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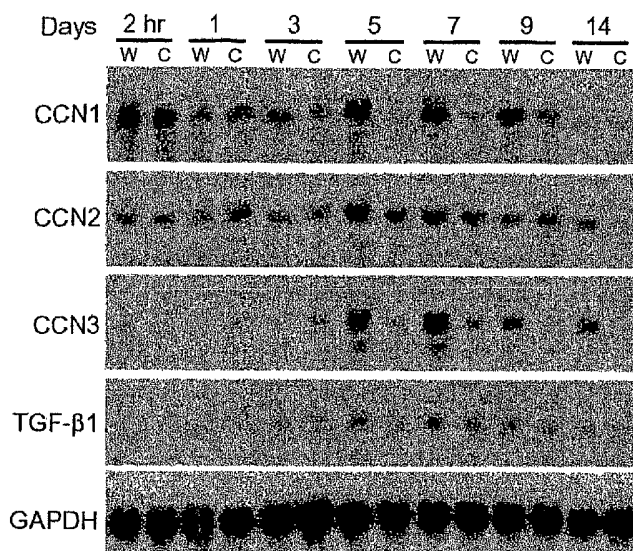
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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING TISSUE REGENERATION AND CHEMOTACTIC RESPONSES



(57) Abstract: The present invention relates to the fields of monitoring and modulating wound healing responses, controlling stem cell homing *in vivo* and tissue construction *in vitro*. Specifically, the present specification discloses that monitoring CCN3 expression during wound healing can be used for diagnostic purposes of detecting abnormal wound repair. The compositions blocking CCN3 activity for the methods of inhibiting wound repair are also disclosed, as well as compositions of expressing CCN3 polypeptide or its functional fragment for the methods of stimulating wound repair. Furthermore, the instant specification also discloses that CCN3 can be added to stem cells to retain the cells in a particular area and, therefore, accomplish stem cell homing. Finally, the instant invention also discloses that CCN3 polypeptide can be used for controlling cell migration during tissue construction *in vitro*.

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COMPOSITIONS AND METHODS FOR MODULATING TISSUE REGENERATION
AND CHEMOTACTIC RESPONSES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/615,213, filed September 28, 2004, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0002] The present invention relates generally to tissue regeneration and chemotactic responses. More specifically, the present invention relates to extracellular matrix signaling molecules such as CCN3-related polypeptides.

2. Description of Related Art

[0003] In a normal wound healing response to physical disruption of tissue such as trauma or surgery, tissue is regenerated through a complex process requiring the orchestration of many different types of cells. After hemostasis, inflammation and cell proliferation and migration follow. Inflammation is characterized by vasodilation, increased vascular permeability, leukocyte infiltration, bacterial killing, and macrophage-based stimulation of cellular proliferation and protein synthesis. In cell proliferation and migration, fibroblasts appear within 2-3 days and dominate wound cell population during the first week. For the initial 2-3 days, their activity is confined to fibroblast replication and migration. Cell migration is based on chemotaxis, which is the movement of cells with or against a chemical gradient. At days 4-5 fibroblasts begin to synthesize and secrete extracellular collagen. The collagen is polymerized and cross-linked to increase the tensile strength of the tissue. Granulation tissue forms at days 5-7 and contains numerous capillaries. Granulation tissue has a support matrix rich in fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and pericytes. Later stages of wound healing may continue for years, depending on the severity of the wound.

[0004] Angiogenesis is essential to wound repair and scar formation. Capillary proliferation is required to support fibroblast migration into the wound and to fulfill fibroblast metabolic requirements. In the absence of angiogenesis, such as in ischemic ulcers or arteriosclerosis

obliterans, fibroblast migration arrests and fails to proceed. Angiogenesis has the steps of cell attachment, basement membrane degradation and migration, proliferation and differentiation, and is associated with epithelial cell migration.

[0005] If a patient's injury is severe, or if the patient suffers from a condition in which the wound healing response is delayed or inhibited, a treatment that would improve or accelerate tissue regeneration would be indicated. For example, in patients with diabetes, liver failure, renal impairment, peripheral vascular disease, or in patients taking drugs that inhibit healing such as corticosteroids or immunosuppressive agents, a therapy to accelerate wound healing could forestall serious consequences of persistent wounds such as infection or tissue necrosis, and could reduce the need for amputation.

[0006] Under other circumstances, suppression or inhibition of the wound healing response would be appropriate. For example, over-expression of the wound healing response could result in conditions such as arthrofibrosis, scleroderma, Dupuytren's contracture, peritoneal adhesions, frozen shoulder, and keloid formation. For other therapeutic reasons, it may also be desirable to suppress or inhibit a normal wound healing response in some patients.

SUMMARY OF THE INVENTION

[0007] Compositions and methods are provided for monitoring the progression of wound healing and modulating tissue regeneration responses. Tissue regeneration may be stimulated by administering to a patient compositions comprising a CCN3 polypeptide or a nucleic acid encoding CCN3 that is operably linked to a control element allowing expression of the polypeptide. Tissue regeneration may be inhibited by administering to a patient a composition comprising an inhibitory polypeptide that selectively interferes with binding of CCN3 to its target receptors. Tissue regeneration may also be inhibited in a patient by administering to the patient a composition comprising an antibody that selectively binds to CCN3.

[0008] Compositions and methods are provided for monitoring wound repair in a patient by measuring the level of expression of CCN3 mRNA and/or CCN3 protein in a sample obtained from the patient, and comparing the level of expression to the level of expression in a control.

[0009] Methods are also provided for inducing chemotaxis of stem cells to a desired location, such as a wound site, in which stem cells are administered to a patient in combination with a CCN3 polypeptide.

[0010] Methods and compositions are also provided for aiding in tissue construction *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **Figure 1.** Expression of *CCN1*, *CCN2*, and *CCN3* during cutaneous wound healing. A, RNA blot of wounded skin. Full-thickness cutaneous excisional wounds were created on the back of 2 month old CD-1 mice using a biopsy instrument. Mice were sacrificed from 2 hours through 15 days post injury as indicated. Total RNA was isolated from wounded (W) and control (C, uninjured skin adjacent to the wound site) tissue and analyzed by RNA blotting with radiolabeled probes of indicated genes. B, immuostaining of skin wounds. Mice treated with cutaneous wounding as described above were sacrificed at indicated days post-injury. Cryosections of frozen tissues embedded in OCT were analyzed by immunostaining affinity-purified polyclonal anti-CCN3 antibodies. For negative controls, no primary antibody was added (PBS), or anti-CCN3 antibodies (1:1000) were blocked with the CCN3 antigen (0.5 g/ml; Anti-CCN3 +GST-CCN3) prior to application on tissue sections. Micrographs were taken at 100 x (days 0, 1, and 7) or at 200X magnification (day 5).

[0012] **Figure 2.** Fibroblast adhesion to CCN3. A, 1064SK skin fibroblasts were plated in microtiter wells pre-coated with the indicated amount of CCN3. After incubation at 37°C for 30 min., adherent cells were fixed, stained with methylene blue, and extracted dye was quantified by absorbance at 620 nm. B, cells were incubated with GRGDSP, GRGESP polypeptides (2.0 mM), 0.1 µg/ml heparin or in combination prior to plating on microtiter wells coated with CCN3, FN. Data shown are mean ± SD of triplicate determinations and are representative of three experiments.

[0013] **Figure 3.** CCN3 supports fibroblast adhesion through integrins $\alpha_6\beta_1$ and $\alpha_5\beta_1$. Fibroblast adhesion was performed as described in Figure 2. Microtiter wells were coated with either 3 µg/ml CCN3, 0.5 µg/ml VN, 2 µg/ml FN, 5 µg/ml LN, or 0.5 µg/ml Type 1 collagen as indicated. Cells were incubated with 50 µg/ml anti- $\alpha_5\beta_1$ mAb JBS5 (A), 50 µg/ml anti- $\alpha_v\beta_3$ mAb LM609 (B), 20 µg/ml anti- α_6 mAbs (C), or 40 µg/ml anti- β_1 mAb P4C10 for 1 hour prior to plating. Data shown are mean ± SD of triplicate determinations and are representative of three experiments.

[0014] **Figure 4.** CCN3 induces chemotaxis in fibroblasts. 1064SK fibroblast migration was monitored using a modified Boyden chamber assay. Test protein was added to the bottom

chamber (unless otherwise indicated) and covered by a gelatinized polycarbonate filter. Cells were added to wells in the upper chamber and allowed to migrate for 6 hours at 37°C before being fixed and stained. Cells that migrated into the lower chamber were counted in ten random high power fields. A, cell migration in response to varying concentrations of CCN3 as indicated. B, migration of fibroblasts was measured in a checkerboard-type analysis. CCN3 or bFGF (10 ng/ml) was added to the lower chamber, the upper chamber, neither chamber, or both chambers as indicated. Data shown are mean $\pm$ SD of triplicate determinations and are representative of three experiments.

[0015] Figure 5. Fibroblast migration to CCN3 is $\alpha_v\beta_5$ -dependent. Migration assays were performed using a modified Boyden chamber. As chemoattractants, CCN3 (2 μ g/ml), vitronectin (10 μ g/ml), and FN (10 μ g/ml) were placed in the bottom chamber. Cells were treated with anti- α_v mAb AV1 (50 μ g/ml), anti- $\alpha_v\beta_5$ mAb P1F6 (50 μ g/ml), anti- $\alpha_v\beta_3$ mAb LM609 (40 μ g/ml) or anti-integrin β_5 mAb (P1D6, 40 μ g/ml) for 1 hour prior to chamber loading. Data shown are mean $\pm$ SD of triplicate determinations and are representative of three experiments.

[0016] Figure 6. Direct binding of CCN3 to integrin $\alpha_v\beta_5$. A, microtiter wells were coated with purified integrin $\alpha_v\beta_5$ (1 μ g/ml) and blocked with 1% BSA. Binding of varying concentrations of CCN3 was detected using anti-CCN3 antibodies by ELISA. B-D, microtiter wells were coated with CCN3 (10 μ g/ml) or VN (1 μ g/ml) and blocked with BSA, and binding of purified $\alpha_v\beta_5$ (1 μ g/ml) was observed by ELISA using anti- α_v antibodies. Effects of pre-incubation of coated proteins with anti-CCN3 antibodies, anti- $\alpha_v\beta_5$ mAb P1F6 (50 μ g/ml), or 20 μ g/ml normal mouse IgG prior to addition and binding of integrin $\alpha_v\beta_5$ was observed. In panel C, integrin $\alpha_v\beta_5$ was incubated with 5 mM EDTA, EDTA + 10 mM Mg^{2+} , 0.2 mM GRGDSF polypeptide, or 0.2 mM GRGESF polypeptide for 30 min. at 4°C prior to addition into microtiter wells. Data shown are from three separate experiments and represented as mean $\pm$ SD of triplicate determinations in each experiment.

[0017] Figure 7. CCN3 enhances bFGF-induced DNA synthesis. The effect of soluble CCN3 on bFGF-induced mitogenesis under serum-free conditions was assessed on fibroblasts attached to 24-well plates. Serum-starved cells were treated with 10 ng/ml bFGF, the indicated concentration of CCN3, and [3 H]thymidine for 21 hours before incorporation was measured.

