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(54) **Titre : FRAGMENTS PEPTIDIQUES DESTINES A INDUIRE LA SYNTHESE DE PROTEINES MATRICIELLES EXTRACELLULAIRES**
(54) **Title: PEPTIDE FRAGMENTS FOR INDUCING SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS**

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGVI GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGPPGPP GIPGCNGIKG ERGPLGPPGL PGFAGNPGPP
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGTPGPPGLP GLQGPVGPPG
FTGPPGPPGP PGPPGEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEPGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEPGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEPGP KGFPGLPGQP GPPGLPVPGQ AGAPGFPGER GEKGDRGFPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYTNG IVECQPGPPG DQGPPGIPGQ
PGFIGEIGEK GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPGL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGMPGLP GPKGSPGSVG LKGERGPPGG VGFPGSRGDT
GPPGPPGYGP AGPIGDKGQA GFPGGPGSPG LPGPKGEPGK IVPLPGPPGA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGQ KGEPGVGLPG LKGLPGLPGI
PGTPGEKGSI GVPGVPGEHG AIGPPGLQGI RGEPGPPGLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGIPGQP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPGKD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPGLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGDK GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPGSDGIPG SAGEKGEPGL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPGLPGLP
GPMGPPGLPG IDGVKGDKGN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIIKGEPG LPGPEGPPGL KGLQGLPGPK GQQGVIGLVG IPGPPGIPGF
DGAPGQKGEM GPAGPTGPRG FPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQGNE RAHGQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCAV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT

(57) **Abrégé/Abstract:**
[0049] Short biologically active tetrapeptides are disclosed that are comprised of the sequences GxxG and PxxP where G (glycine) and P (proline) are maintained and x is a variable amino acid. The peptides can be used singly or in combination to stimulate production of extracellular matrix proteins in skin. A rapid, low-cost method of producing heterogenous formulations of tetrapeptides is disclosed.

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[Continued on next page]

(54) Title: PEPTIDE FRAGMENTS FOR INDUCING SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS

(57) Abstract: [0049] Short biologically active tetrapeptides are disclosed that are comprised of the sequences GxxG and PxxP where G (glycine) and P (proline) are maintained and x is a variable amino acid. The peptides can be used singly or in combination to stimulate production of extracellular matrix proteins in skin. A rapid, low-cost method of producing heterogenous formulations of tetrapeptides is disclosed.

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGVI GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGPPGPP GIPGCNGTKG ERGPLGPPGL PGFAGNPGPP
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGTPGPPGLP GLQGPVGPPG
FTGPPGPPGP PGPPGEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEPGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEPGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEPGP KGFPGLPGQP GPPGLPVPGQ AGAPGFPGER GEKGDRGFPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYTNG IVECQPGPPG DQGPPGIPGQ
PGFIGEIGEK GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPGL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGHPGLP GPKGSPGSVG LKGERGPPGG VGFPGSRGDT
GPPGPPGYGP AGPIGDKGQA GFPGGPGSPG LPGPKGEPGK IVPLPGPPGA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGQ KGEPGVGLPG LKGLPGLPGI
PGTPGEKGSI GVPGVPGEHG AIGPPGLQGI RGEPGPPGLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGTPGQP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPGKD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPGLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGDK GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPGSDGIPG SAGEKGEPGL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPGLPGLP
GPMGPPGLPG IDGVKGDKGN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIIKGEPG LPGPEGPPGL KGLQGLPGPK GQQGVTGLVG IPGPPGIPGF
DGAPGQKGEM GPAGPTGPRG FPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQGNE RAHGQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCAV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT

FIG. 1

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PEPTIDE FRAGMENTS FOR INDUCING SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS

[0001]

FIELD OF THE INVENTION

[0002] The invention relates to tetrapeptides with the amino acid motif GxxG or PxxP, where G (glycine) and P (proline) are maintained and x is a variable amino acid. The invention also relates to frame shift active tetrapeptides which are tetrapeptide sequences shifted one frame from a GxxG or PxxP tetrapeptide in an ECM protein. In particular, the invention relates to GxxG, PxxP, or frame shift active peptides that stimulate production of extracellular matrix proteins and enhance wound closure of the epithelial cell monolayer of scratch-wounded human skin. The peptide compositions may be used in formulations for repairing damaged skin or maintaining healthy skin.

BACKGROUND OF THE INVENTION

[0003] Skin aging is commonly viewed as wrinkle formation and impaired wound healing. A wound is defined as a break in the epithelial integrity of the skin. Normal wound healing involves a complex and dynamic but superbly orchestrated series of events leading to the repair of injured tissues. The largest component of normal skin is the extracellular matrix (ECM), a gel-like matrix produced by the cells that it surrounds. The ECM is composed of two major classes including fibrous structural proteins and proteoglycans. Changes in the composition and crosslinked state of the ECM are known to be associated with aging and a range of acquired and heritable skin disorders. It has been well documented that ECM not only provides structural support, but also influences cellular behavior such as differentiation and proliferation. Also, more and more research suggests that the matrix components may be a source of cell signals to facilitate epithelial cell proliferation and migration and thus enhance wound healing.

[0004] The largest class of fibrous ECM molecules is the collagen family, which includes at least 16 different types of collagen. Collagen in the dermal matrix is composed primarily of type I (80-85%) and type III (8-11%) collagens, both of which are fibrillar, or rod-shaped, collagens. The tensile strength of skin is due predominately to these fibrillar collagen molecules, which self-assemble into microfibrils in a head-to-tail and staggered side-to-side lateral arrangement.

Collagen molecules become cross-linked to adjacent collagen molecules, creating additional strength and stability in collagen fibers. Damage to the collagen network (e.g. by enzymes or physical destruction), or its total collapse causes healing to take place by repair.

[0005] Various bioactive peptides that stimulate production of ECM proteins have been reported in both the scientific literature and in issued patents. Peptides historically have been isolated from natural sources and have recently been the subject of structure-function relationship studies. Natural peptides have also served as starting points for the design of synthetic peptide analogs.

[0006] Specific sequences within ECM proteins can stimulate useful elements in skin, such as type I collagen, type III collagen, and fibronectin (Katayama et. al., J. BIOL. CHEM. 288:9941-9944 (1983)). Katayama et al. identified the pentapeptide, KTTKS (SEQ ID NO:17), within the carboxy-terminal propeptide (residues 197-241) of type I collagen. The propeptide is cleaved during production of the mature collagen protein. The cleaved propeptide may participate in regulating collagen production via a biosynthesis feedback mechanism, with the KTTKS segment playing an active role. Maquart et al. (J SOC BIOL. 193:423-28 (1999)) reported that the peptides GHK and CNYYSNS also stimulate ECM synthesis. These sequences may be released during ECM turnover, thereby signaling the need for ECM repair. The short peptide sequences liberated by either mechanism are often called "matrikines" (Maquart et al., J. SOC. BIOL. 193:423-28 (1999)).

