

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 September 2008 (12.09.2008)

PCT

(10) International Publication Number
WO 2008/109794 A2

(51) International Patent Classification:

A61K 48/00 (2006.01)

(21) International Application Number:

PCT/US2008/056142

(22) International Filing Date: 7 March 2008 (07.03.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/905,780 8 March 2007 (08.03.2007) US

(71) Applicant (for all designated States except US): **TUFTS MEDICAL CENTER** [US/US]; 750 Washington Street, Boston, MA 02111 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **KAYYALI, Usamah** [US/US]; 54 Benjamin Road, Belmont, MA 02478 (US).

(74) Agent: **KEOWN, Wayne, A.**; Keown & Zuccherro, LLP, 500 West Cummings Park, Suite 1200, Woburn, MA 01801 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

(54) Title: THE ROLE OF THE MK2 PATHWAY IN TREATING FIBROSIS AND SCARRING

(57) Abstract: The invention provides methods for treating or preventing fibrosis or scarring in tissue of a mammal by increasing the expression and/or activity of MK2 in cells of fibrotic or injured tissue in the mammal.



WO 2008/109794 A2

THE ROLE OF THE MK2 PATHWAY IN TREATING FIBROSIS AND SCARRING
(Atty. Docket No. NEMC-002PC)

Government Funding

This invention was made with government support under HL-79320 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Summary of the related art

Pulmonary fibrosis as a major component of interstitial lung disease is characterized by abnormal fibroblast proliferation and deposition of extracellular matrix proteins that remodel the normal pulmonary tissue structure and compromise its function. In some forms of interstitial lung disease, e.g., idiopathic pulmonary fibrosis (IPF) the mechanism by which fibrosis arises remains poorly understood (1, 2). Pulmonary hypertension is a disease characterized by narrowing and increased resistance of the pulmonary arteries whose etiology is not well understood. While the disease can be primary or secondary to other conditions, a common feature in different types of pulmonary hypertension is vascular remodeling (for review, see Humbert et al., 2004). In particular, extension of smooth muscle cells to parts of pulmonary arteries that are not usually muscularized, and thickening of the walls of pulmonary arteries by proliferation of fibroblasts that produce extracellular matrix proteins are prominent features of the disease. Indeed fibroblasts have been reported to constitute the first type of cells to respond to pulmonary hypertension producing stimuli, e.g., chronic hypoxia in animal models (Stenmark et al., 2002) (Humbert et al., 2004).

Fibrosis arises in several tissues in response to inflammation and chemical injury, or idiopathically, leading to accumulation of fibroblasts which deposit extracellular matrix (ECM) proteins. Such processes are part of normal wound healing, and are often reversible by apoptosis of accumulating fibroblasts and resorption of ECM proteins. However, in fibrosis the proliferation of fibroblasts and ECM deposits appear excessive

and dysregulated, resulting in the gradual displacement of normal tissue by fibrotic foci that disrupt tissue function. Differentiation of fibroblasts into myofibroblasts has long been believed to be an important event in many conditions such as wound repair and fibrosis. For example, it has been reported that myofibroblasts occur in areas of active fibrosis and are responsible for production and deposition of extracellular matrix proteins in pulmonary fibrosis (3). Thus, one of the important cells in pulmonary fibrosis is the myofibroblast, which occurs in areas of active fibrosis and is responsible for production and deposition of extracellular matrix proteins (Vyalov et al., 1993). The factors that determine the path to resolution versus fibrosis are not fully understood, however considerable focus has been placed on signals that cause fibroblasts to differentiate into myofibroblasts such as those generated by TGF β (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993; Roy et al., 2001; Yokozeki et al., 1997).

Myofibroblasts, which are believed to play a role in wound contraction, are more contractile than fibroblasts due to increased expression of the smooth muscle-specific actin α -SMA (for review, see 14). Studies into the mechanism by which myofibroblast differentiation occurs focused on signaling pathways activated by TGF β in fibroblasts. In the lungs of IPF patients, TGF β (5), IL-1 β and TNF α (6, 7) were reported to be elevated. Indeed TGF β is believed to play a pivotal role in fibrosis and its overexpression in mice is sufficient to cause pulmonary fibrosis independent of injury and inflammation (8, 9). TGF β is a cytokine that can exert many actions and has been implicated in diseases ranging from cancer to pulmonary hypertension. One action of TGF β that has been of interest in fibrosis research is its causing fibroblasts to differentiate into myofibroblasts which are known to accumulate in sites of fibrosis (3, 10-13).

Several signaling pathways have been proposed to mediate the actions of TGF β on fibroblasts including the MAP kinase p38. p38 is a stress-activated kinase that is activated in response to stimuli such as ultraviolet radiation and hyperosmolarity. Once p38 is activated, it phosphorylates a variety of substrates, including the kinase MK2. Upon its phosphorylation by p38, MK2 becomes activated, and in turn, phosphorylates additional substrates, such as the small heat shock protein HSP27. When phosphorylated, HSP27 no longer blocks the polymerization of actin, thus resulting in the stabilization of actin fibers (Benndorf et al., 1994).

As described above, one of the characteristics of fibroblasts is their ability to differentiate into myofibroblasts, a contractile form that is identified by increased production of smooth muscle α -actin (sm α or α -SMA). Thus MK2 might be a target that is perturbed in pulmonary and other types of fibrosis. There is, therefore, a need for the ability to restore and/or increase MK2 activity.

BRIEF SUMMARY OF THE INVENTION

In a first aspect the invention provides a method for therapeutically treating a vertebrate having fibrosis or scarring as a result of injury or some other condition. The method in this aspect of the invention comprises increasing the expression and/or activity of MK2 in fibrotic tissue of a mammal. In certain embodiments of the invention the mammal is a human. In certain embodiments, the fibrotic tissue is lung tissue.

In a second aspect the invention provides a method for preventing fibrosis or scarring after injury in a vertebrate. The method in this aspect of the invention comprises increasing the expression and/or activity of MK2 in injured tissue of a mammal. In certain embodiments of the invention, the mammal is a human. In certain embodiments, the injured tissue is lung tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: MK2^{-/-} lungs produce more collagen in response to bleomycin treatment than wild type lungs. Bleomycin caused an increase in lung collagen deposition in both wild type and MK2^{-/-} lungs. However, the collagen deposition was significantly higher in MK2^{-/-} bleomycin-treated lungs than in wild type bleomycin-treated lungs. * indicates statistically significant difference from saline-treated wild type mean, # indicates statistically significant difference from saline-treated MK2^{-/-} mean, ^ indicates statistically significant difference from bleomycin-treated wild type mean. P<0.05 in ANOVA and Holm-Sidak post-hoc analysis.

Figure 2: MK2^{-/-} mice develop more severe fibrosis in response to bleomycin than wild type mice. Treatment of wild type mice with bleomycin (B) cause significant cellularity and collagen deposition (blue staining) relative to saline-treated wild type mice (A). MK2^{-/-} mice exhibited more severe cellularity and collagen deposition in response to bleomycin (D), compared to saline-treated MK2^{-/-} mice (C) and bleomycin-treated wild type mice (B). Several slides were blindly ranked for fibrosis by 3 investigators and scored as described in Examples. Average scores demonstrate that bleomycin-treated MK2^{-/-} mice develop more severe fibrosis than bleomycin-treated wild type mice (E). * indicates statistically significant difference from saline-treated wild type mean, # indicates statistically significant difference from saline-treated MK2^{-/-} mean, ^ indicates statistically significant difference from bleomycin-treated wild type mean. P<0.05 in ANOVA and Holm-Sidak post-hoc analysis.

Figure 3: Knocking down MK2 also reduces α -SMA expression. Transfection with siRNA against MK2 reduced the level of MK2 and α -SMA relative to mock siRNA transfection in a dose-dependent manner. MK2 siRNA transfection had no effect on total actin expression.