Controls include samples treated with either BSA or 20 μ g/ml CCN3 in the absence of bFGF.

Data shown for all panels are mean $\pm$ S.D. of triplicate determinations and are representative of duplicate experiments.

[0018] Figure 8. CCN3 induced gene expression in fibroblasts is modulated by TGF- β 1.

Serum-starved human skin fibroblasts were treated for 24 hours with various concentration (from 0 to 10 μ g/ml) of CCN3, either in the presence or absence of 20 ng/ml of TGF- β 1. Total RNA was isolated and analyzed by RNA blotting. Expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed and served as sample loading control. Serum-starved fibroblasts were treated with 10 ng/ml TGF- β 1 alone or in combination with varying concentrations of CCN3 for 24 hours.

DETAILED DESCRIPTION

[0019] Before the present compounds, products and compositions and methods are disclosed and described, it is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the context clearly dictates otherwise.

1. Definitions

[0020] As used herein, the term "analog", when used in the context of a peptide or polypeptide, means a peptide or polypeptide comprising one or more non-standard amino acids or other structural variations from the conventional set of amino acids.

[0021] As used herein, the term "antibody" means an antibody of class IgG, IgM, IgA, IgD or IgE, or fragments or derivatives thereof, including Fab, F(ab')₂, Fd, and single chain antibodies, diabodies, bispecific antibodies, bifunctional antibodies, and derivatives thereof. The antibody may be a monoclonal antibody, polyclonal antibody, affinity purified antibody, "humanized" antibody products, CDR-grafted antibody products, or mixtures thereof which exhibit sufficient binding specificity to a desired epitope, or a sequence derived therefrom. The antibody may also be a chimeric antibody. Also contemplated are antibody fragments. The antibody products include the aforementioned types of antibody products used as isolated antibodies or as antibodies attached to labels. Labels can be signal-generating enzymes, antigens, other antibodies, lectins, carbohydrates, biotin, avidin, radioisotopes, toxins, heavy metals, and other

~~compositions known in the art.~~ The antibody may be derivatized by the attachment of one or more chemical, polypeptide, or polypeptide moieties known in the art. The antibody may be conjugated with a chemical moiety.

[0022] As used herein “biological activity of CCN3” includes, but is not limited to, the activities of full-length CCN3 described herein, and the ability to be bound by an antibody specific for CCN3.

[0023] As used herein, the term “derivative”, when used in the context of a peptide or polypeptide, means a peptide or polypeptide different other than in primary structure (amino acids and amino acid analogs). By way of illustration, derivatives may differ by being glycosylated, one form of post-translational modification. For example, peptides or polypeptides may exhibit glycosylation patterns due to expression in heterologous systems. If at least one biological activity is retained, then these peptides or polypeptides are derivatives according to the invention. Other derivatives include, but are not limited to, immunogenic carriers such as Keyhole Limpet Hemocyanin, radiolabelled peptides or polypeptides, fusion peptides or fusion polypeptides having a covalently modified N- or C-terminus, PEGylated peptides or polypeptides, peptides or polypeptides associated with lipid moieties, alkylated peptides or polypeptides, peptides or polypeptides linked via an amino acid side-chain functional group to other peptides, polypeptides or chemicals, and additional modifications as would be understood in the art.

[0024] As used herein, the term “fragment”, when used in the context of a peptide or polypeptide, means a peptide of from about 8 to about 50 amino acids in length. The fragment may be 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acids in length.

[0025] As used herein, the term “homolog”, when used in the context of a peptide or polypeptide, means a peptide or polypeptide sharing a common evolutionary ancestor.

[0026] As used herein, “tissue regeneration” means the growth or regrowth of tissue, either *in vivo*, *ex vivo*, or *in vitro*.

[0027] As used herein, the term “treat” or “treating” when referring to protection of a mammal from a condition, means preventing, suppressing, repressing, or eliminating the condition. Preventing the condition involves administering a composition of the present invention to a mammal prior to onset of the condition. Suppressing the condition involves administering a

composition of the present invention to a mammal after induction of the condition but before its clinical appearances. Repressing the condition involves administering a composition of the present invention to a mammal after clinical appearance of the condition such that the condition is reduced or maintained. Elimination of the condition involves administering a composition of the present invention to a mammal after clinical appearance of the condition such that the mammal no longer suffers the condition.

[0028] As used herein, the term “stem cells” refers to highly proliferative cells that can give rise to daughter cells with more than one fate, that is they are pluripotent. Stem cells may be autologous or non-autologous.

[0029] As used herein, the term “variant”, when used in the context of a peptide or polypeptide, means a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retains at least one biological activity.

2. Modulation of Tissue Regeneration Using CCN3

[0030] CCN3 is shown herein to promote tissue regeneration. As a result, CCN3 polypeptides may be used to increase tissue regeneration. CCN3 inhibitory polypeptides may be used to decrease tissue regeneration. CCN3 polypeptides and inhibitory polypeptides may be used alternatively or in combination, depending on the patient's indications and whether an increase or decrease of tissue regeneration is desired.

[0031] Modulators of tissue regeneration, such as CCN3 polypeptides, may be used for treating wound healing disorders including, but not limited to, diabetic foot and leg ulcerations, including neuropathic ulcerations, decubitus lesions, and necrobiosis lipoidica diabetorum; vascular ulcerations, including venous stasis ulceration, arterial ulcerations, varicose vein ulcerations, post-thrombotic ulcerations, atrophie blanche ulcerations, congenital absence of veins/ulcerations, congenital or traumatic arteriovenous anastomosis, temporal arteritis, atherosclerosis, hypertension (Martorell's ulcerations), thrombosis, embolism, platelet agglutination, ankle blow-out syndrome, or hemangiomas; decubitus ulcers or pressure sores (e.g., with bed rest); traumatic ulcerations, such as those caused by external injuries, burns, scalds, chemical injuries, post-surgical injuries, self-inflicted injuries, lesions at an injection site, neonatal or perinatal trauma, or sucking blisters; infestations and bites, such as those caused by spiders, scorpions, snakes, or fly larvae; cold injury, such as perniosis (erythrocyanosis frigida), or cryoglobulinemic ulcerations; neoplastic ulceration, such as those caused by basal cell

carcinomas, squamous cell carcinomas, malignant melanomas, lymphoma, leukemia, Kaposi's sarcoma, tumor erosion, midline lethal granuloma, or Wegener's granulomatosis; blood diseases with ulcerations, such as polycythemia, spherocytosis, or sickle cell anemia; skin diseases with ulcerations, such as tinea, psoriasis, pemphigoid, pemphigus, neurotic excoriations, trichotillomania, erosive lichen planus, or chronic bullous dermatosis of childhood; metabolic disease ulcerations, such as those associated with diabetes mellitus or gout (hyperuricemia); neuropathic ulcerations, such as those associated with diabetes mellitus, tabes dorsalis, or syringomyelia; ischemic ulcerations, such as those associated with scars, fibrosis, or radiation dermatitis; vasculitis ulcerations, such as those associated with lupus erythematosus, rheumatoid arthritis, scleroderma, immune complex disease, pyoderma gangrenosum, or ulceration associated with lipodermatosclerosis; infectious ulcerations, such as: viral ulcerations, e.g. those associated with Herpes simplex or Herpes zoster in an immunocompromised or normal individual; bacterial infections with ulcerations, such as those associated with tuberculosis, leprosy, swimming pool granuloma, ulceration over osteomyelitis, Buruli ulcer, gas gangrene, Meleny's ulcer, bacterial gangrene associated with other bacterial infection (e.g., streptococcal infection), scalded skin syndrome, ecthyma gangrenosum (such as can occur in children infected with *Pseudomonas aeruginosa*), and toxic epidermal necrolysis; mycotic ulcerations, such as those associated with superficial fungal infection or deep fungal infection; spirochetal ulcerations, such as those associated with syphilis or yaws; leishmaniasis; mydriasis; or cellulitis; surgical ulcerations, such as those associated with closed incisions or excisions, open incisions or excisions, stab wounds, necrotic incisions or excisions, skin grafts, or donor sites; or other ulcerations, such as those associated with skin tears (traumatic ulcerations), fistula, peristomal ulcerations, ulcerations associated with aplasia cutis congenita, ulcerations associated with epidermolysis bullosa, ulcerations associated with ectodermal dysplasias, ulcerations associated with congenital protein C or S deficiency, ulcerations associated with congenital erosive and vesicular dermatosis, ulcerations associated with acrodermatitis enteropathica, and amputation stump ulcerations.

[0032] Modulators of tissue regeneration, for example, CCN3 inhibitory polypeptides, may be used in treatment of disorders such as arthrofibrosis, Dupuytren's contracture, peritoneal adhesions, frozen shoulder, scleroderma, and keloid formation.

[0033] Modulators of tissue regeneration may also be used in tissue and organ construction *in vitro*.

a. CCN3

[0034] During embryonic development, CCN3 is widely expressed in derivatives of all three germ layers, with high levels of expression in skeletal muscle, smooth muscle of vessel walls, the nervous system, adrenal cortex, and differentiating chondrocytes. Consistent with a role in development, CCN3 interacts with the epidermal growth factor-like domain of Notch1 and regulates Notch signaling. CCN3 is associated with the ECM upon secretion, interacts with fibulin in a yeast two-hybrid system and may regulate calcium signaling. Aberrant expression of CCN3 has been identified in a variety of tumors and in vascular injury. CCN3 can induce angiogenesis *in vivo* and promote pro-angiogenic activities in endothelial cells in culture. Thus, CCN3 supports cell adhesion, induces chemotaxis, enhances growth factor-induced DNA synthesis, and promotes cell survival in vascular endothelial cells. Mechanistically, these activities are mediated through interactions with integrin receptors in a context-dependent manner. Endothelial cell adhesion to CCN3 is mediated through integrins $\alpha_v\beta_3$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$, with HSPGs serving as coreceptors of $\alpha_6\beta_1$. CCN3-induced endothelial cell chemotaxis is mediated through $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Although CCN3 does not contain an RGD sequence, it is a direct ligand of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ as demonstrated by solid phase binding assays.

[0035] Since CCN3 is expressed in hypertrophic cartilage, where vessel growth is required for the formation of a scaffold onto which the osteoblasts settle and deposit bone matrix, CCN3-induced angiogenesis may be important in endochondral ossification. During nephrogenesis, CCN3 is localized to the metanephric mesenchyme into which endothelial cells are recruited. These endothelial cells then proliferate and form a capillary network as the metanephric mesenchyme develops to form the glomeruli, the basic units of filtration.

[0036] CCN3 expression is also detected in various tumors, including Wilm's tumors, and benign adrenocortical tumors. It is well established that tumor growth beyond ~1 mm in size requires the growth of new vessels to provide the necessary blood supply. Furthermore, the Wilms' Tumor suppressor gene (WT1) negatively regulates CCN3 expression. As part of its tumor suppressing function, WT1 down-regulates the angiogenic inducer CCN3.