[0007] While a number of natural and synthetic peptides exist, there is a need for improved biologically active peptides and methods for their use.

SUMMARY OF THE INVENTION

[0007a] Certain exemplary embodiments provide a tetrapeptide of the formula GxxG that induces the production of an extracellular matrix protein, wherein the tetrapeptide is SEQ ID NO: 5 (GEPG), SEQ ID NO: 8 (GEKG) or SEQ ID NO: 3 (GSPG).

[0007b] Other exemplary embodiments provide a tetrapeptide of the formula GxxG that induces the production of an extracellular matrix protein wherein the tetrapeptide is SEQ ID NO: 5 (GEPG), SEQ ID NO: 8 (GEKG), SEQ ID NO: 3 (GSPG) or SEQ ID NO: 7 (GPPG), for use in the manufacture of a medicament or cosmetic for treating damaged skin or maintaining healthy skin.

[0007c] Other exemplary embodiments provide a tetrapeptide capable of inducing the production of an extracellular matrix protein wherein the tetrapeptide is SEQ ID NO: 5 (GEPG) or SEQ ID NO: 8 (GEKG).

[0007d] Other exemplary embodiments provide a composition comprising a mixture of the tetrapeptides of SEQ ID NO:5 and SEQ ID NO:8.

[0007e] Other exemplary embodiments provide a cosmetic composition comprising a mixture of the tetrapeptides of SEQ ID NO:5 and SEQ ID NO:8.

[0007f] Further, exemplary embodiments include a medicament composition useful for treating damaged skin comprising the tetrapeptide or tetrapeptides and a pharmaceutically acceptable carrier.

[0008] Tetrapeptides are disclosed that are characterized by the amino acid sequence motif GxxG or PxxP, where G (glycine) and P (proline) residues are maintained and x is a variable amino acid. The tetrapeptides are derived from sequences that occur multiple times throughout the primary sequence of the ECM protein, type IV collagen. The disclosed sequences induce production of all forms of collagen more than previously known peptide sequences, including KTTKS, sold under the trademark MATRIXYL™ by SEDERMA SAS (France). Further, a composition comprising a combination of various multiply-repeating sequences elicits an even greater collagen-producing response. Additional benefits may be expected from peptide combinations present in a variety of ECM proteins.

[0009] Producing a specific combination of tetrapeptides for ECM rebuilding can be commercially cost-prohibitive. A relatively simple and cost-effective means of producing a diverse combination of biologically active tetrapeptides is disclosed. By producing a combinatorial library of tetrapeptides with the GxxG or PxxP motif, a variety of biologically active tetrapeptides can be generated in the same manufacturing run (*e.g.*, GEPG, GPEG, GPPG, and GEEG). The combination of tetrapeptides may induce more formation of ECM proteins than single peptides. Compositions comprising the disclosed tetrapepides, alone or in combination, are useful in skin care markets including, but not limited to, those that address skin wrinkling, toning, firmness, or sagging. The stimulation of collagen by the disclosed tetrapeptides can significantly improve the health and appearance of damaged and aged skin.

BRIEF DESCRIPTION OF THE FIGURES

[0010] FIG. 1 is SEQ ID NO:45 which is the Collagen IV amino acid sequence illustrating the occurrences of GxxG tetrapeptides. All bold sequences are underlined and overlapping sequences are double-underlined.

[0011] FIG. 2 is SEQ ID NO:46 which is the Collagen III amino acid sequence illustrating the occurrences of the frame shift actives PGPR and GAGP. All frame shift active sequences are bold and underlined and the GxxG sequences occurring one frame shift away are double-underlined.

[0012] FIG. 3 is also SEQ ID NO:45, the Collagen IV amino acid sequence, illustrating the occurrences of the tetrapeptide PGPP.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The invention is generally directed towards tetrapeptides that stimulate production of ECM proteins and modulate wound healing, and uses of such tetrapeptides.

Peptides

[0014] One embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GxxG or PxxP. In this embodiment G (glycine) or P (proline) is maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12. SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16.

[0015] Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GxPG, where x is P at either variable position, or both. In this embodiment, G (glycine) and P (proline) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7.

[0016] Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GExG. In this embodiment, G (glycine) and E (glutamic acid) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:5 or SEQ ID NO:8.

[0017] Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif PGxP. In this embodiment, P (proline) and G (glycine) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16.

[0018] Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif PExP. In this embodiment, P (proline) and E (glutamic acid) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:1 or SEQ ID NO:9.

[0019] Another embodiment of the invention is directed towards a frame shift active tetrapeptide. In this embodiment, the tetrapeptide occurs one frame shift from either a GxxG or PxxP tetrapeptide in an ECM protein. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:4 or SEQ ID NO:6.

[0020] Each of the above-described peptides can comprise D- or L-amino acids. The peptides can comprise all D-amino acids or L-amino acids. The peptides can have an acid C-terminus (-CO_2H) or, preferably, an amide C-terminus (-$CONH_2$, -CONHR, or -$CONR_2$). The peptides may be further augmented or modified, either chemically or enzymatically. For example, the peptides may be amidated (-NH_2) on the C-terminus, which may render the tetrapeptide less susceptible to protease degradation and increase their solubility compared to the free acid forms. The peptides may also be lipidated which may provide for enhanced skin penetration.

[0021] The above-described peptides may contain the following amino acids: R (arginine), L (leucine), P (proline), F (phenylalanine), Q (glutamine), E (glutamic acid), I (isoleucine), K

(lysine), S (serine), V (valine), A (alanine), N (asparagine), D (aspartic acid), T (threonine), Y (tyrosine) and G (glycine). The above-described peptides do not include the following M (methionine), C (cysteine), H (histidine) or W (tryptophan). Accordingly, in one embodiment, x is not selected from either (methionine), C (cysteine), H (histidine) or W (tryptophan).

Methods of Use

[0022] An additional embodiment of the invention is directed towards methods of using the above-described peptides. The methods of use may involve the use of a single peptide, or may involve the use of two or more peptides in combination.