Figure 4: Fibrotic lesions in MK2^{-/-} mice contain few α -SMA -positive cells compared to wild type fibrotic lesions. Lung sections were stained with an anti- α -SMA antibody. Both

wild type and MK2^{-/-} sections expressed α -SMA in smooth muscle cells (thick arrows). However, α -SMA -positive fibroblasts (thin arrows) were much more prominent in fibrotic regions of bleomycin-treated wild type lung sections. (A) Saline-treated wild type, (B) bleomycin-treated wild type, (C) saline-treated MK2^{-/-}, (D) bleomycin-treated MK2^{-/-}. Blind scoring of several slides from each treatment as described in Examples demonstrates that, excluding smooth muscle cells in airways and blood vessels, bleomycin-treated MK2^{-/-} lung sections contain significantly less α -SMA -positive cells than bleomycin-treated wild type lung sections (E). * indicates statistically significant difference from saline-treated wild type mean, # indicates statistically significant difference from bleomycin-treated MK2^{-/-} mean. P<0.05 in ANOVA and Holm-Sidak post-hoc analysis.

Figure 5: Fibrotic lesions in MK2^{-/-} mice contain many vimentin-positive cells compared to wild type fibrotic lesions: Lung sections were stained with an anti-vimentin antibody. Both wild type and MK2^{-/-} sections expressed vimentin in fibrotic lesions. However, vimentin-positive fibroblasts were much more prominent in fibrotic regions of bleomycin-treated MK2^{-/-} lung sections, reflecting a higher degree of fibrosis. (A) Saline-treated wild type, (B) bleomycin-treated wild type, (C) saline-treated MK2^{-/-}, (D) bleomycin-treated MK2^{-/-}. Blind scoring of several slides from each treatment as described in Examples demonstrates that, bleomycin-treated MK2^{-/-} lung sections contain significantly more vimentin-positive cells than bleomycin-treated wild type lung sections (E). * indicates statistically significant difference from saline-treated wild type mean, # indicates statistically significant difference from saline-treated MK2^{-/-} mean, ^ indicates statistically significant difference from bleomycin-treated wild type mean. P<0.05 in ANOVA and Holm-Sidak post-hoc analysis.

Figure 6: MK2^{-/-} MEF proliferate faster than wild type MEF. When wild type and MK2^{-/-} MEF were seeded at equal density, and counted after 24, 48 and 72 hours, MK2^{-/-} MEF cell count increased faster than wild type MEF cell count.

Figure 7: MK2^{-/-} MEF produce more collagen than wild type MEF. MEF from wild type and MK2^{-/-} mice were seeded at equal density in 1% serum. After 24 hours collagen was assayed as described in Examples in conditioned media, as well as in attached cells. MK2^{-/-} MEF produced more collagen than their wild type counterparts. * indicates statistically significant difference from wild type mean, P<0.05 in t-test.

Figure 8: MK2^{-/-} MEF are slower than wild type MEF in repopulating a wounded part of the culture dish. Cells were scraped off sub-confluent MEF culture dishes of equal density using a sterile pipette tip. After 24 hours more wild type MEF than MK2^{-/-} MEF were observed in the wounded area. Over expressing phosphomimicking pmHSP27 in MK2^{-/-} cells did not reverse the defective migratory properties of MK2^{-/-} MEF.

Figure 9: MK2 is not upstream of Smad signaling in response to TGFβ: A: TGFβ causes phosphorylation of both Smad2/3 and p38 in wild type and MK2^{-/-} MEF. Smad2/3 phosphorylation is observed before p38 phosphorylation. B: Inhibiting the TGFβ Type I receptor kinase by SB431542 blocked Smad2/3 phosphorylation in both wild type and MK2^{-/-} MEF. SB431542 also blocked the activation of p38 by TGFβ.

Figure 10: Consequences of injury in the presence and absence of MK2 are shown. In the presence of MK2 myofibroblast differentiation, resolution or scar formation occurs. In the absence of MK2, fibroblast proliferation and fibrosis or severe scarring occurs.

Figure 11: Immunological staining of histological lung sections from GFP-nirus-infected or MK2-virus infected MK2^{-/-} mice are shown.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to therapeutic approaches to managing conditions related to MK2 activity. The patents and publications cited herein reflect the level of knowledge in the art and are hereby incorporated by reference in their entirety. Any conflict between the teachings of these patents and publications and this specification shall be resolved in favor of the latter.

Research on the role played by fibroblasts in fibrosis has increasingly focused on a differentiated form of fibroblasts, the myofibroblast. Because of their expression of smooth muscle-specific proteins, such as α -SMA, their role in wound contraction and cytokine production, as well as their localization near fibrotic foci, myofibroblasts were suspected of promoting fibrosis. Surprisingly, the present inventors have discovered that mitogen-activated protein kinase-activated protein kinase 2 (MK2) and myofibroblast formation appear to play a role in protection against, rather than in causing fibrosis.

In a first aspect the invention provides a method for therapeutically treating a mammal having fibrosis or scarring as a result of injury or some other condition. The method in this aspect of the invention comprises increasing the expression and/or activity of MK2 in cells in fibrotic tissue in a mammal. In certain embodiments of the invention the mammal is a human. In certain embodiments, the fibrotic tissue is lung tissue.

As used herein, the terms “fibrosis” and “fibrotic” refer to the formation or development of excess fibrous connective tissue in an organ or tissue. Fibrosis often occurs in multiple organs including, but is not limited to, the lungs, heart, liver, kidney and skin. Fibrosis occurs in different organs in response to different types of injury, or it can arise without a clear stimulus as in the case of i.e., idiopathic pulmonary fibrosis (IPF). Such stimuli include, but are not limited to, chemotherapeutic agents, sarcoidosis, asbestos, silica dust, and connective tissue diseases such as scleroderma. As used herein, the term “scarring” means the formation or development of scar or fibrous tissue replacing normal tissues destroyed by injury or disease.

In the methods according to the invention, MK2 expression and/or activity can be increased in fibrotic tissue by various means.

In certain embodiments, MK2 expression is increased in cells of fibrotic tissues by transfecting the cells with a virus or other expression vector comprising an MK2 transgene.

In certain embodiments, MK2 activity in cells of fibrotic tissue is increased by inhibiting the activity of an inhibitor of MK2 activity. For example, the therapeutic composition useful for treating fibrosis or scarring includes, but is not limited to, small molecules that inhibit the activity of the autoinhibitory peptide of MK2 preventing it from binding MK2 and thus resulting in MK2 activation. Such small molecules include the peptide HPRNPARRTPGTRRGAPQEFGAA from human herpes virus 8 and fragments, derivatives, analogs or mimetics thereof. Mutant variants of the autoinhibitory peptide itself may also be used for this purpose.

Thus, in certain embodiments, MK2 expression and/or activity in cells of fibrotic tissue is increased by contacting the cells with small molecule that increases the expression and/or activity of MK2, or that decreases the expression or activity of an inhibitor of MK2 expression or activity.

In the methods according to this aspect of the invention, administration of therapeutic compositions comprising a virus, expression vector or small molecule can be by any suitable route, including, without limitation, parenteral, oral, sublingual, transdermal, topical, inhalation, intranasal, aerosol, intraocular, intratracheal, intrarectal or vaginal. Administration of the therapeutic compositions of the invention can be carried out using known procedures at dosages and for periods of time effective to treat fibrosis or scarring. The term an "effective amount" or a "sufficient amount" generally refers to an amount sufficient to affect a desired biological effect, such as beneficial results. Thus, an "effective amount" or "sufficient amount" will depend upon the context in which it is being administered. It may be desirable to administer simultaneously, or sequentially a therapeutically effective amount of one or more of the therapeutic compositions of the invention to an individual as a single treatment episode.

In any of the methods according to this aspect of the invention, the therapeutic composition can be administered in combination with any other agent useful for treating fibrosis or scarring that does not diminish the effect of the therapeutic composition. In addition the methods could include agents that activate p38 MAP kinase, the upstream

activator of MK2, such as hyperosmolar solutions, low oxygen, and sodium arsenite which are expected to cause activation of MK2, as well as agents that inhibit p38, such as SB203580, which are expected to inhibit MK2.

The term "in combination with" generally means in the course of treating the same condition in the same patient, and includes administering a compound and/or an agent in any order, including simultaneous administration, as well as temporally spaced order of up to several days apart. Such combination treatment may also include more than a single administration of the compound according to the invention, and/or independently an agent. The administration of the compound and/or agent may be by the same or different routes.