[0037] In contrast to CCN1 and CCN2, which are transcriptionally activated by mitogenic growth factors and repressed under conditions of growth arrest, CCN3 is induced by growth

" arrest and down-regulated by growth factors. TGF- β 1 strongly induces *CCN1* and *CCN2*, while it represses *CCN3*. Thus, *CCN3* is regulated in an antithetical manner compared to *CCN1* and *CCN2*, suggesting that they may serve opposing functions.

[0038] As shown in the examples, CCN3 regulates angiogenesis and fibroblast functions during wound healing. Specifically, the expression of CCN3 is upregulated during wound healing, with peak levels observed 5-7 days after wounding. Furthermore, CCN3 functions through specific integrins to enhance growth factor-induced DNA synthesis, support fibroblast adhesion, and induce fibroblast chemotaxis.

[0039] Additionally, CCN3 cooperates with TGF- β 1 in an antagonistic or synergistic manner in the regulation of specific genes. CCN3 induces neovascularization *in vivo*, and promotes pro-angiogenic activities in endothelial cells. In contrast to CCN1 and CCN2, CCN3 does not upregulate VEGF-A expression in human skin fibroblasts. While the potential role of VEGF-A upregulation in the angiogenic function of CCN1 and CCN2 has not been established, the angiogenic function of CCN3 is unlikely to be mediated through VEGF-A. However, CCN3 is able to upregulate MMP-1 (Figure 8) and MMP-3 proteases that play a role in matrix remodeling and angiogenesis during wound healing. The ability of TGF- β to repress MMP-1 is dominant over the upregulation of MMP-1 by CCN3, and CCN3 works synergistically with TGF- β to upregulate PAI-1 expression (Figure 8). Thus, the bioavailability of CCN3 and/or TGF- β in the cellular microenvironment may profoundly influence the pattern of gene expression in various cell types that participate in wound healing. This microenvironment may also be dynamic, thus allowing for finely tuned up- or down-regulation of genes such as MMP-1.

[0040] Many of the cellular processes that occur in wound healing are also observed in the tumor microenvironment, leading to the proposed notion that tumor stroma is "normal wound healing gone awry". Consistently, CCN3 is not only expressed during wound healing, but is associated with adrenocortical tumors, cartilage neoplasia, hepatocellular carcinomas, musculoskeletal tumors, prostate cancer, and Wilm's tumors. It is likely that both the angiogenic activity of CCN3 and its activities upon stromal fibroblasts play a role in tumor growth as well as wound healing. CCN3 also interacts with Notch1 through its CT domain and modulate Notch signaling. Notch1 plays a critical role in cell fate determination and lymphocyte development, and is implicated in keratinocyte differentiation during wound healing. Thus, CCN3 may regulate Notch signaling in the context of both embryonic development and tissue repair.

(1) CCN3 Polypeptides and Inhibitory Polypeptides

[0041] CCN3 polypeptides include, but are not limited to, polypeptides comprising SEQ ID NO:1, as well as analogs, derivatives, fragments, homologs and variants thereof that are at least 75%, 80%, 85%, 90%, 95%, 97%, 98% or 99% identical to SEQ ID NO: 1. The CCN3 polypeptides may comprise the amino acid sequence GQKCIVQTTSWSQCSKS (SEQ ID NO: 3). The polypeptides may be natural, recombinant or synthetic. The polypeptides may have at least one biological activity of CCN3. The polypeptides may also be inhibitory polypeptides that are inhibitors or antagonists of CCN3 activity. The CCN3 polypeptides may also be antibodies that specifically bind to CCN3.

[0042] The CCN3 polypeptides may comprise one or more of the following CCN3 domains: insulin growth factor-binding protein (amino acids 47-94), von Willebrand (vWE) type C domain (amino acids 110-170) or C-terminal cysteine knot-like domain (amino acids 269-338).

[0043] The polypeptide may comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-5, 3-7, 6-10, 8-12, 11-15, 13-17, 16-20, 18-22, 21-25, 23-27, 26-30, 28-32, 31-35, 33-37, 36-40, 38-42, 41-45, 43-47, 46-50, 48-52, 51-55, 53-57, 56-60, 58-62, 61-65, 63-67, 66-70, 68-72, 71-75, 73-77, 76-80, 78-82, 81-85, 83-87, 86-90, 88-92, 91-95, 93-97, 96-100, 98-102, 101-105, 103-107, 106-110, 108-112, 111-115, 113-117, 116-120, 118-122, 121-125, 123-127, 126-130, 128-132, 131-135, 133-137, 136-140, 138-142, 141-145, 143-147, 146-150, 148-152, 151-155, 153-157, 156-160, 158-162, 161-165, 163-167, 166-170, 168-172, 171-175, 173-177, 176-180, 178-182, 181-185, 183-187, 186-190, 188-192, 191-195, 193-197, 196-200, 198-202, 201-205, 203-207, 206-210, 208-212, 211-215, 213-217, 216-220, 218-222, 221-225, 223-227, 226-230, 228-232, 231-235, 233-237, 236-240, 238-242, 241-245, 243-247, 246-250, 248-252, 251-255, 253-257, 256-260, 258-262, 261-265, 263-267, 266-270, 268-272, 271-275, 273-277, 276-280, 278-282, 281-285, 283-287, 286-290, 288-292, 291-295, 293-297, 296-300, 298-302, 301-305, 303-307, 306-310, 308-312, 311-315, 313-317, 316-320, 318-322, 321-325, 323-327, 326-330, 328-332, 331-335, 333-337, 336-340, 338-342, 341-345, 343-347, 346-350, 348-352, 351-355, and 353-357.

[0044] The polypeptide may also comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-10, 6-19, 11-20, 16-29, 21-30, 26-39, 31-40, 36-49, 41-50, 46-59, 51-60, 56-69, 61-70, 66-79, 71-80, 76-89, 81-90, 86-99, 91-100, 96-109, 101-110, 106-119, 111-120, 116-129, 121-130, 126-139, 131-140, 136-149, 141-150, 146-159, 151-160, 156-169, 161-170,

66-179, 171-180, 176-189, 181-190, 186-199, 191-200, 196-209, 201-210, 206-219, 211-220, 216-229, 221-230, 226-239, 231-240, 236-249, 241-250, 246-259, 251-260, 256-269, 261-270, 266-279, 271-280, 276-289, 281-290, 286-299, 291-300, 296-309, 301-310, 306-319, 311-320, 316-329, 321-330, 326-339, 331-340, 336-349, 341-350, and 346-357.

[0045] The polypeptide may also comprise amino acids from SEQ ID NO: 1 selected from the group consisting of 1-15, 8-22, 16-30, 23-37, 31-45, 38-52, 46-60, 53-67, 61-75, 68-82, 76-90, 83-97, 91-105, 98-112, 106-120, 113-127, 121-135, 128-142, 136-150, 143-157, 151-165, 158-172, 166-180, 173-187, 181-195, 188-202, 196-210, 203-217, 211-225, 218-232, 226-240, 233-247, 241-255, 248-262, 256-270, 263-277, 271-285, 278-292, 286-300, 293-307, 301-315, 308-322, 316-330, 323-337, 331-345, 338-352, and 346-357.

(2) Formulations of CCN3 polypeptides

[0046] Compositions comprising a CCN3 polypeptide may further comprise one or more pharmaceutically acceptable additional ingredients such as carriers, excipients, diluents such as water or nonaqueous vehicles, antimicrobial agents, and the like. The compositions may contain between about 5% and 60% of an active component by weight.

[0047] Compositions may be in the form of tablets, capsules, dispersible powders, granules, or lozenges formulated in a conventional manner. For example, tablets and capsules for oral administration may contain conventional excipients including, but not limited to, binding agents, fillers, lubricants, disintegrants, wetting agents, buffers, flavoring agents, and coloring agents. Binding agents include, but are not limited to, syrup, acacia, gelatin, sorbitol, tragacanth, mucilage of starch and polyvinylpyrrolidone. Fillers include, but are not limited to lactose, sugar, microcrystalline cellulose, maize starch, calcium phosphate, and sorbitol. Lubricants include, but are not limited to, magnesium stearate, stearic acid, talc, polyethylene glycol, and silica. Disintegrants include, but are not limited to, potato starch and sodium starch glycolate. Wetting agents include, but are not limited to, sodium lauryl sulfate. Tablets may be coated according to methods known in the art.

[0048] Compositions may also be liquid formulations including, but not limited to, aqueous or oily suspensions, solutions, emulsions, syrups, and elixirs. The compositions may also be formulated as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain additives including, but not limited to, suspending agents, emulsifying agents, buffers, flavoring agents, coloring agents, nonaqueous vehicles and

preservatives. Suspending agents include, but are not limited to, sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminum stearate gel, and hydrogenated edible fats. Suspensions may contain, for example, from about 0.05% to 5% of suspending agents. Syrups may contain, for example, from about 10% to 50% of sugar. Emulsifying agents include, but are not limited to, lecithin, sorbitan monooleate, and acacia. Nonaqueous vehicles include, but are not limited to, edible oils, almond oil, fractionated coconut oil, oily esters, propylene glycol, and ethyl alcohol. Elixirs may contain, for example, from about 20% to 50% of ethanol. Preservatives include, but are not limited to, methyl or propyl p-hydroxybenzoate and sorbic acid.

[0049] Compositions may also be formulated as suppositories which may contain suppository bases including, but not limited to, cocoa butter or glycerides. Compositions may also be formulated for inhalation, which may be in a form including, but not limited to, a solution, suspension, or emulsion that may be administered as a dry powder or in the form of an aerosol using a propellant, such as dichlorodifluoromethane or trichlorofluoromethane. Compositions may also be formulated in transdermal formulations comprising aqueous or nonaqueous vehicles including, but not limited to, creams, ointments, lotions, pastes, medicated plaster, patch, or membranes.

[0050] Compositions may also be formulated for parenteral administration including, but not limited to, by injection or continuous infusion. Formulations for injection may be in the form of suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain buffers or formulation agents including, but not limited to, suspending, stabilizing, and dispersing agents. Suspensions may contain from about 0.05% to about 5% suspending agent in an isotonic medium. Formulations for injection may also include adjuvants. The composition may also be provided in a powder form for reconstitution with a suitable vehicle including, but not limited to, sterile, pyrogen-free water.

[0051] Compositions may also be formulated as a depot preparation. The liposome preparation may comprise liposomes which penetrate the cells of interest, and fuse with the cell membrane, resulting in delivery of the contents of the liposome into the cell. For example, liposomes such as those described in U.S. Pat. No. 5,077,211 to Yarosh, U.S. Pat. No. 4,621,023 to Redziniak, et al., or U.S. Pat. No. 4,508,703 to Redziniak, et al. can be used. Other suitable formulations can employ niosomes. Niosomes are lipid vesicles similar to liposomes with membranes consisting

"largely or non-ionic lipids," some forms of which are effective for transporting compounds across the stratum corneum.

b. Treatment

(1) Inducing tissue regeneration.

[0052] Tissue regeneration may be induced or accelerated by the administration of CCN3 polypeptides to a patient whose symptoms so indicate. Upon administration, the CCN3 polypeptide may replace or augment the patient's native CCN3 with respect to the fibroblast interactions and angiogenesis required for wound healing. The CCN3 polypeptide may be administered locally or systemically in any of the formulations or by any of the methods of administration described above.

