680533

AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST: STANDARD PATENT

I/We, the Applicant(s)/Nominated Person(s) specified below, request I/We be granted a patent for the invention disclosed in the accompanying standard complete specification.

[70,71] Applicant(s)/Nominated Person(s):

American Cyanamid Company, of One Cyanamid Plaza, Wayne, New Jersey, 07470, UNITED STATES OF AMERICA

[54] Invention Title:

Heparin Binding Site Structural Analogues of Fibroblast Growth Factors

[72] Inventor(s):

Lu-Yuan Li, Andrew Peter Seddon, Yakov Gluzman and Peter Bohlen

[74] Address for service in Australia:

Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia (Code **SF**)

Details of Basic Application(s):

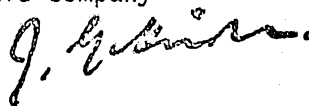| [31] Appl'n No(s):	[33] Country:	[32] Application Date:
126,974	US	24 September 1993

Basic Applicant(s): Lu-Yuan Li, Andrew Peter Seddon, Yakov Gluzman and Peter Bohlen

In accordance with Regulation 3.25, samples of materials deposited in accordance with the Budapest Treaty in relation to this Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

DATED this TWENTY SECOND day of SEPTEMBER 1994

American Cyanamid Company

By:

S 049646 230994

Registered Patent Attorney

IRN: 282384   INSTR CODE: 50380

5.45/2

SPRUSON & FERGUSON

Australia

Patents Act 1990

Notice Of Entitlement

I, Alphonse R Noë, of 470 Haviland Road, Stamford, Connecticut, United States of America, being authorised by the Applicant/Nominated Person in respect of an application entitled:

Heparin Binding Site Structural Analogues of Fibroblast Growth Factors

state the following:-

The Applicant/Nominated Person has entitlement from the actual inventors as follows:-

The Applicant/Nominated Person is the assignee of the actual inventors.

The Applicant/Nominated Person is entitled to rely on the basic application listed on the Patent Request as follows:

The Applicant/Nominated Person is the assignee of the basic applicants.

The basic application listed on the Patent Request is the first application made in a Convention country in respect of the invention.

The Applicant/Nominated Person is the depositor of the following deposits with the American Type Culture Collection (ATCC), of 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America:

Microorganism	Deposit Date	Accession No.
Escherichia coli strain BL21 haboring plasmids pLysS and FGF-2 ($E^{2,5}$Q128,138), FGF-2 Q128,138,BL21pLysS	13 September 1993	ATCC 69418
Escherichia coli, strain BL21 harboring plasmids pLYsS and pET FGF-2 ($E^{3,5}$Q128), FGF-2 Q128 BL21 pLysS	13 September 1993	ATCC 69419
Escherichia coli strain BL21 harboring plasmids pLysS and pET FGF-2 ($E^{2,5}$Q138), FGF-2 Q138 BL21 pLysS	13 September 1993	ATCC 69420

Dated ⎯ *October 10, 1994* ⎯

⎯⎯⎯⎯⎯⎯⎯

Alphonse R Noë

IRN: 282384

INSTR CODE: 50380

[N:\LIBW]07803:GSA

American *Type Culture* Collection

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

American Cyanamid Company
Attention: Andrew P. Seddon
Lederle Labs (205/224)
N. Middletown Road
Pearl River, NY 10965

Deposited on Behalf of: American Cyanamid Company

Identification Reference by Depositor:	ATCC Designation
Escherichia coli strain DH5α harboring plasmid	
pET FGF-2LI (E ^{3,5}A^{116}Q^{110}F^{117}P^{119}N^{118}), FGF-2LI DH5α	69417
Escherichia coli strain BL21 harboring plasmids	
pLysS and FGF-2 (E^{3,5}Q128,138), FGF-2 Q128,138,BL21pLysS	69418
Escherichia coli strain BL21 harboring plasmids	
pLysS and pET FGF-2 (E^{4,5}Q128), FGF-2 Q128 BL21 pLysS	69419
Escherichia coli strain BL21 harboring plasmids	
pLysS and pET FGF-2 (E^{4,5}Q138), FGF-2 Q138 BL21 pLysS	69420

The deposits were accompanied by: ___ a scientific description _X_ a proposed taxonomic description indicated above.

The deposits were received _September 13, 1993_ by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

_X_   We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested _September 17, 1993_. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbi A. Brandon   Date: _September 17, 1993_

Bobbi A. Brandon, Head, ATCC Patent Depository

cc: E.J. Tsevdos

(12) PATENT ABRIDGMENT   (11) Document No. AU-B-74185/94
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(54) Title
      HEPARIN BINDING SITE STRUCTURAL ANALOGUES OF FIBROBLAST GROWTH FACTORS

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(71) Applicant(s)
      AMERICAN CYANAMID COMPANY

(72) Inventor(s)
      LU-YUAN LI; ANDREW PETER SEDDON; YAKOV GLUZMAN; PETER BOHLEN

(74) Attorney or Agent
      SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001

(56) Prior Art Documents
      WO 89/04832
      EP 298723

(57) Claim

1.   A fibroblast growth factor-2(FGF-2) structural analogue selected from $Gln^{134}FGF$-2, $Gln^{138}FGF$-2, $Gln^{128,138}FGF$-2, and $Gln^{129,134}FGF$-2.

HEPARIN BINDING SITE STRUCTURAL ANALOGUES

OF FIBROBLAST GROWTH FACTORS

Technical Field of the Invention

This invention relates to fibroblast growth factor structural analogues, notably fibroblast growth factor-2 (formerly called basic fibroblast growth factor) analogues,
5 which have point mutations in a heparin binding site, e.g., one or more basic residues replaced by other residues such as neutral residues. The analogues exhibit binding affinities to cell surface receptors similar to the native growth factor, but possess different heparin binding and
10 biological properties from the native factor.

Background of the Invention

Polypeptide growth factors are hormone-like modulators of cell proliferation and differentiation. Growth factors are responsible for the regulation of a variety of physio-
15 logical processes, including development, regeneration, and wound repair, and have been associated with normal as well as with pathophysiological processes. Numerous growth factors have been identified in various tissues and cells, and names that have been applied to these factors include epi-
20 dermal growth factor, platelet-derived growth factor, nerve growth factor, hematopoietic growth factor, and fibroblast growth factor.

Fibroblast growth factor (FGF) was first described as
25 an activity derived from bovine brain or pituitary tissue

which was mitogenic for fibroblasts and endothelial cells.
It was later noted that the primary mitogen from brain was
different from that isolated from pituitary. These two fac-
tors were named acidic and basic FGF, respectively. Acidic

5 and basic FGF are proteins containing approximately 154
amino acids. Their amino acid sequences exhibit an approxi-
mate 55% sequence homology between them.

Acidic and basic fibroblast growth factors are now

10 known to be members of a larger family of heparin-binding
growth factors which collectively trigger a variety of bio-
logical responses in many cell types, including those of
mesoderm and neuroectoderm origin, such as endothelial
cells, smooth muscle cells, adrenal cortex cells, prostatic

15 and retina epithelial cells, oligodendrocytes, astrocytes,
chrondocytes, myoblasts, and osteoblasts. As original
family members, acidic and basic FGF are now denoted FGF-1
and FGF-2, respectively. Seven other members of the family
have been identified on the basis of their modulation of

20 cell proliferation and differentiation, and sequence homo-
logy to other FGFs.

In addition to eliciting a mitogenic response that
stimulates cell growth, fibroblast growth factors can stimu-
late a large number of cell types to respond in a non-mito-

25 genic manner. These activities include promotion of cell
migration into wound areas (chemotaxis), initiation of new
blood vessel formation (angiogenesis), modulation of nerve
regeneration and survival (neurotrophism), modulation of en-
docrine functions, and stimulation or suppression of spe-

30 cific cellular protein expression, extracellular matrix pro-
duction and cell survival (Baird, A., and Böhlen, P., *Hand-
book of Exp. Pharmacol. 95*(1): 369-418, Springer, 1990).
These properties provide a basis for using fibroblast growth
factors in therapeutic approaches to accelerate wound heal-

35 ing, nerve repair, collateral blood vessel formation, and

the like. For example, fibroblast growth factors have been suggested to minimize myocardium damage in heart disease and surgery (e.g., U.S. Pat. No. 4,378,347 to Franco).

