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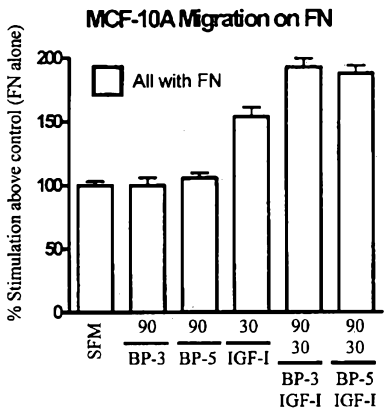

FIGURE 2

(57) **Abstract**: Isolated protein complexes are provided comprising growth factors such as IGF-I, IGF-II, EGF, bFGF, or KGF and fibronectin, or at least domains thereof that enable binding to and activation of both a growth factor receptor, and an integrin receptor-binding domain of fibronectin. These protein complexes include synthetic proteins where the growth factor and fibronectin sequences are joined by a linker sequence. Also provided are uses of these protein complexes for stimulating or inducing cell migration and/or proliferation in wound healing, tissue engineering, cosmetic and therapeutic treatments such as skin replacement, skin replenishment and treatment of burns where epithelial cell migration is required. In other embodiments, the invention provides inhibition of cancer cell metastasis, particularly in relation to breast cancer.

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TITLE

FIBRONECTIN: GROWTH FACTOR CHIMERAS

FIELD OF THE INVENTION

THIS INVENTION relates to protein complexes having respective domains that enable binding to and activation of both a growth factor receptor and an integrin receptor for fibronectin. In particular embodiments, this invention relates to chimeric proteins comprising growth factors such as insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or keratinocyte growth factor (KGF) receptor-binding domains and an integrin receptor-binding domain of fibronectin (FN). More particularly, this invention relates to protein complexes that stimulate cell migration and to compositions and methods that promote or induce cell migration and/or proliferation. These compositions and methods have use in wound healing, tissue engineering, cosmetic and therapeutic treatments such as skin replacement, and skin replenishment and treatment of burns where epithelial cell migration and/or proliferation is required. In other embodiments, the invention provides treatment provided by the present invention related to prevention or inhibition of cancer cell metastasis, particularly in relation to breast cancer.

BACKGROUND OF THE INVENTION

A number of peptide growth factors involved in a broad range of cellular processes including hyperplasia, DNA synthesis, differentiation, cell cycle progression, and inhibition of apoptosis are known, and include the insulin-like growth factors (IGFs, *e.g.*, IGF-I and IGF-II) (Jones & Clemmons, 1995, Endocrine Rev. **16** 3; Wood & Yee, 2000, J. Mammary Gland Biology and Neoplasia **5** 1), EGF (Heldin *et al.*, 1981, Science **4** 1122), bFGF (Taraboletti *et al.*, 1997, Cell Growth. Differ. **8** 471), and KGF (Marchese *et al.*, 1990, J. Cell Physiol. **144** 326). These effects are mediated through binding to their cognate tyrosine-kinase linked cell surface receptors, the type 1 IGF receptor (IGF-IR), EGF receptor, bFGF receptor, and KGF receptor, respectively. The IGFs are also tightly regulated by a family of specific binding proteins, termed IGFBPs, whose primary role is to bind free IGFs

and thereby moderate their half-life, specificity and activity (Clemmons, 1998, Mol. Cell. Endocrinol. **140** 19).

Fibronectin is a high molecular mass adhesive glycoprotein found in all vertebrates. Fibronectin plays a role in cell adhesion, cell morphology and surface architecture. It's main function seems to be its involvement in cellular migration during development, tissue repair and wound healing, regulation of cell growth, and differentiation (Alitalo & Vaheri, 1982, Adv. Cancer Res. **37** 111; Yamada, 1983, Annu. Rev. Biochem. **62** 761; Hynes, 1985, Annu. Rev. Cell Biol. **1** 67). Fibronectin polymorphism is due to alternative splicing patterns in three regions (ED-A, ED-B and IIICS) of the single fibronectin primary transcript (Petersen *et al.*, 1983, Proc. Natl. Acad. Sci. USA **80** 137; Schwarzbauer *et al.*, 1983, Cell **35** 421; Kornblihtt *et al.*, 1984, Nucleic Acids Res. **12** 5853). The exact composition of fibronectin depends on the tissue type and/or cellular conditions. In humans, there are potentially 20 different forms of fibronectin, most arising from alternative splicing of some type 3 modules (Potts and Campbell, 1994, Curr. Opin. Cell Biol. **6** 648). Expression of fibronectin splicing variants appears to be both developmentally regulated and tissue-specific.

Fibronectin has the ability to bind a number of extracellular molecules, including heparin, collagen and hyaluronic acid. Fibronectin organizes cell-cell interactions and cellular interaction with the extracellular matrix by binding to different components of the extracellular matrix and to membrane-bound fibronectin receptors (integrins) on cell surfaces.

However, the relative contributions of growth factors and fibronectin, and their respective domains, present in protein complexes, in terms of stimulating biological responses such as cell migration and/or proliferation, have remained elusive.

SUMMARY OF THE INVENTION

The present inventors have discovered that protein complexes in the form of synthetic chimeras comprising growth factors such as IGF-I, IGF-II, EGF, bFGF, or KGF and FN stimulate cell migration and/or proliferation by binding and

synergistically co-activating cognate growth factor receptors and FN-binding integrin receptors.

Therefore, the invention is broadly directed to isolated protein complexes that comprise a receptor-binding domain of a growth factor domain and at least a domain of fibronectin that is capable of binding an integrin receptor, wherein the isolated protein complex can co-activate the growth factor and integrin receptor to thereby elicit a biological response.

In a first aspect, the invention provides an isolated protein complex in the form of a synthetic chimeric protein comprising an amino acid sequence of:

(i) a growth factor, or at least a domain of a growth factor which is capable of binding a cognate growth factor receptor; and

(ii) fibronectin, or a fragment of fibronectin comprising at least an integrin-binding domain of fibronectin.

Preferably, according to the aforementioned aspects the growth factor is IGF-I, IGF-II, EGF, bFGF, or KGF.

Preferably, the integrin receptor is an α_1 or an α_4 integrin.

This aspect of the invention also contemplates an amino acid sequence of one or more additional fragments of fibronectin in the synthetic chimeric protein.

This aspect of the invention also includes within its scope amino acid deletions, additions, substitutions and/or mutations of amino acid sequences corresponding to (i) and (ii) above, as well as amino acid sequences corresponding to the one or more additional fragments of fibronectin.

In a second aspect, the invention provides an isolated nucleic acid encoding the isolated protein complex of the first aspect.

In a third aspect, the invention provides a genetic construct comprising the isolated nucleic acid of the second aspect operably linked to one or more regulatory sequences in an expression vector.

Preferably, the genetic construct is an expression construct.

In a fourth aspect, the invention provides a host cell comprising the genetic construct of the third aspect.

In a fifth aspect, the invention provides a pharmaceutical composition comprising the isolated protein complex of the first aspect and a pharmaceutically-acceptable carrier, diluent or excipient.

This aspect of the invention also contemplates a pharmaceutical composition comprising the host cell of the fourth aspect, which cell expresses said synthetic protein(s).

In a sixth aspect, the invention provides an antibody specific for the synthetic protein of the first aspect.

In a seventh aspect, the invention provides a method of promoting cell migration including the step of using a synthetic protein to bind both a growth factor receptor and an integrin receptor.

Preferably, the growth factor receptor is IGF-IR, EGF receptor, bFGF receptor, or KGF receptor.

Preferably, the integrin receptor is an α_1 or an α_4 integrin.

In a preferred embodiment, this aspect of the invention relates to promotion or induction of epithelial/keratinocyte/fibroblast cell migration and/or proliferation to facilitate wound healing in mammals, preferably humans.

Preferably, said synthetic protein is as according to the first aspect of the invention.

In an eighth aspect, the invention provides a method of preventing cell migration and/or proliferation, including the step of preventing, inhibiting or otherwise reducing binding of both a growth factor receptor and an integrin receptor by a complex comprising a growth factor and fibronectin.