[0053] In addition, tissue regeneration can be stimulated by administering to a patient a nucleic acid encoding a CCN3 polypeptide. The nucleic acid may be operably linked to a promoter which controls expression of CCN3 polypeptide directly in patient's cells at a wound site. The promoter may be selected from promoters that are either specific for a particular type of human cells (*e.g.*, K14 promoter) or promoters that ensure expression of transgenic CCN3 in all cell types found at a wound site (*e.g.*, thymidine kinase promoter).

[0054] Tissue regeneration may also be stimulated by the administration of stem cells to a patient in combination with the administration of a CCN3 polypeptide. The CCN3 polypeptide may be administered in any manner that leads to delivery of the CCN3 polypeptide to the desired site. For example, the CCN3 polypeptide may be injected directly to the desired site or may be conjugated with a targeting agent that allows targeting of the CCN3 polypeptide to the desired site. Upon delivery of the CCN3 to the desired site, the CCN3 polypeptide may stimulate the stem cells to chemotax toward the site.

[0055] Cells such as fibroblasts or endothelial cells may be retained at a wound site or desired site by the delivery of CCN3 polypeptide to the site. Other types of cells, including but not limited to stem cells, may also be retained at a site by the delivery of CCN3 polypeptides.

(2) Inhibiting tissue regeneration by blocking CCN3 activities.

[0056] CCN3 inhibitory polypeptides may be administered to a patient whose symptoms indicate the need to inhibit tissue regeneration. Upon the administration of CCN3 inhibitory polypeptides, the polypeptides may compete with the patient's native CCN3 for receptor binding sites. The angiogenesis and cell migration activities of CCN3 may be decreased accordingly,

and the patient's CCN3 tissue regeneration activity may thereby be diminished or appropriately modulated. Inhibitory polypeptides may be administered directly, or DNA encoding the inhibitory polypeptides may be administered as gene therapy.

[0057] Tissue regeneration may also be inhibited by blocking CCN3 activity with a pharmaceutical composition comprising an antibody that specifically binds to CCN3 and blocks its activity.

c. Methods of diagnosis/screening of CCN3 levels in monitoring wound healing

[0058] Levels of CCN3 may be screened or monitored to evaluate a patient's need for intervention in treating a wound or an area of tissue regeneration. A biopsy may be taken from a wound site or another site of interest. Levels of the CCN3 protein may be measured in the sample and compared with CCN3 expression in a control, such as a sample from a healthy patient. Protein levels may be measured by Western blot, histological immunostaining, ELISA, or by another suitable method known to those of skill in the art. Alternatively, levels of mRNA encoding CCN3 from the patient's biopsy may be measured against those of a control. Levels of mRNA may be measured by Northern hybridization, RNA dot-blot, RT-PCR, in situ hybridization, or by another suitable method known to those of skill in the art.

d. Tissue Construction *in vitro*.

[0059] In an *in vitro* tissue culture, CCN3 polypeptide may be added to induce or accelerate one or more CCN3 activities such as angiogenesis or cell migration. Cultured tissue for use in treatment of wounds may require cells to mature and segregate properly to form the tissue components that exist at the wound site. To this end, administration of a CCN3 polypeptide may facilitate angiogenesis. Likewise, CCN3 may also be used to facilitate cell migration in cultures where the localization of cells (*e.g.*, fibroblasts) is desirable, or for the production of tissue having a particular shape. The addition of a CCN3 polypeptide may also prevent cells from migrating uncontrollably during tissue construction. Tissue cultured by this method may be used to treat wounds, including but not limited to surgical wounds, burns, or other injuries, or to treat patients in need of tissue replacement such as joint replacement or heart valve replacement.

(3) Administration

[0060] Compositions may be administered in any manner including, but not limited to, orally, parenterally, sublingually, transdermally, rectally, transmucosally, topically, via inhalation, via buccal administration, or combinations thereof. Parenteral administration includes but is not

limited to, intravenous, intraarterial, intraperitoneal, subcutaneous, intramuscular, intrathecal, and intraarticular. The compositions may also be administered in the form of an implant, which allows slow release of the compositions as well as a slow, controlled intravenous infusion.

(4) Dosage

[0061] The effective dosage of an active ingredient employed may vary depending on the particular composition employed, the mode of administration and the severity of the condition being treated, and is ultimately determined by the attendant physician. However, in general, satisfactory results are obtained when the compositions of the invention are administered at a daily dosage from about 0.5 to about 500 mg/kg of animal body weight. Dosage forms suitable for internal use comprise from about 0.5 to 500 mg of the active composition in admixture with a solid or liquid pharmaceutically acceptable carrier. This dosage regimen may be adjusted to provide the optimal therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

[0062] A number of factors may lead to the polypeptides being administered at a wide range of dosages. When given in combination with other therapeutics, the dosage of the composition may be given at relatively lower dosages. In addition, the use of targeting agents may allow the necessary dosage to be relatively low.

[0063] The present invention has multiple aspects, illustrated by the following non-limiting examples. Example 1 teaches cloning, expression and purification of recombinant CCN3. Example 2 discloses the production of anti-CCN3 antibodies. Example 3 demonstrates that CCN3 induces neovascularization. Example 4 discloses kinetics of CCN3 expression during wound healing. Example 5 discloses that CCN3 mediates fibroblast adhesion through integrins $\alpha_5\beta_1$, $\alpha_6\beta_1$ and HSPGs. Example 6 teaches how CCN3 controls chemotactic responses. Example 7 provides a method for CCN3 dependent control of chemotactic responses. Example 8 teaches that CCN3 enhances bFGF-induced DNA synthesis in fibroblasts. Example 9 teaches that CCN3 can regulate genes that control matrix remodeling. These examples are intended to be illustrative and should not be construed to limit the scope of the invention.

Example 1

Purification of Recombinant CCN3

[0064] Human *CCN3* cDNA was constructed by ligation of a 5' (nt 72-654, Genbank X96584) and a 3' (nt 654-1653) fragments, and the resulting full-length cDNA was cloned into pKS+ and verified by sequencing. The 5' fragment (nt 72-654) was obtained by reverse transcriptase-polymerase chain reaction using total RNA isolated from serum-starved human skin fibroblasts using the primer set 5'-AGCAGTGCCAATCTACAGC-3' (SEQ ID NO: 4) and 5'-CAGCATCTCACATTGACGG-3' (SEQ ID NO: 5). The RT-PCR product was digested with SphI and StyI to yield a fragment containing nt 72-654. The 3' fragment (nt 654-1653) was generated by restriction digestion of IMAGE clone #49415 (human neonatal brain, nt 590-1653) with StyI and XbaI. To produce recombinant CCN3 protein, the full-length *CCN3* cDNA was cloned into the baculovirus expression vector pBlueBac 4.5 (Invitrogen, Carlsbad, CA). The vector was modified to encode an enterokinase histidine tag linked to the C-terminus of CCN3.

[0065] CCN3 was produced in serum-free baculovirus expression system using High Five insect cells as described. Briefly, High Five cells were maintained in serum-free EX-CELL 400 medium (JRH Bioscience, Lenexa, KS) at 27°C and infected at a multiplicity of infection of 10. Conditioned medium was collected at 38 h post-infection, adjusted to 20 mM sodium phosphate and applied to a Sepharose SP (Sigma-Aldrich, St. Louis, MO) column at 4°C. After washing with a 20 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl, bound proteins were eluted with a linear gradient of NaCl (0.4 - 1 M) in phosphate buffer. Fractions containing CCN3 as judged by SDS-PAGE were pooled and further purified on a nickel-agarose column. Fractions were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining and immunoblotting (Figure 1).

Example 2

Preparation of anti-CCN3 Antibodies

[0066] The second domain of CCN3 (von Willebrand type C repeat) and the central variable region were cloned separately as glutathione S-transferase (GST) fusion proteins and used as antigens to immunize New Zealand white rabbits. DNA fragments were generated by polymerase chain reactions using primer sets 5'-CGCGGATCCGCGGTAGAGGGAGATAACTGTG-3' (SEQ ID NO: 6) and 5'-

CCGGAATTCAGCTGCAAGGGTAAGGCCTCC-3' (SEQ ID NO: 7) (encoding a.a. 104-188), and 5'-GATGAGGAGGATTCACTGGGA-3' (SEQ ID NO: 8) and 5'-

AATGCAGTTGACACTTGAG-3' (SEQ ID NO: 9) (encoding a.a. 176-207). To facilitate cloning, the forward primers start with a BamHI site and the reverse primers end with an EcoRI site. The resulting cDNA fragments were cloned directionally into the PGEX-2T vector (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and confirmed by sequence analysis. The GST-fusion proteins were purified on a glutathione-S sepharose column and used as antigens.

[0067] Antisera and affinity-purified antibodies were produced according to standard protocol (40). IgG was purified from antisera using protein A column chromatography (Pierce Biotechnology, Rockford, IL). For affinity purification, antisera were first passed through a GST column to remove antibodies against GST, and then purified through a GST-CCN3 (VWC domain)-affinity column. Anti-CCN3 antibodies did not cross react with CCN1 or VN (data not shown) by ELISA.

Example 3

Cornea Assay

[0068] **CCN3 induces neovascularization *in vivo*.** The ability of CCN3 to promote endothelial cell adhesion, migration, and survival are consistent with properties of an angiogenic inducer. CCN3-induced neovascularization was examined *in vivo* by implanting Hydron pellets, formulated with test substances, into rat corneas essentially as described. Briefly, male Sprague-Dawley rats were anesthetized and Hydron pellets (Interferon Sciences, Inc., New Brunswick, NJ) containing test substances were implanted into micropockets made in the corneal stroma 1 to 1.5 mm from the corneal limbus. Where indicated, CCN3 and bFGF were incubated with anti-CCN3 antibodies for 1 hour at RT prior to being incorporated into the Hydron pellet. 7 days post implantation, rats were perfused with India ink with heparin (100-U bolus) and neovascularization was examined and scored.

[0069] As shown in Figure 9, CCN3 induced neovascularization when implanted into rat cornea, whereas the vehicle did not induce any response (Table 1). Neovascularization was also observed in corneas implanted with Hydron pellets containing bFGF, a known potent angiogenic inducer. Pre-incubation of CCN3 with anti-CCN3 antibodies obliterated CCN3-induced

neovascularization, indicating that the angiogenic activities observed can be ascribed to the CCN3 polypeptide.

Example 4

Kinetics of CCN3 Expression During Wound Healing

[0070] Excisional Wounds and Immunohistochemistry. Female CD-1 mice at 2 months of age were anesthetized using I.P.-injected Nembutal, and two standard, full-thickness, round wounds of 6 mm diameter were created on the back of each animal using a disposable biopsy instrument (Miltex Inc., Bethpage, NY). Mice were sacrificed, and normal skin and wounds were harvested at different time points throughout the experiment, ranging from 2 hours to 15 days. Tissues were rinsed in cold PBS and fixed in 4% paraformaldehyde overnight at 4°C, followed by rinses in PBS and incubation in 0.5M sucrose overnight at 4°C. The tissues were frozen and embedded in OCT media and cryosectioned at 16 µm. Immunohistochemistry was performed on cryosectioned tissue using protein A purified antibodies generated against the central variable domain of CCN3. The histostain-SAP kit (Broad Spectrum, BCIP/NBT) kit (Zymed) was used as described by the supplier with minor modifications. To avoid non-specific staining due to addition of streptavidin conjugated to alkaline phosphatase, a pre-conjugated goat anti-rabbit secondary antibody (Zymed) was used in place of the antibody supplied by Zymed.