Thus, current research regarding FGF-2 and other FGFs
5 centers on the molecular details of the receptor-mediated pathways by which the diverse physiological activities of the factors are expressed, as a way to gain information for the design of therapeutically useful agents that can either mimic or inhibit the action of the factors. The biological
10 responses of FGF are mediated by the heparin sulfate-dependent binding of the growth factor to specific cell surface receptors, yet the molecular interactions of heparin and receptor with FGF and the exact nature of the events of the signal transduction pathway are unknown. Since the primary
15 structure of FGF-2 isolated from a variety of sources is known, and bovine and human FGF-2 have been cloned and expressed in *E. coli* and *S. cervisiae*, recent attention has focused on the elucidation of secondary and tertiary structure and on the activity of structural analogues.

20 Presta and coworkers found that replacement of arginine-118, lysine-119, lysine-128, and arginine-129 of FGF-2 with glutamine by site-directed mutagenesis led to a mutant denoted by the investigators as M6B-bFGF that exhibited similar receptor binding and mitogenic activity to native
25 growth factor, but a reduced affinity for cell surface heparin-like low affinity binding sites, a reduced chemotactic activity, and a reduced capacity to induce the production of urokinase-type plasminogen activator in cultured endothelial cells (Presta, M., et al., Biochem. Biophys.
30 Res. Com. 185: 1098-1107 (1992)). In addition, the mutant lacked a significant angiogenic activity in vivo. Replacement of lysine-128 and arginine-129 of FGF-2 by the same researchers, on the other hand, yielded a mutant denoted M6A-bFGF that exhibited different properties, i.e., a

70% inhibition of cell receptor binding (Eur. Pat. Ap. Pub. No. 363,675 to Bergonzoni, L., et al.).

Studies employing synthetic peptides related to the FGF sequence showed that FGF-2 (33-77) and (102-129) bind to
5 heparin and act as weak partial agonists and antagonists in a mitogenic assay of FGF activity (Baird, A., et al. Proc. Nat. Acad. Sci. USA 85: 2324-2328 (1988)). The same study identified a sequence, FGF-2 (115-124), involved in receptor binding. From the crystal structure of FGF-2, a cluster of
10 basic residues appear to play a role in the binding of anionic heparin sulfate to the molecule (Zhang, J., et al., Proc. Nat. Acad. Sci. USA 88: 3446-3450 (1991) and Eriksson, E.A., et al., Proc. Nat. Acad. Sci. USA 88: 3441- 3445 (1991)). Two pairs of basic residues, lysine-128
15 and lysine-138 and arginine-129 and lysine-134, respectively (denoted lysine-119 and lysine-129 and arginine-120 and lysine-125 by the investigators since they employed an active truncated form of the polypeptide corresponding to residues 9-154 of the full-length sequence), were found to
20 form ionic contacts with two ordered sulfate ions in the crystal structure (Zhang, cited above). These may mimic heparan sulfate moieties. So it is possible that the two binding sites are involved in heparin binding to FGF.

Summary of the Invention

25 It is an objective of the present invention to provide novel structural analogues of fibroblast growth factors, especially analogues of human fibroblast growth factor-2.

It is a further and more specific objective of the present invention to provide fibroblast growth factor struc-
30 tural analogues which exhibit, relative to the native factor, similar binding to cell surface receptors but different biological activities, such as decreased mitogenic activity,

thereby providing fibroblast growth factor antagonists. Fibroblast growth factor antagonists are useful as potential antiangiogenesis, anticancer, antitumor, and antiproliferative agents.

5   These and other objects are accomplished by the present invention, that provides fibroblast growtu factor structural analogues which have at least one point mutation in a putative heparin binding site, typically a deletion or replacement of a basic residue with another residue such as
10   a neutral or acidic residue. Some embodiments have at least one point mutation in one heparin sulfate binding site, and no mutation in another heparin binding site. Preferred analogues exhibit different heparin binding and biological activity from native growth factor; especially preferred
15   analogues exhibit decreased affinity toward heparin and a decreased capacity to induce cell proliferation. Some embodiments exhibit a decreased angiogenesis in *in vitro* assays. Some embodiments exhibit a decreased capacity and others an increased capacity to induce the production of
20   urokinase-type plasminogen activator. Some embodiments exhibit a decreased capacity to induce tube-like structures in cultured endothelial cells compared to native factor; others exhibit an increased capacity of the same activity. Particularly preferred analogues have at least one or two
25   basic residues such as lysine residues replaced by glutamine.

Fibroblast growth factor-2 structural analogues of the invention include those having either lysine-128, or lysine-138, or lysine-128 and lysine-138, or arginine-129, or lysine-134, or arginine-129 and lysine-134, replaced by glu-
30   tamine residues. Particularly preferred fibroblast-2 structural analogues have lysine-128 or lysine-138 or both replaced by glutamine.

The invention also provides DNA encoding the fibro-blast growth factor derivatives, biologically functional circular plasmid or viral DNA vectors comprising the DNA, and procaryotic or eucaryotic host cells such as *E. coli* transformed or transfected with the vectors in a manner allowing the host cell to express the new factors.

According t ¬ne aspect of the invention, there is provided a fibroblast growth factor-2(FGF-2) . ictural analogue selected from $Gln^{134}FGF-2$, $Gln^{138}FGF-2$, $Gln^{128,138}FGF-2$, and $Gln^{129,134}FGF-2$.

Brief Description of the Figures

Figures 1A and 1B plot fluorescence intensities of FGF-2 (1A) and $gln^{128,138}FGF-2$ (1B) against concentrations of heparin.

Figure 2 plots radioactivity associated with ^{125}I-labelled FGF-2 bound to high affinity FGF-receptors on baby hamster kidney (BHK) cells against concentrations of FGF-2 and the mutants. The open circles and squares plot FGF-2 data; the closed squares plot $gln^{138}FGF-2$ data; the closed circles plot $gln^{128,138}FGF-2$ data; and the closed triangles plot $gln^{128}FGF-2$ data as described hereinafter.

Figures 3A and 3B plot cell counts as a function of FGF-2 and mutant concentrations. Figure 3A shows site 1 mutants (residues lysine-128 and lysine-138), and Figure 3B shows site 2 mutants (residues arginine-129 and lysine-134).

Figure 4 shows wild-type or mutant FGF-2 (10ng/ml) induction of urokinase-type plasminogen activator.

Figure 5 is a bar graph depicting the potency of *in vitro* angiogenic activities of wild-type or mutant FGF-2 (10ng/ml) as measured by induction of the formation of tube-like structures by adult bovine aortic endothelial cells cultured on a type I collagen gel.

Detailed Description of the Invention

This invention is based upon the finding that replacement of certain basic residues in fibroblast growth factors with neutral residues give mutants which exhibit, in com-
5 parison to native factor, similar affinities to cell surface receptors. Some of these analogues exhibit different heparin binding and biological activities.

A protein is defined herein as a fibroblast growth factor (FGF) if it shows FGF activity in *in vitro* or
10 *in vivo* assays and binds to heparin or heparin-like substances. By "heparin" is meant the heterogeneous, sulfated anionic polysaccharide composed of D-glucuronic acid and D-glucosamine, bound to a protein core as the "proteoglycan" or in a free form as the "aglycan", that has potent
15 anticoagulant properties. "Heparin-like" substances are molecules that have oligosaccharide structures related to the heparins, but may or may not have anticoagulant activity. Any type of fibroblast growth factor or mutant factor (mutein) or derivative is encompassed by this invention,
20 particularly human fibroblast growth factor.

The fibroblast growth factor analogues of this invention are structural analogues of fibroblast growth factor having at least one basic amino acid residue deleted or replaced by another residue. Typically, the analogues have at
25 least one one point mutation in a putative heparin binding site; many analogues have no mutation in another heparin binding site. For example, where certain basic residues are deleted or replaced by neutral residues, the analogues obtained exhibit binding affinity to baby hamster kidney (BHK)
30 cell surface receptors similar to native growth factor. Preferred analogues exhibit binding and biological activities that are pharmacologically dissociated, so that the heparin binding and biological activity are different from the na-

tive factor. As set out in greater detail below, in many cases the biological activities exhibited by the analogues are pharmacologically dissociated. Many mutants exhibit some biological activities reduced, but others not. Pre-
5 ferred embodiments exhibit similar or superior binding to baby hamster kidney (BHK) cell receptors when compared to native fibroblast growth factor.

Especially preferred analogues have one or two basic residues such as lysine, arginine or a mixture of lysine and
10 arginine replaced by a neutral residue such as glutamine. In some embodiments, the analogues exhibit decreased affi- nity toward heparin from the native factor. Some embodi- ments also exhibit a decreased capacity to induce urokinase- type plasminogen activator production and decreased angio-
15 genesis, exhibited as a decrease in the capacity to induce in vitro capillary formation. Preferred embodiments exhibit a decreased mitogenic activity exhibited as a de- creased capacity to induce cell proliferation. Other differences in the binding affinity or biological activity
20 exhibited by the analogues in comparison to native factor are discussed hereinafter.