Preferably, the growth factor receptor is IGF-IR, EGF receptor, bFGF receptor, or KGF receptor.

Preferably, the integrin receptor is an α_1 or an α_4 integrin.

In a preferred embodiment, this aspect of the invention relates to prevention or inhibition of metastatic cancer cell migration and/or proliferation in mammals, preferably humans.

A particular example contemplated by this aspect of the invention is prevention or inhibition of breast cancer metastasis.

It will also be appreciated that the methods of the seventh and eighth aspects may encompass prophylactic and therapeutic methods of treatment.

In a ninth aspect, the invention provides use of the isolated protein complex of the first aspect to produce a molecule that:

(i) is an agonist of protein complexes comprising a growth factor and fibronectin; or

(ii) is an antagonist of protein complexes comprising a growth factor and fibronectin.

In one embodiment, the invention provides use of the synthetic protein of the first aspect to produce a molecule that:

(i) is an agonist of IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGF-I:IGFBP:FN protein complexes; or

(ii) is an antagonist of IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGF-I:IGFBP:FN protein complexes.

Agonists and/or antagonists produced according to this aspect of the invention may have particular efficacy in promoting wound healing, tissue engineering, skin regeneration and/or prevention of cancer cell metastasis or hyperproliferative disorders of the skin, such as scarring and psoriasis.

In a tenth aspect, the invention provides a biomaterial that comprises the isolated protein complex of the first aspect.

In particular embodiments the biomaterial may be a surgical implant, prosthesis, scaffold, wound or burn dressing, or the like suitably impregnated, coated or otherwise comprising an isolated protein complex of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Amino acid sequence of (A) human fibronectin (SEQ ID NO:1), (B) mature IGF-I (SEQ ID NO:2), (C) mature IGF-II (SEQ ID NO:3), (D) mature EGF (SEQ ID NO:4), (E) mature bFGF (SEQ ID NO:5), (F) mature KGF (SEQ ID NO:6), and (G) preferred linker sequences (SEQ ID NOs:7-12).

Figure 2. IGF-I, IGFBP and FN protein complexes stimulate breast cancer cell migration. MCF-10A cells were seeded onto Transwells that had been coated with FN (1 μg/mL) and increasing concentrations of IGF-I prebound in the presence

of IGFBP-3 or -5. The cells where allowed to migrate for 5 hours. The number of cells traversing the membrane in response to each treatment was then expressed as a percentage of those that migrated on FN only (SFM). MCF-10 data are pooled from three experiments with treatments tested in four wells in each replicate experiment. Error bars indicate SEM. SFM = Serum-free media.

DETAILED DESCRIPTION OF THE INVENTION

The present invention has arisen from the discovery that synthetic chimeras comprising growth factors such as IGF-I, IGF-II, EGF, bFGF, or KGF and FN bind and exert their biological effect on cell migration through their cognate growth factor receptors and the FN-binding integrin receptor expressed by responsive cells. More particularly, this dual binding event synergistically stimulates cell migration and/or proliferation. These stable, biologically active single-chain chimeric molecules comprise at least the minimal domain or region of a growth factor capable of binding its cognate receptor in combination with one or more type-III domains of FN comprising at least an integrin-binding domain of FN.

This discovery has led the present inventors to provide an isolated protein complex that comprises at least the minimal domain or region of IGF-I, IGF-II, EGF, bFGF, or KGF, for example, capable of binding their cognate receptors in combination with the integrin-binding domain of FN. Even more particularly, a single, contiguous protein may be produced which comprises these domains.

Such protein complexes, in the form of a single synthetic protein, coordinately bind or co-ligate their cognate receptors and the FN-binding integrin receptor and are therefore useful agents for the promotion of cell migration and/or proliferation and wound healing. Analogously, prevention of cognate receptor and FN-binding integrin receptor co-ligation can be used to prevent cancer cell metastasis.

Throughout this specification, unless otherwise indicated, "*comprise*", "*comprises*" and "*comprising*" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

In the particular context of growth factor receptor-binding domains and integrin-binding domains, such a domain will comprise an amino acid sequence of the domain, together with other, additional amino acids as desired.

It will be understood also that such a domain may "*consist essentially of*" the amino acid sequence of the domain, together with no more than ten, preferably no more than five or even more preferably no more than four, three, two, or one additional amino acids.

It will be understood also that such a domain may "*consist of*" the amino acid sequence of the domain, in the absence of any additional amino acids.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

As used herein, by "*synthetic*" is meant not naturally occurring but made through human technical intervention. In the context of synthetic proteins and nucleic acids, this encompasses molecules produced by recombinant, chemical synthetic or combinatorial techniques as are well understood in the art.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art. The term "*protein*" also includes and encompasses such terms as "*glycoprotein*", "*lipoprotein*" and the like, as are commonly used in the art.

A "*peptide*" is a protein having less than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

As hereinbefore described, the present invention provides, in one particular aspect, an isolated protein complex in the form of a synthetic chimeric protein comprising an amino acid sequence of:

(i) a growth factor, or at least a domain of a growth factor which is capable of binding a cognate growth factor receptor; and

(ii) fibronectin, or a fragment of fibronectin comprising at least an integrin-binding domain of fibronectin.

As used herein, a "*chimeric protein*", comprises a contiguous sequence of amino acids, the amino acids derived from an integrin receptor-binding domain of fibronectin, optionally, additional domains of fibronectin, and a growth factor or at least a receptor-binding domain of a growth factor.

As used herein, a "*growth factor*" is a biologically active protein that is capable of regulating cell growth, differentiation, survival and/or migration *in vitro* and/or *in vivo*.

Exemplary growth factors include, but are not limited to, IGFs (Jones & Clemmons, 1995, Endocrine Rev. **16** 3; Wood & Yee, 2000, J. Mammary Gland Biology and Neoplasia **5** 1; Keiss *et al.*, 1994, Hormone Research **41** 66), such as IGF-I (UniProtKB/Swiss-Prot: #P05019, mature protein comprises amino acid residues 49-118 of the complete sequence) and IGF-II (UniProtKB/Swiss-Prot: #P01344, mature protein comprises amino acid residues 25-91 of the complete sequence), VEGF (Neufeld *et al.*, 1999, FASEB J. **13** 9-22), PDGF (Heldin, 1992, EMBO J. **11** 4251-4259), EGF (Heldin *et al.*, 1981, Science **4** 1122-1123; UniProtKB/Swiss-Prot: #P01133, mature protein comprises amino acid residues 971-1023 of the complete sequence), fibroblast growth factor (FGF; Nurcombe *et al.*, 2000, J. Biol. Chem. **275** 30009-30018), bFGF (Taraboletti *et al.*, 1997, Cell Growth. Differ. **8** 471-479; UniProtKB/Swiss-Prot: #P09038, mature protein comprises amino acid residues 143-288 of the complete sequence), osteopontin (Nam *et al.*, 2000, Endocrinol. **141** 1100), thrombospondin-1 (Nam *et al.*, 2000, *supra*), tenascin-C (Arai *et al.*, 1996, J. Biol. Chem. **271** 6099), PAI-1 (Nam *et al.*, 1997, Endocrinol. **138** 2972), plasminogen (Campbell *et al.*, 1998, Am. J. Physiol. **275** E321), fibrinogen (Campbell *et al.*, 1999, J. Biol. Chem **274** 30215), fibrin (Campbell *et al.*, 1999, *supra*), transferrin (Weinzimer *et al.*, 2001, J. Clin. Endocrinol. Metab. **86** 1806), and KGF (Marchese *et al.*, 1990, J. Cell Physiol. **144** 326-32; UniProtKB/Swiss-Prot: #P21781, mature protein comprises amino acid residues 32-194 of the complete sequence).

Isolated protein complexes in the form of synthetic chimeric proteins of the invention comprise a growth factor or at least a domain of a growth factor of a growth factor which is capable of binding a cognate growth factor receptor.