[0071] RNA Isolation and RNA Blot Analysis. To study the effects of CCN3 and TGF-β1 on fibroblast gene expression, 1064SK fibroblasts were serum-starved for 24 hours, then treated with soluble CCN3 (from 0.1 to 10 µg/ml) or TGF-β1 (20 ng/ml) in serum-free media for 24 hours. Total cellular RNA was isolated, resolved on agarose-formaldehyde gel, blotted and probed with ³²P-dCTP-incorporated cDNA according to standard protocol (44). Blots were washed at high stringency (0.1× SSC, 0.1% SDS at 65°C) and analyzed by a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

[0072] To study gene expression in skin wounds, full thickness skin excisional wounds were created on mice and the wound tissue harvested as described. The tissue was immediately lysed by grinding in lysis buffer on ice (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium N-lauroylsarcosine, 0.1 M 2-mercaptoethanol) and RNA was extracted by acid-phenol protocol (45). The cDNA clones of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and matrix metalloproteinase-1 (MMP-1) were obtained from ATCC. Partial cDNAs

corresponding to human VEGF-A, PAI-1 and mouse TGF- β 1 were generated by RT-PCR reactions.

[0073] In reverse transcription reactions total RNAs isolated from human skin fibroblasts or mouse embryonic fibroblasts were used as templates and oligo-dT nucleotide as primer. The primer set for VEGF-A in the PCR reaction corresponds to nucleotide position 198-226 and 622-590 in the human VEGF-A cDNA sequence (GenBank #M32977). PCR primers for human PAI-1 cDNA correspond to nucleotide position 359-381 and 1121-1098 (GenBank #X04429). Primers for mouse TGF- β 1 cDNA correspond to nucleotide position 873-899 and 1072-1049 (GenBank #M13177).

[0074] **Expression of CCN family members during cutaneous wound healing.** *CCN3* has been shown to be regulated in an antithetical manner compared to *CCN1* and *CCN2* in fibroblasts under conditions of mitogen stimulation or growth arrest (31,33-36). Whereas TGF- β 1 strongly induces *CCN1* and *CCN2*, it potently represses *CCN3* in fibroblasts (34,36). The effects of TGF β 1 on *CCN1* and *CCN2*, as opposed to *CCN3*, may represent a broader pattern of expression. Based on this observation of the pattern of expression, *CCN3* may play different or opposing roles to those of *CCN1* and *CCN2* in biological processes regulated by TGF-1, such as cutaneous wound healing.

[0075] To compare the expression of *CCN3* in wound healing against other CCN family members, full-thickness excisional cutaneous wounds were made on female CD-1 mice at 2 months of age. The wound tissues were isolated and analyzed at various times post injury. Skin tissues adjacent to the wound were used as control, and were thus subjected to similar mechanical trauma experienced by the wound tissue but without the actual injury.

[0076] As shown in Figure 1A, *CCN1* expression was induced 2 hours post-wounding, but declined to basal level one day after injury. *CCN1* expression became elevated again 5 days post-injury, and remained high until a gradual decline was noted beyond 9 days. *CCN2* expression was not elevated until 5 days after injury, and declined to near basal level after 9 days. *CCN3* mRNA level was sharply elevated 5 days post-wounding, peaked on day 7, and declined after 9 days but remained above the control level for at least 14 days. TGF- β 1 expression was also elevated between day 5-7 post injury.

[0077] These results surprisingly show that all three members of the CCN family are induced during skin wound repair. Although each of these genes has a slightly different expression

profile through time, their expression overlaps during days 5-7 post-injury during granulation tissue growth, coincident with the expression of TGF- β .

[0078] The pattern of CCN3 message expression was substantiated by immunostaining for CCN3 protein in cryosectioned skin wound tissues. CCN3 was prominently localized in fibroblasts and endothelial cells in the granulation tissue 5-7 days post-injury (Figure 1B). CCN3 was also detected in migrating keratinocytes that re-epithelialized the wounded skin, but not in quiescent keratinocytes in the adjacent uninjured skin. Thus, both CCN3 mRNA and protein accumulate in the skin wound 5-7 days post-injury.

Example 5

CCN3 Mediates Fibroblast Adhesion Through Integrins $\alpha\beta_1$, $\alpha\beta_1$ and HSPGs.

[0079] **Antibodies, polypeptides and reagents.** Function-blocking monoclonal Abs against various integrins (P1D6, anti- α_5 ; AV1, anti- α_v ; LM609, anti- $\alpha_v\beta_3$; JBS5, anti- $\alpha_5\beta_1$) and purified integrin $\alpha_v\beta_5$ were from Chemicon (Temecula, CA). GoH3 (anti- α_6) was from Beckman-Coulter, Inc. (Fullerton, CA) and P4C10 (anti- β_1) was from Invitrogen (Carlsbad, CA). Normal mouse IgG was from Zymed Laboratories, Inc. (South San Francisco, CA), and normal rabbit serum was from Sigma-Aldrich (St. Louis, MO). GRGDSP and GRGESp polypeptides were purchased from Invitrogen (Carlsbad, CA). Heparin (sodium salt, from porcine intestinal mucosa) was from Sigma-Aldrich (St. Louis, MO). FN, VN, LN, and bFGF were from Invitrogen (Carlsbad, CA); type I collagen was from Becton Dickinson (Franklin Lakes, NJ).

[0080] **Cell Culture and Adhesion Assay.** Normal human skin fibroblasts (1064SK) were obtained from American Type Culture Collection (ATCC #CRL-2076), and maintained in Iscove's modified Dulbecco's medium (Invitrogen/GIBCO-BRL) with 10% fetal bovine serum (Intergen, Purchase, NY) at 37°C with 5% CO₂ and used for experiments before passage 8. Briefly, test proteins were diluted in PBS and coated onto 96-well microtiter plates (50 μ l per well) with incubation at 4°C for 16 hours. Wells were rinsed with PBS and blocked with 1% BSA at RT for 1 hour. Skin fibroblasts were harvested in PBS containing 2.5 mM EDTA, washed and resuspended at 2.5 x 10⁵ cells/ml in serum-free Iscove's modified Dulbecco's medium containing 1% BSA. Where indicated, cells were mixed with EDTA, Ca²⁺, Mg²⁺, polypeptides, or heparin before plating, or incubated with antibodies for 1 h at RT prior to plating. Cell suspension (50 μ l) was added to each well, and adherent cells were fixed in 10%

10 minutes after 30 min incubation at 37°C. Cells were stained with methylene blue, and adhesion was quantified by dye extraction and measurement of absorbance at 620 nm.

[0081] Solid phase integrin binding assay. The binding of CCN3 to purified integrin $\alpha_v\beta_5$ was measured by solid-phase receptor binding assay as described previously with modifications (43). For CCN3 binding to immobilized integrin, microtiter wells (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with purified integrin (1 $\mu\text{g/ml}$) and incubated at 4°C overnight. The wells were washed and blocked with 1% heat-inactivated BSA for 2 hours at room temperature. Soluble CCN3 was added and allowed to bind at 4°C for 16 hours; bound ligand was detected using affinity-purified anti-CCN3 antibodies (1:1000). For integrin binding to immobilized ligands, microtiter wells were coated with 10 $\mu\text{g/ml}$ CCN3 or 1 $\mu\text{g/ml}$ VN as above. Where indicated, coated wells were pre-incubated with affinity-purified anti-CCN3 antibodies or normal rabbit serum for 2 hours at RT. After washing, purified integrin $\alpha_v\beta_5$ (1 $\mu\text{g/ml}$ in buffer with 25 mM octylglucoside) was added and incubated overnight at 4°C. Where indicated, soluble integrin was either mixed with EDTA, Mg^{2+} , or polypeptides prior to plating, or incubated with function-blocking monoclonal antibodies for 30 minutes at 4°C prior to plating. After washing, bound integrins were detected with polyclonal antibodies (AB1930) or mAb (P3G8) against integrin α_v (Chemicon, Temecula, CA). After washing, wells were incubated with horseradish peroxidase-conjugated secondary antibody (1:2500), and color reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc., South San Francisco, CA) with absorbance measured at 420 nm.

[0082] CCN3 mediates fibroblast adhesion through integrins $\alpha_5\beta_1$, $\alpha_6\beta_1$, and HSPGs.

Immobilized CCN3 supports fibroblast adhesion in a dose-dependent manner, with maximal adhesion achieved at a coating concentration of 1-2 $\mu\text{g/ml}$ (Figure 2A). Cell adhesion to CCN3 was blocked by the presence of EDTA (5 mM) and restored by the addition of 10 mM Mg^{2+} or Ca^{2+} , consistent with the involvement of integrins. To assess which integrins are involved in fibroblast adhesion, we investigated the inhibitory effect of RGD-containing polypeptides. The addition of GRGDSP polypeptide (2 mM), but not the control polypeptide GRGESp, significantly blocked cell adhesion to CCN3 (Figure 2B). As expected, fibroblast adhesion to FN was completely abrogated. These results suggest that fibroblasts adhere to CCN3 in part through RGD-sensitive integrins, such as α_v integrins and $\alpha_5\beta_1$ integrins.

[0083] To identify the integrins involved in CCN3-supported fibroblast adhesion, cells were incubated with anti- $\alpha_v\beta_3$ mAb LM609 prior to plating. Whereas LM609 partially blocked adhesion to vitronectin, an $\alpha_v\beta_3$ ligand, it did not inhibit adhesion to CCN3 (Figure 3B). As expected, cell adhesion to FN, which binds $\alpha_5\beta_1$, was unaffected by LM609 (Figure 3B). However, the anti- $\alpha_5\beta_1$ mAb JBS5 partially blocked adhesion to CCN3 and FN, but not VN (Figure 3A), indicating that fibroblast adhesion to CCN3 is mediated in part through integrin $\alpha_5\beta_1$.

[0084] Incubation of cells with mAbs against integrin α_6 (GoH3) or β_1 (P4C10) blocked fibroblast adhesion to CCN3, indicating that integrin $\alpha_6\beta_1$ is an adhesion receptor for CCN3. As expected, control experiments showed that anti- α_6 mAb blocked adhesion to laminin but not vitronectin, and anti- β_1 mAb blocked adhesion to type 1 collagen but not vitronectin (Figure 3C, D). Likewise, when added to cells prior to plating, as little as 50 ng/ml of soluble low molecular weight heparin partially but significantly inhibited adhesion of fibroblasts to CCN3 but had no effect on cell adhesion to fibronectin (Figure 2B). These results show the involvement of integrin $\alpha_6\beta_1$ and HSPGs in fibroblast adhesion to CCN3. The addition of GRGDSP polypeptide and soluble heparin (50 ng/ml) together abolished cell adhesion CCN3 completely, suggesting that the combination of the $\alpha_5\beta_1$ and $\alpha_6\beta_1$ -HSPG coreceptors can account for fibroblast adhesion to CCN3.