[0023] An embodiment of the invention is a method of promoting repair of damaged skin and maintenance of healthy skin using tetrapeptides that stimulate production of ECM proteins. The method generally is directed towards contacting dermal (skin) cells with a composition containing the peptide. The compositions can be an aerosol, emulsion, liquid, lotion, cream, paste, ointment, foam, or other pharmaceutically acceptable formulation. Generally, a pharmaceutically acceptable formulation would include any acceptable carrier suitable for use on human skin, e.g. cosmetically acceptable carrier and dermatological acceptable carrier. The compositions may contain other biologically active agents such as retinoids or other peptides. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The contacting step can be performed *in vivo*, *in situ*, *in vitro*, or by any method known to those of skill in the art. Most preferably, the contacting step is to be performed topically at a concentration sufficient to elicit a stimulatory response. The concentration of the peptide in the composition can be about 0.01 μg/mL to about 100 μg/mL, about 0.1 μg/mL to about 50 μg/mL, and about 0.1 μg/mL to about 1 μg/mL. The contacting step can be performed on a mammal, a cat, a dog, a cow, a horse, a pig, or a human. A preferred composition for promoting ECM protein production comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

[0024] An additional embodiment of the invention is directed towards a method for promoting wound healing of skin damaged by normal aging, disease, injury, trauma, or by surgery or other medical procedures. The method can comprise administering to the wound of an animal a composition, wherein the composition comprises any of the above-described peptides, singularly

or in combination. The compositions can be a liquid, lotion, cream, paste, ointment, foam, or any other pharmaceutically acceptable formulation. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The compositions may contain other biologically active agents such as antimicrobial agents or growth factors. The compositions may also be used in combination with other therapeutic agents such as tissue grafts, tissue culture products, oxygen or dressings. The concentration of the peptide in the composition can be about 0.01 μg/mL to about 100 μg/mL, about 0.1 μg/mL to about 50 μg/mL, and about 0.1 μg/mL to about 1 μg/mL. The composition can be administered to the wound topically. The animal can generally be any kind of animal, and preferably is a mammal, and more preferably is a human, cow, horse, cat, dog, pig, goat, or sheep. A preferred composition for wound healing applications in which ECM protein production is promoted comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

[0025] An additional embodiment of the invention is directed towards a method for reducing scarring of skin damaged by normal aging, disease, injury, trauma, or by surgery or other medical procedures. The method can comprise administering to the wound of an animal a composition, wherein the composition comprises any of the above-described peptides, singularly or in combination. The compositions can be a liquid, lotion, cream, paste, ointment, foam, or other pharmaceutically acceptable formulation. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The compositions may contain other biologically active agents such as antimicrobial agents or growth factors. The compositions may also be used in combination with other therapeutic agents such as tissue grafts, tissue culture products, oxygen or dressings. The concentration of the peptide in the composition can be about 0.01 μg/mL to about 100 μg/mL, about 0.1 μg/mL to about 50 μg/mL, and about 0.1 μg/mL to about 1 μg/mL. The composition can be administered to the wound topically. The animal can generally be any kind of animal, and preferably is a mammal, and more preferably is a human, cow, horse, cat, dog, pig, goat, or sheep. A preferred composition for wound healing applications in which ECM protein production is promoted comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a

most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

[0026] A further embodiment of the invention is directed towards a method for producing the disclosed tetrapeptides in combination. The peptides may be produced using any method known to those skilled in the art such as those disclosed in Merrifield, R.B., *Solid Phase Peptide Synthesis I.,* J. AM. CHEM. SOC. 85:2149-2154 (1963); Carpino, L.A. et al., *[(9-Fluorenylmethyl)Oxy] Carbonyl (Fmoc) Amino Acid Chlorides: Synthesis, Characterization, And Application To The Rapid Synthesis Of Short Peptides*, J. ORG. CHEM. 37:51:3732-3734; Merrifield, R.B. et al., *Instrument For Automated Synthesis Of Peptides*, ANAL. CHEM. 38:1905-1914 (1966); or Kent, S.B.H. et al., *High Yield Chemical Synthesis Of Biologically Active Peptides On An Automated Peptide Synthesizer Of Novel Design*, IN: PEPTIDES 1984 (Ragnarsson U., ed.) Almqvist and Wiksell Int., Stockholm (Sweden), pp. 185-188. Preferably, the peptides will be produced by a machine capable of sequential addition of amino acids to a growing peptide chain. However, the peptides may also be manufactured using standard solution phase methodology.

[0027] It has been observed that the addition of a mixture of free amino acids instead of homogenous peptide mixtures during peptide chain synthesis results in varied incorporation of free amino acids such that a combination of peptides results from the synthesis reactions. The relative incorporation frequency of a particular amino acid included in a mixture of two or more amino acids added during synthesis may be adjusted. Adjustment is made possible by modifying the ratio of a free amino acid made available during the synthesis process relative to the other amino acids in the mixture (this is termed an isokinetic mixture).

[0028] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example1: Identification of repeat tetrapeptide sequences in collagen

[0029] A relatively high proportion of collagen IV tetrapeptide repeat sequences have the motif GxxG (where x is any amino acid). A number of these are shown *in situ* as part of the full collagen IV sequence illustrated in Figure 1 as SEQ ID NO:45. Collagen IV was examined first due to its role of interacting with other specialized ECM components (*See* Gregory Schultz et al., 2005). There are eleven sequences with the GxxG motif in collagen IV that appear more than ten times (GxxG where xx is represented by: vp, ek, fp, lp, pp, sp, ep, ip, pk, qp and tp). Of these tetrapeptide sequences, eight of eleven sequences contain proline in position 3, two of eleven sequences contain P in position 2, one of eleven sequences contains proline in positions 2 and 3, and one of eleven sequences contains no proline. The disclosed sequences are referred to as REPLIKINES™. "REPLIKINE" is defined as a short sequence within ECM proteins that occurs multiple times (i.e., is replicated). This sequence may be present in one ECM protein (e.g., collagen IV). Preferably, the sequence is present in multiple ECM proteins (e.g., all collagens, elastin, laminin, etc.). The presence of the sequence in multiple ECM proteins increases the likelihood that the fragment may be able to promote ECM synthesis or repair.

[0030] The eleven GxxG sequences appearing in collagen IV listed above are highlighted in the human collagen IV sequence illustrated in Figure 1. In this figure, all bold sequences are underlined and overlapping sequences are double-underlined. All but one of these sequences also appears in collagens I, II, III, and V. This fact contributes to the ability of the disclosed peptides to stimulate the production of all collagen types, particularly when the peptides are used in combination. Table 1 shows the frequency of several tetrapeptide repeats in ECM proteins. Bold sequences in Table 1 are those that appear in collagen IV ten or more times.

Table 1: Frequency of tetrapeptides in ECM proteins

SEQ. ID NO	Sequence	Collagen I	Collagen II	Collagen III	Collagen IV	Collagen V	Elastin	Elastin Precursor
19	GAAG	10	5	7		2	4	5
20	GAKG	3	4	3	5	5		
21	GAPG	13	21	25	6	9		
22	GDKG	2	2	4	9	3		
23	GDRG	2	5	2	4	1		
8	**GEKG**	**3**	**5**	**4**	**22**	**15**		
5	**GEPG**	**11**	**15**	**10**	**11**	**4**		
24	GERG	10	11	14	6	7		
2	**GFPG**	**4**	**8**	**6**	**22**	**5**	**1**	**1**
25	GIPG	2	2	6	14	6	5	5
26	GKDG	1	4	5	2	2		
27	GKPG	2	3	3	4	1		