In this context, by "*domain*" is meant at least that portion or region of a growth factor that is capable of binding a cognate growth factor receptor. Typically, although not exclusively, the cognate growth factor receptor is expressed by a cell and binding or ligation of said cognate growth factor receptor by said at least a domain of a growth factor elicits a cellular response such as cell growth, differentiation, survival and/or migration.

With particular regard to IGF-I, said domain suitably comprises amino acid residue 24, which is not a leucine residue.

Typically, said residue is tyrosine.

With particular regard to IGF-II, said domain suitably comprises amino acid residue 27, which is not a leucine residue.

Typically, said residue is tyrosine.

With particular regard to IGF-I, in one embodiment said domain consists of residues 1 to 70 of IGF-I.

In another embodiment, said domain consists of residues 4 to 70 of IGF-I.

It will also be understood that another component of isolated protein complexes of the invention is at least an integrin-binding domain of fibronectin.

Preferably, the integrin receptor is an α_1 or an α_4 integrin.

Although not wishing to be bound by any particular theory, it is proposed that synthetic chimeric proteins are able to co-ligate and co-activate a cognate receptor for said growth factor and an integrin receptor for fibronectin to thereby stimulate, induce, augment, or otherwise promote cell migration.

An advantage of chimeric proteins according to the invention is that they are readily produced by chemical synthetic or recombinant means and are expected to be more stable *in vivo*, as they do not rely on maintaining the protein-protein interactions that are required in non-covalently associated oligo-protein complexes.

In this regard, although isolated protein complexes that comprise receptor-binding domains of IGF-I would also comprise an IGFBP, it is proposed that

according to the aforementioned mode of action, an IGFBP is preferably not present in an IGF-I/FN synthetic chimera.

In other embodiments, the invention provides isolated protein complexes, such as in the form of synthetic chimeric proteins, comprising IGF-I, IGF-II, EGF, bFGF, or KGF and FN, or a fragment of FN that comprises at least an integrin-binding domain of FN.

Preferably, the integrin receptor is an α_1 or an α_4 integrin receptor.

In this context, by "*fragment*" is meant a domain, sub-sequence or portion of fibronectin. The fragment preferably constitutes less than 500, less than 400, less than 300 or more preferably about 80-280 contiguous amino acids of a mature fibronectin sequence. Multiple fragments of fibronectin are also contemplated.

The integrin-binding domain of fibronectin suitably comprises an RGD sequence. The RGD sequence is located in fibronectin type III domains 8 to 10 (amino acids 1266-1536 of the fibronectin sequence). More specifically, the RGD sequence is present in the fibronectin type III domain 10, defined by amino acids 1447-1536 of the fibronectin sequence, although secondary integrin-binding sites may be present across the larger 8 to 10 domain region.

Accordingly, in one particular embodiment, the synthetic chimera comprises a fibronectin fragment comprising an RGD sequence, wherein the fragment comprises or consists of at least 6, at least 10, at least 20, at least 50, at least 60, at least 70, at least 80, or all of amino acids 1447-1536 of a fibronectin amino acid sequence.

In another particular embodiment, the synthetic chimera comprises a fibronectin fragment comprising an RGD sequence, said fragment comprising or consisting of an amino acid sequence of at least 6, at least 10, at least 20, at least 50, at least 100, at least 150, at least 200, at least 250, at least 260, or all of amino acids 1266-1536 of a fibronectin amino acid sequence.

In yet another particular embodiment, the synthetic chimera comprises a fibronectin fragment comprising an RGD sequence according to the aforementioned embodiments, wherein said synthetic chimera further comprises at least 10, 20, 50, 100, 200, 300, 500, 800, or 1000 amino acids of a fibronectin amino acid sequence, for example N-terminal of residue 1266 and/or C-terminal of residue 1536. Thus, said synthetic chimera can include fibronectin type I and/or type II domains, such as,

for example, a fibronectin fragment comprising or consisting of at least 6, at least 10, at least 20, at least 50, at least 100, at least 150, at least 200, or all of amino acids 50-273 of a fibronectin amino acid sequence.

In still another particular embodiment, the synthetic chimera comprises a fibronectin fragment comprising or consisting of an amino acid sequence of at least 6, at least 10, at least 20, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, or all of amino acids 1173 to 1536 of a fibronectin amino acid sequence.

It will be appreciated that the foregoing fibronectin sequence numbering is made with reference to the fibronectin sequence shown in FIG. 1. This fibronectin sequence is derived from the UniProtKB Protein Database, protein accession number P02751. Fibronectin domains and regions are set forth in Table I.

Preferably, synthetic chimeras comprising fibronectin or a fragment comprising an integrin-binding domain do not comprise an IGFBP amino acid sequence.

Preferably, synthetic chimeric proteins as hereinbefore described further comprise a "*linker sequence*" located between and contiguous with a growth factor sequence and a fibronectin amino acid sequence.

In one embodiment, said linker sequence comprises one or more glycine residues and one or more serine residues.

Particular examples of linker sequences may be selected from; Gly_4 Ser (SEQ ID NO:7); Gly_4 Ser_3 (SEQ ID NO:8); $(Gly_4$ $Ser)_3$ (SEQ ID NO:9); and $(Gly_4$ $Ser)_4$ (SEQ ID NO:10), although without limitation thereto.

In another embodiment, the linker sequence includes a Plasmin Cleavage Recognition Site (Sakiyama-Elbert *et al.*, 2001, FASEB **15** 1300), such as according to the sequence:

Leu Ile Lys Met Lys Pro (SEQ ID NO:11)

In yet another embodiment, the linker sequence includes a Collagenase-3 Cleavage Recognition Site (Kim & Healy, 2003, Biomacromolecules **4** 1214), such as according to the sequence:

Gln Pro Gln Gly Leu Ala Lys (SEQ ID NO:12)

The invention also extends to use of biologically-active fragments of the synthetic chimeric proteins of the invention and/or to use of biologically-active fragments of the particular growth factor receptor-binding domains and integrin-binding domains exemplified herein.

In one embodiment, said "*biologically-active fragment*" has no less than 10%, preferably no less than 25%, more preferably no less than 50% and even more preferably no less than 75%, 80%, 85%, 90%, or 95% of a biological activity of a protein from which it is derived.

In another embodiment, said "*biologically-active fragment*" has no less than 10%, preferably no less than 25%, more preferably no less than 50% and even more preferably no less than 75%, 80%, 85%, 90%, or 95% of a contiguous amino acid sequence of a protein from which it is derived.

Also contemplated are variant protein complexes of the invention.

Typically, and in relation to proteins, a "*variant*" protein has one or more amino acids that have been replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the protein (conservative substitutions).

It will be appreciated that one or more amino acid residues of a reference sequence, such as a growth factor, receptor-binding domain of a growth factor, an integrin-binding domain of fibronectin, IGFBPs, or one or more corresponding residues present in a synthetic chimeric protein, may be modified or deleted, or additional sequences added, without substantially altering the biological activity of the isolated protein complex of the invention.

In one embodiment, a protein variant shares at least 70%, preferably at least 80% or 85% and more preferably at least 90%, 95%, 98%, or 99% sequence identity with a reference amino acid sequence.

Preferably, sequence identify is measured over at least 60%, more preferably over at least 75%, more preferably over at least 90% or more preferably over at least 95%, 98% or substantially the full length of the reference sequence.

In order to determine percent sequence identity, optimal alignment of amino acid and/or nucleotide sequences may be conducted by computerised

implementations of algorithms (Geneworks program by Intelligenetics; GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, Nucl. Acids Res. **25** 3389.

In another example, "sequence identity" may be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA).

A detailed discussion of sequence analysis can be found in Unit 19.3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons Inc NY, 1995-1999).

The invention also contemplates derivatives of a receptor-binding domain of a growth factor, an integrin-binding domain of fibronectin or an isolated protein complex comprising the same.