Example 6

CCN3 Induces a Positive Chemotactic Response in Fibroblasts.

[0085] **Cell migration assay.** A 48-well modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD) was used to assay cell migration as described with modifications (5). Fibroblasts were harvested with trypsin, washed and resuspended at 5×10^5 cells/ml in DMEM containing 0.1% BSA. Test proteins were loaded into wells of the lower chamber; the wells were then covered with a gelatinized polycarbonate filter (5 μ m pore diameter, Nuclepore, Newton, MA) followed by the upper chamber. Cells were then loaded into the upper chamber. Where indicated, cells were either mixed with polypeptides or incubated with antibodies (1 hour at RT) prior to loading. After a 6 hour incubation at 37°C, the membrane was removed and stained using a Diff-Quik Kit (Dade-Behring, Deerfield, IL). Cell migration was monitored by

counting the total number of cells migrated in 10 randomly selected microscope fields at 400 X magnification.

[0086] Fibroblasts migrate to CCN3 through integrin $\alpha_v\beta_5$. As shown in Figure 4A, CCN3 stimulates migration of neonatal primary human foreskin fibroblasts in a dose-dependent manner, reaching maximal level at 2 $\mu\text{g/ml}$. Higher concentrations of CCN3 were less effective in promoting cell migration, such that the dose response curve formed a bell shaped curve typical of many chemotactic factors. In order to determine whether CCN3 induces chemotaxis (directed migration) or chemokinesis (random cell movement) in fibroblasts, a checkerboard type analysis was performed. Cell migration was assessed with CCN3 placed in the upper chamber (with cells), the lower chamber (opposite side to cells), in both chambers, or in neither. As shown in Figure 4B, addition of CCN3 to the lower chamber induces maximal level of cell migration, while addition of CCN3 with cells to the upper chamber produced no effect, indicating that CCN3 does not induce a chemokinetic response. Addition of CCN3 to both chambers reduces the level of fibroblast migration, suggesting that fibroblasts are sensitive to the gradient of CCN3 protein. These results show that CCN3 is a chemotactic factor in fibroblasts.

[0087] Incubation of cells with either anti- α_v mAb (AV1) or anti-integrin $\alpha_v\beta_5$ mAb (P1F6) blocked fibroblast migration to CCN3 completely (Figure 5A). As expected, these antibodies blocked migration of fibroblasts to VN but not to FN. Neither LM609 nor P1D6 (anti- α_5 mAb) inhibited CCN3-induced migration, whereas LM609 blocked cell migration to vitronectin and P1D6 inhibited migration to FN (Figure 5B, C). These results indicate that CCN3 mediates migration of fibroblasts specifically through integrin $\alpha_v\beta_5$.

Example 7

CCN3-induced Chemotaxis is Mediated Through Direct Interaction of CCN3 With $\alpha_v\beta_5$.

[0088] Purified integrin $\alpha_v\beta_5$ was immobilized on microtiter wells, onto which CCN3 was added in varying concentrations and binding was detected using anti-CCN3 antibodies. As shown in Figure 6A, CCN3 binds immobilized integrin $\alpha_v\beta_5$ in a dose-dependent and saturable manner, with half maximal binding occurring at 1.5 $\mu\text{g/ml}$ (40 nM) CCN3. Binding can also be observed when CCN3 was immobilized on microtiter wells and integrin $\alpha_v\beta_5$ added in soluble form, detected using antibodies against integrin α_v subunits (Figures 6B-6D). To probe the specificity of CCN3 binding to $\alpha_v\beta_5$, a variety of antagonists was used. The binding of $\alpha_v\beta_5$ to immobilized

CCN3, but not to VN, was blocked by anti-CCN3 antibodies (Figure 6B). EDTA completely abrogated the binding of integrin $\alpha_v\beta_5$ to CCN3 and to VN, and as expected, binding was restored by the addition of $MgCl_2$ (Figure 6C). GRGDSP polypeptide (SEQ ID NO: 2), but not GRGESp polypeptide (SEQ ID NO: 10), was able to inhibit the binding of $\alpha_v\beta_5$ to CCN3 and to positive control VN. Finally, mAb against integrin $\alpha_v\beta_5$ (P1F6) blocked binding of integrin $\alpha_v\beta_5$ to CCN3 and VN (Figure 6D). Taken together, these results show that CCN3 is a novel ligand of and binds directly and specifically to integrin $\alpha_v\beta_5$.

Example 8

CCN3 Enhances bFGF-Induced DNA Synthesis in Fibroblasts.

[0089] Thymidine Incorporation Assay. 1064SK fibroblasts were plated on 24-well plates at 1×10^4 cells/well and grown in Iscove's modified Dulbecco's medium with 10% fetal bovine serum for 48 hours, rinsed with PBS, and incubated with serum-free medium containing 0.5% BSA for an additional 48 hours. Fresh serum-free medium containing designated proteins and 1 μ Ci/well [3 H]thymidine were added simultaneously, and after incubation for 21 hours, cells were washed with phosphate-buffered saline and fixed with 10% trichloroacetic acid. DNA was dissolved in 0.1 N NaOH and incorporated thymidine measured using a scintillation counter.

[0090] Enhancement of bFGF-induced DNA synthesis. Both CCN1 and CCN2 have been shown to potentiate the activities of mitogenic growth factors and enhance DNA synthesis without being mitogenic on their own. Likewise, CCN3 enhanced bFGF-induced fibroblast DNA synthesis in a dose-dependent manner as judged by a [3 H]thymidine incorporation assay, whereas CCN3 by itself had no effect (Figure 7). Thus, while CCN3 is not mitogenic on its own, it can enhance bFGF-induced DNA synthesis in fibroblasts.

Example 9

CCN3 Can Regulate Genes That Control Matrix Remodeling And Its Effects May Be Modulated By TGF- β 1.

[0091] As described above, CCN3 supports fibroblast adhesion, induces chemotaxis, and enhances mitogenesis, consistent with its expression in granulation tissue and a potential role in wound repair. Thus, CCN3 may regulate the expression of genes controlling processes such as angiogenesis and matrix remodeling.

[0092] Various amounts of CCN3 were added in a soluble form to serum-starved fibroblasts for 24 hours, both in the presence or absence of TGF- β 1 (Figure 8). CCN3 strongly upregulated MMP-1 expression at 10 μ g/ml, but had only a modest effect on PAI-1. VEGF-A was not regulated by CCN3 under these conditions. TGF- β 1 strongly represses MMP-1 expression, and its effect is dominant over that of CCN3 when presented in combination, completely negating CCN3-dependent upregulation of MMP-1. However, the effects of TGF- β 1 and CCN3 on PAI-1 were synergistic, leading to a higher level of expression than detected with either inducer alone. Thus, CCN3 can regulate genes involved in matrix remodeling, and its effect may be modulated by TGF- β 1.

CLAIMS

What is claimed is:

1. A method of modulating wound healing in a patient comprising administering to a patient in need thereof a pharmaceutical composition comprising a CCN3 polypeptide.
2. A method of modulating wound healing in a patient comprising administering to a patient in need thereof a pharmaceutical composition comprising an antibody to a polypeptide comprising a sequence of SEQ ID NO:1.
3. A method of modulating wound healing in a patient comprising administering to a patient in need thereof a pharmaceutical composition comprising a nucleic acid encoding a polypeptide comprising a sequence of SEQ ID NO: 1 or a sequence at least 75% identical thereto.
4. The method of claim 3 wherein said nucleic acid is operably linked to a promoter.
5. The methods of claim 1, 2, 3, or 4 wherein said pharmaceutical composition is administered locally to a wound site in a form selected from the group consisting of patches, creams, gels and injectable solutions.
6. A method of diagnosing a wound healing disorder comprising:
 - 1) measuring the level of mRNA, wherein said mRNA encodes a polypeptide comprising a sequence of SEQ ID NO: 2 or a sequence at least 75% identical thereto in a sample obtained from a wound of a patient; and
 - 2) comparing the level of mRNA in said sample to a control, whereby a difference in expression indicates that said patient is affected by a wound healing disorder.
7. The method of claim 6 wherein said mRNA is measured by a procedure selected from the group consisting of: Northern Hybridization, RNA dot-blot, RT-PCR and *in situ* hybridization.
8. The method of claim 6 wherein said control sample is obtained from a source selected from the group consisting of: a wound of a patient who does not suffer a wound healing disorder and a sample that contains a known quantity of said mRNA.

9. A method of diagnosing a wound healing disorder comprising:
- 1) measuring expression of a polypeptide comprising SEQ ID NO: 1 or a sequence at least 75% identical thereto in a sample obtained from a wound of a patient; and
 - 2) comparing the level of expression of said protein to a control, whereby a difference in expression indicates that said patient is affected by a wound healing disorder.
10. The method of claim 9 wherein said protein expression is measured by a method selected from the group consisting of Western blot analysis, histological immunostaining and ELISA.
11. A method of increasing chemotaxis of stem cells to a site in a patient comprising locally administering to the patient a composition comprising stem cells and a polypeptide comprising a sequence of SEQ ID NO: 1 or a sequence at least 75% identical thereto.
12. The method of claim 11, wherein said patient is a cancer patient.
13. A method of *in vitro* tissue construction comprising incubating cells in a tissue culture with a nucleic acid encoding a polypeptide comprising the sequence of SEQ ID NO: 1 or a sequence at least 75% identical thereto operably linked to a promoter.