In a second aspect the invention provides a method for preventing fibrosis or scarring in a mammal. The method in this aspect of the invention comprises increasing the expression and/or activity of MK2 in cells of tissue in a mammal that have been injured, but which is not yet fibrotic, or only partially fibrotic. In certain embodiments of the invention the mammal is a human. In certain embodiments, the injured tissue is lung tissue. In these embodiments of the invention, the expression and/or activity of MK2 is achieved by contacting the cells of the injured tissue with a therapeutic composition according to the invention, as described in the first aspect above. Administration of the therapeutic composition, alone or in combination with another agent, is carried out as described for the first aspect of the invention.

As used herein, the term "pharmaceutical composition" refers to the combination of a virus, expression vector, or small molecule that increases the expression and/or activity of MK2 and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable" refers to a material that does not interfere with the effectiveness of the compositions of the first, second or third aspects of the invention and is compatible with a biological system such as a cell, cell culture, tissue, or organism. In certain embodiments, the biological system is a living organism, such as a vertebrate.

As used herein, the term “carrier” encompasses any excipient, diluent, filler, salt, buffer, stabilizer, solubilizer, lipid, or other material well known in the art for use in pharmaceutical formulations. It will be understood that the characteristics of the carrier, excipient, or diluent will depend on the route of administration for a particular application. The preparation of pharmaceutically acceptable formulations containing these materials is described in, e.g., Remington’s Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990, ISBN: 0-912734-04-3.

The examples below are intended to further illustrate certain preferred embodiments of the invention, and are not intended to limit the scope of the invention.

Data are presented as means $\pm$ standard deviation (SD). Statistical differences were determined by either Student’s t-test for comparison of two sample means or analysis of variance (ANOVA) for comparison of more than two sample means followed by Holm-Sidak post-hoc testing for multiple comparisons between two sample means ($P < 0.05$ was considered statistically significant).

Example 1

Materials

Reagents: Media and supplements (D-MEM), fetal bovine serum (FBS), penicillin G potassium, streptomycin, fungizone, and glutamine were purchased from Invitrogen (Carlsbad, CA). TGF β was purchased from R&D Systems (Minneapolis, MN) and activated before use according to manufacturers instructions. SB431542 and all other reagents and drugs were obtained from Sigma (St. Louis, MO). On the day of the experiments, all drugs were diluted in serum-free medium.

Animals: Mice that lack MK2 (MK2^{-/-}) mice were generated as described earlier (42). In brief, a neo selection cassette, which contains translational stop codons in all three reading frames, was inserted into the Sac I site located in the exon that encodes

subdomains V and VI of MK2. MK2^{-/-} mice were then back-crossed into the C57BL/6J, and lack of expression of MK2 was verified by PCR and immunoblotting as described earlier (4). Wild type C57BL/6J from Charles River Laboratories (Wilmington, MA) were used as controls for the described experiments. These mice were anesthetized with xylozine-ketamine, and then saline or bleomycin (3 U/kg in 50 µL) were introduced into their lungs by insufflation.

Cell Culture: Immortalized mouse embryonic fibroblasts (MEF) from wild type and MK2^{-/-} knockout mice were prepared as described earlier [Kotlyarov et al., 1999]. Primary embryonic fibroblasts from C57BL/6J wild type or MK2^{-/-} mice, were co-transfected with pSV40Tag encoding the SV40 large T antigen and the pREP8 plasmid, followed by selection and expansion of histidinol-resistant colonies. MEF cells were maintained in DMEM containing 10% FBS, penicillin, streptomycin, fungizone and glutamine at 37 °C in humidified air containing 5 % CO₂. MEF were passaged in 0.25 % trypsin-0.02 % ethylenediaminetetraacetic acid (EDTA) solution, and one day prior to the experiments, cells were maintained in serum-free or 1% serum-containing media.

For studying the effects of HSP27 phosphorylation, wild type and MK2^{-/-} MEF, were transfected with the pcDNA3-HSP27PM (43) to express HSP27 in which the residues, S15D, S78D, S82D, were mutated to mimic phosphorylated HSP27 (pmHSP27). The pmHSP27 vector was introduced alone in the case of wild type MEF, and co-transfected with pMEpuro (a selection vector which confers resistance to puromycin to eukaryotic cells) in the case of MK2^{-/-} MEF. The vectors were introduced (5 µg of pmHSP27, and 0.5 µg pMEpuro) into MEF using lipofectamine. Stable transfected wild type MEF cell lines were obtained by selection with geneticin, and resistant colonies were isolated, expanded, and then screened for pmHSP27 by immunoblotting of cell lysates with anti HSP27 antibody. As MK2^{-/-} MEF were already resistant to geneticin, stable transfectants produced by co-transfection with pMEpuro were selected with puromycin, and resistant clones were isolated and expanded as above.

For proliferation assays, cells were seeded in 12-well dishes and cultured for 24, 48 and 72h. Counting was performed after trypsinizing the cells using a Coulter counter apparatus according to manufacturer's instructions.

For migration assays, cells were plated in 35 mm dishes, after 24h the monolayer was scratched with a sterile tip to make the wounding gap (time 0 h). Cells were cultured for another 24 h in 10% serum media and then fixed with 4% formaldehyde for 10 min. Four pictures were randomly taken from duplicate dishes and wound closure was compared with time 0 h to assess cell migration.

Example 2

Collagen Assay: It has been recently described that embryonic fibroblasts (MEF) isolated from MK2^{-/-} mice expressed less α -SMA than their wild type counterparts either at baseline in response to TGF β (4). The relevance of these findings to the whole animal was ascertained by assessing the development of pulmonary fibrosis in MK2^{-/-} mice compared to WT mice.

Animals from each group were exposed to either saline or bleomycin, a chemotherapeutic associated with pulmonary fibrosis in human patients that has been used extensively to model pulmonary fibrosis in animals. After two weeks of exposure to a single dose of saline or bleomycin (3 u/kg insufflation), mice were evaluated for the amount of acid-soluble collagen in their lungs using the Sircol Assay (Biocolor Ltd, Newtownabbey, United Kingdom). In brief, lungs were minced into small cubes and incubated overnight in 10 volumes of 0.5 M acetic acid. Next homogenates were incubated with an aliquot of Sircol Dye reagent (Sirius Red in picric acid) for 30 min. The collagen-dye complex was then pelleted by centrifugation at 10,000 x g for 10 minutes. The dye was then extracted with 0.5 M NaOH, and the absorbance of samples along with known collagen standards was measured at 540 nm in a Tecan Spectrafluor plate reader. The same assay was used to assay collagen in conditioned media from MEF cells (without the acid step), and in cell lysates (scraped into 0.5 M acetic acid).

An increase in collagen deposition is associated with fibrosis. As shown in Figure 1, treatment with bleomycin caused a significant increase in lung collagen content in both wild type and MK2^{-/-} mice. However, the increase in collagen in response to bleomycin

was significantly higher in MK2^{-/-} mice (180%) than in wild type mice (77%). These data suggested that MK2^{-/-} mice might be more susceptible to fibrosis than wild type mice.

Example 3

Histochemistry and immunohistochemistry: To confirm the findings of the collagen assay, lungs from the mice treated as described above were examined histochemically. After perfusion and fixation, lung sections were stained with Eosin/hematoxylin and Gomori's Trichrome stain which stains collagen. In brief, lungs were instilled intratracheally with 4% formaldehyde at 21 cm H₂O pressure. The tissue blocks were then embedded in paraffin, and 4 µm sections were cut for staining. Gomori's Trichrome staining was used to highlight collagen deposition. Other sections were immunostained with antibodies against α-SMA (1A4, Biogenex, San Ramon, CA) and vimentin (VIM 3B4, Ventana, Tucson, AZ) using an automated immunostainer (Ventana, Tucson, AZ) according to manufacturer's instructions. For quantitation of fibrosis or immunolabeling, a scale of intensity was employed by three different researchers blindly, such that, each slide was given a value as 0, 1, or 2 according to the intensity of target staining.