Structural analogues of FGF-2 are preferred in many embodiments. By "FGF-2" is meant any fibroblast growth factor-2 exhibiting a biological activity, including the
25 146-amino acid polypeptide originally isolated and sequen- ced, the 154 amino acid form currently thought to be the full polypeptide, truncated forms exhibiting activity, ex- tended forms such as placental FGF, higher molecular weight forms described in the literature and analogues including
30 derivatives and muteins of any of these. The term specifi- cally includes natural FGF-2 extracted from mammalian tissue as well as recombinant polypeptides expressed from cloned DNA in E. coli or S. cervisiae from any species or expressed in insect or mammalian cells with appropriate vectors, de- noted herein as "native" FGF-2.

Human FGF-2 is preferred in many embodiments. Human
FGF-2 includes both native FGF-2 described above, as well as
previously described structural analogues such as those
having amino acid additions, amino acid substitutions, and
5 amino acid deletions, including deletions of portions of the
amino or carboxyl terminus, and chimeric proteins containing
FGF at the N- or C- terminus of another protein. Example
FGF-2s which can be further modified according to the inven-
tion include those having cysteine substituted with a neu-
10 tral amino acid such as serine, or aspartic acid, arginine,
glycine, serine, or valine substituted with other acids
suggested to have enhanced stability in Eur. Pat. Ap. Pub.
No. 281,822 to Seno, et al.; muteins formed by re-
placing at least one, and more preferably two, of the cys-
15 teines found in natural FGF-2 with a different amino acid
residue to yield a more stable analogue (Eur. Pat. Ap. Pub.
No. 320,148 to Arakawa and Fox); muteins lacking 7 to 46
amino acids from the carboxyl terminus and, optionally,
having amino acid replacements suggested to have improved
20 stability while retaining activity in Eur. Pat. Ap. Pub. No.
326,907 to Seno, et al.; muteins having point mutations
or an N-terminal deletion suggested in Eur. Pat. Ap. Pub.
No. 298,723 to Fiddes, et al.; the M1-bFGF to M6-bFGF
muteins containing missing and substituted amino acids
25 disclosed in Eur. Pat. Ap. Pub. No. 363,675 to Bergonzoni,
cited above; readily expressed FGF prepared by replacement
of Ala-3 and Ser-5 of recombinant FGF with Glu as described
in Seddon, cited above and analogues thereof; analogues
having an amino acid deletion, insertion or replacement in
30 the surface loop that extends from the ninth ß-strand to the
tenth ß-strand and having the overall secondary and tertiary
structure of the original factor and the like.

In the practice of this invention, a fibroblast growth factor derivative of this invention such as a human FGF-2 structural analogue is prepared by deleting, or, preferably, substituting, a basic amino acid residue with another resi-

5   due. Typical replacements involve the replacement of a lysine or an arginine amino acid residue with another residue such as a neutral or acidic amino acid residue. Replacement amino acid residues include, but are not limited to, alamine, glycine, valine, leucine, isoleucine, aspartic

10   acid, serine, threonine and glutamine. Glutamine is employed in one embodiment.

In preferred embodiments, the substitution takes place in a basic residue implicated in the binding of a sulfate moiety of heparin. Preferably, a point mutation in one

15   putative heparin binding site occurs; in many embodiments, there is no mutation in another heparin binding site. Heparin, a highly sulfated anionic polysaccharide, protects both FGF-1 and FGF-2 from inactivation by elevated temperature, acidic conditions, and digestion from proteases, and poten-

20   tiates the mitogenic activity of FGF-1. Interaction of FGF with cell surface proteoglycans has been suggested to be necessary for the binding of FGF-2 to its high affinity cell membrane receptors, and hence modulation of FGF activities.

Especially preferred FGF-2 structural analogues of

25   this invention have at least one and preferably two point mutations such as a substitution of a lysine with glutamine in the site 2 binding site for sulfates described by Zhang, cited above. Example structural analogues of FGF-2 of this invention thus include FGF-2 having lysine-128 replaced by

30   glutamine (herein denoted $gln^{128}FGF-2$), FGF-2 having lysine-138 replaced by glutamine (herein denoted $gln^{138}FGF-2$), and FGF-2 having both lysine-128 and lysine-138 replaced by glutamine (herein denoted $gln^{128,138}FGF-2$).

$Gln^{128,138}FGF-2$ is preferred in one embodiment. Binding affinities of this analogue and others to cell surface receptors are similar to that of native or wild-type FGF-2, but significant changes in heparin binding and bio-
5   logical activities are also observed. Dissociation constants for heparin binding for $gln^{128}FGF-2$ and $gln^{138}FGF-2$, for example, are increased by about 10-fold, and that for $gln^{128,138}FGF-2$, by about 100-fold. The marked reduction in binding affinity does not appear to result from perturbation
10 of the structural integrity of the protein since BHK receptor binding is not significantly affected.

Diminished heparin binding is, however, associated with a significant decline in several of the biological activities of FGF-2 attributable to angiogenic responses,
15 particularly the capacity to induce the formation of capillary-like structures by cultured endothelial cells. The mitogenic activities of $qln^{128}-FGF-2$ and $gln^{128,138}-FGF-2$, for example, are about 10-fold lower than that of the wild-type protein, whereas that of $gln^{138}FGF-2$ is unchanged.
20 In addition, the capacity of the mutant $gln^{128,138}FGF-2$ to induce urokinase-type plasminogen activator in adult bovine aortic endothelial (ABAE) cells is markedly reduced. This analogue also exhibits a diminished capacity to induce ABAE cells to form capillary-like structures in an *in vitro*
25 angiogenesis model described more fully hereinafter. $Gln^{128}-FGF-2$, on the other hand, exhibits tube formation and urokinase-type plasminogen activator activity similar to native fibroblast growth factor.

Structural analogues of FGF-2 having point mutations
30 in site 1, on the other hand, exhibit a greater similarity to native FGF-2. For example, FGF-2 having arginine-129 replaced by glutamine (herein denoted $gln^{129}FGF-2$), FGF-2 having lysine-134 replaced by glutamine (herein denoted $gln^{134}FGF-2$), and FGF-2 having both arginine-129 and

lysine-134 replaced by glutamine (herein denoted $gln^{129,134}$-FGF-2) exhibit binding affinities to cell surface receptors, heparin binding properties and biological activities similar to native FGF-2.

5   The structural analogues of this invention may be prepared using any chemical or biochemical means known to those skilled in the art, such as preparation by assembling polypeptides from constituent amino acids or peptides and polypeptides. Alternatively, the analogues may be prepared by

10   recombinant protein synthesis involving preparation of DNA encoding analogue FGF, insertion of that DNA into a vector, expression of the vector in host cells, and isolation of the recombinant FGF thereby produced.

      DNA encoding the FGF analogues of this invention may

15   be prepared by altering a gene of a FGF by nucleotide deletions, nucleotide additions, and/or point mutations produced using standard means. Illustrations using cassette mutagenesis are given hereinafter. Because of the degeneracy of the genetic code, a variety of codon change combinations can be

20   selected to form DNA that encodes the FGF analogues, so that any nucleotide deletion(s), addition(s) and/or point mutations that result in a DNA encoding an analogue are encompassed by this invention. Since certain codons are more efficient for polypeptide expression in certain types of organ-

25   isms, the selection of fibroblast gene alterations to yield DNA material that codes for the analogues of this invention are preferably those that yield the most efficient expression in the type of organism which is to serve as the host of the recombinant vector. Altered codon selection may also

30   depend upon vector construction considerations.

      Fibroblast growth factor DNA starting material that is altered to form DNA coding for the FGF analogues of the invention may be natural, recombinant or synthetic. Thus,

DNA starting material is isolated from tissue or tissue cul-
ture, constructed from oligonucleotides using conventional
methods, obtained commercially, or prepared by isolating RNA
encoding FGF from fibroblasts, and using the RNA so obtained
5   to synthesize single-stranded cDNA which is used as a tem-
plate to synthesize the corresponding double stranded DNA.

Illustrating the present invention are cloned comple-
mentary DNA sequences defining human FGF-2 analogues such as
that constructed in Example 1.   Also encompassed are DNA se-
10 quences homologous or closely related to complementary DNA
described herein, namely DNA sequences which hybridize, par-
ticularly under stringent conditions resulting in pairing
only between nucleic acid fragments that having a high fre-
quency of complementary base sequences, to FGF analogue
15 cDNA, and RNA corresponding thereto.   In addition to the
FGF-encoding sequences, DNA encompassed by this invention
may contain additional sequences, depending upon vector
construction sequences, that facilitate expression of the
gene.