FIGURE 1A

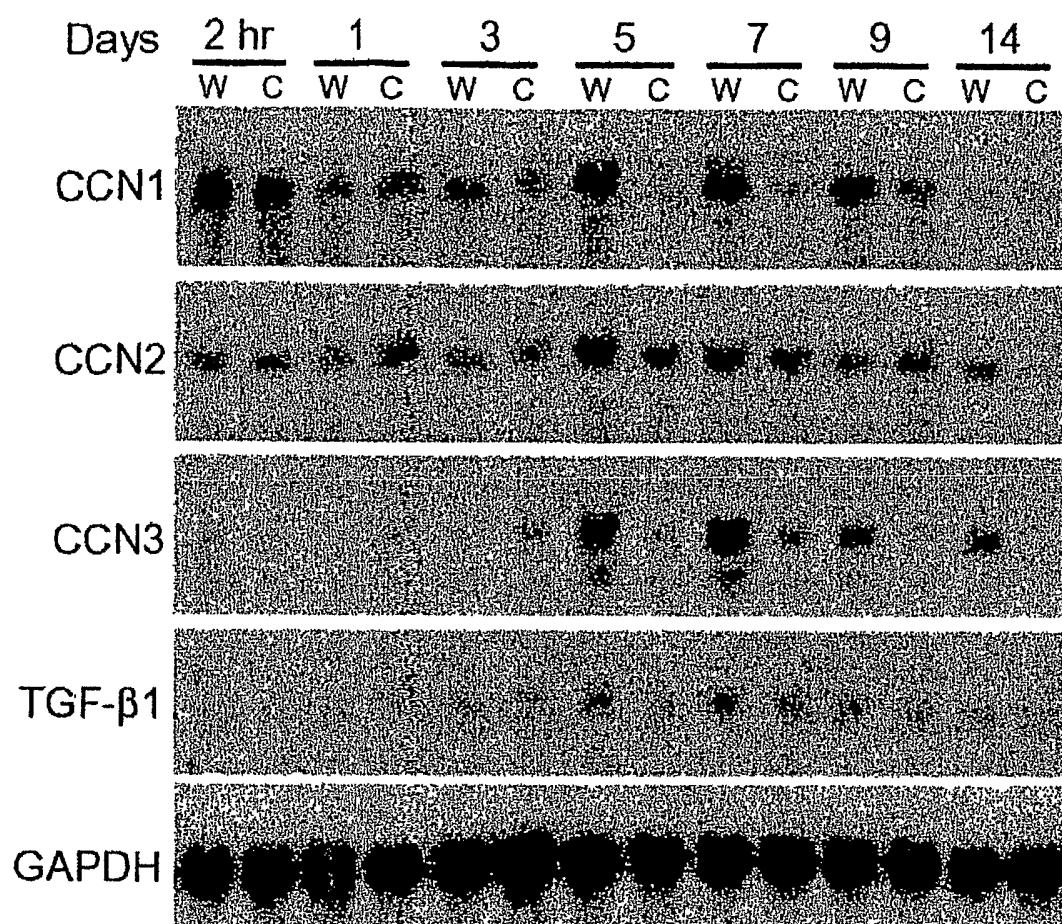


FIGURE 1B

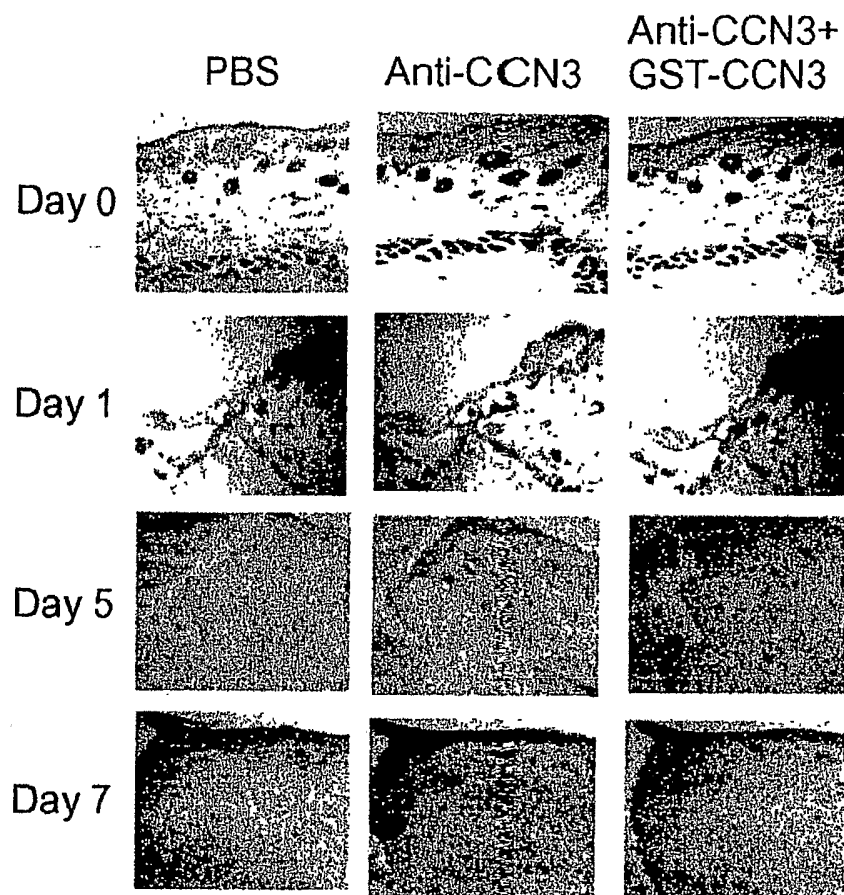


FIGURE 2

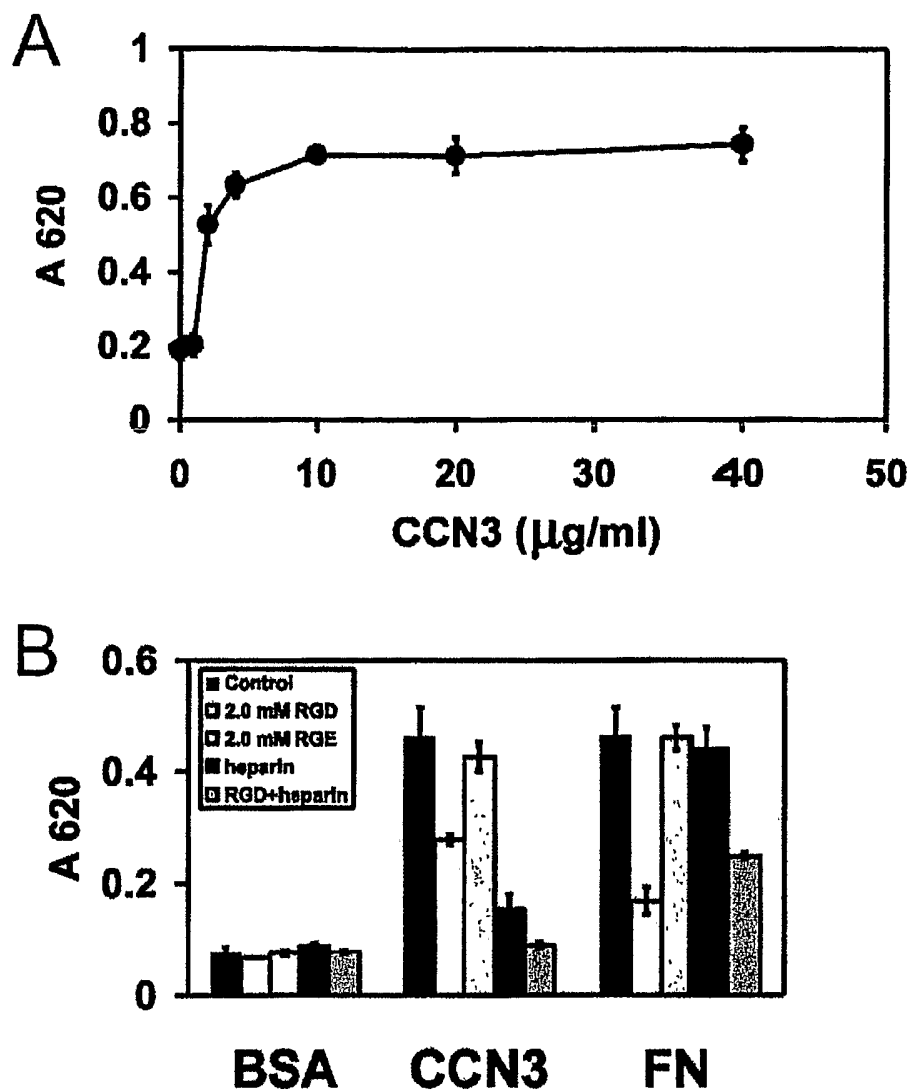


FIGURE 3

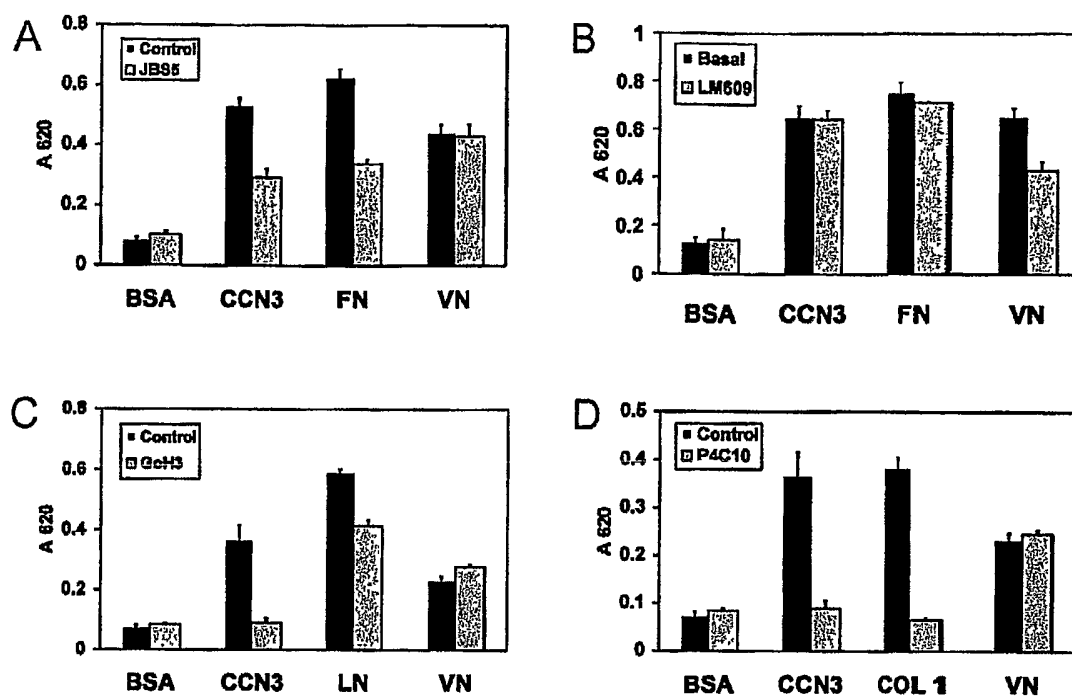


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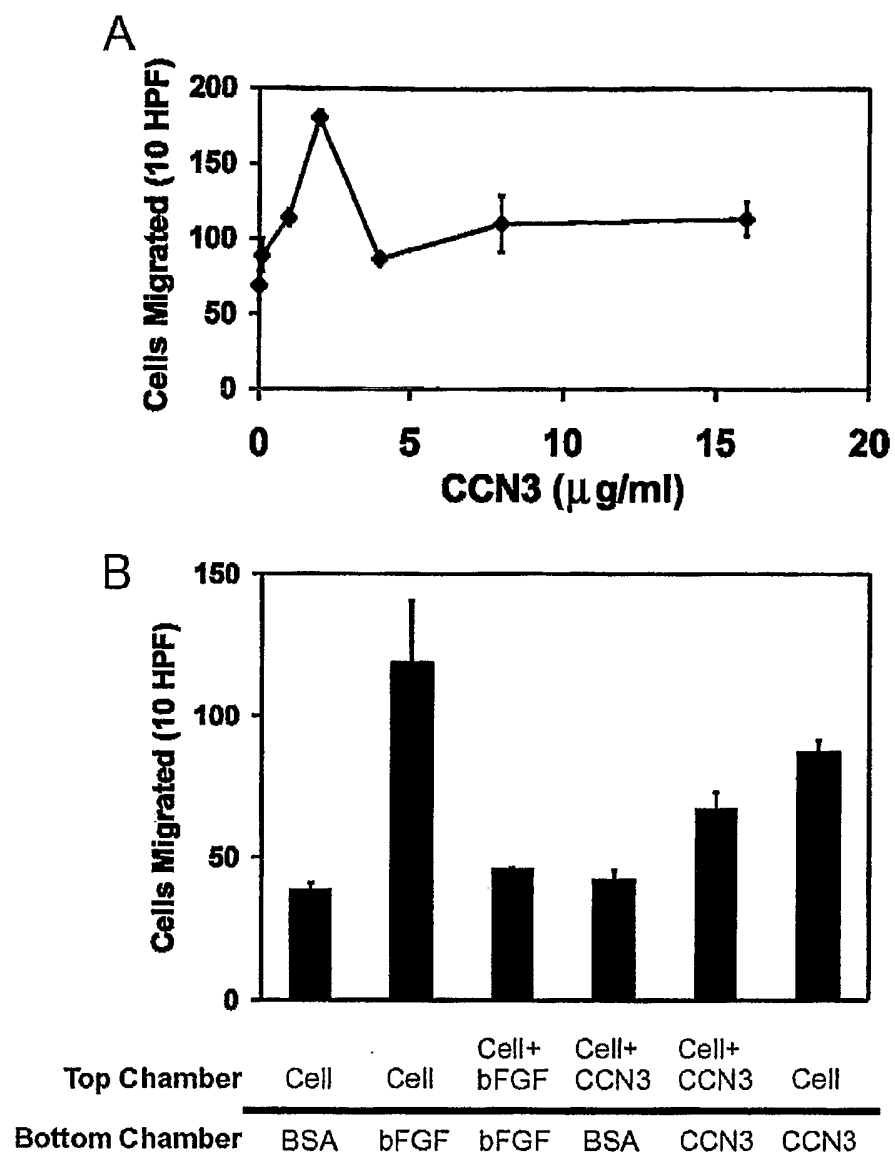