As shown in Figure 2 (A-D) the bleomycin treated animals had significant cellularity in regions that should correspond to airspaces. The Trichrome stain also revealed increased collagen deposition in interstitial spaces of bleomycin-treated mice compared to saline-treated mice (Figure 2A-D). Furthermore, the bleomycin-treated MK2^{-/-} mice developed more severe fibrosis compared to the wild type mice. To quantify the fibrosis in these mice, several Trichrome-stained sections from differently treated mice were ranked as follows: 0 for no fibrosis, 2 for significant fibrosis, and 1 for intermediate fibrosis. The slides were blindly scored by three different researches. The table in Figure 2E shows the average and standard deviations of the pooled scores. Both wild type and MK2^{-/-} bleomycin-treated groups had significantly higher fibrosis scores than the saline-treated corresponding groups. Moreover, there was a statistically significant difference between the wild type and MK2^{-/-} bleomycin-treated groups, indicating that MK2^{-/-} mice developed more severe pulmonary fibrosis than wild type mice.

Example 4

siRNA Transfection: As stated above, early data demonstrated that MEF from MK2^{-/-} mice express much less α -SMA than wild type mice. Since different populations of fibroblasts have been reported to produce different amounts of α -SMA, the possibility that these earlier findings were due to isolation of different populations of MEF from MK2^{-/-} and wild type mice need to be determined. Furthermore, it need to be determined if the observed reduction in α -SMA in MK2^{-/-} MEF was due to a developmental effect in MK2^{-/-} mice. Thus siRNA against MK2 were used to suppress the expression of MK2 in wild type MEF. In brief, 80%-90% confluent cells were transfected with MK2 siRNA (Cat # 16704 Ambion, CA) and mock control siRNA (Cat # 4611 Ambion, CA) with Lipofectamine 2000 (Invitrogen, CA) at indicated concentrations for 30 h according to the manufacturer's instructions. Western blotting was then performed to test the silencing effect of siRNA on the expression levels of target proteins.

As shown in Figure 3, MK2 siRNA transfection caused a significant reduction in the level of MK2 expressed in wild type MEF compared to mock transfected MEF. When these cells were probed for the expression of α -SMA, it was observed that the MK2 siRNA transfection also inhibited the expression of α -SMA (Figure 3). These findings indicate that MK2 plays an important role in myofibroblast differentiation regardless of fibroblast population or developmental effects.

Example 5

SDS-PAGE and Immunoblotting:

The expression of α -SMA in the lungs of MK2^{-/-} and wild type mice was evaluated. Thus lung sections from mice treated with saline or bleomycin as described above were stained with an antibody against α -SMA. In brief, cell lysates were assayed for protein using the Bradford protein assay [Bradford, 1976] and then diluted with 2 $\times$ Laemmli loading buffer for SDS-PAGE [Laemmli, 1970]. Equal amounts of protein were then loaded in 4-20% Tris/glycine gels, and electrophoresed for 90 min at 125 V constant voltage. Next, the gel was blotted onto an Immobilon-P membrane by electrophoretic transfer at 25 V constant voltage overnight. The membrane was then

washed, blocked with 5% milk, and probed with antibodies against MK2, p38 and phospho-p38 (Cell Signaling, Beverly, MA); α -SMA (1A4, Sigma, St. Louis, MO); pan-actin (C-2, Santa Cruz, Santa Cruz, CA); smad2/3(18, BD-Transduction Laboratories, San Jose, CA); and phospho-smad2 (138D4, Cell Signaling, Beverly, MA). Appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL), and a chemiluminescent substrate according to the manufacturer's instructions (SuperSignal, Pierce, Rockford, IL) were used to visualize immunoreactive bands.

Smooth muscle cells in wild type and MK2^{-/-} both stained positively for α -SMA in the vasculature and airways independently of the treatment (Figure 4) suggesting that MK2 disruption has no effect on α -SMA expression in smooth muscle cells. However in the bleomycin-treated mice, a different staining pattern for fibroblasts emerged. In lung sections from wild type bleomycin-treated mice there were many cells in areas of fibrosis that stained positively for α -SMA actin (Figure 4B), consistent with the presence of myofibroblasts in these lesions. However, areas of extensive fibrosis in the MK2^{-/-} bleomycin-treated mice contained minimal staining for α -SMA (Figure 4D). Differences in α -SMA staining were quantified by having 3 investigators blindly rank several slides for the degree of α -SMA labeling in fibrotic areas. As shown in Figure 4E, MK2^{-/-} sections contained significantly less α -SMA -positive fibroblasts than wild type sections. Furthermore, the number of α -SMA -positive cells populating areas of fibrosis in bleomycin-treated MK2^{-/-} sections were not significantly different from either wild type or MK2^{-/-} saline-treated sections. The presence of fibroblasts in these areas was confirmed using an antibody against vimentin, which revealed many vimentin-positive cells in areas of fibrosis in both wild type and MK2^{-/-} bleomycin-treated mice (Figure 5). Indeed scoring of vimentin slides in the manner followed with α -SMA staining revealed more vimentin staining in bleomycin-treated MK2^{-/-} sections than in bleomycin-treated wild type sections (Figure 5E). These findings indicate that fibroblasts and extensive fibrosis occur to a more severe degree when myofibroblasts are not found in the lesions. They also suggest that the widely held view that myofibroblasts play an important role in active fibrosis might not be true.

Example 6

Embryonic fibroblasts from MK2^{-/-} mice exhibit a higher proliferative rate and secrete more collagen than wild type fibroblasts:

Since both MEF and whole animals exhibit the same characteristic inhibition of α -SMA in response to MK2 disruption, other characteristics of fibroblasts in culture that might explain the observations made in bleomycin-treated mice were investigated. When seeded at equal density mouse embryonic fibroblasts from MK2^{-/-} mice grew at a faster rate than their wild type counterparts. This effect was observed at 10% serum and in the absence of serum, such that MK2^{-/-} were more tolerant to serum deprivation. As shown in Figure 6, MK2^{-/-} MEF cell count increases faster than wild type MEF. Such increased proliferation rate might explain the excessive cellularity observed in fibrotic foci in bleomycin-treated MK2^{-/-} mouse lungs.

Collagen in wild type and MK2^{-/-} MEF was assayed after seeding at equal density and maintaining in 1% serum for 24 hours. As shown in Figure 7, MK2^{-/-} fibroblasts produced more collagen than their corresponding wild type fibroblasts. These data are consistent with the increased collagen deposition observed in the lungs of bleomycin-treated MK2^{-/-} mice relative to wild type mice (Figure 1)

Example 7

MK^{-/-} fibroblasts are defective in migratory activity-Reduced migratory behavior in MK^{-/-} fibroblasts is not due to lack of phosphorylated HSP27:

MK2^{-/-} MEF have been described to migrate more slowly than their wild type counterparts (23). The ability of MEF to repopulate a wound made by a sterile pipette in a tissue culture dish was assayed. As seen in Figure 8, wild type MEF repopulated the wounded area much faster than the MK2^{-/-} cells. As MK2 is known to alter the actin cytoskeleton through its direct action on the small heat shock protein HSP27, stably transfected phosphomimicking (pm) HSP27 MK2^{-/-} MEF were generated. It was previously demonstrated that expressing this construct stably in endothelial cells causes an increase in actin stress fibers, which is believed to represent a more contractile phenotype (24). While transfected MK2^{-/-} MEF expressed phosphomimicking HSP27,

they were still defective in migration compared to wild type cells (Figure 8). Furthermore, since some reports demonstrated that increased physical contractility can in turn induce the expression of α -SMA, whatever increased contractility overexpressing pmHSP27 could cause, had no effect on the expression of α -SMA in MK2^{-/-} MEF. These results indicate that MK2 affects migration of fibroblasts, not through its effect on HSP27 phosphorylation, but possibly through a direct effect on α -SMA expression.