20   DNA encoding the FGF analogues of this invention, or
RNA corresponding thereto, are then inserted into a vector,
e.g., a pBR, pUC, pUB or pET series plasmid, and the recom-
binant vector used to transform microbial host organisms.
Host organisms useful in the invention are bacterial (e.g.,
25 E. coli or B. subtilis), yeast (e.g., S. cervisiae), mamma-
lian (e.g., mouse fibroblast), or insect cells.   This inven-
tion thus also provides novel, biologically functional viral
and circular plasmid RNA and DNA vectors incorporating RNA
and DNA sequences describing the FGF analogues generated.
30 Culture of host organisms stably transformed or transfected
with such vectors under conditions facilitative of large
scale expression of the exogenous, vector-borne DNA or RNA
sequences and isolation of the desired polypeptides from the
growth medium, cellular lysates, or cellular membrane

fractions yields the desired products. An example of ex-
pression of FGF-2 analogues in *E. coli* is given in
Example 1.

The present invention provides for the total and/or
5   partial manufacture of DNA sequences coding for FGF-2 ana-
logues, and including such advantageous characteristics as
incorporation of codons preferred for expression by selected
non-mammalian hosts, provision of sites of cleavage by
restriction by endonuclease enzymes, and provision of addi-
10   tional initial, terminal or intermediate DNA sequences which
facilitate construction of readily expressed vectors. Cor-
respondingly, the present invention provides for manufacture
(and development by site specific mutagenesis of cDNA and
genomic DNA) of DNA sequences coding for microbial expres-
15   sion of FGF analogues which differ from the forms specifi-
cally described herein in terms of identity or location of
one or more amino acid residues (i.e., deletion analogues
containing less than all of the residues specified for human
FGF-2, and/or substitution analogues wherein one or more
20   residues are added to a terminal or medial portion of the
polypeptide), and which share the biological properties of
FGF-2 analogues described herein.

DNA (and RNA) sequences of this invention code for all
sequences useful in securing expression in procaryotic or
25   eucaryotic host cells of polypeptide products having at
least a part of the primary structural conformation, and one
or more of the biological properties of FGF analogues which
are comprehended by: (a) the DNA sequences encoding FGF-2
analogues as described herein, or complementary strands;
30   (b) DNA sequences which hybridize to DNA sequences defined
in (a) or fragments thereof; and (c) DNA sequences which,
but for the degeneracy of the genetic code, would hybridize
to the DNA sequences defined in (a) and (b) above. Specifi-
cally comprehended are genomic DNA sequences encoding alle-

lic variant forms of FGF analogues included therein, and
sequences encoding analogue RNA, fragments thereof, and RNA
or DNA sequences that incorporate codons facilitating trans-
cription or RNA replication of messenger RNA in non-verte-
5 brate hosts.

Isolation and purification of microbially expressed
polypeptides provided by the invention are by conventional
means including, for example, preparative chromatographic
separations such as that illustrated in Example 1 utilizing
10 heparin, and immunological separations, including monoclonal
and/or polyclonal antibody preparations.

As summarized above and described in more detail in
the examples below, mutations involving Arg-129 and Lys-134
in FGF-2 result in little change with respect to any of the
15 parameters measured, including heparin binding. A mutation
involving Lys-138 exhibits decreased heparin binding, but no
significant change in endothelial cell proliferation com-
pared to native factor. The double mutant $gln^{129,134}FGF-2$
apparently exhibits enhanced urokinase and tube-formation
20 activities. These analogues may thus be used as FGF-2
agonists. By "agonist" is meant any substance that has
affinity for and stimulates physiological activity at cell
receptors normally stimulated by naturally occurring
substances. The FGF analogues that are agonists of FGF
25 activity can promote vascularization, cell growth, and/or
cell survival, and thus have application in tissue repair
such as healing of wounds, burns, bone fractures, surgical
abrasions, ulcers, and the like as well as tissue repair
during ischemia and myocardial infarction.

30 In contrast, as summarized above and described in more
detail in the examples below, mutations involving Arg-128
and Lys-138 such as $gln^{128,138}FGF-2$ exhibit reduced he-
parin binding, reduced mitogenic activity, reduced capacity

to induce urokinase-type plasminogen activator, and reduced
angiogenesis in vitro. Thus, reduced heparin binding
seems cause a deficiency in the signal transducing pathways
which exert FGF-2 activities, such as induction of cell
5 proliferation, urokinase production, and in vitro
capillary formation. As such, the analogue is expected to
exhibit an antagonist activity.

By "antagonist" is meant any substance that tends to
nullify the action of another, as one, for example, that
10 binds to a cell receptor without eliciting a biological
response. For FGFs, antagonists include, but are not limi-
ted to, substances which bind to FGF receptors but do not
stimulate proliferation or migration of fibroblasts, endo-
thelial cells and astroglial cells. FGF antagonists are
15 useful in the treatment of certain neoplasms, particularly
angiogenic neoplasms, as a valuable tool for inhibiting
tumoral angiogenesis as well as an anticancer and anti-
proliferative agent. The antagonists additionally act as
angiogenesis inhibitors and, therefore, are useful for the
20 treatment of diseases where neovascularization is dominant
in the pathology, such as retinopathies of the eye, neovas-
cular glaucoma, skin disorders, chronic inflammation, rheu-
matoid arthritis, and the like. As used herein, the terms
agonist and antagonist are not limiting, and a single factor
25 may exhibit antagonism to one biological function mediated
by native factor, and agonism to another function. Where
this occurs, the biological activities of native factor are
said to be pharmacologically dissociated in the analogue.

The following examples are presented to further illus-
30 trate and explain the present invention and should not be
taken as limiting in any regard.

Example 1

Human FGF-2 analogues are constructed, expressed and purified.

A gene encoding human glu^{3,5}FGF-2 is first prepared as described in Seddon, A.P., et al., Annals N.Y. Acad. Sci.
5   638: 98-108 (1991). Briefly stated, a synthetic gene encoding the 155 amino acid form of human FGF-2 cloned into pUC 18 is purchased from British Bio-technology, Oxford, UK. The nucleotide sequence (2-49) to be changed is excised from pUC 18 with restriction endonucleases HindIII and BspMII and a
10 synthetic fragment encoding the first 5 N-terminal amino acids of FGF-1 and containing an internal NdeI site is cloned into pUC 18, yielding a construct encoding glutamic acid at positions 3 and 5. The cDNA encoding FGF-2 is then excised from pUC 18 with NdeI and BamHl and cloned into the NdeI and BamHl
15 site of the expression vector pET-(M13) $\triangle$PS, a derivative of pET-3a (Rosenberg, A.H., et al., Gene 56: 125-135 (1987)).

The plasmid is modified by site-directed mutagenesis using single-stranded DNA and two primers, 5'-GTCTAGAAAA TACACCAGTT GGTACGTAGC ACTGCAGCGA ACCGGTCAGT ATAAA-3' (SEQ ID
20 NO 1) and 5'-CTTGGTTCCC AAACAGGGCC CGGGCAGAAA GCTA-3' (SEQ ID NO 2) to obtain the gln^{128}-FGF-2 mutant gene, that contains newly introduced unique restriction sites SnaB1, Pst1 and Apal (underlined), respectively. These flanking restriction sites in the plasmid are digested with the cog-
25 nate restriction enzymes so that short synthetic duplex DNA cassettes can be inserted using cassette-directed mutagenesis as described by Wells, et al. (Gene 34: 315-323 (1985)).

Cassette directed mutagenesis of this variant gene
30 using SnaB1 and Apal and two sets of annealed oligo-nucleotides 5'-GTAGCACTTA AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC-3' (SEQ ID NO 3) and 5'-CTGTTTGGGA ACCAAGCTTA

TACTGCCCCG TACGCTTAAG TGCTAC-3' (SEQ ID NO 4), and 5'-
GTAGCACTGC AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC-3'
(SEQ ID NO 5) and 5'-CTGTTTGGGA ACCAAGCTTA TACTGCCCCG
TACGCTGCAG TGCTAC-3' (SEQ ID NO 6) gives, respectively, a

5 mutant gln^{138}FGF-2 that has new restriction sites Afl2
and Hind3 (underlined), and a double mutant gln^{128,138}-
FGF-2 that has new restriction site Hind3 (underlined).