SEQ. ID NO	Sequence	Collagen I	Collagen II	Collagen III	Collagen IV	Collagen V	Elastin	Elastin Precursor
28	GLKG	2	1	1	5	4		
29	GLPG	15	10	9	42	15	1	1
30	GNPG	3	5	3	2	1		
31	GPAG	16	20	20	3	6		
32	GPKG	3	11	4	12	9		
7	**GPPG**	**33**	**40**	**40**	**46**	**43**		
33	GPQG	7	11	9	7	5		
34	GPRG	11	13	10	4	7		
35	GPSG	10	11	5	1	5		
36	GPTG	4	3	2	2	6		
37	GPVG	9	3	3	2	5		
38	GQPG	3	4	6	12	7		
39	GRDG	4	2	3	3			
40	GRPG	3	3	4	2	5		
3	**GSPG**	**4**	**6**	**21**	**16**	**3**		
41	GTPG	3	4	2	11	2		
42	GVKG	1	3	2	3	1		
43	GVPG		1	3	10	1	14	15
44	GYPG	1	1	1	4	2		

[0031] As also evident from a review of the collagen IV sequence, SEQ ID NO:45, there are also many occurrences of sequences having the PxxP motif. For example, the sequence PGPP occurs no less than fifteen times as illustrated in Figure 3. Therefore, this disclosed sequence is also referred to as a REPLIKINE™. Preferably, this sequence is present in multiple ECM proteins (e.g., all collagens, elastin, laminin, etc.) as the presence of this sequence in multiple ECM proteins increases the likelihood that the fragment may be able to promote ECM synthesis or repair. The fifteen PGPP sequences appearing in collagen IV listed above are highlighted and underlined in the human collagen IV sequence illustrated in Figure 3.

Example 2: Identification of frame shift actives

[0032] In addition to the relatively high proportion of collagen IV tetrapeptide repeat sequences with the motif GxxG, other tetrapeptide sequences occurring one amino acid frame shift away from a GxxG or PxxP tetrapeptide sequence have been identified. These sequences may repeat or occur only once within an ECM protein and may be located one amino acid position away from either a GxxG or PxxP tetrapeptide sequence as described herein. These tetrapeptide sequences are referred to as frame shift actives. Such frame shift actives may accordingly contain either a G or a P in either the second or third position depending on the direction of frame shift. It has been further recognized that frame shift actives may be combined with other

tetrapeptide sequences disclosed in this application forming a combikine. An example of such a combikine is H06 and H15.

[0033] One example of a frame shift active is GAGP or H12 (SEQ ID NO:6). H12 (GAGP) appears one residue (or frame) shift from the GxxG tetrapeptide GGAG in Collagen III (SEQ ID NO:46) as illustrated in Figure 2. In this figure, all frame shift active sequences are bold and underlined and the GxxG sequences occurring one frame shift away are double-underlined. Furthermore, as shown in Table 5, this tetrapeptide (GAGP) achieves good results for collagen production at 48 hours. Another example is the sequence PGPR, which is H10 (SEQ ID NO:4) which occurs eleven times in Collagens I-IV. As it appears multiple times in an individual ECM protein, this tetrapeptide would further be considered a REPLIKINE. Figure 2 (SEQ ID NO:46) illustrates several instances of this tetrapeptide with each occurring one frame shift from the GxxG tetrapeptide GPRG. This particular frame shift active appears in multiple ECM proteins and therefore increases the likelihood that the fragment may be able to promote ECM synthesis or repair.

Example 3: Identification of repeat sequences that stimulate collagen production

[0034] Several sequences identified in Examples 1 and 2 were synthesized using standard peptide chemistry and assayed for the stimulation of collagen from dermal fibroblasts. The synthesized peptides were amidated at the C-terminus, which rendered the tetrapeptides less susceptible to protease degradation and increased their solubility compared to the free acid forms. Human dermal fibroblasts were incubated in 96-well plates at 37 °C and 5% CO_2 for 24 and 48 hours in 150 µL complete cell culture media (Cascade Biologics, Portland, OR; Cat. No. M-106-500), supplemented with Low Serum Growth Supplement (Cascade Biologics, Portland, OR; Cat. No. S-003-10) containing sample peptides at a final peptide concentration of 50 µg/mL. Each well was seeded with 10,000 cells. Following the incubation, 100-µL medium samples were recovered from each well and assayed for collagen production

[0035] The assays were performed by Tebu-bio Laboratories (France) using the SIRCOL™ Collagen Assay Kit (Biocolor Assays, UK) following the manufacturer's protocol. The SIRCOL™ Collagen Assay is a quantitative dye-binding method designed for the analysis of soluble collagens released into culture medium by mammalian cells during *in vitro* culture. The collagen of the tested samples binds to the anionic SIRCOL™ dye. The collagen-dye complexes precipitate out of solution and are pelleted by centrifugation. The recovered collagen-dye pellet

was dissolved in an alkaline solution prior to absorbance measurements. Duplicate measurements were taken at the 24 and 48 hour times from two separate samples. The four measurements for each sample were averaged. The absorbance of reagent blanks, collagen standards, and samples were measured at 560 nm. The reagent blank absorbance was subtracted from the absorbance from each sample at 24 and 48 hours.

[0036] Two separate data sets were used to generate two collagen standard calibration curves. The first calibration curve was generated for purposes of calculating the quantity of collagen in samples H6 (combination of SEQ ID NOs:1-4), H7-H14 (SEQ ID NOs:1-8, respectively) and H15 (combination of SEQ ID NOs:5-8). The second calibration curve was generated for calculating the quantity of collagen in samples H16 (SEQ ID NO:9), H21-23 (SEQ ID NOs:10-12, respectively), H25-26 (SEQ ID NOs:13-14, respectively), or H29-30 (SEQ ID NOs:15-16, respectively), H32 (SEQ ID NO:17), H33 (combination of SEQ ID NOs:9-12), H34 (combination of SEQ ID NOs:11-14), H35 (combination of SEQ ID NOs:13-16), H36 (combination of SEQ ID NOs:1, 6, 5, 8), H37 (SEQ ID NO:17) and H38 (SEQ ID NO:8) from the absorbance measurements was created by plotting the Abs_{560nm} of the known collagen standards versus the respective concentrations of the collagen standards (in micrograms) each time a series of assays were performed. With respect to each data set, the same calibration curve was used for samples taken at the 24 and 48 hour times (Tables 2A and 2B). Accordingly, different standard curves were prepared immediately prior to performing each series of assays.