Example 8

Disruption of MK2 does not affect Smad signaling which appears to be upstream of p38:

Fibroblast proliferation and α -SMA expression have been linked to growth factors such as TGF β , which are known to exert many of its effects through Smad signaling. Thus this signaling pathway in MK2^{-/-} versus wild type MEF was examined. After treatment with TGF β (1 ng/mL) for different times, Smad2 phosphorylation was assayed. As shown in Figure 9A, Smad2 phosphorylation is activated by TGF β similarly in wild type or MK2^{-/-} MEF, indicating that MK2 is not upstream of Smad in TGF β -activated signaling. The activation of p38 by TGF β in both cell types was examined, and demonstrated that it is indeed activated, albeit at a later time-course than Smad2 (Figure 9A). Preincubating wild type MEF with 1 μ M SB202190 an inhibitor of p38 MAP kinase which is an upstream activator of MK2, had no effect on activation of Smad2 by TGF β (data not shown). This result is consistent with the absence of differences in Smad2 activation between MK2^{-/-} and wild type MEF. On the other hand, when wild type or MK2^{-/-} fibroblasts were preincubated with an inhibitor of TGF β Type I receptor kinase inhibitor SB431542 (10 μ M), Smad2 activation by TGF β was inhibited in both cell types. Furthermore, inhibiting Smad2 activation by SB431542 also inhibited p38 activation (Figure 9B). These findings suggest that Smad2 signals upstream of MK2 and that the effects of MK2 on fibroblast physiology and fibrosis are not due to altered downstream Smad signaling.

Example 9

Expression of MK2 in cells of mouse lung tissue

The retroviral construct pMMP-IRES-GFP MK2 (Roninca et al., *Mol Cell Biol* 27:170-181, 2007) was propagated in HEK293 cells and titrated. Next, MK2^{-/-} mice were intubated and the viral supernatant was instilled with a syringe through the tube, followed by brief ventilation to insure delivery of the virus to the lung. Some mice received the control pMMP-IRES-GFP (GFP-virus) and others received the pMMP-IRES-GFP-MK2 (MK2 virus). After 48 hours, lungs were immunostained with either an antibody against MK2 or control IgG. The Vector (Vector, Burlingame, CA) avidin biotin system was used for secondary antibody labeling, followed by the NovaRed horse radish peroxidase substrate. Slides were then counterstained with hematoxylin. As shown in Figure 11, lungs of mice infected with MK2-encoding virus exhibited stronger antiMK2 labeling than either IgG controls or GFP-virus-infected mouse lungs. The staining appeared strongest in epithelial cells which would be the first to encounter the virus, but was also visible in the parenchyma. These results demonstrate that these viral vectors can be used successfully to overexpress MK2 in mouse lungs.

Example 10

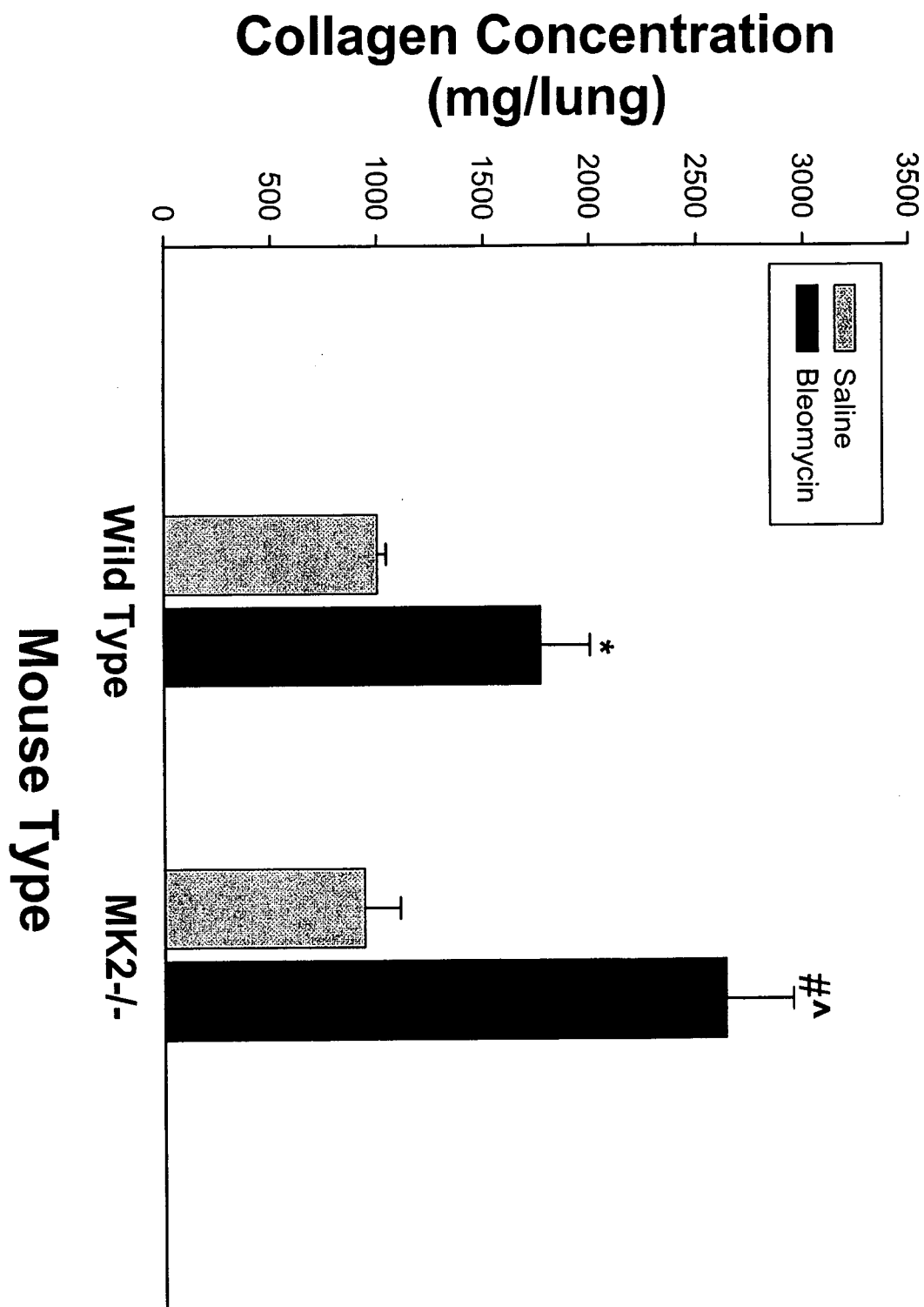
Effect of MK2 overexpression on bleomycin induced pulmonary fibrosis in MK2^{-/-} mice

MK2^{-/-} mice are transfected with pMMP-IRES-GFP MK2 as described in Example 9. After 48 hours, transfected mice are challenged with bleomycin as described in Example 2, and histological lung samples are analysed for fibrosis, also as described in Example 2. It is expected that mice transfected with pMMP-IRES-GFP MK2 will develop less fibrosis than mice transfected with pMMP-IRES-GFP or untransfected mice.