As used herein, "*derivative*" proteins of the invention have been altered, for example by addition, conjugation or complexing with other chemical moieties or by post-translational modification techniques as are well understood in the art

"*Additions*" of amino acids may include fusion of the polypeptides or variants thereof with other polypeptides or proteins. The other protein may, by way of example, assist in the purification of the protein. For instance, these include a polyhistidine tag, maltose binding protein, green fluorescent protein (GFP), Protein A, or glutathione S-transferase (GST).

Other derivatives contemplated by the invention include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the polypeptides, fragments and variants of the invention. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride, acylation of amino groups with

succinic anhydride and tetrahydrophthalic anhydride, amidination with methylacetimidate, carbamoylation of amino groups with cyanate, pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$, reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivitization, by way of example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid, formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials, formation of a mixed disulphides with other thiol compounds, reaction with maleimide, maleic anhydride or other substituted maleimide, carboxymethylation with iodoacetic acid or iodoacetamide, and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides, or by oxidation with N-bromosuccinimide.

Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

Examples of incorporating non-natural amino acids and derivatives during peptide synthesis include, but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline,

phenylglycine, ornithine, sarcosine, 2-thienyl alanine, and/or D-isomers of amino acids.

An example of methods suitable for chemical derivatization of proteins is provided in Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et. al.*, John Wiley & Sons NY (1995-2001).

Isolated protein complexes, and individual protein components thereof, (inclusive of fragments, variants, derivatives, and homologs) may be prepared by any suitable procedure known to those of skill in the art.

In one embodiment, proteins of the invention are produced by chemical synthesis. Chemical synthesis techniques are well known in the art, although the skilled person may refer to Chapter 18 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et. al.*, John Wiley & Sons NY (1995-2001) for examples of suitable methodology.

In another embodiment, proteins may be prepared as recombinant proteins.

While production of recombinant proteins is well known in the art, the skilled person may refer to standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), in particular Sections 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. 1995-1999), in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. 1995-1999), in particular Chapters 1, 5 and 6.

In one embodiment, a recombinant protein is produced by a method including the steps of:

(i) preparing an expression construct which comprises a nucleic acid encoding said protein, operably linked to one or more regulatory nucleotide sequences in an expression vector;

(ii) transfecting or transforming a host cell with the expression construct; and

(iii) expressing the recombinant protein in said host cell.

An "*expression vector*" may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome.

By "*operably linked*" or "*operably connected*" is meant that said regulatory nucleotide sequence(s) is/are positioned relative to the recombinant nucleic acid of the invention to initiate, regulate or otherwise control transcription of the nucleic acid, or translation of a protein encoded by the nucleic acid.

Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells.

Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, splice donor/acceptor sequences, and enhancer or activator sequences.

Constitutive promoters (such as CMV, RSV, adenovirus, SV40, and human elongation factor promoters) and inducible/repressible promoters (such as *tet*-repressible promoters and IPTG-, metallothionine- or ecdysone-inducible promoters) are well known in the art and are contemplated by the invention. It will also be appreciated that promoters may be hybrid promoters that combine elements of more than one promoter.

The expression construct may also include a fusion partner (typically provided by the expression vector) so that the recombinant protein of the invention is expressed as a fusion polypeptide with said fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of said fusion protein.

Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP), and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion protein by affinity chromatography. For the purposes of fusion protein purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners and the Pharmacia GST purification system.

In some cases, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion protein of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated protein can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, haemagglutinin and FLAG tags.

Suitable host cells for expression may be prokaryotic or eukaryotic, such as *Escherichia coli* (DH5α for example), yeast cells, Sf9 cells utilized with a baculovirus expression system, CHO cells, COS, CV-1, NIH 3T3, and 293 cells, although without limitation thereto.

Expression constructs may also include one or more selection marker nucleotide sequences that confer transformed host cell resistance to a selection agent. Selection markers useful for the purposes of selection of transformed bacteria include *bla*, *kanR* and *tetR*, while transformed eukaryotic cells may be selected by markers such as hygromycin, G418 and puromycin, although without limitation thereto.

With regard to introducing genetic material into host cells, the terms "*transforming*" and "*transfecting*" are used generally to describe introduction of genetic material into a host cell. There are many well known methods for introducing foreign genetic material into a host cell including, but not limited to, calcium phosphate precipitation, electroporation, delivery by lipofectamine, lipofectin and other lipophilic agents, calcium phosphate precipitation, DEAE-Dextran transfection, microparticle bombardment, microinjection, and protoplast fusion.

The invention provides an isolated nucleic acid that encodes a synthetic chimeric protein of the invention, including variants and homologs thereof.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNAi, and DNA, inclusive of cDNA and genomic DNA and DNA-RNA hybrids.

A "*polynucleotide*" is a nucleic acid having eighty (80) or more contiguous nucleotides, while an "*oligonucleotide*" has less than eighty (80) contiguous nucleotides.

A "*probe*" may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

A "*primer*" is usually a single-stranded oligonucleotide, preferably having 15-50 contiguous nucleotides, which is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

Synthetic nucleic acids of the invention may be produced by chemical synthetic approaches or by recombinant methods that utilize nucleic acid sequence amplification techniques, or a combination thereof, as are well known in the art.

Chemically synthesized primers and oligonucleotides, synthesizers and associated technologies useful according to the present invention are typically available in most laboratories or may be purchased from commercial sources.

Suitable nucleic acid amplification techniques are well known to the skilled person, and include polymerase chain reaction (PCR) and ligase chain reaction (LCR) as for example described in Chapter 15 of Ausubel *et al. supra*; strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252; rolling circle replication (RCR) as for example described in Liu *et al.*, 1996, J. Am. Chem. Soc. **118** 1587, International application WO 92/01813 and International Application WO 97/19193; nucleic acid sequence-based amplification (NASBA) as for example described by Sooknanan *et al.*,1994, Biotechniques **17** 1077; and Q-β replicase amplification as for example described by Tyagi *et al.*, 1996, Proc. Natl. Acad. Sci. USA **93** 5395, although without limitation thereto.

A preferred nucleic acid sequence amplification technique is PCR.

As used herein, an "*amplification product*" refers to a nucleic acid product generated by a nucleic acid amplification technique.

In producing and expressing nucleic acids of the invention, it will also be appreciated that advantage may be taken with respect to codon sequence redundancy,

such that the nucleic acids exemplified herein may be readily modified without changing an amino acid sequence encoded thereby.

In particular embodiments, nucleic acids may be optimized according to preferred "*codon usage*" of a host cell to be used for recombinant expression, as is well known in the art. This can effectively "tailor" a nucleic acid for optimal expression in a particular organism, or cells thereof, where preferential codon usage affects protein expression.

Therefore, the invention includes synthetic nucleic acids that are homologous to the nucleic acids exemplified herein.

In one embodiment, nucleic acid homologs share at least 70%, preferably at least 80%, more preferably at least 90%, and even more preferably at least 95% sequence identity with a nucleic acid encoding any one of the synthetic chimeric protein constructs described herein.

Preferably, sequence identity is measured over at least 70%, more preferably at least 80%, even more preferably at least 90%, 95% or advantageously over substantially the full length of the encoding nucleic acid of the invention.

In another embodiment, nucleic acid homologs hybridize to a nucleic acid encoding any one of the synthetic chimeric protein constructs described herein under high stringency conditions.

"*Hybridize*" and "*Hybridization*" are used herein to denote the pairing of at least partly complementary nucleotide sequences to produce a DNA-DNA, RNA-RNA or DNA-RNA duplex. Hybridized sequences occur through base-pairing between complementary purines and pyrimidines as is well known in the art.

In this regard, it will be appreciated that modified purines (for example, inosine, methylinosine and methyladenosine) and modified pyrimidines (thiouridine and methylcytosine) may also engage in base pairing.

"*Stringency*" as used herein, refers to temperature and ionic strength conditions, and presence or absence of certain organic solvents and/or detergents during hybridisation. The higher the stringency, the higher will be the required level of complementarity between hybridizing nucleotide sequences.

"*Stringent conditions*" designates those conditions under which only nucleic acid having a high frequency of complementary bases will hybridize.