Casette mutagenesis of gln^{138}FGF-2 using restricting
10 sites SnaBl and Hind3 and annealed oligonucleotides 5'-
GTAGCACTGC AGCAGACGGG GCAGTATC-3' (SEQ ID NO 7), and 5'-
AGCTGATACT GCCCCGTCTG CTGCAGTGCT AC-3' (SEQ ID NO 8) results in
a quadruple mutant gln^{128,129,134,138}FGF-2, that has a new
restriction site Pstl (underlined). Cassette mutagenesis of
15 the gln^{128}-FGF-2-K138Q mutant using unique restriction sites
SnaBl and Apal and annealed oligonucleotides 5'-GTAGCACTTA
AGCAGACGGG GCAGTATCAG CTTGGTTCCA AAACAGGGCC-3' (SEQ ID NO 9),
and 5'-CTGTTTTGGA ACCAAGCTGA TACTGCCCCG TCTGCTTAAG TGCTAC-3'
(SEQ ID NO 10) produces another double mutant gln^{129,134}FGF-2,
20 that has a new restriction site Afl2 (underlined). This mutant
is further used to generate two more variants, again by cassette-
directed mutagenesis, using sites Afl2 and Apal and annealed
oligonucleotides 5'-TAAGCAGAC GGGGCAGTAT AAGCTTGGTT CCAAAACAGG
GCC-3' (SEQ ID NO 11) and 5'-CTGTTTTGGA ACCAAGCTTA TACTGCCCCG
25 TCTGC-3' (SEQ ID NO 12) to give mutant gln^{129}-FGF-2 with a new
restriction site Hind3 (underlined), and 5'-TTAAGCGTAC
GG GCAGTAT CAGCTTGGTT CCAAAACAGG GCC-3' (SEQ ID NO 13), 5'-
CTGTTTTGGA ACCAAGCTGAT ACTGCCCCGT ACGC-3' (SEQ ID NO 14) to give
gln^{134}FGF-2 with new restriction site Spll (underlined). The
30 desired mutant plasmids are selected on the basis of suscepti-
bility to cleavage at the newly introduced restriction sites
and confirmed by complete sequencing.

The mutants are then expressed and purified. The
35 plasmids containing the genes encoding the seven mutants
described above are transformed into competent E. coli
BL21 plys S and cultured at 37°C in Luria broth containing

$50\mu g/ml$ ampicillin and $30\mu g/ml$ chloramphenicol until absorbance at 600nm of 0.4 is reached. Expression of the recombinant protein is induced by the addition of 2mM isopropylthiogalactoside for 2 hours at 37°C. Cells from a 1 litre culture are harvested by centrifugation, resuspended in 30ml of 50mM tris-HCl, pH 7.5, containing 0.1mM

5 EDTA and 0.6M NaCl, and disrupted by treatment with lysozyme ($10\mu g/ml$) for 20 minutes at 4°C followed by sonication (6 x 30 sec pulses). The lysates are clarified by centrifugation (10,000 x g, 20 minutes), and the supernatant solutions incubated with 5ml of hydrated heparin Sepharose (Pharmacia/LKB) at 4°C for 1 hour with constant rotation. The resin is isolated by filtration on $0.8\mu m$ filter apparatus (Nalgene), washed

10 extensively with 10mM Tris-HCl pH 7.4, containing 0.6M NaCl, and bound protein is eluted with Tris buffer containing 3M NaCl (25ml). The 3M NaCl eluent is diluted 6-fold with Tris buffer and loaded onto a TSK heparin 5PW column (0.21 x 15cm; the Nest Group, MA) and the column developed using a linear gradient (0.6-2M NaCl) in 90 minutes at a flow rate of 3ml/minute.

15   *E. coli* (BL21 plys S) strains harbouring the mutants were deposited in the American Type Culture Collection (A.T.C.C.) under the provisions of the Budapest Treaty on 13 September 1993. A strain harbouring gln^{128}-FGF-2 bears A.T.C.C. accession number 69419, a strain harbouring gln^{138}-FGF-2 bears A.T.C.C. accession number 69420, and a strain harbouring $gln^{128,138}$-FGF-2 bears A.T.C.C. accession

20 number 69418.

Example 2

Thermodynamic dissociation constants for heparin binding and the stoichiometry of heparin binding to the FGF-2 analogues of Example 1 are obtained.

FGF-2 contains a single tryptophan residue at position 123. Crystallographic data

25 suggest that this tryptophan residue is located close to a cluster of basic residues

above), suggesting that the fluorescence emission of the tryptophan residue may be used to report changes in protein conformation upon the binding of various ligands. Therefore, the fluorescence properties of FGF-2 and FGF-2 mutants

5   in the presence and absence of heparin and a heparin mimic, ß-cyclodextran tetradecasulfate (BCDS) are evaluated in this example.

Fluorescence titration measurements are conducted in a Perkin-Elmer LS-5 fluorospectrometer using excitation and

10 emission wavelengths of 290 nm and 350 nm, respectively. Two milliliters of FGF solution in 10 mM Tris-HCl, pH 7.4, containing 2 mM of dithiothreitol are placed in a cuvette. A small aliquot (~2-5 ul) of heparin (Hepar Industries, Franklin, OH) or BCDS (American Maize-Products, Hammand, IN)

15 is added to the cuvette each time before the fluorescence intensity of the solution is read against a solution of tryptophan that gives comparable fluorescence intensity and to which an identical amount of heparin or BCDS is added each time. Concentrations of FGF-2 and $\text{gln}^{128,138}$FGF-2

20 are 370 nM and 280 nM, respectively. The results are plotted in Figures 1A and 1B, and show that tryptophan fluorescence emission is quenched upon addition of heparin to the protein solution. An equivalent tryptophan residue, Trp122, exists in acidic FGF (FGF-1), for which similar

25 fluorescence quenching is obtained. The addition of BCDS to the protein solution also results in quenching of fluorescence of FGF-2, whereas the addition of the nonsulfated compound (BCD) shows no spectral effects, indicating that sulfation of the ligand is required for binding and change

30 in fluorescence properties of the protein.

The thermodynamic dissociation constant (K_D) is obtained from plots of fluorescence intensity versus ligand concentration. The fluorescence intensity data are fitted by non-linear regression by using equation 1 below, where

35 F_O is observed fluorescence intensity, F_I is initial

intensity, ΔF is the difference between final and the initial fluorescence intensities, C_t is the total FGF protein concentration, K_D is the thermodynamic dissociation constant, and L_t is the total heparin concentration. It is assumed in equation 1 that the stoichiometry of FGF and heparin in the complex is 1:1. A graphic software Kaleidagraph is used for handling data.

(1)
$$F_0 = F_I + \frac{\Delta F((C_t+K_D+L_t) - \sqrt{(C_t+K_D+L_t)^2-4C_tL_t}}{2C_t}$$

However, in the fluorescence titration experiments the titration curves shown in Figure 1 cannot be fitted directly using equation 1. This indicates that the stoichiometry of the protein-ligand complex is not equal to unity. Since the heparin preparation used here is composed of an average of 20 sulfated monosaccharide units per chain, it is likely that more than one FGF molecule is bound per oligosaccharide chain. The extensively positive charges on the surface of the protein and the strong negative charges of the ligands suggest that ionic interactions appear to be the main driving force in the binding of heparin to FGF. Moreover, it is reasonable to assume that, in the absence of any data to indicate otherwise, the binding sites on heparin are independent and the binding affinities are similar. By the same token, BCDS is a seven-membered cyclic oligosaccharide with 14 negatively charged sulfate groups and could possess several binding sites for FGF. Under this assumption, the effective concentration of the ligand needs to be adjusted by the multiplication of the actual concentrations by the number of possible binding sites. If n is the average number of FGF molecules bound per oligosaccharide, then equation 1 can be modified to give

(2)
$$F_0 = F_I + \frac{\Delta F((C_t+K_D+nL_t) - \sqrt{(C_t+K_D+nL_t)^2-4nC_tL_t}}{2C_t}$$

in which the effective concentration of the ligand is
increased by n times.

Equation 2 is used to fit the data shown in Figure 1
and the values of the two variables, K_D and n, are deter-
mined by non-linear regression of at least three titration
curves for each molecule. An average of four (n = 4) FGF-2
molecules interacting with heparin is obtained. Four bind-
ing sites on each heparin molecule are assumed for mutant
$gln^{128,138}$FGF-2. Since the molecular weight of heparin
used in the experiments is about 5000, which has an average
of 20 monosaccharide units, about 4 to 6 monosaccharide
units may interact with each FGF molecule. Two to four FGF
molecules can bind to one BCDS (which has a calculated mole-
cular weight of 2226). This estimate is in good agreement
with that reported by Mach, et al. (Biochemistry 32: 5480-
5489 (1993)) of a stoichiometry of 1:4-6 determined by a
variety of different approaches including light scattering
and analytical ultracentrifugation analyses.

Table I presents the thermodynamic dissociation con-
stants obtained from fluorescence quenching experiments for
the wild-type FGF and the mutants using heparin or BCDS.