Table 2A: Calibration curve for assaying collagen production by peptides H6-H15

Collagen standards (μg)	$A_{560\,nm}$ 24h test	A_{560nm} 48h test
0	0.00	0.00
5	0.08	0.10
10	0.11	0.15
25	0.32	0.35
50	0.66	0.65

Table 2B: Calibration curve for assaying collagen production by peptides H16, H21-23, H25-26, and H29-38

Collagen Standards (μg)	A_{560nm} Assay date 1	A_{560nm} Assay date 2
0	0.00	0.00
5	0.12	0.09
10	0.14	0.15
25	0.48	0.42
50	0.88	0.80

[0037] A linear regression was performed from plotting the Abs_{560nm} values versus concentrations of the respective collagen standards using MICROSOFT EXCEL™. The regression resulted in a lines described by the formula $y = 0.013x$ for both incubation times noted in Table 2A. As the results were identical, only the 24-hour time period was used for the second series calibration curves. The formula of the line obtained on assay date 1 and assay date 2 of the second series of samples was $y = 0.0178x$ and $y = 0.0162x$, respectively. The peptide LL-37 (SEQ ID NO:18) was used as a positive control as it has been widely reported to have an impact upon wound healing in man (Heilborn et al., The Cathelicidin Anti-Microbial Peptide LL-37 Is Involved In The Re-Epithelialization Of Human Skin Wounds And Is Lacking In Chronic Ulcer Epithelium, *J. Invest. Dermato.* 120:379-89 (2003)). The assay detection limit defined by the manufacturer is 2.5 μg.

[0038] The total amount of collagen produced in samples containing peptides was calculated from the averaged absorbance values taken at 24 hours (Table 3A) and 48 hours (Table 3B) using the linear equation derived from the standard curve. The total amount of collagen produced in samples containing peptides H16 (SEQ ID NO:9), H21-23 (SEQ ID NOs:10-12, respectively), H25-26 (SEQ ID NOs:13-14, respectively), or H29-30 (SEQ ID NOs:15-16, respectively), H32 (SEQ ID NO:17), H33 (combination of SEQ ID NOs:9-12), H34 (combination of SEQ ID NOs:11-14), H35 (combination of SEQ ID NOs:13-16), H36 (combination of SEQ ID NOs:1, 6, 5, 8), H37 (SEQ ID NO:17) and H38 (SEQ ID NO:8) was calculated from the absorbance values taken at 24 hours (Table 4A) and 48 hours (Table 4B) using the linear equation derived from the standard curve. These values were compared with peptide LL37 (SEQ ID NO:18), a peptide known to stimulate collagen. In each table, samples marked by an asterisk (*) may not be significant as the assay detection limit is 2.5 μg.

Table 3A: Absorbance measurements and quantification of collagen in test samples H6-H15 at 24 hours.

SEQ ID NO	Peptides	A_{560nm}		Average	Average minus blank	Collagen (µg)
18	LL37	0.102	0.136	0.12	0.04	3.0
-	H6	0.084	0.140	0.11	0.03	**2.5**
1	H7	0.098	0.063	0.08	0.00	0.0*
2	H8	0.122	0.078	0.10	0.02	1.5*
3	H9	0.147	0.104	0.13	0.05	**3.5**
4	H10	0.103	0.146	0.12	0.04	**3.4**
5	H11	0.110	0.168	0.14	0.06	**4.5**
6	H12	0.063	0.101	0.08	0.00	0.2*
7	H13	0.114	0.093	0.10	0.02	1.8*
8	H14	0.115	0.122	0.12	0.04	**3.0**
-	H15	0.132	0.093	0.11	0.03	**2.5**
-	Blank	0.074	0.076	0.08	0.00	0.0

Table 3B: Absorbance measurements and quantification of collagen in test samples H6-H15 at 48 hours.

SEQ ID NO	Peptides	A_{560nm}		Average	Average minus blank	Collagen (µg)
18	LL37	0.262	0.113	0.19	0.07	**5.2**
-	H6	0.086	0.189	0.14	0.02	1.3*
1	H7	0.192	0.189	0.19	0.07	**5.4**
2	H8	0.137	0.126	0.13	0.01	0.9*
3	H9	0.117	0.061	0.09	0.00	0.0*
4	H10	0.136	0.085	0.11	0.00	0.0*
5	H11	0.113	0.181	0.15	0.03	2.1*
6	H12	0.106	0.231	0.17	0.05	**3.7**
7	H13	0.100	0.145	0.12	0.00	0.2*
8	H14	0.132	0.176	0.15	0.03	**2.6**
-	H15	0.177	0.174	0.18	0.06	**4.3**
-	Blank	0.120	0.115	0.12	0.00	0.0

Table 4A: Absorbance measurements and quantification of collagen in test samples H16, H21-23, H25-26, or H29-38 at 24 hours.

SEQ ID NO	Peptides	A_{560nm}		Average	Average minus blank	Collagen (µg)
9	H16	0.133	0.137	0.14	0.06	**3.1**
10	H21	0.129	0.119	0.12	0.04	**2.5**
11	H22	0.192	0.085	0.14	0.06	**3.3**
12	H23	0.090	0.073	0.08	0.00	0.1*
13	H25	0.129	0.076	0.10	0.02	1.3*

SEQ ID NO	Peptides	A_{560nm}		Average	Average minus blank	Collagen (µg)
14	H26	0.114	0.149	0.13	0.05	2.9
15	H29	0.111	0.063	0.09	0.01	0.4*
16	H30	0.099	0.092	0.10	0.02	0.9*
17	H32 (crystals and cell toxicity)	0.087	0.055	0.07	-0.01	-0.5*
-	H33	0.086	0.125	0.11	0.03	1.4*
-	H34	0.117	0.120	0.12	0.04	2.2*
-	H35	0.103	0.090	0.10	0.02	0.9*
-	H36	0.105	0.128	0.12	0.04	2.1*
17	H37	0.099	0.100	0.10	0.02	1.1*
8	H38	0.103	0.159	0.13	0.05	2.9
-	Blank	0.072	0.086	0.08	0.00	0.0

Table 4B: Absorbance measurements and quantification of collagen in test samples H16, H21-23, H25-26, or H29-38 at 48 hours.

SEQ ID NO	Peptides	A_{560nm}		Average	Average minus blank	Collagen (µg)
9	H16	0.065	0.064	0.06	0.00	0.3*
10	H21	0.089	0.126	0.11	0.05	2.9
11	H22	0.102	0.087	0.09	0.03	2.1*
12	H23	0.093	0.082	0.09	0.03	1.7*
13	H25	0.059	0.084	0.07	0.01	0.7*
14	H26	0.081	0.153	0.12	0.06	3.5
15	H29	0.086	0.094	0.09	0.03	1.9*
16	H30	0.083	0.101	0.09	0.03	2.0*
17	H32 (crystals and cell toxicity)	0.088	0.072	0.08	0.02	1.2*
-	H33	0.096	0.092	0.09	0.03	2.1*
-	H34	0.076	0.155	0.12	0.06	3.4
-	H35	0.120	0.074	0.10	0.04	2.3*
-	H36	0.154	0.082	0.12	0.06	3.6
17	H37	0.078	0.114	0.10	0.04	2.2*
8	H38	0.123	0.089	0.11	0.05	2.8
-	Blank	0.106	0.0106	0.06	0.00	0.0

[0039] Because sample sizes were 100 µL, the concentration of collagen produced in each sample in micrograms per milliliter is determined by multiplying the amount of collagen detected by ten. The results of all samples tested are summarized in Table 5.