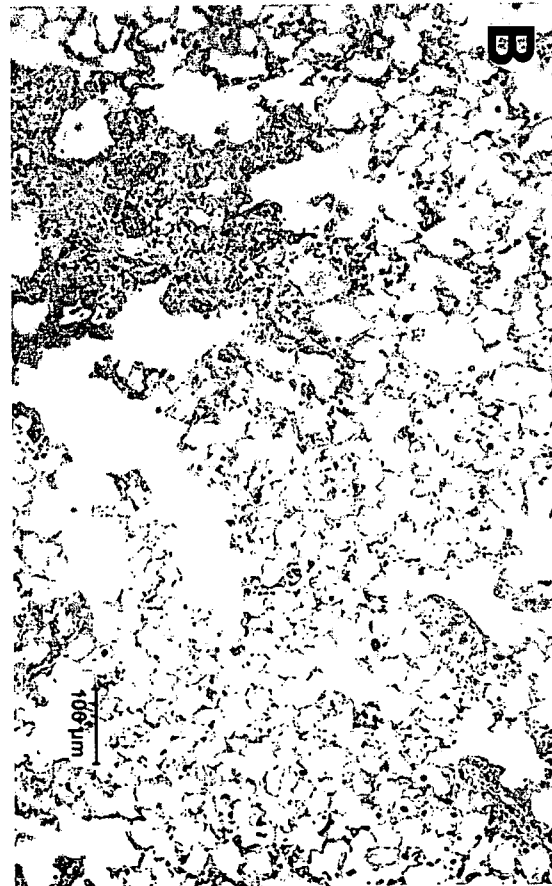
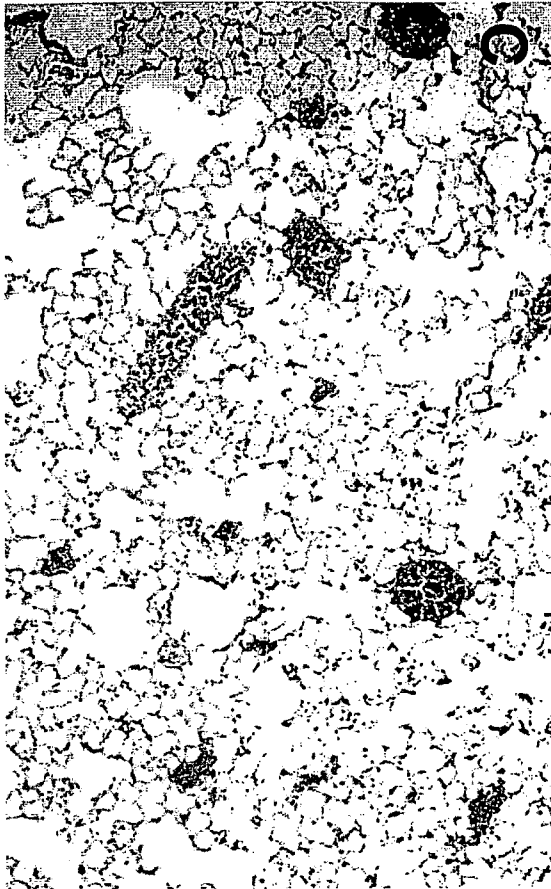
What is Claimed is:

1. A method for therapeutically treating a mammal having fibrosis or scarring comprising increasing the expression and/or activity of MK2 in cells in fibrotic tissue in the mammal.
2. The method according to claim 1 wherein MK2 is overexpressed by transfecting cells in the mammal with a vector that overexpresses MK2.
3. The method according to claim 1, wherein the activity of MK2 in cells of the mammal is increased by inhibiting the activity of an inhibitor of MK2.
4. The method according to claim 1, wherein the fibrotic tissue is lung tissue.
5. The method according to claim 1, wherein the mammal is a human.
6. A method for preventing fibrosis or scarring in a mammal having injured tissue comprising increasing the expression and/or activity of MK2 in cells in the injured tissue in the mammal.
7. The method according to claim 6 wherein MK2 is overexpressed by transfecting cells in the mammal with a vector that overexpresses MK2.
8. The method according to claim 6, wherein the activity of MK2 in cells of the mammal is increased by inhibiting the activity of an inhibitor of MK2.
9. The method according to claim 6, wherein the fibrotic tissue is lung tissue.
10. The method according to claim 6, wherein the mammal is a human.

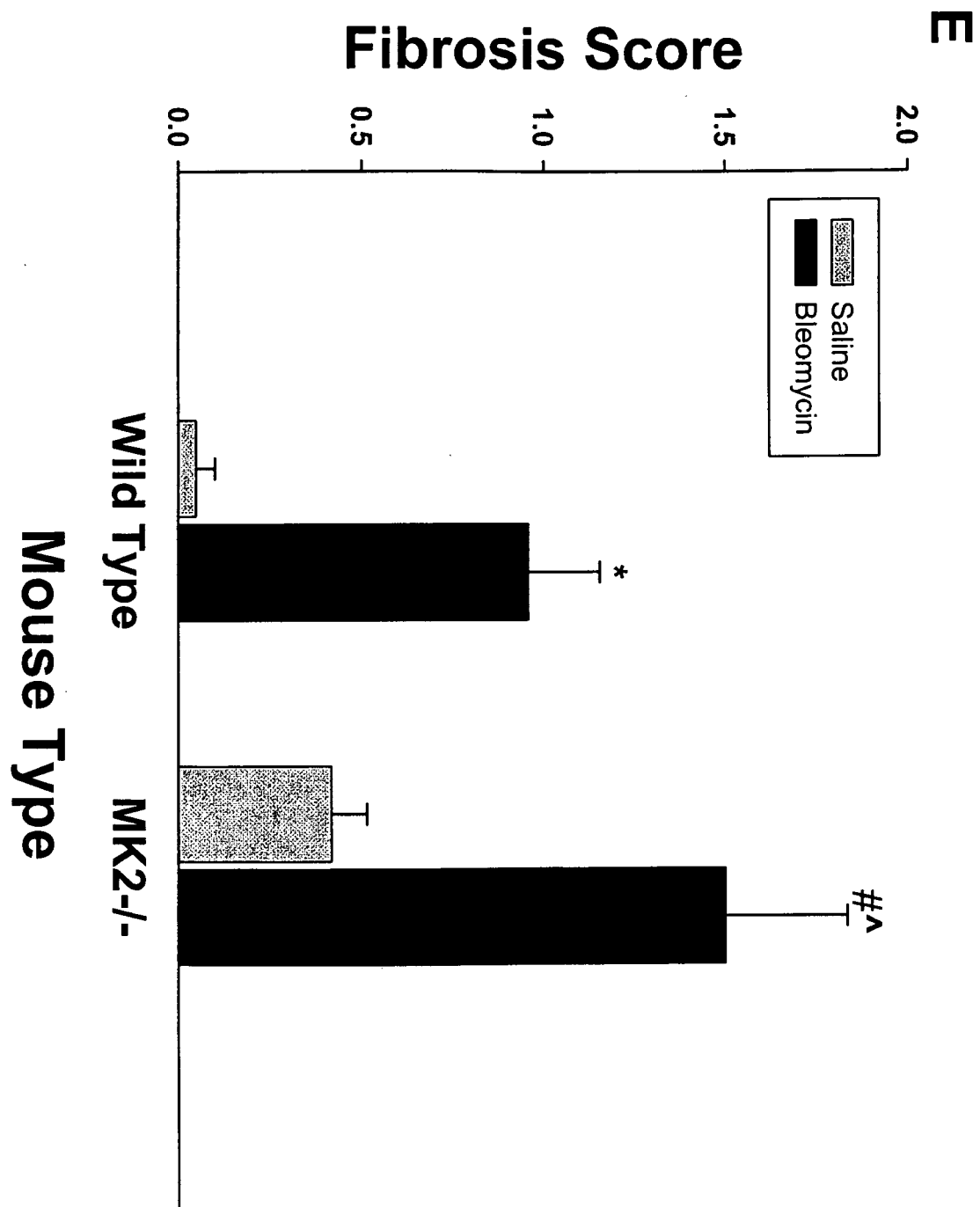
1/15



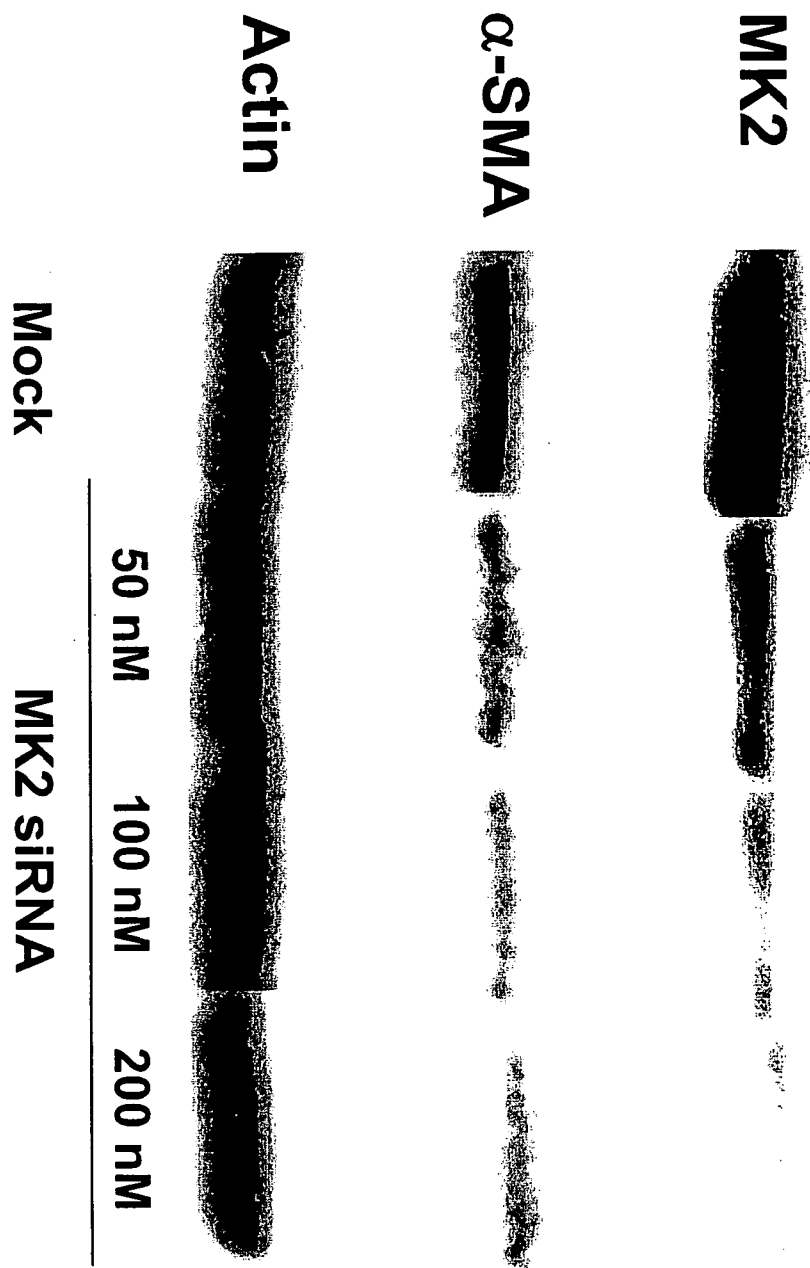
2/15

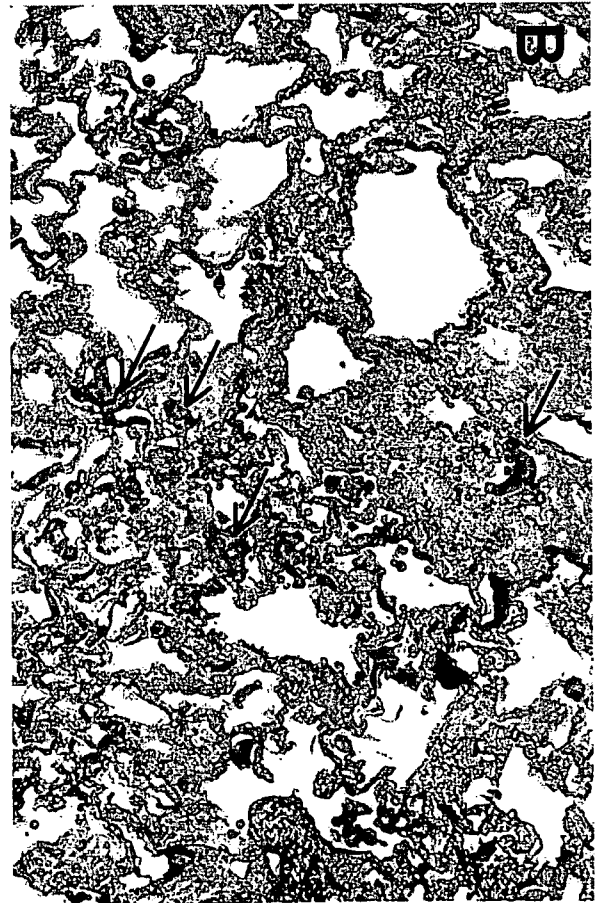
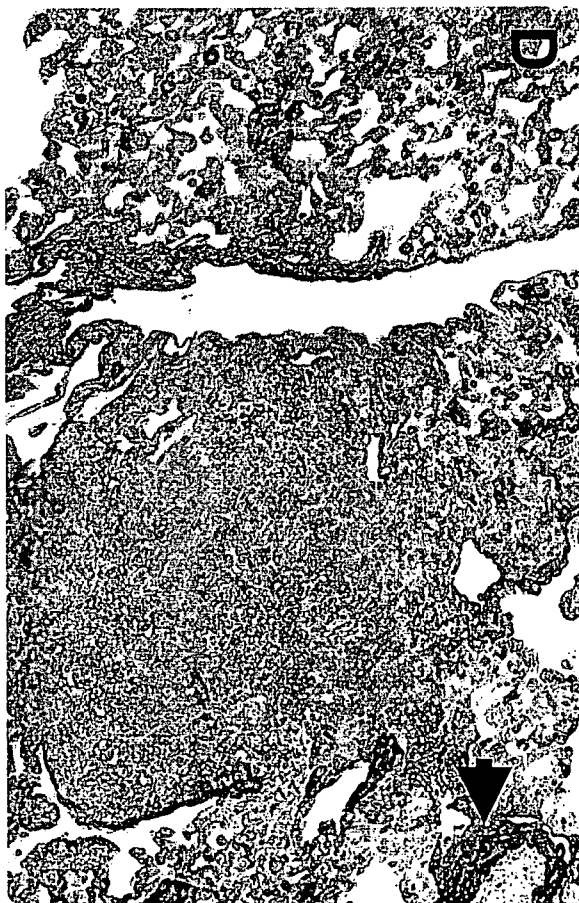
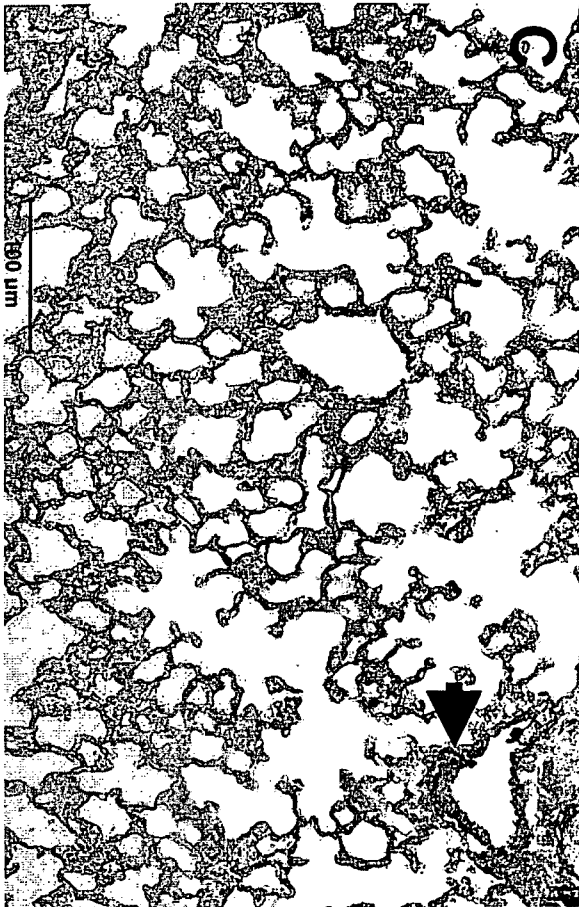