Reference herein to high stringency conditions includes and encompasses:

(i) from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridisation at 42°C, and at least about 0.01 M to at least about 0.15 M salt for washing at 42°C;

(ii) 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS for hybridization at 65°C, and (a) 0.1 x SSC, 0.1% SDS; or (b) 0.5% BSA, 1mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 1% SDS for washing at a temperature in excess of 65°C for about one hour; and

(iii) 0.2 x SSC, 0.1% SDS for washing at or above 68°C for about 20 minutes.

In general, washing is carried out at T_m = 69.3 + 0.41 (G + C) % -12°C. In general, the T_m of a duplex DNA decreases by about 1°C with every increase of 1% in the number of mismatched bases.

Notwithstanding the above, stringent conditions are well known in the art, such as described in Chapters 2.9 and 2.10 of. Ausubel *et al.*, *supra* and in particular at pages 2.9.1 through 2.9.20.

The invention also contemplates antibodies against a synthetic chimeric protein of the invention, inclusive of chimeric proteins, or fragments, variants and/or derivatives thereof. Antibodies of the invention may be polyclonal or monoclonal. Well-known protocols applicable to antibody production, purification and use may be found, for example, in Chapter 2 of Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY (John Wiley & Sons NY, 1991-1994) and Harlow, E. & Lane, D. Antibodies: A Laboratory Manual, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988.

Generally, antibodies of the invention bind to or conjugate with a polypeptide, fragment, variant or derivative of the invention. For example, the antibodies may comprise polyclonal antibodies. Such antibodies may be prepared for example by injecting a polypeptide, fragment, variant or derivative of the invention into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are described for example in Coligan *et*

al., CURRENT PROTOCOLS IN IMMUNOLOGY, supra, and in Harlow & Lane, 1988, supra.

In lieu of the polyclonal antisera obtained in the production species, monoclonal antibodies may be produced using the standard method as for example, described by Köhler & Milstein (1975, Nature **256**, 495), or by more recent modifications thereof as, for example, described in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, supra by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the polypeptides, fragments, variants or derivatives of the invention.

The invention also includes within its scope antibodies which comprise Fc or Fab fragments of the polyclonal or monoclonal antibodies referred to above. Alternatively, the antibodies may comprise single chain Fv antibodies (scFvs) against the proteins of the invention. Such scFvs may be prepared, for example, in accordance with the methods described respectively in U.S. Patent 5,091,513, European Patent 239,400 or the article by Winter & Milstein (1991, Nature **349** 293).

Labels may be associated with the antibody or antibody fragment.

The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu^{34}), a radioisotope, and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

A large number of enzymes useful as labels are disclosed in U.S. Patent 4,366,241, U.S. Patent 4,843,000 and U.S. Patent 4,849,338. Enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, b-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase, and the like. The enzyme label may be used alone or in combination with a second enzyme in solution.

By way of example, the fluorophore may be fluorescein isothiocyanate (FITC), oregon green, tetramethylrhodamine isothiocyanate (TRITL), allophycocyanin (APC), and R-Phycoerythrin (RPE), although without limitation thereto.

The invention also provides pharmaceutical compositions that comprise an isolated protein complex of the invention, inclusive of variants and derivatives thereof.

Such isolated protein complex may be in any form, inclusive of synthetic chimeric proteins of the invention, although without limitation thereto.

Pharmaceutical compositions of the invention may be used to promote or otherwise facilitate cell migration, tissue regeneration and wound healing. Alternatively, pharmaceutical compositions may be administered to prevent tumour metastasis by preventing or inhibiting tumour cell migration to a secondary site.

The composition may be used in therapeutic or prophylactic treatments as required. For example, pharmaceutical compositions may be applied in the form of therapeutic or cosmetic preparations for skin repair, wound healing, healing of burns and other dermatological treatments.

In this regard, pharmaceutical compositions may be administered in association with, or as a component of, a biomaterial, biopolymer, inorganic material such as hydroxyapatite or derivates thereof, surgical implant, prosthesis, wound or burn dressing, compress, bandage, or the like suitably impregnated, coated or otherwise comprising the pharmaceutical composition.

Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

Preferably, the pharmaceutically-acceptable carrier, diluent or excipient is suitable for administration to mammals, and more preferably, to humans.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates, and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991).

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal, and the like may be employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches, and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids, and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated, inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

With regard to pharmaceutical compositions for wound healing, particular reference is made to U.S. Patent 5,936,064 and International Publication WO 99/62536.

Pharmaceutical compositions of the invention may also include expression vectors such as viral vectors such as vaccinia, and viral vectors useful in gene therapy. The latter include adenovirus and adenovirus-associated viruses (AAV) such as described in Braun-Falco *et al.* (1999, Gene Ther. **6** 432), retroviral and lentiviral

vectors such as described in Buchshacher *et al.* (2000, Blood **95** 2499) and vectors derived from herpes simplex virus and cytomegalovirus. A general overview of viral vectors useful in endocrine gene therapy is provided in Stone *et al.* (2000, J. Endocrinol. **164** 103).

The present invention may also utilize specific expression vectors which target gene expression to epidermal cells, such as described in U.S. Patent 5,958,764 and for *in vivo* wound healing applications, such as described in U.S. Patent 5,962,427.

The invention provides methods of treatment using isolated protein complexes, inclusive of synthetic chimeric proteins of the invention. These methods are particularly aimed at therapeutic and/or prophylactic treatment of mammals, and more particularly, humans.

However, therapeutic uses according to the invention may also be applicable to mammals such as domestic and companion animals, performance animals such as horses, camels and greyhounds, livestock, laboratory animals and animals used as sources of cells, organs and tissues for xenotransplantation.

The invention also contemplates methods of cosmetic treatment where isolated protein complexes, inclusive of synthetic chimeric proteins of the invention, are administered to improve or enhance skin quality or skin appearance.

Such treatments may include prevention or remediation of skin disorders such as psoriasis and hypertrophic scarring that result from aberrant skin cell proliferation.

Alternatively, methods of treatment are contemplated whereby tumour metastasis is prevented or inhibited by blocking tumour cell migration to a secondary site. In addition, methods of treating cancer by blocking cell proliferation also contemplated.

In particular embodiments, therapeutic and/or prophylactic treatments may utilize an isolated protein complex, inclusive of synthetic chimeric proteins of the invention, in association with, or as a component of, a biomaterial, biopolymer, inorganic material such as fluorohydroxyapatite, surgical implant, prosthesis, wound or burn dressing, compress, bandage, or the like suitably impregnated, coated or otherwise comprising the isolated protein complex.

Such methods include administration of pharmaceutical compositions as hereinbefore defined, and may be by way of microneedle injection into specific tissue sites, such as described in U.S. Patent 6,090,790, topical creams, lotions or sealant dressings applied to wounds, burns or ulcers, such as described in U.S. Patent 6,054,122 or implants which release the composition such as described in International Publication WO 99/47070.

Gene therapy is also applicable in this regard, such as according to methods set forth in U.S. Patent 5,929,040 and U.S. Patent 5,962,427.

There also exist methods by which skin cells can be genetically modified for the purpose of creating skin substitutes, such as by genetically engineering desired growth factor expression (Supp *et al.*, 2000, J. Invest. Dermatol. **114** 5). An example of a review of this field is provided in Bevan *et al.* (Biotechnol. Gent. Eng. Rev. **16** 231).

Also contemplated is "seeding" a recipient with transfected or transformed cells, such as described in International Publication WO 99/11789.

These methods can be used to stimulate cell migration and thereby facilitate or progress wound and burn healing, repair of skin lesions such as ulcers, tissue replacement and grafting such as by *in vitro* culturing of autologous skin, re-epithelialization of internal organs such as kidney and lung and repair of damaged nerve tissue.

Skin replacement therapy has become well known in the art, and may employ use of co-cultured epithelial/keratinocyte cell lines, for example as described in Kehe *et al.* (1999, Arch. Dermatol. Res. **291** 600) or *in vitro* culture of primary (usually autologous) epidermal, dermal and/or keratinocyte cells. These techniques may also utilize engineered biomaterials and synthetic polymer "scaffolds".