Table 5: Collagen synthesis induced by peptides

				Collagen produced (µg/mL)	
SEQ ID NO	Name	Primary sequence	[Peptide] (µg/mL).	24hrs	48hrs
1	H07	PEGP	50	0	54
2	H08	GFPG	50	15	9
3	H09	GSPG	50	35	0
4	H10	PGPR	50	34	0
-	H06	H7, H8, H9, H10 (SEQ ID NOs:1, 2, 3, 4)	50	25	13
5	H11	GEPG	50	45	21
6	H12	GAGP	50	2	37
7	H13	GPPG	50	18	2
8	H14	GEKG	50	30	26
8	H38	GEKG	0.3	29	28
-	H15	H11, H12, H13, H14 (SEQ ID NOs:5, 6, 7, 8)	50	25	43
9	H16	PEKP	50	31	3
10	H21	PKGP	50	25	29
11	H22	PGQP	50	33	21
12	H23	PGTP	50	1	17
13	H25	PMGP	50	13	7
14	H26	PGPP	50	29	35
15	H29	PQGP	50	4	19
16	H30	PGNP	50	9	20
17	H32	KTTKS (SEDERMA™ peptide)	50	na	12
17	H37	KTTKS (SEDERMA™ peptide)	0.3	11	22
-	H33	H16, H21, H22, H23 (SEQ ID NOs:9, 10, 11, 12)	50	14	21
-	H34	H22, H23, H25, H26 (SEQ ID NOs:11, 12, 13, 14)	50	22	34
-	H35	H25, H26, H29, H30 (SEQ ID NOs:13, 14, 15, 16)	50	9	23
-	H36	H7, H12, H11, H14 (SEQ ID NOs:1, 6, 5, 8)	50	21	36
18	LL37	LLGDFFRKSKEKIGKEFKRIVQRID FLRNLVPRTES	50	30	52

[0040] All tetrapeptides tested stimulated the production of soluble collagen. Of the sequences tested, GxxG tetrapeptides with a glutamic acid in position 2 best stimulate collagen at both 24 and 48 hour time-points. These sequences are H11 (GEPG; SEQ ID NO:5), H14 (GEKG; SEQ ID NO:8) and H38 (GEKG; SEQ ID NO:8). The peptides were initially screened using a peptide concentration of 50 µg/mL. To survey the concentration effective for stimulating collagen production, H14 (SEQ ID NO:8) was also tested at 0.3 µg/mL as H38. As shown in Table 5,

H38-induced collagen stimulation was not diminished at the lower concentration, indicating that the maximal stimulating concentration of SEQ ID NO:8 is at or below 0.3 μg/mL.

[0041] To test its efficacy, SEQ ID NO:8 (H14 and H38) was compared to the peptide, LL37, (SEQ ID NO:18) which is known to stimulate collagen production. Based on the amount of collagen released by fibroblasts in response to LL37, 25 μg/mL was considered a significant amount of collagen released due to contact with a tetrapeptide. SEQ ID NO:8 induced about the same amount of collagen as LL37 (SEQ ID NO:18) at 24 hours. Importantly, collagen produced as a result of contact with SEQ ID NO:8 was substantially maintained for at least 48 hours. SEQ ID NO:8 was also compared to a leading skin care peptide known to stimulate collagen production, KTTKS (SEQ ID NO:17) (Katayama et. al., J. BIOL. CHEM. 288:9941-9944 (1983)). KTTKS is an ingredient in the product MATRIXYL™ (SEDERMA SAS, France). SEQ ID NO:8 stimulated more collagen production than the KTTKS (SEQ ID NO:17) peptide (Table 5) at 24 and 48 hours.

Example 4: Identification of peptide combinations that synergistically enhance collagen stimulation – COMBIKINES

[0042] Heterogeneous populations of active tetrapeptides may stimulate collagen production at a higher level than homogenous samples of tetrapeptides. The components of the heterogeneous composition are called COMBIKINES™. COMBIKINES are a group of REPLIKINES combined to produce a greater or broader effect upon one or more target cell types. The peptides H11 (SEQ ID NO:5), H12 (SEQ ID NO:6), H13 (SEQ ID NO:7), and H14 (SEQ ID NO:8) were combined to a final concentration of 50 μg/mL and assayed using the same protocol as for the individual peptides. As expected, the result obtained at the 24 hour time point equaled the mean of the individual induction scores. The combination of peptides at 48 hours, however, induced collagen to a level of 43 μg/mL. Surprisingly, this amount was far in excess of the anticipated mean (21 μg/mL) of the four individual peptides (see Table 5). Thus, specific combinations of peptides may stimulate collagen production to a greater degree than the individual peptides at the same concentration. Further, tetrapeptides from a variety of ECM sources such as collagen, laminin, and elastin may produce enhanced induction of a variety of ECM proteins (see Tables 1 and 5).

Example 5: Cost-effective COMBIKINE manufacturing for enhancing stimulation of collagen production

[0043] The high cost of peptide synthesis limits the feasibility of producing of heterogeneous compositions of bioactive peptides. The present invention greatly mitigates this limitation. Because the presently disclosed sequences have a commonality (*e.g.*, a glycine or proline at both termini), a range of tetrapeptides varied at positions 2 and 3 can be synthesized in a single manufacturing run. The synthetic peptides can be made by any method known in the art. (Benoiton, N., *Chemistry of Peptide Synthesis*, CRC (2005)). During manufacture of the peptides, amino acid mixtures are added instead of homogenous samples. The chemistry for determining the correct ratios of amino acid concentrations added at the mixed positions to gain the desired ratio of resulting peptides has been described previously (Greenbaum et al., *Molecular and Cellular Proteomics* 1:60-68, 2002; Krstenansky et al., *Letters in Drug Design and Discovery* 1:6-13, 2004. Using this methodology, a library of heterogeneous peptides can be made for nearly the same cost of synthesizing one peptide.

[0044] The application of this manufacturing process enables the cost-effective production of bioactive combikines. This is made possible by the unique composition of the disclosed tetrapeptides. The tetrapeptide mixtures are better suited for incorporation into topical use formulations than longer peptides. Because of their length, tetrapeptides have practical and chemical advantages over longer peptides, including the following: easier incorporation and dissolution into formulations, higher skin and pore permeability, and higher production yields with easier methods of manufacturing combinations of peptides. Although not required, the ideal formulations of tetrapeptides, singly or in combination, are formulations that maintain significant collagen production at 24 hours for up to 48 hours. More preferably, the formulations would induce synthesis of ECM for the entire 48 hour period such that more collagen is produced by 48 hours than at 24 hours. Although within the scope of the current invention, tetrapeptides that promote production of ECM proteins at 24 hours, but show diminished production at 48 hours, are less favored. In this regard, Table 6 shows the results of the currently disclosed peptides. Preferred peptides are in bold.