Table I Thermodynamic dissociation constants (K_D)
associated with the binding of heparin to FGF-2 and the
mutants.

FGF	K_D Heparin (nM)	K_D BCDS (nM)
FGF-1	2 ± 0.2	4 ± 2
FGF-2	14 ± 6	14 ± 7
gln^{128}FGF-2	234 ± 34	178 ± 44
gln^{138}FGF-2	126 ± 53	228 ± 23
$gln^{128,138}$FGF-2	1280 ± 380	2700 ± 600
gln^{129}FGF-2	3 ± 3	3 ± 1
gln^{134}	2 ± 0.2	12 ± 0.1
$gln^{129,134}$FGF-2	8 ± 6	130 ± 27

It can be seen from the data that the K_D values change dramatically for site 1 mutants but not for the proteins containing mutations at site 2. The affinity toward heparin decreases about 10-fold for mutant gln^{128}-

5 FGF-2 and gln^{138}FGF-2 and about 100-fold for the double mutant $gln^{128,138}$FGF-2. Similar results are observed with BCDS.

In an investigation of the concentrations of NaCl required to elute the FGF and mutant proteins from heparin

10 Sepharose resin, gln^{128}FGF-2 is the only mutant protein that required a lower salt concentration to be eluted.

Example 3

Binding and relevant biological activity studies using the FGF-2 mutants isolated and purified in Example 1 are

15 made.

The mutant proteins are tested for their capacity to compete for the binding of ^{125}I-FGF-2 (Amersham Corp.) to baby hamster kidney (BHK) cells, which express high numbers of FGF receptors, as described by Moscatelli (*J. Cell*

20 *Physiol.* *131*: 123-130 (1987)). Briefly, cells plated on 24-well plates are incubated with 50 pM ^{125}I-FGF-2 with serial dilutions of unlabelled FGF-2 or the mutants at room temperature for 1 hour. The cells are then incubated at 4°C for 30 minutes, washed twice with phosphate-buffered saline

25 and treated with 20 mM Hepes, pH 7.5, containing 2 M NaCl to remove ^{125}I-FGF-2 bound to low affinity heparan sulfate binding sites. Receptor-bound FGF-2 is recovered by treatment of the cells with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8, and counted in a gamma-counter. Assays are

30 conducted in duplicate. The results are plotted in Figure 2. No significant difference is observed for any of the FGF-2 mutants as compared to the wild type.

The mitogenic activity of FGF-2 and mutants are deter-
mined using bovine vascular endothelial cells derived from
adult aortic arch as described by Gospardarowicz, et al.
(Proc. Nat. Acad. Sci. 81: 6963-6967 (1984)). Briefly,
cells are seeded at an initial density of 8000 cells per
24-well plate in 0.5 ml Dulbecco's modified Eagle's medium
(DMEM) containing 10% calf serum (Hyclone, Logan, UT) sup-
plemented with penicillin (100 units/ml), streptomycin (100
ug/ml) and L-glutamine (2 mM). Two hours after plating,
20 ul aliquots of serial dilutions of FGF-2 and mutants
in DMEM are added. After 5 days in culture, duplicate
plates are trypsinized and cell densities determined by cell
counting in a Coulter counter. Determinations are conducted
in duplicate. The results are plotted in Figure 3.

The activity of FGF-2 and the mutants to induce pro-
liferation of ABAE cells is determined as a function of
added FGF concentrations in the culture media. The EC_{50}
values for mutants $gln^{128}FGF-2$ and $gln^{128,138}FGF-2$ in
the site 1 group (Figure 3A) show about a 10-fold increase,
from 0.5 ng/ml to 5.0 ng/ml, whereas mutant $gln^{138}FGF-2$
and all site-2 mutants show no significant change as com-
pared to the wild-type value (Figure 3B).

Urokinase-type plasminogen activator induction by the
FGF mutants is evaluated. Adult bovine aortic endothelial
(ABAE) cells are seeded at 20,000 cells/well in 96-well
plates and maintained in DMEM containing 10% calf serum
(Hyclone, Logan, UT) supplemented with penicillin (100
units/ml), streptomycin (100 ug/ml) and L-glutamine (2
mM) and different concentrations of FGF-2 or mutants. After
24 hours, the cells are washed with phosphate-buffered
saline and lysed in 60 mM Tris-HCl, pH 8.5, containing 0.05%
Triton X-100. Cell-associated urokinase-type plasminogen
activator (uPA) activity is measured as described by Presta,
et al., cited above, using the plasmin chromogenic substrate

D-norleucyl-hexahydrotyrosyl-lysine p-nitroanilide acetate
(American Diagnostics, Greenwich, CT). Cell-associated
protein concentrations are determined using Coomassie blue
- binding to protein. The results are plotted in Figure 4.
5 Mutant gln^{128,138}FGF-2 is the only mutant that exhibits a
markedly reduced capability to induce uPA.

In vitro angiogenesis evaluations are made by
observing whether the mutants induce capillary-like struc-
tures in ABAE cells cultured on a 3-dimensional collagen
10 gel. Three-dimensional collagen gel plates (24-well) are
prepared by adding 0.5 ml chilled solution of 0.7 mg/ml
rat-tail type I collagen (Becton Dickinson Labwares, Bed-
ford, MA) containing 1X DMEM and adjusting to neutral pH
with NaHCO$_3$ to each well. After formation of collagen gel
15 (about 1-2 mm thickness), ABAE cells are seeded at 50,000
cells/well. The cultures are maintained at 37°C in DMEM
containing 10% calf serum (Hyclone, Logan, UT) supplemented
with penicillin (100 units/ml), streptomycin (100 ug/ml)
and L-glutamine (2 mM) until the cultures reach confluency,
20 usually in 5 days by which time the cells form a monolayer
on the gel. The medium is then replaced with fresh medium
containing different concentrations of FGF-2 or mutants.
The cultures are maintained at 37°C for 48 hours, then dis-
continued by fixation with cold methanol (-20°C).

25   The abundance of the capillary-like structures formed
by ABAE cells is analyzed by using a Kontron IBAS Image
Analyzer assisted with a Hamamatsu C2400 video camera and a
Zeiss Axioshop microscope. The phase-contrast image of each
field obtained with a video camera is converted to a binary
30 image in which the areas occupied by capillary-like struc-
tures are white and the rest is a black background. The
extent of in vitro angiogenesis is then measured as a
percentage of the white areas divided by the total area
measured. Cell cultures of identical conditions are main-

tained in duplicate wells. Three to four areas of each well
are examined by image analysis and the mean value and
standard deviation are determined. The results from this
computer-assisted quantitation method are set out in Figure

5   5. Tube formation induced by mutant $gln^{128,138}$FGF-2 is
much less than that indicated by wild-type FGF-2 and other
mutants.

        The above description is for the purpose of teaching
the person of ordinary skill in the art how to practice the

10 present invention, and it is not intended to detail all
those obvious modifications and variations of it which will
become apparent to the skilled worker upon reading the
description. It is intended, however, that all such obvious
modifications and variations be included within the scope of

15 the present invention as defined in the appended claims.

INFORMATION FOR SEQ ID NO: 1

    (i)   SEQUENCE CHARACTERISTICS

          (A) LENGTH: 55 bases

          (B) TYPE: nucleic acid

5   (C) STRANDEDNESS: single

          (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE

          (A) DESCRIPTION: oligonucleotide

    (v)   FRAGMENT TYPE: synthetic DNA

10 (ix)   FEATURE

          (D) INFORMATION: primer used in preparing constructs

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 1:

GTCTAGAAAA TACACCAGTT GGTACGTAGC ACTGCAGCGA ACCGGTCAGT ATAAA

INFORMATION FOR SEQ ID NO: 2

15 (i)   SEQUENCE CHARACTERISTICS

          (A) LENGTH: 34 bases

          (B) TYPE: nucleic acid

          (C) STRANDEDNESS: single

          (D) TOPOLOGY: linear

20 (ii)   MOLECULE TYPE

          (A) DESCRIPTION: oligonucleotide

    (v)   FRAGMENT TYPE: synthetic DNA

    (ix)   FEATURE

          (D) INFORMATION: primer used in preparing constructs

25 (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 2:

CTTGGTTCCC AAACAGGGCC CGGGCAGAAA GCTA

INFORMATION FOR SEQ ID NO: 3

    (i)   SEQUENCE CHARACTERISTICS

          (A) LENGTH: 50 bases

30   (B) TYPE: nucleic acid

          (C) STRANDEDNESS: single

          (D) TOPOLOGY: linear

    (ii)   MOLECULE TYPE

          (A) DESCRIPTION: oligonucleotide

35 (v)   FRAGMENT TYPE: synthetic DNA

    (ix)   FEATURE

          (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 4

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 3:

GTAGCACTTA AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC

INFORMATION FOR SEQ ID NO: 4

  (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 46 bases

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 3

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 4:

CTGTTTGGGA ACCAAGCTTA TACTGCCCCG TACGCTTAAG TGCTAC

INFORMATION FOR SEQ ID NO: 5

  (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 50 bases

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 6

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 5:

GTAGCACTGC AGCGTACGGG GCAGTATAAG CTTGGTTCCC AAACAGGGCC

INFORMATION FOR SEQ ID NO: 6

  (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 46 bases

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 5

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 6:

CTGTTTGGGA ACCAAGCTTA TACTGCCCCG TACGCTGCAG TGCTAC

INFORMATION FOR SEQ ID NO: 7

   (i)   SEQUENCE CHARACTERISTICS

       (A) LENGTH: 28 bases

       (B) TYPE: nucleic acid

5   (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

       (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

10 (ix)   FEATURE

       (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 8

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 7:

GTAGCACTGC AGCAGACGGG GCAGTATC

INFORMATION FOR SEQ ID NO: 8

15 (i)   SEQUENCE CHARACTERISTICS

       (A) LENGTH: 32 bases

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

20 (ii)   MOLECULE TYPE

       (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

       (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 7

25 (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 8:

AGCTGATACT GCCCCGTCTG CTGCAGTGCT AC

INFORMATION FOR SEQ ID NO: 9

   (i)   SEQUENCE CHARACTERISTICS

       (A) LENGTH: 50 bases

30   (B) TYPE: nucleic acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

       (A) DESCRIPTION: oligonucleotide

35 (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

       (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 10

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 9:

GTAGCACTTA AGCAGACGGG GCAGTATCAG CTTGGTTCCA AAACAGGGCC

INFORMATION FOR SEQ ID NO: 10

  (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 46 bases

        (B) TYPE: nucleic acid

5   (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

10 (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 9

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 10:

CTGTTTTGGA ACCAAGCTGA TACTGCCCCG TCTGCTTAAG TGCTAC

INFORMATION FOR SEQ ID NO: 11

15 (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 43 bases

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

20 (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

  (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 12

25 :i)   SEQUENCE DESCRIPTION: SEQ ID NO 11:

TTAAGCAGAC GGGGCAGTAT AAGCTTGGTT CCAAAACAGG GCC

INFORMATION FOR SEQ ID NO: 12

  (i)   SEQUENCE CHARACTERISTICS

        (A) LENGTH: 35 bases

30   (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

  (ii)   MOLECULE TYPE

        (A) DESCRIPTION: oligonucleotide

35 (v)   FRAGMENT TYPE: synthetic DNA

  (ix)   FEATURE

        (D) INFORMATION: used in preparing constructs annealed with SEQ ID NO 11

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO 12:

CTGTTTTGGA ACCAAGCTTA TACTGCCCCG TCTGC

INFORMATION FOR SEQ ID NO: 13

(i)   SEQUENCE CHARACTERISTICS

    (A) LENGTH: 43 bases

    (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

(ii)   MOLECULE TYPE

    (A) DESCRIPTION: oligonucleotide

(v)   FRAGMENT TYPE: synthetic DNA

(ix)   FEATURE

    (D) INFORMATION: used in preparing constructs

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO 13:

TTAAGCGTAC GGGGCAGTAT CAGCTTGGTT CCAAAACAGG GCC

INFORMATION FOR SEQ ID NO: 14

(i)   SEQUENCE CHARACTERISTICS

    (A) LENGTH: 34 bases

    (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

(ii)   MOLECULE TYPE

    (A) DESCRIPTION: oligonucleotide

(v)   FRAGMENT TYPE: synthetic DNA

(ix)   FEATURE

    (D) INFORMATION: used in preparing constructs

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO 14:

CTGTTTTGGA ACCAAGCTGAT ACTGCCCCGT ACGC

BIBLIOGRAPHY

Arakawa, T. and Fox G.M., *Eur. Pat. Ap. No.* 320,148 (1989).

Baird, A., and Böhlen, P., *Handbook of Exp. Pharmacol. 95*(1): 369-418, Springer, 1990.

Baird, A., *et al.*, *Proc. Nat. Acad. Sci. USA 85*: 2324-2328 (1988).

Bergonzoni, L., *et al.*, Eur. Pat. Ap. Pub. No. 363,675, 18 April 1990.

Eriksson, E.A., *et al.*, *Proc. Nat. Acad. Sci. USA 88*: 3441-3445 (1991).

Fiddes, J.C., *et al.*, Eur. Pat. Ap. Pub. No. 298,723 (1989).

Franco, W.P., U.S. Pat. No. 4,378,347, Mar. 29, 1983.

Gospardarowicz, D., *et al.*, *Proc. Nat. Acad. Sci. 81*: 6963-6967 (1984).

Mach, H., *et al.*, *Biochemistry 32*: 5480-5489 (1993).

Moscatelli, D., *J. Cell. Physiol. 131*: 123-130 (1987).

Presta, M., *et al.*, *Biochem. Biophys. Res. Commun. 185*: 1098-1107 (1992).

Rosenberg, A.H., *et al.*, *Gene 56*: 125-135 (1987).

Seddon, A.P., *et al.*, *Annals N.Y. Acad. Sci. 638*: 98-108 (1991).

Seno, M., *et al.*, Eur. Pat. Ap. Pub. Nos. 281,822 (1988) and 326,907 (1989).

Wells, J.A., *et al.*, *Gene 34*: 315-323 (1985).

Zhang, J., *et al.*, *Proc. Nat. Acad. Sci. USA 88*: 3446-3450 (1991).

The claims defining the invention are as follows:

1.　　A fibroblast growth factor-2(FGF-2) structural analogue selected from $Gln^{134}FGF-2$, $Gln^{138}FGF-2$, $Gln^{128,138}FGF-2$, and $Gln^{129,134}FGF-2$.

2.　　A DNA sequence encoding said FGF-2 structural analogue according to claim 1.

3.　　A method for the treatment or prophylaxis of angiogenic neoplasms in a mammal requiring said treatment or prophylaxis, which method comprises administering to said mammal an effective amount of the $Gln^{128,138}FGF-2$ structural analogue of claim 1.

4.　　A method for the treatment of tissue damage in a mammal requiring said treatment, which method comprises administering to said mammal an effective amount of the $Gln^{129,134}FGF-2$ structural analogue of claim 1.