Examples of reviews of the field in general are provided in Terskikh & Vasiliev (1999, Int. Rev. Cytol. **188** 41) and Eaglestein & Falanga (1998, Cutis **62** 1).

More particularly, the production of replacement oral mucosa useful in craniofacial surgery is described in Izumi *et al.* (2000, J. Dent. Res. **79** 798). Fetal keratinocytes and dermal fibroblasts can be expanded *in vitro* to produce skin for grafting to treat skin lesions, such as described in Fauza *et al.* (J. Pediatr. Surg. **33** 357), while skin substitutes from dermal and epidermal skin elements cultured *in*

vitro on hyaluronic acid-derived biomaterials have been shown to be potentially useful in the treatment of burns (Zacchi *et al.*, 1998, J. Biomed. Mater. Res. **40** 187).

Polymer scaffolds are also contemplated for the purpose of facilitating replacement skin engineering, as for example described in Sheridan *et al.* (2000, J. Control Release **14** 91) and Fauza *et al.* (1998, *supra*), as are microspheres as agents for the delivery of skin cells to wounds and burns (LaFrance & Armstrong, 1999, Tissue Eng. **5** 153).

The invention contemplates use of isolated protein complexes, inclusive of synthetic chimeric proteins of the invention, to identify, screen, design or otherwise produce agonists or antagonists of complexes comprising a growth factor and fibronectin, such as IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGF-I:IGFBP:FN complexes. Such agents may be a "*mimetic*". The term "*mimetic*" is used herein to refer to molecules that are designed to resemble particular functional regions of proteins or peptides, and includes within its scope the terms "*agonist*", "*analogue*" and "*antagonist*" as are well understood in the art.

In one embodiment, agonists are produced that mimic the binding of the cognate growth factor receptors and FN receptors by IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGF-I:IGFBP:FN complexes. Such molecules may have utility as stimulators of cell migration such as required for wound healing, skin regeneration and the like.

In another embodiment, antagonists are produced that prevent or inhibit the binding of the cognate growth factor receptors and integrin receptors by IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGFII:IGFBP:FN complexes. Such molecules may have utility as inhibitors of cell migration and/or cell proliferation and thereby constitute useful anti-tumour agents and also in treatments of skin disorders such as psoriasis and hypertrophic scarring that result from aberrant cell proliferation.

The aforementioned mimetics, agonists, antagonists, and analogues may be peptides, polypeptides or other organic molecules, preferably small organic molecules, with a desired biological activity and half-life.

Computer-assisted structural database searching is becoming increasingly utilized as a procedure for identifying mimetics. Database searching methods which, in principle, may be suitable for identifying mimetics, may be found in International

Publication WO 94/18232 (directed to producing HIV antigen mimetics), U.S. Patent 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics).

Other methods include a variety of biophysical techniques which identify molecular interactions. These allow for the screening of candidate molecules according to whether said candidate molecule affects formation of IGF-I:FN, IGF-II:FN, EGF:FN, bFGF:FN, KGF:FN, or IGF-IGFBP-FN complexes, for example. Methods applicable to potentially useful techniques such as competitive radioligand binding assays (see, Upton *et al.*, 1999, *supra* for a relevant method), analytical ultracentrifugation, microcalorimetry, surface plasmon resonance, and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997).

So that the present invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

EXAMPLE 1

IGF-I, IGFBPs and FN stimulate cell migration

MCF-10A cells were seeded onto Transwells that had been coated with FN (1 ug/mL) and increasing concentrations of IGF-I prebound in the presence of IGFBP-3 or -5. The cells where allowed to migrate for 5 hours. The number of cells traversing the membrane in response to each treatment was then expressed as a percentage of those that migrated on FN only (SFM). MCF-10 data are pooled from three experiments with treatments tested in four wells in each replicate experiment and shown in FIG. 2. Error bars indicate SEM. SFM = Serum-free media. IGF-I:FN, IGF-I:IGFBP-3:FN and IGF-I:IGFBP-5:FN were able to stimulate significantly increased migration above that of FN alone control wells (responses of 153.7 +/- 7.3%, 192.5 +/- 6.8% and 187.5 +/- 6.5% of the FN control wells, respectively) ($p<0.05$). The response of the MCF7-10A cells to IGF-I:IGFBP-3:FN and IGF-I:IGFBP-5:FN treatments was also significantly greater than those obtained with either IGFBP or IGF-I alone with FN ($p<0.05$). This data indicates that maximal responses occur

when the trimeric IGF-I:IGFBP-3/5:FN complexes are present. This suggests that chimeras containing IGF-I linked to FN activate the FN binding integrins and the cognate growth factor receptor.

EXAMPLE 2

Synthetic chimeric fibronectin:growth factor proteins

Provided herein are examples of synthetic chimeric proteins of the invention, in the form of FN:growth factor (*e.g.*, IGF-I, IGF-II, EGF, bFGF, and KGF) chimeras.

The synthetic chimeric proteins include any full-length or truncated forms of FN fused with a growth factor, with or without amino acid residue modifications. In addition, FN and the growth factors may be fused with or without the various peptide linkers.

A series of chimeric expression constructs are designed in which various lengths of the FN protein are linked to the full-length mature IGF-I, IGF-II, EGF, bFGF, or KGF proteins, or at least a domain of the IGF-I, IGF-II, EGF, bFGF, or KGF proteins capable of binding a cognate growth factor receptor. In each case, the FN segments are preferably linked to the IGF-I, IGF-II, EGF, bFGF, or KGF sequence via a linker, for example, a Gly_4 Ser (SEQ ID NO:7) linker, a Gly_4 Ser_3 (SEQ ID NO:8) linker, a $(Gly_4\ Ser)_3$ (SEQ ID NO:9) linker, or a $(Gly_4\ Ser)_4$ (SEQ ID NO:10) linker.

Exemplary synthetic chimeric proteins include, but are not limited to:

A) FN type-III Domain 8 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

B) FN type-III Domains 8-9 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

C) FN type-III Domains 8-10 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

D) FN type-III Domain 9 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

E) FN type-III Domains 9-10 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

F) FN type-III Domain 10 [Linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

G) FN type-I Domains 1-5 [linker] FN type-III Domain 8 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

H) FN type-I Domains 1-5 [linker] FN type-III Domains 8-9 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

I) FN type-I Domains 1-5 [linker] FN type-III Domains 8-10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

J) FN type-I Domains 1-5 [linker] FN type-III Domain 9 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

K) FN type-I Domains 1-5 [linker] FN type-III Domains 9-10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

L) FN type-I Domains 1-5 [linker] FN type-III Domain 10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

M) FN type-I Domains 4-5 [linker] FN type-III Domain 8 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

N) FN type-I Domains 4-5 [linker] FN type-III Domains 8-9 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

O) FN type-I Domains 4-5 [linker] FN type-III Domains 8-10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

P) FN type-I Domains 4-5 [linker] FN type-III Domain 9 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

Q) FN type-I Domains 4-5 [linker] FN type-III Domains 9-10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF);

R) FN type-I Domains 4-5 [linker] FN type-III Domain 10 [linker] Growth Factor (IGF-I, IGF-II, EGF, bFGF, or KGF).

Human FN, IGF-I, IGF-II, EGF, bFGF, and KGF gene DNA sequences (SEQ ID NOs: 1-6, respectively) can be codon-optimised for expression in *Spodoptera frugiperda*. The coding sequences can then be cloned into an expression vector incorporating a poly-histidine affinity tag to aid in the purification of the chimeras (*e.g.*, the pIB/V5-His expression vector (Invitrogen)). A nucleotide sequence encoding an amino acid linker as discussed above can be inserted via site-directed

mutagenesis PCR. The addition of an Asn to the C-terminus of the linker sequence can be used to generate a Asn-Gly motif with Gly being the first amino acid of the growth factor protein. This motif enables hydroxylamine induced cleavage of the growth factor protein from the chimeras.

The resulting constructs will encode various lengths of the FN protein linked by a linker to the full-length mature IGF-I, IGF-II, EGF, bFGF, or KGF proteins, or at least a domain of the IGF-I, IGF-II, EGF, bFGF, or KGF proteins capable of binding a cognate growth factor receptor. The DNA sequence of all constructs can be verified to ensure that the fidelity of the desired DNA sequences are maintained.

Clones in the pIB/V5-His vector can be used to transfect Sf9 insect cells and transiently-expressed secreted protein is detected in the conditioned media, as assessed by immunoblotting. Briefly, the samples are resolved on SDS-PAGE under reducing conditions and the proteins are transferred onto a nitrocellulose membrane using a semi-dry transfer method. The membrane is interrogated with poly-clonal anti-FN antibodies and the target protein species are then visualized using enhanced chemiluminescence.

Purification of the chimeric proteins is based on Ni-NTA Superflow Agarose (QIAGEN, Australia) affinity chromatography performed according to the manufacturer's instructions. The chimeric proteins are monitored throughout the purification process by SDS-PAGE and western blot analysis using a poly-clonal anti-FN antibody (Calbiochem).

Cells, such as MCF-10A cells, MCF-7 cells, and isolated human epithelial cells, keratinocytes and fibroblasts can be used to examine the effects of the synthetic chimeric proteins on cell migration and/or proliferation. For example, cell migration can be assessed using Transwell™ migration assays, while cell proliferation can be determined using cell proliferation assays well known to one of skill in the art.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can

be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein are incorporated herein by reference.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Table I. *Fibronectin domains and regions*

Position	Length	Description
50-90	41	Fibronectin type-I 1
95-138	44	Fibronectin type-I 2
139-182	44	Fibronectin type-I 3
184-228	45	Fibronectin type-I 4
229-273	45	Fibronectin type-I 5
306-345	40	Fibronectin type-I 6
355-403	49	Fibronectin type-II 1
415-463	49	Fibronectin type-II 2
468-511	44	Fibronectin type-I 7
516-558	43	Fibronectin type-I 8
559-602	44	Fibronectin type-I 9
607-699	93	Fibronectin type-III 1
720-809	90	Fibronectin type-III 2
811-898	88	Fibronectin type-III 3
908-995	88	Fibronectin type-III 4
996-1084	89	Fibronectin type-III 5
1087-1172	86	Fibronectin type-III 6
1173-1265	93	Fibronectin type-III 7
1266-1356	91	Fibronectin type-III 8
1357-1446	90	Fibronectin type-III 9
1447-1536	90	Fibronectin type-III 10
1541-1630	90	Fibronectin type-III 11
1631-1720	90	Fibronectin type-III 12
1723-1810	88	Fibronectin type-III 13
1813-1901	89	Fibronectin type-III 14
1902-1991	90	Fibronectin type-III 15
2100-2190	91	Fibronectin type-III 16
2204-2248	45	Fibronectin type-I 10
2249-2291	43	Fibronectin type-I 11
2293-2336	44	Fibronectin type-I 12
907-1172	266	DNA-binding
52-272	221	Fibrin- and-heparin-binding 1
308-608	301	Collagen-binding
464-477	14	Critical for collagen binding
1267-1540	274	Cell-attachment
1721-1991	271	Heparin-binding 2
1813-1991	179	Binds to FBLN1
1992-2102	111	Connecting strand 3 (CS-3) (V region)
2206-2337	132	Fibrin-binding 2

CLAIMS

1. An isolated protein complex in the form of a synthetic chimeric protein, comprising an amino acid sequence of:
   (i) a growth factor or at least a domain of said growth factor which is capable of binding a cognate growth factor receptor; and
   (ii) one or more type-III domains of fibronectin (FN), wherein the one or more type-III domains comprise a sequence corresponding to amino acids 1266-1536 of SEQ ID NO:1, or a variant comprising at least 70% sequence identity to that sequence.

2. The isolated protein complex of Claim 1, wherein said growth factor is selected from insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and keratinocyte growth factor (KGF).

3. The isolated protein complex of Claim 1 or Claim 2, wherein the integrin-binding domain is an α_1 integrin-binding domain or an α_4 integrin-binding domain.

4. The isolated protein complex of Claim 1 or Claim 2, wherein the one or more type-III domains of FN comprise amino acids 1173-1536 of a FN sequence (SEQ ID NO:1).

5. The isolated protein complex of any one of Claims 1-4, which does not comprise an IGFBP amino acid sequence.

6. The isolated protein complex of any one of the preceding claims, further comprising an additional fragment of FN.

7. The isolated protein complex of Claim 6, wherein the additional fragment of FN comprises amino acids 50-273 of a FN sequence (SEQ ID NO:1).

8. The isolated protein complex of Claim 6, wherein the additional fragment of FN comprises amino acids 184-273 of a FN sequence (SEQ ID NO:1).

9. The isolated protein complex of any one of the preceding claims, further comprising at least one linker sequence.

10. The isolated protein complex of Claim 9, wherein the linker sequence comprises a protease cleavage site.

11. The isolated protein complex of Claim 9, wherein the linker sequence is selected from the group consisting of:

(i) Gly_4 Ser (SEQ ID NO:7);

(ii) Gly_4 Ser_3 (SEQ ID NO:8);

(iii) $(Gly_4$ $Ser)_3$ (SEQ ID NO:9);

(iv) $(Gly_4$ $Ser)_4$ (SEQ ID NO:10);

(v) Leu Ile Lys Met Lys Pro (SEQ ID NO:11); and

(vi) Gln Pro Gln Gly Leu Ala Lys (SEQ ID NO:12).

12. An isolated nucleic acid encoding the isolated protein complex of any one of the preceding claims.

13. A genetic construct, comprising the isolated nucleic acid of Claim 10 operably linked to one or more regulatory nucleotide sequences in a vector.

14. The genetic construct of Claim 11, which is an expression construct, wherein the isolated nucleic acid is operably linked to a promoter.

15. A host cell, comprising the genetic construct of Claim 11.

16. A pharmaceutical composition, comprising the isolated protein complex of any one of Claims 1-11 and a pharmaceutically-acceptable carrier, diluent or excipient.

17. A surgical implant, scaffold or prosthesis impregnated, coated or otherwise comprising the isolated protein complex of any one of Claims 1-11.

18. A wound or burn dressing, comprising the isolated protein complex of any one of Claims 1-11.

2010324554 13 May 2014

19. A method of promoting cell migration and/or proliferation, including the step of using the isolated protein complex of any one of Claims 1-11 to bind both a growth factor receptor and an integrin receptor expressed by a cell to thereby induce, augment or otherwise promote migration and/or proliferation of said cell.

20. The method of Claim 19, wherein the isolated protein complex is administered to an animal to promote cell migration and/or proliferation *in situ*.

21. The method of Claim 19, for prophylactically or therapeutically to induce, augment or otherwise promote epithelial cell migration and/or proliferation to thereby facilitate wound healing *in situ*.

22. The method of Claim 20 or Claim 21, wherein the animal is a human.

23. The method of Claim 19, wherein the isolated protein complex is administered to one or more cells or tissues *in vitro*.

24. The complex of any one of claims 1-11, or the nucleic acid of claim 12, or the genetic construct of claim 13 or claim 14, or the host cell of claim 15, or the pharmaceutical composition of claim 16, or the surgical implant, scaffold or prosthesis of claim 17, or the dressing of claim 18, or the method of any one of claims 19-23, substantially as hereinbefore described with reference to the figures and/or examples.

A) FN AA sequence

```
1     mlrgpgpgll llavqclgta vpstgasksk rqaqqmvqpq spvavsqskp gcydngkhyq
61    inqqwertyl gnalvctcyg gsrgfncesk peaeetcfdk ytgntyrvgd tyerpkdsmi
121   wdctcigagr grisctianr cheggqsyki gdtwrrphet ggymlecvcl gngkgewtck
181   piaekcfdha agtsyvvget wekpyqgwmm vdctclgegs gritctsrnr cndqdtrtsy
241   rigdtwskkd nrgnllqcic tgngrgewkc erhtsvqtts sgsgpftdvr aavyqpqphp
301   qpppyghcvt dsgvvysvgm qwlktqgnkq mlctclgngv scqetavtqt yggnsngepc
361   vlpftyngrt fyscttegrq dghlwcstts nyeqdqkysf ctdhtvlvqt qqgnsngalc
421   hfpflynnhn ytdctsegrr dnmkwcgttq nydadqkfgf cpmaaheeic ttnegvmyri
481   gdqwdkqhdm ghmmrctcvg ngrgewtcia ysqlrdqciv dditynvndt fhkrheeghm
541   lnctcfgqgr grwkcdpvdq cqdsetgtfy qigdswekyv hgvryqcycy grgigewhcq
601   plqtypsssg pvevfitetp sqpnshpiqw napqpshisk yilrwrpkns vgrwkeatip
661   ghlnsytikg lkpgvvyegq lisiqqyghq evtrfdfttt ststpvtsnt vtgettpfsp
721   lvatsesvte itassfvvsw vsasdtvsgf rveyelseeg depqyldlps tatsvnipdl
781   lpgrkyivnv yqisedgeqs lilstsqtta pdappdptvd qvddtsivvr wsrpqapitg
841   yrivyspsve gsstelnlpe tansvtlsdl qpgvqyniti yaveenqest pvviqqettg
901   tprsdtvpsp rdlqfvevtd vkvtimwtpp esavtgyrvd vipvnlpgeh gqrlpisrnt
961   faevtglspg vtyyfkvfav shgreskplt aqqttkldap tnlqfvnetd stvlvrwtpp
1021  raqitgyrlt vgltrrgqpr qynvgpsvsk yplrnlqpas eytvslvaik gnqespkatg
1081  vfttlqpgss ippyntevte ttivitwtpa prigfklgvr psqggeapre vtsdsgsivv
1141  sgltpgveyv ytiqvlrdgq erdapivnkv vtplspptnl hleanpdtgv ltvswerstt
1201  pditgyritt tptngqqgns leevvhadqs sctfdnlspg leynvsvytv kddkesvpis
1261  dtiipavppp tdlrftnigp dtmrvtwapp psidltnflv ryspvkneed vaelsispsd
1321  navvltnllp gteyvvsvss vyeqhestpl rgrqktglds ptgidfsdit ansftvhwia
1381  pratitgyri rhhpehfsgr predrvphsr nsitltnltp gteyvvsiva lngreespll
1441  igqqstvsdv prdlevvaat ptslliswda pavtvryyri tygetggnsp vqeftvpgsk
1501  statisglkp gvdytitvya vtgrgdspas skpisinyrt eidkpsqmqv tdvqdnsisv
1561  kwlpssspvt gyrvtttpkn gpgptktkta gpdqtemtie glqptveyvv svyaqnpsge
1621  sqplvqtavt nidrpkglaf tdvdvdsiki awespqgqvs ryrvtysspe dgihelfpap
1681  dgeedtaelq glrpgseytv svvalhddme sqpligtqst aipaptdlkf tqvtptslsa
1741  qwtppnvqlt gyrvrvtpke ktgpmkeinl apdsssvvvs glmvatkyev svyalkdtlt
1801  srpaqgvvtt lenvspprra rvtdatetti tiswrtktet itgfqvdavp angqtpiqrt
1861  ikpdvrsyti tglqpgtdyk iylytlndna rsspvvidas taidapsnlr flattpnsll
1921  vswqpprari tgyiikyekp gspprevvpr prpgvteati tglepgteyt iyvialknnq
1981  ksepligrkk tdelpqlvtl phpnlhgpei ldvpstvqkt pfvthpgydt gngiqlpgts
2041  gqqpsvgqqm ifeehgfrrt tppttatpir hrprpyppnv geeiqighip redvdyhlyp
2101  hgpglnpnas tgqealsqtt iswapfqdts eyiischpvg tdeeplqfrv pgtstsatlt
2161  gltrgatyni ivealkdqqr hkvreevvtv gnsvneglnq ptddscfdpy tvshyavgde
2221  wermsesgfk llcqclgfgs ghfrcdssrw chdngvnyki gekwdrqgen gqmmsctclg
2281  ngkgefkcdp heatcyddgk tyhvgeqwqk eylgaicsct cfgggrgwrc dncrrpggep
2341  spegttgqsy nqysqryhqr tntnvncpie cfmpldvqad redsre (SEQ ID NO:1)
```

B) IGF-I AA sequence

GPETLCGAEL VDALQFVCGD RGFYFNKPTG YGSSSRRAPQ TGIVDECCFR SCDLRRLEMY CAPLKPAKSA (SEQ ID NO:2)

C) IGF-II AA sequence

AYRPSETLCG GELVDTLQFV CGDRGFYFSR PASRVSRRSR GIVEECCFRS CDLALLETYC ATPAKSE (SEQ ID NO:3)

FIGURE 1

D) EGF AA sequence

NSDSECPLSH DGYCLHDGVC MYIEALDKYA CNCVVGYIGE RCQYRDLKWW ELR (SEQ ID NO:4)

E) bFGF AA sequence

PALPEDGGSG AFPPGHFKDP KRLYCKNGGF FLRIHPDGRV DGVREKSDPH IKLQLQAEER GVVSIKGVCA NRYLAMKEDG RLLASKCVTD ECFFFERLES NNYNTYRSRK YTSWYVALKR TGQYKLGSKT GPGQKAILFL PMSAKS (SEQ ID NO:5)

F) KGF AA sequence

CNDMTPEQMA TNVNCSSPER HTRSYDYMEG GDIRVRRLFC RTQWYLRIDK RGKVKGTQEM KNNYNIMEIR TVAVGIVAIK GVESEFYLAM NKEGKLYAKK ECNEDCNFKE LILENHYNTY ASAKWTHNGG EMFVALNQKG IPVRGKKTKK EQKTAHFLPM AIT (SEQ ID NO:6)

G) Linker Sequences

1) Gly_4 Ser (SEQ ID NO:7)

2) Gly_4 Ser_3 (SEQ ID NO:8)

3) $(Gly_4$ $Ser)_3$ (SEQ ID NO:9)

4) $(Gly_4$ $Ser)_4$ (SEQ ID NO:10)

5) Leu Ile Lys Met Lys Pro (SEQ ID NO:11)

6) Gln Pro Gln Gly Leu Ala Lys (SEQ ID NO:12)

FIGURE 1 cont'd

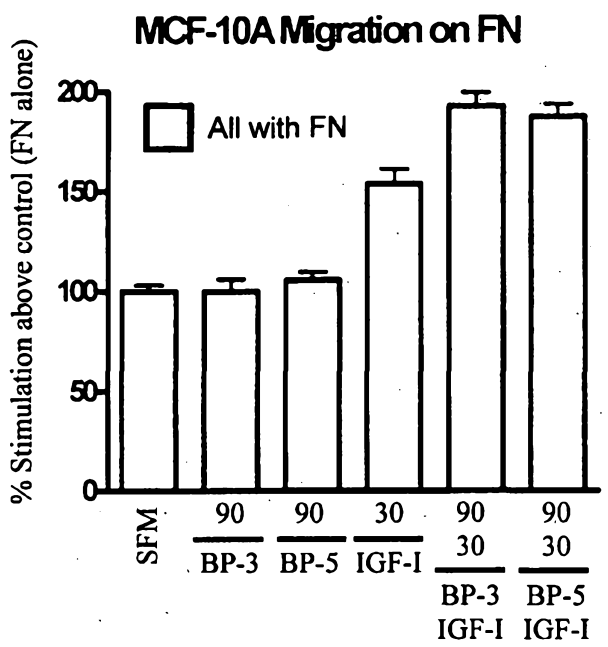
MCF-10A Migration on FN
All with FN
% Stimulation above control (FN alone)
200
150
100
50
0
SFM
90
BP-3
90
BP-5
30
IGF-I
90
30
BP-3
IGF-I
90
30
BP-5
IGF-I

FIGURE 2