Table 6: Disclosed peptides

SEQ ID NO	Peptides	Released collagen (µg/mL) 24h	Released collagen (µg/mL) 48h	Significant release of collagen at 24h and 48h	Increase in collagen release at 48h v. 24h	Decrease in collagen release at 48h v. 24h
18	LL37	30	52	√	√	
-	H6	25	13			
1	H7	0	54		√	
2	H8	15	9			
3	H9	35	0			√
4	H10	34	0			√
5	H11	45	21			√
6	H12	2	37		√	
7	H13	18	2			
8	**H14**	**30**	**26**	√		
8	**H38**	**29**	**28**	√		
-	**H15**	**25**	**43**	√	√	
9	H16	31	3			√
10	**H21**	**25**	**29**	√		
11	H22	33	21			√
12	H23	1	17		√	
13	H25	13	7			√
14	**H26**	**29**	**35**	√		
15	H29	4	19		√	
16	H30	9	20		√	
17	H32 (crystals and cell toxicity)	NA	12			
17	H37	11	22		√	
-	H33	14	21		√	
-	H34	22	34		√	
-	H35	9	23		√	
-	H36	21	36		√	

Example 6: Collagen stimulators also serve as multi-effector molecules enhancing skin epithelial cell wound closer

[0045] Collagens are key components of all phases of wound healing. Stimulation of collagen production reflects that damage has occurred to the collagen network (e.g. by enzymes or physical destruction). Indeed, the total collapse of the collagen network in fact causes healing to take place. Therefore a collagen stimulator may also serve as a multi-effector molecule orchestrating certain matrix remodeling and enhancing wound healing.

[0046] Wound healing experiments were performed on monolayers of human skin epithelial cells (CRL-2592) plated onto 12-well plates. Cells were serum-starved for 24 hours before experimentation. Confluent monolayers of CRL-2592 were wounded using a P200 (200-µL) pipette tip. The wounds were washed and picture-documented prior to peptide treatment. Peptides were added to a final concentration from 20 to 40 µg/ml. Cells were kept in an incubator at 37°C, 5% CO_2, and 92% humidity, except when images were being captured for a short period at room temperature. Wound closure was followed at 6-hour and 10-hour time points. PBS-treated wounds were used as negative controls for comparison purposes.

Table 7: Effect of peptides on human skin epithelial wound closure *in vitro*

	0hr	6hr		10hr	
Compound	W-size*	W-size	% closure	W-size	% closure
PBS-1	36	29	19.40%	21	41.70%
PBS-2	52	42	19.20%	30	42.30%
SEQ ID NO:14	25	12	52%	2.75	89%
SEQ ID NO:5	48	39	19%	30	37.50%

* W-size: wound size (arbitrary)

[0047] In vitro monolayer wound closure is a result of cell migration, which is important in many biological processes such as embryogenesis, angiogenesis, inflammatory reactions and wound repair. These processes are thought to be regulated by interactions with other cells, cytokines and ECM proteins. As shown in Table 7, SEQ ID NO:14 significantly induces wound closure compared to the effects of PBS alone. Such activity is peptide-specific as well as cell type-specific since SEQ ID NO:14 does not induce wound closure in a human skin fibroblast monolayer (data not shown). SEQ ID NO:5 is also a collagen inducer, but does not enhance wound closure or epithelial cell migration to any great extent compared to the effects of PBS alone. The fact that SEQ ID NO:14 induced cell migration or wound closure in a manner specific to skin epithelial cells (i.e. does not recruit fibroblasts) may add an advantage to using this peptide for skin care, since it is believed that the recruitment of large numbers of active fibroblasts to a wound site results in excess deposition and contraction of tissue resulting in scarring.

Claims:

1. A tetrapeptide capable of inducing the production of an extracellular matrix protein wherein the tetrapeptide is SEQ ID NO: 5 (GEPG) or SEQ ID NO: 8 (GEKG).

2. The tetrapeptide of claim 1 for use in the manufacture of a medicament for treating damaged skin.

3. The tetrapeptide of any one of claims 1 or 2, wherein the tetrapeptide is amidated at the carboxy-terminus.

4. A medicament composition useful for treating damaged skin comprising the tetrapeptide according to any one of claims 1 to 3, and a pharmaceutically acceptable carrier.

5. The medicament composition of claim 4, wherein the tetrapeptide is present in concentration ranging from 0.10 μg/mL to 100 μg/mL.

6. The medicament composition of claim 4, wherein the composition is in the form of an aerosol, emulsion, liquid, lotion, cream, paste, ointment, or foam.

7. A medicament composition for treating damaged skin comprising a mixture of the tetrapeptides of SEQ ID NO: 5 and SEQ ID NO: 8.

8. The composition of claim 7 which further comprises the tetrapeptides of SEQ ID NO: 7 and SEQ ID NO: 6.

9. The medicament composition of any one of claims 4 to 8 for use in skin care.

10. The medicament composition of claim 9, wherein said skin care addresses skin wrinkling and sagging.

11. The medicament composition of any one of claims 4 to 9, wherein the tetrapeptide is useful to stimulate collagen production when applied to the skin.

12. The medicament composition of any one of claims 4 to 9 for use in treatment of a skin wound.

13. The medicament composition of claim 12, wherein said skin wound is a result of aging, disease, injury, trauma, or surgery.

14. Use of the tetrapeptide according to any one of claims 1 to 3, or a mixture of the tetrapeptide of SEQ ID NO: 5 and the tetrapeptide of SEQ ID NO: 8, to stimulate production of collagen by a collagen-producing cell.

15. The use of claim 14, wherein the collagen-producing cell is a fibroblast cell.

16. A cosmetic composition useful for maintaining healthy skin comprising the tetrapeptide according to any one of claims 1 to 3, and a cosmetically acceptable carrier.

17. The cosmetic composition of claim 16, wherein the tetrapeptide is present in concentration ranging from 0.10 μg/mL to 100 μg/mL.

18. The cosmetic composition of claim 16, wherein the composition is in the form of an aerosol, emulsion, liquid, lotion, cream, paste, ointment, or foam.

19. A cosmetic composition comprising a mixture of the tetrapeptides of SEQ ID NO: 5 and SEQ ID NO: 8.

20. The cosmetic composition of claim 19 which further comprises the tetrapeptides of SEQ ID NO: 7 and SEQ ID NO: 6.

21. The cosmetic composition of any one of claims 16 to 20 for use in skin care.

22. The composition of claim 21, wherein said skin care addresses skin wrinkling and sagging.

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGVI GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGPPGPP GIPGCNGTKG ERGPLGPPGL PGFAGNPGPP
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGTPGPPGLP GLQGPVGPPG
FTGPPGPPGP PGPPGEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEPGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEPGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEPGP KGFPGLPGQP GPPGLPVPGQ AGAPGFPGER GEKGDRGFPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYTNG IVECQPGPPG DQGPPGIPGQ
PGFIGEIGEK GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPGL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGHPGLP GPKGSPGSVG LKGERGPPGG VGFPGSRGDT
GPPGPPGYGP AGPIGDKGQA GFPGGPGSPG LPGPKGEPGK IVPLPGPPGA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGQ KGEPGVGLPG LKGLPGLPGI
PGTPGEKGSI GVPGVPGEHG AIGPPGLQGI RGEPGPPGLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGTPGQP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPGKD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPGLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGDK GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPGSDGIPG SAGEKGEPGL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPGLPGLP
GPMGPPGLPG IDGVKGDKGN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIIKGEPG LPGPEGPPGL KGLQGLPGPK GQQGVTGLVG IPGPPGIPGF
DGAPGQKGEM GPAGPTGPRG FPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQGNE RAHGQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCAV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT

FIG. 1

MMSFVQKGSW LLLALLHPTI ILAQQEAVEG GCSHLGQSYA DRDVWKPEPC
QICVCDSGSV LCDDIICDDQ ELDCPNPEIP FGECCAVCPQ PPTAPTRPPN
GQGPQGPKGD PGPPGIPGRN GDPGIPGQPG SPGSPGPPGI CESCPTGPQN
YSPQYDSYDV KSGVAVGGLA GYPGPAGPPG PPGPPGTSGH PGSPGSPGYQ
GPPGEPGQAG PSGPPGPPGA IGPSGPAGKD GESGRPGRPG ERGLPGPPGI
KGPAGIPGFP GMKGHRGFDG RNGEKGETGA PGLKGENGLP GENGAPGPMG
PRGAPGERGR PGLPGAAGAR GNDGARGSDG QPGPPGPPGT AGFPGSPGAK
GEVGPAGSPG SNGAPGQRGE PGPQGHAGAQ GPPGPPGING SPGGKGEMGP
AGIPGAPGLM GARGPPGPAG ANGAPGLRGG AGEPGKNGAK GE**PGPR**GERG
EAGIPGVPGA KGEDGKDGSP GEPGANGLPG AAGERGAPGF RGPAGPNGIP
GEKGPAGERG APGPAGPRGA AGEPGRDGVP GGPGMRGMPG SPGGPGSDGK
PGPPGSQGES GRPGPPGPSG PRGQPGVMGF PGPKGNDGAP GKNGERGGPG
GPGPQGPPGK NGETGPQGPP GPTGPGGDKG DTGPPGPQGL QGLPGTGGPP
GENGKPGEPG PKGDAGAPGA PGGKGDAGAP GERGPPGLAG APGLRG**GAGP**
PGPEGGKGAA GPPGPPGAAG TPGLQGMPGE RGGLGSPGPK GDKGEPGGPG
ADGVPGKDGP RGPTGPIGPP GPAGQPGDKG EGGAPGLPGI AGPRGSPGER
GETGPPGPAG FPGAPGQNGE PGGKGERGAP GEKGEGGPPG VAGPPGGSGP
AGPPGPQGVK GERGSPGGPG AAGFPGARGL PGPPGSNGNP GPPGPSGSPG
KDGPPGPAGN TGAPGSPGVS GPKGDAGQPG EKGSPGAQGP PGAPGPLGIA
GITGARGLAG PPGM**PGPR**GS PGPQGVKGES GKPGANGLSG ERGPPGPQGL
PGLAGTAGEP GRDGNPGSDG LPGRDGSPGG KGDRGENGSP GAPGAPGHPG
PPGPVGPAGK SGDRGESGPA GPAGAPGPAG SRGAPGPQGP RGDKGETGER
GAAGIKGHRG FPGNPGAPGS PGPAGQQGAI GSPGPAGPRG PVGPSGPPGK
DGTSGHPGPI GP**PGPR**GNRG ERGSEGSPGH PGQPGPPGPP GAPGPCCGGV
GAAAIAGIGG EKAGGFAPYY GDEPMDFKIN TDEIMTSLKS VNGQIESLIS
PDGSRKNPAR NCRDLKFCHP ELKSGEYWVD PNQGCKLDAI KVFCNMETGE
TCISANPLNV PRKHWWTDSS AEKKHVWFGE SMDGGFQFSY GNPELPEDVL
DVQLAFLRLL SSRASQNITY HCKNSIAYMD QASGNVKKAL KLMGSNEGEF
KAEGNSKFTY TVLEDGCTKH TGEWSKTVFE YRTRKAVRLP IVDIAPYDIG
GPDQEFGVDV GPVCFL

FIG. 2

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGVI GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGP**PGPP** GIPGCNGTKG ERGPLGPPGL PGFAGN**PGPP**
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGT**PGPP**GLP GLQGPVGPPG
FTGP**PGPP**GP **PGPP**GEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEPGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEPGYP GLIGRQGPQG EKGEAGP**PGP PG**IVIGTGPL GEKGERGYPG
TPGPRGEPGP KGFPGLPGQP GPPGLPVPGQ AGAPGFPGER GEKGDRGFPG
TSLPGPSGRD GL**PGPP**GSPG PPGQPGYTNG IVECQ**PGPP**G DQGPPGIPGQ
PGFIGEIGEK GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPGL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGHPGLP GPKGSPGSVG LKGERGPPGG VGFPGSRGDT
GP**PGPP**GYGP AGPIGDKGQA GFPGGPGSPG LPGPKGEPGK IVPL**PGPP**GA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG F**PGPP**GPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGQ KGEPGVGLPG LKGLPGLPGI
PGTPGEKGSI GVPGVPGEHG AIGPPGLQGI RGE**PGPP**GLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGTPGQP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPGKD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPGLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGDK GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPGSDGIPG SAGEKGEPGL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPGLPGLP
GPMGPPGLPG IDGVKGDKGN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GL**PGPP**GPPG
PYDIIKGEPG LPGPEGPPGL KGLQGLPGPK GQQGVTGLVG I**PGPP**GIPGF
DGAPGQKGEM GPAGPTGPRG F**PGPP**GPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQGNE RAHGQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCAV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT

FIG. 3

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGVI GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGPPGPP GIPGCNGTKG ERGPLGPPGL PGFAGNPGPP
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGTPGPPGLP GLQGPVGPPG
FTGPPGPPGP PGPPGEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEPGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEPGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEPGP KGFPGLPGQP GPPGLPVPGQ AGAPGFPGER GEKGDRGFPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYTNG IVECQPGPPG DQGPPGIPGQ
PGFIGEIGEK GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPGL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGHPGLP GPKGSPGSVG LKGERGPPGG VGFPGSRGDT
GPPGPPGYGP AGPIGDKGQA GFPGGPGSPG LPGPKGEPGK IVPLPGPPGA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGQ KGEPGVGLPG LKGLPGLPGI
PGTPGEKGSI GVPGVPGEHG AIGPPGLQGI RGEPGPPGLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGTPGQP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPGKD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPGLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGDK GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPGSDGIPG SAGEKGEPGL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPGLPGLP
GPMGPPGLPG IDGVKGDKGN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIIKGEPG LPGPEGPPGL KGLQGLPGPK GQQGVTGLVG IPGPPGIPGF
DGAPGQKGEM GPAGPTGPRG FPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQGNE RAHGQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCAV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT