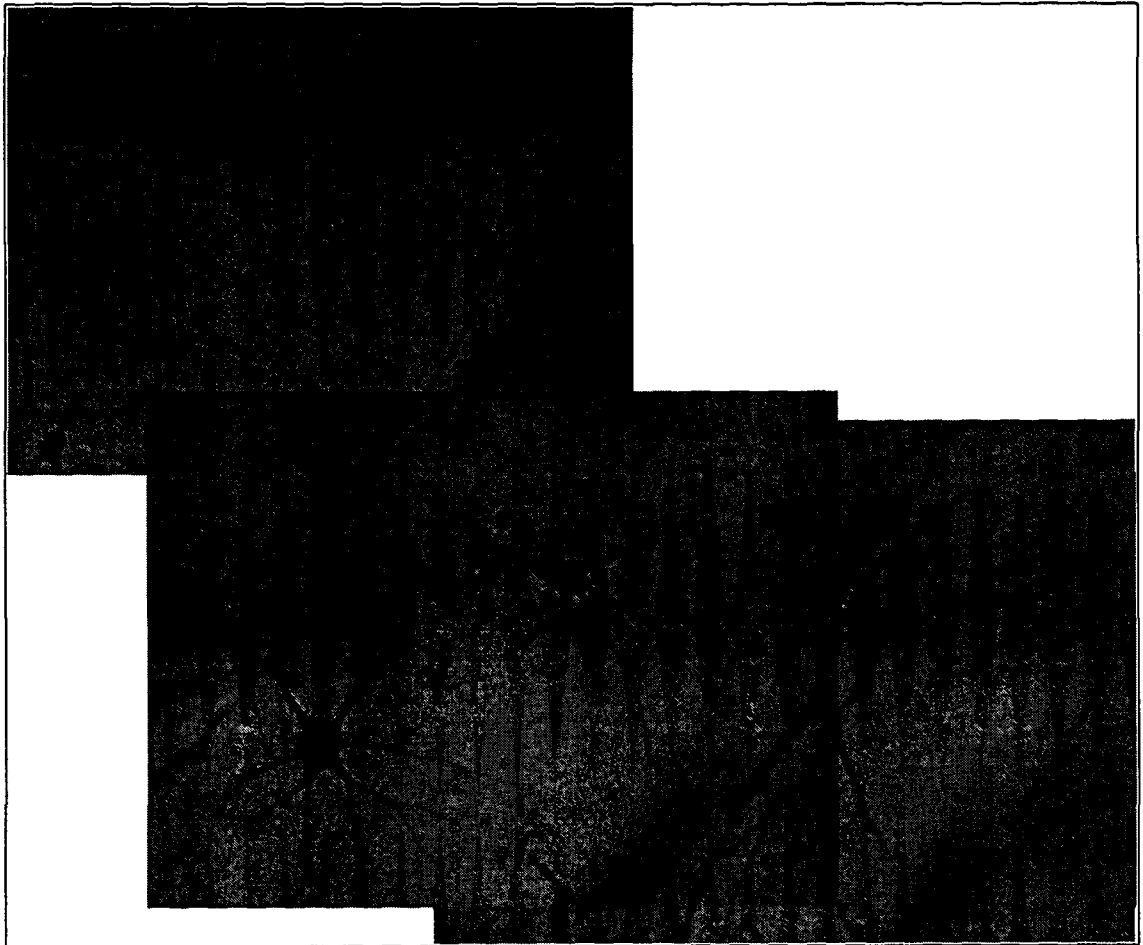


Figure 6

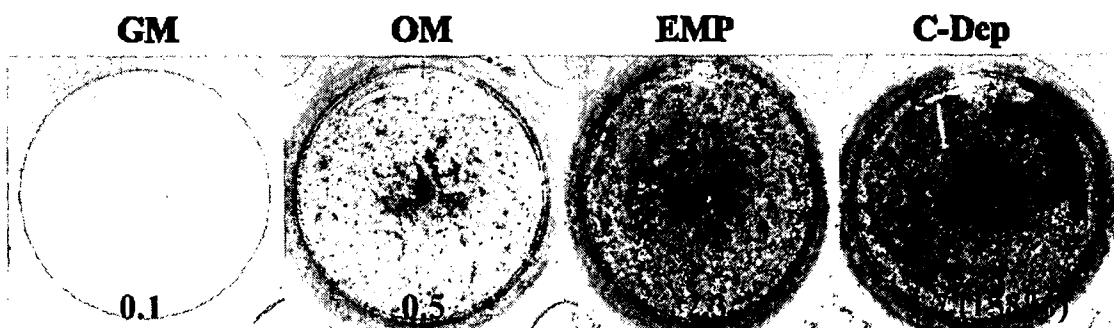


Figure 7

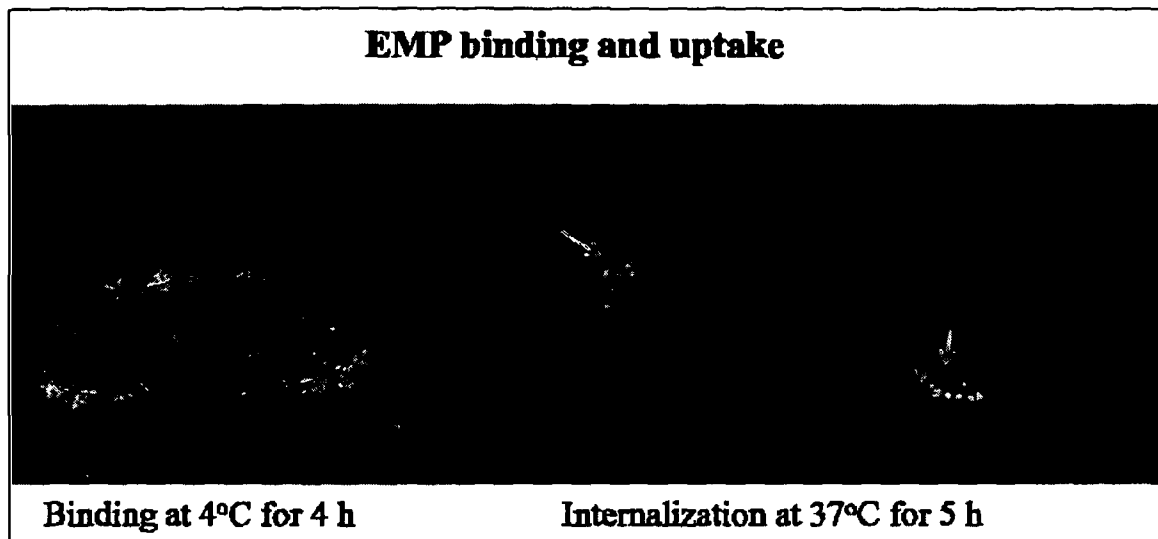


Figure 8

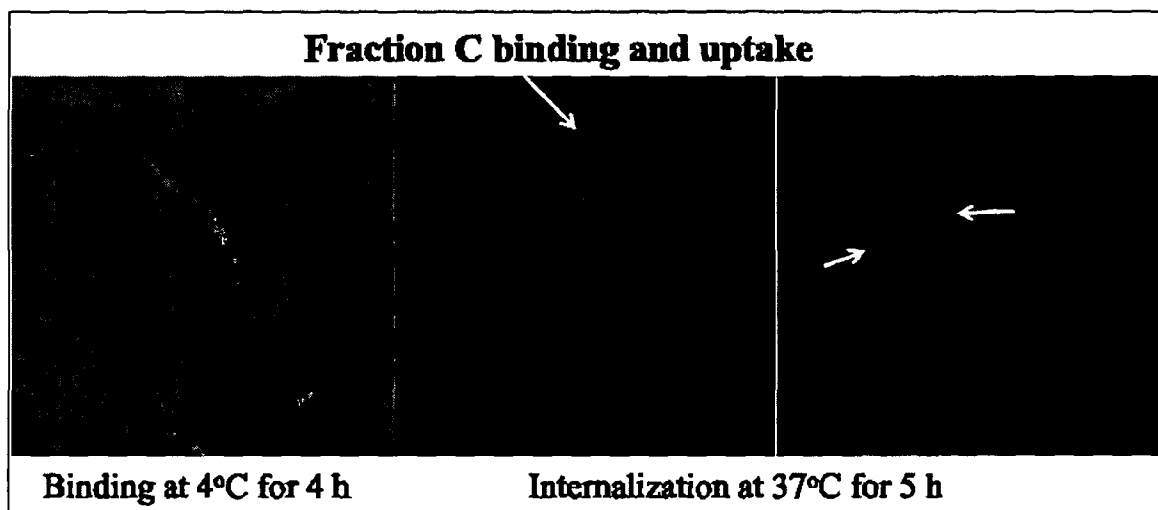


Figure 9 (from Mumulidu 2007)

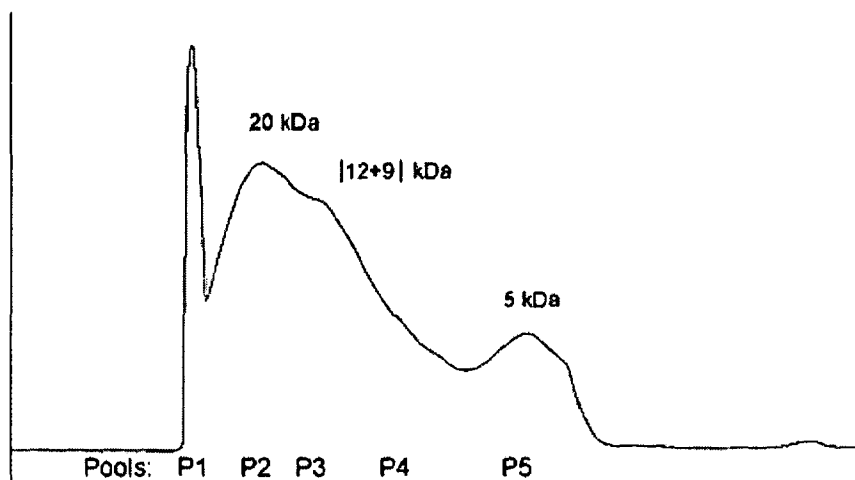


Figure 10 (from Mumulidu 2007)