FIGURE 5

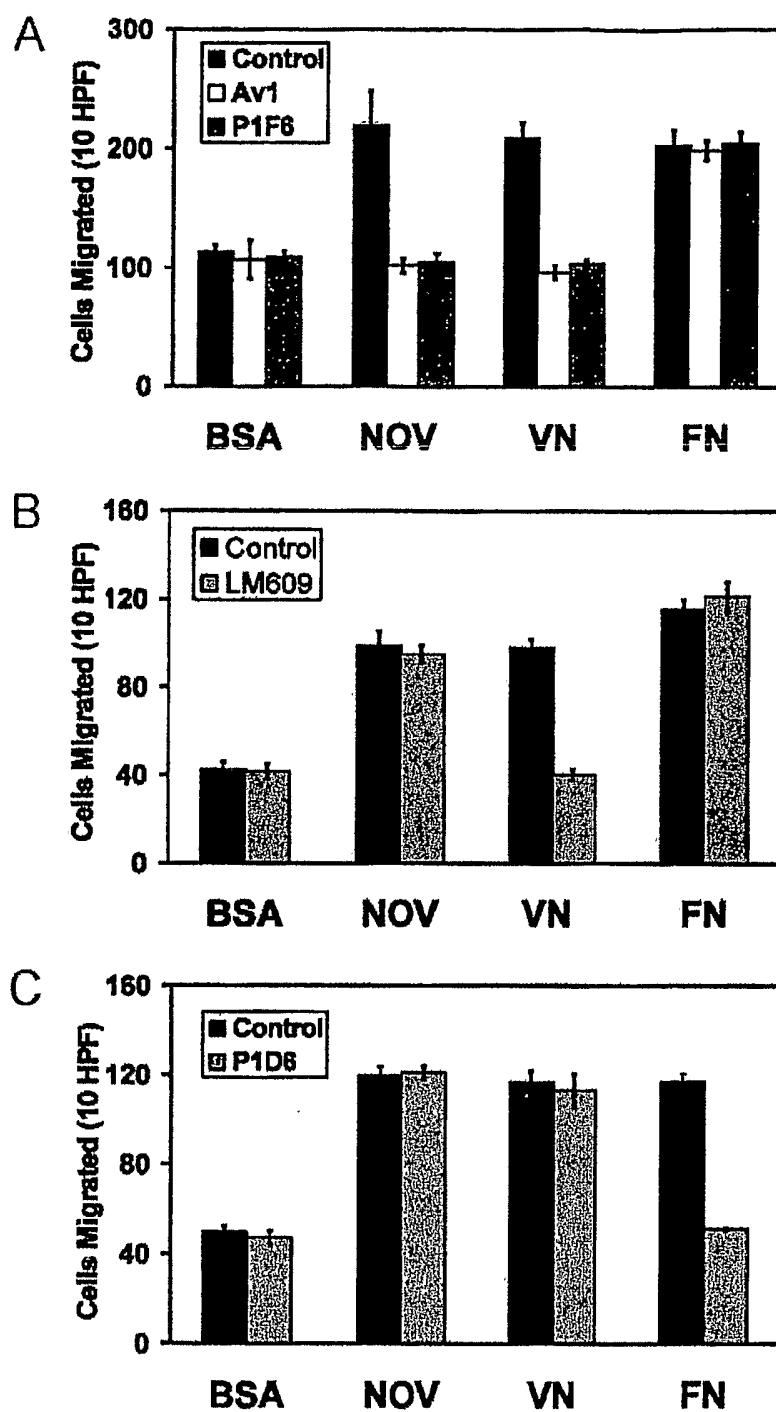


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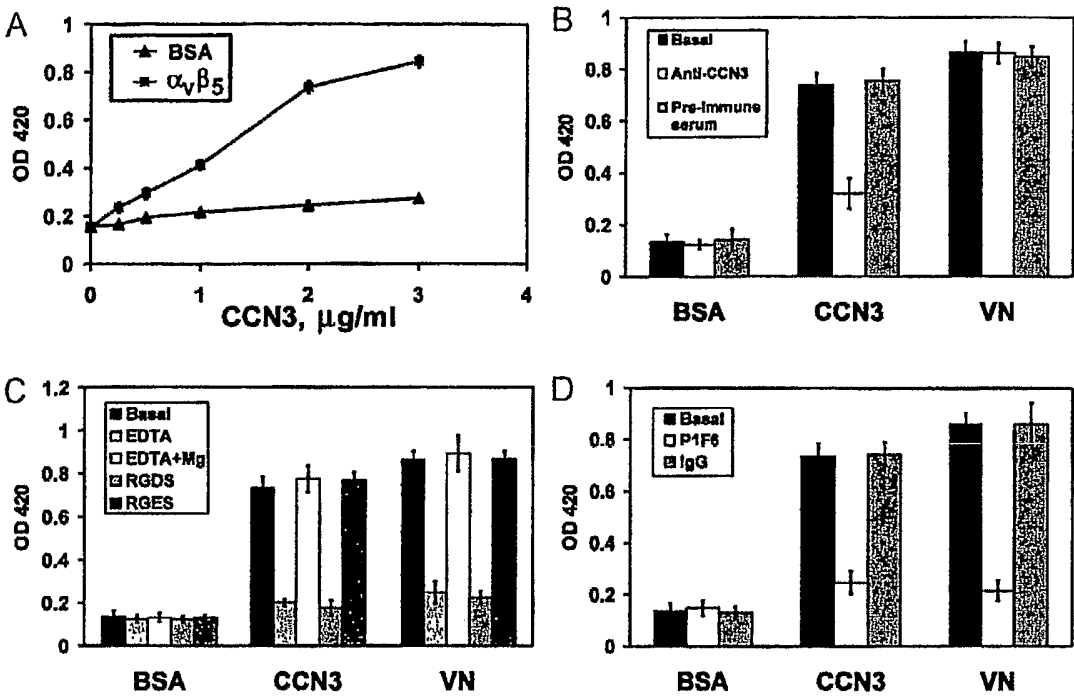


FIGURE 7

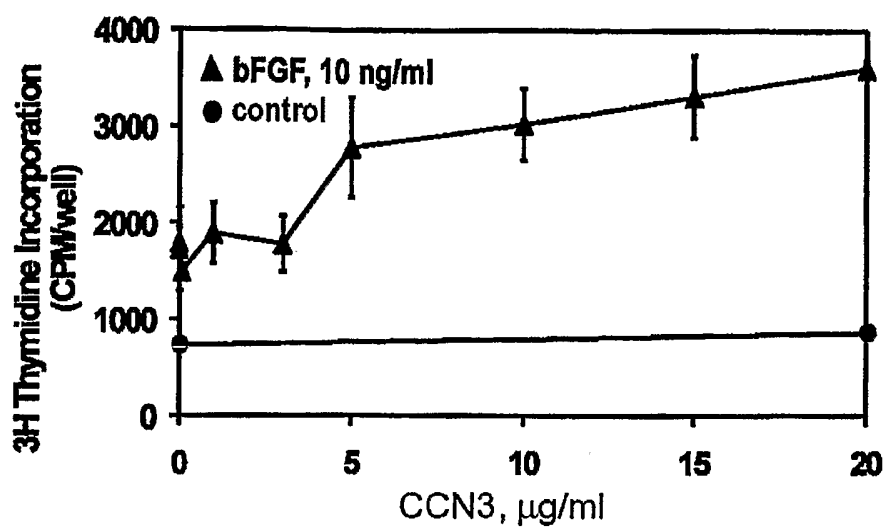
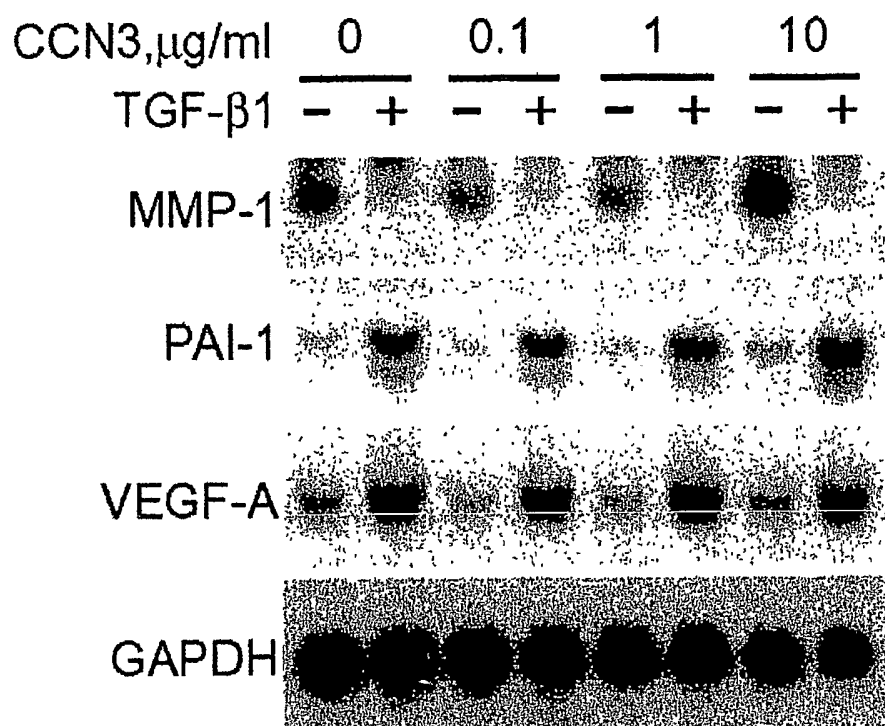


FIGURE 8



SEQUENCE LISTING

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Lau, Lester F.

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