5.　　A hybridoma cell line bearing the ATCC accession number 69420.

6.　　A hybridoma cell line bearing the ATCC accession number 69418.

Dated 15 May, 1997
American Cyanamid Company

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

HEPARIN BINDING SITE STRUCTURAL ANALOGUES

OF FIBROBLAST GROWTH FACTORS

ABSTRACT

Structural analogues of fibroblast growth factors have at least one point mutation in ⹂ heparin binding site. Typical point mutations are deletions or replacements of basic residues with other residues, e.g., neutral or acidic resi-
5 dues. In some embodiments, the analogues have at least one point mutation in a heparin binding site. Preferred analogues have one or two basic residues replaced by glutamine, and exhibit a binding affinity to cell surface receptors similar or superior to native growth factor, but different
10 heparin binding and biological activity from the native growth factor.
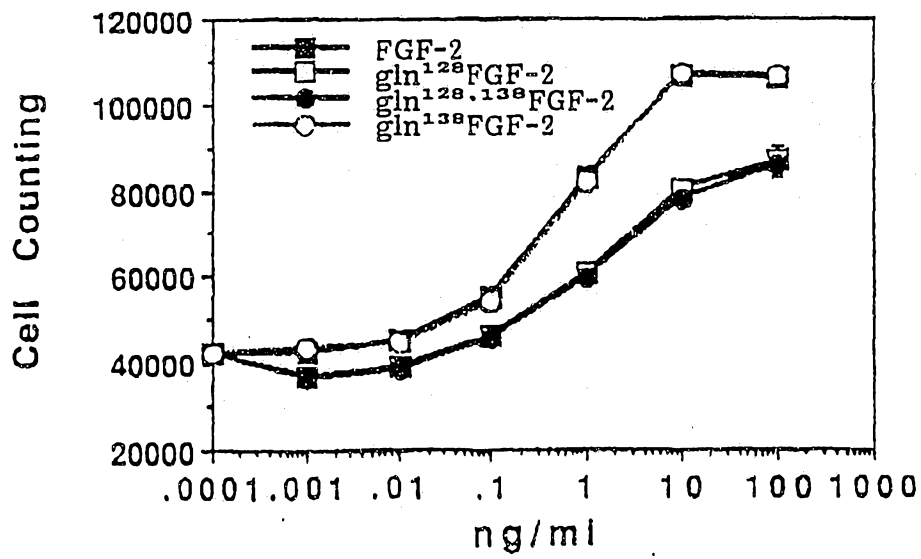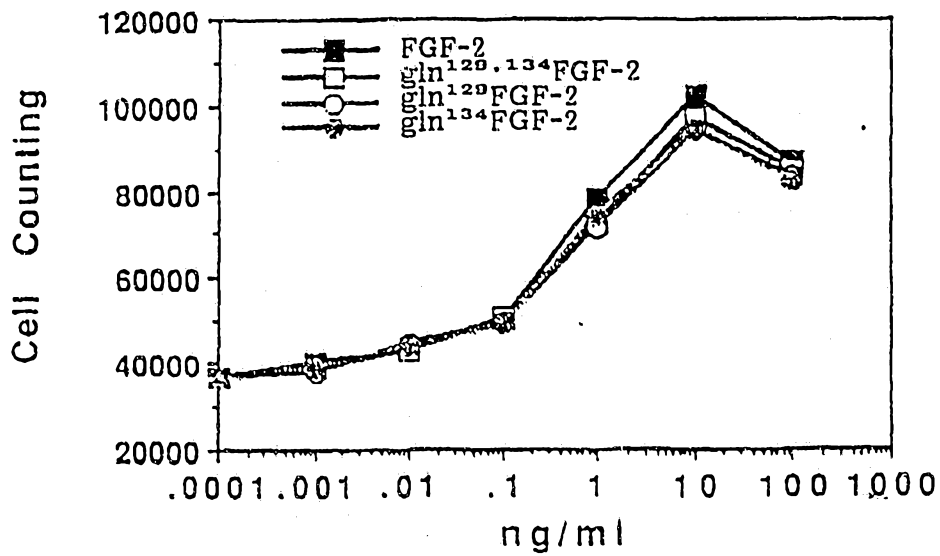
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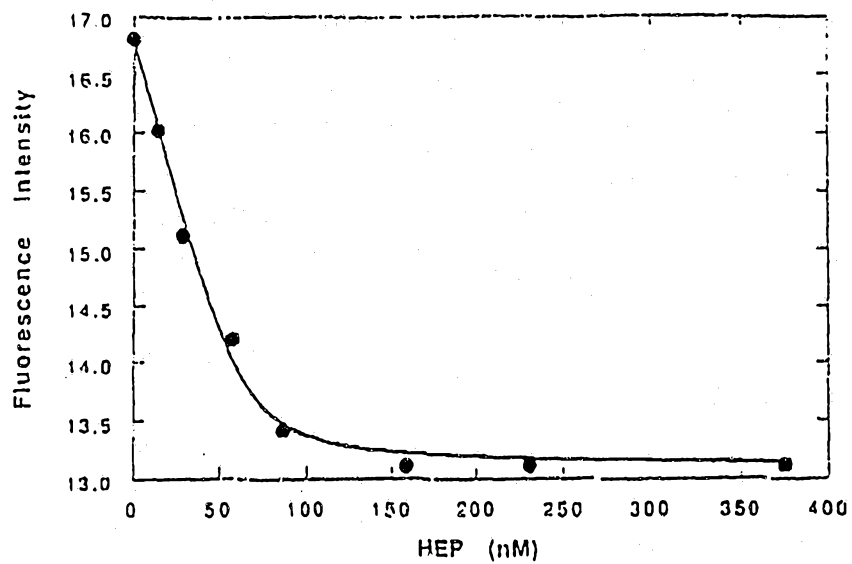
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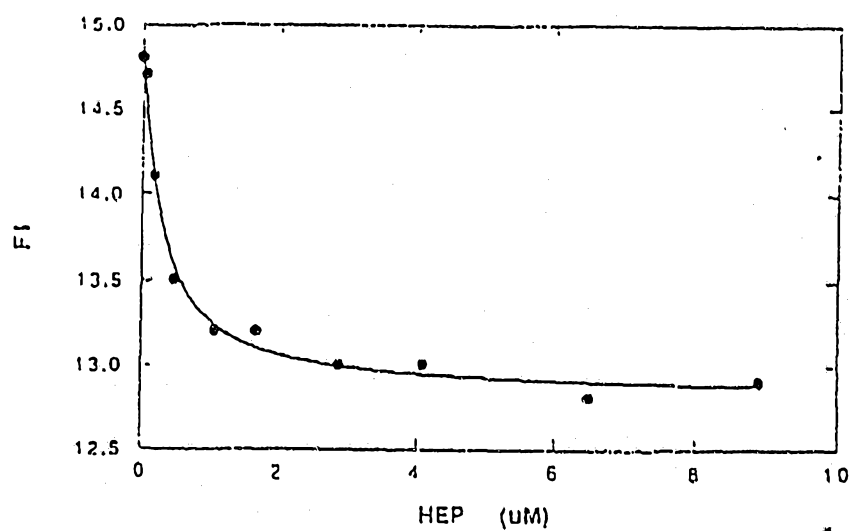
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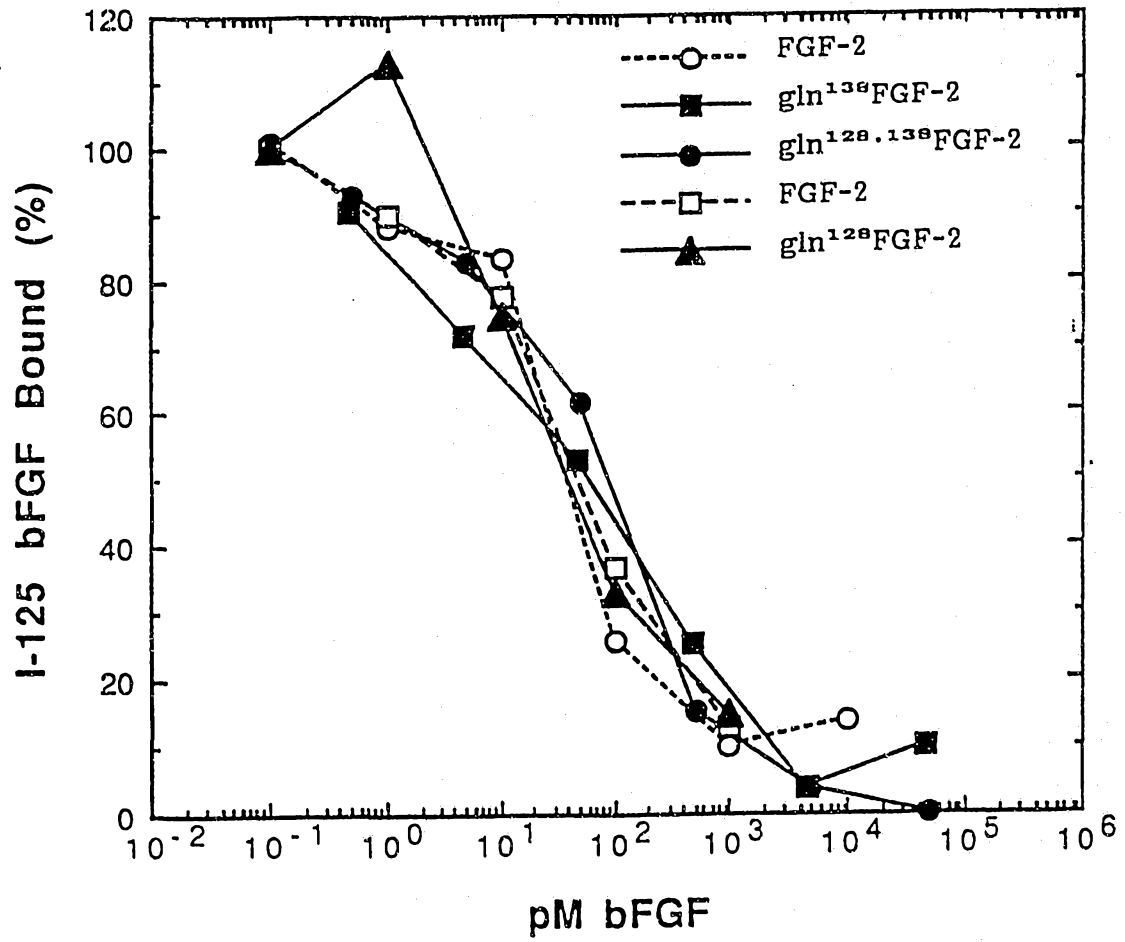
Figure 2

Figure 3A

Figure 3B

Figure 1

Figure 5