3/15



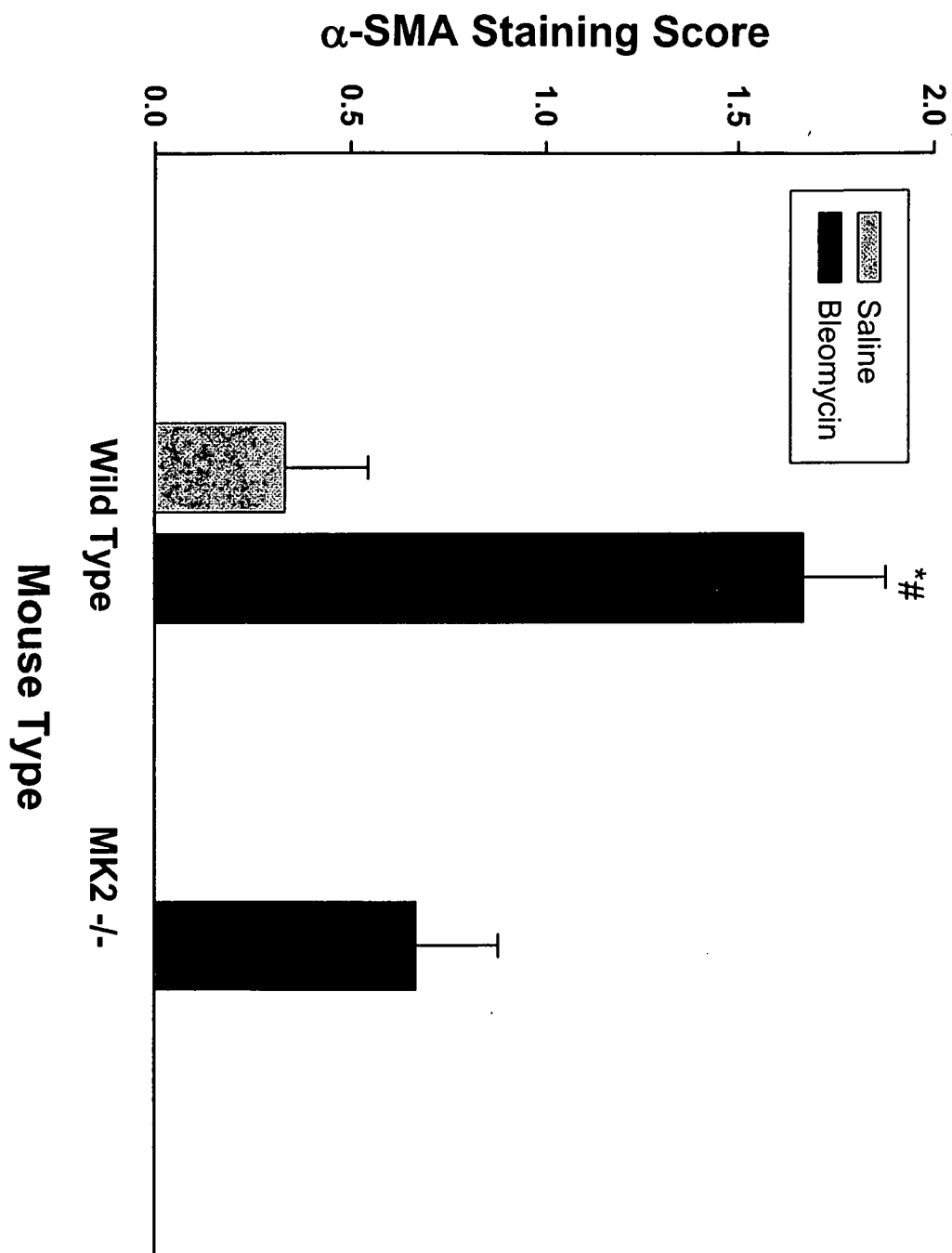
4/15



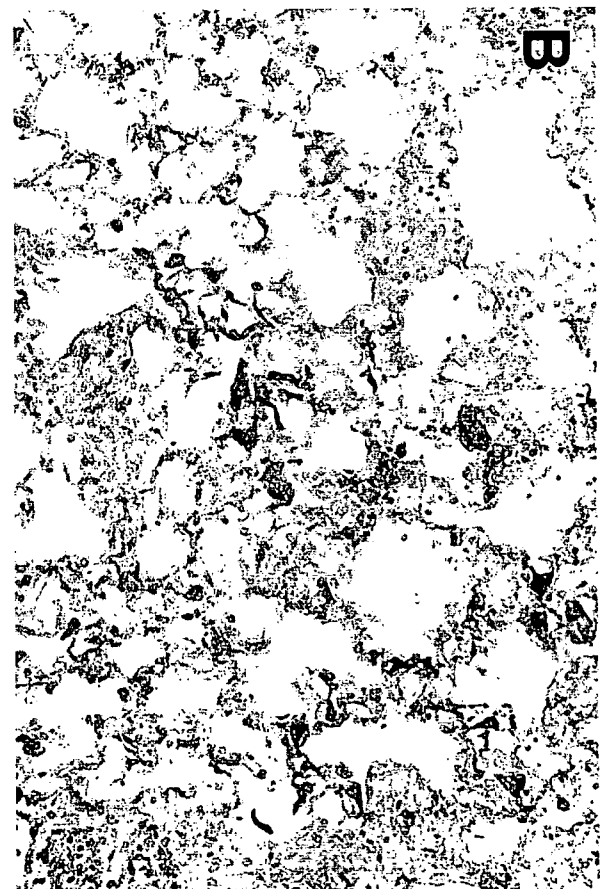
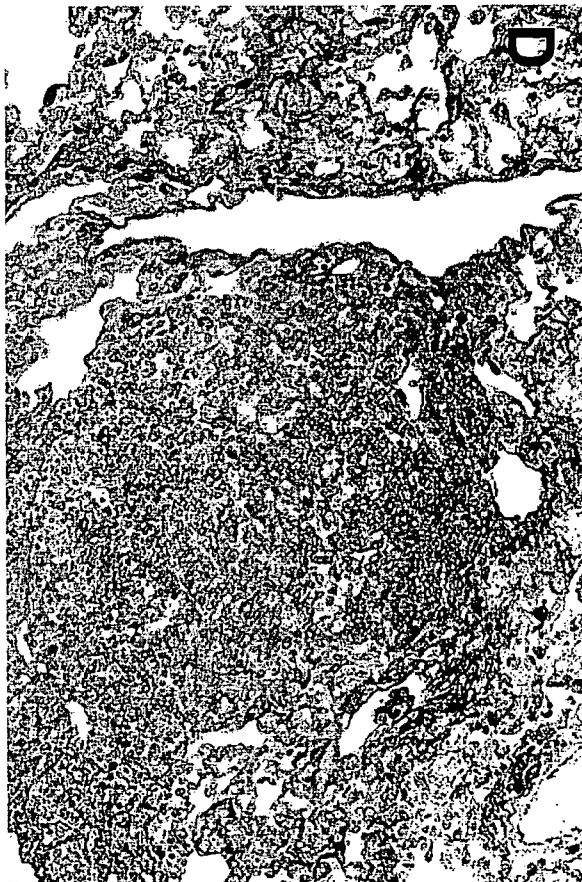
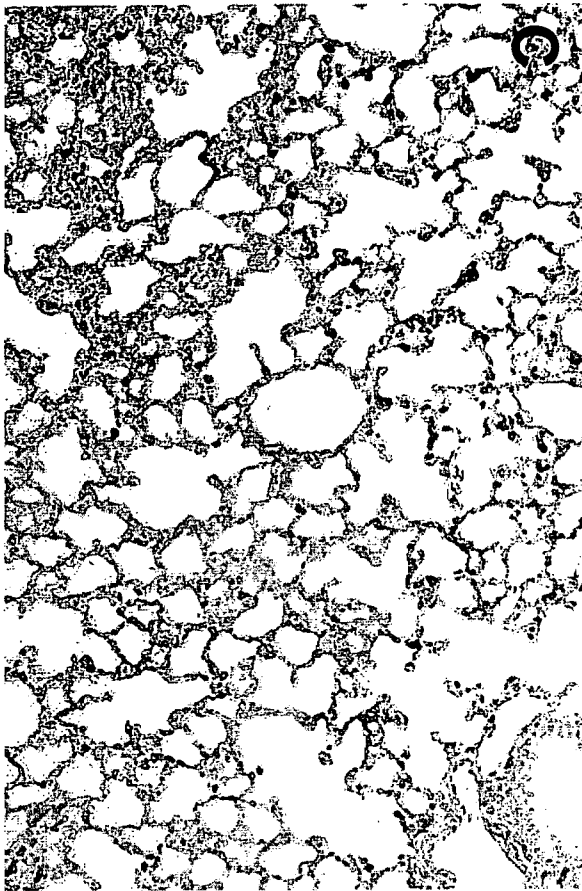


6/15

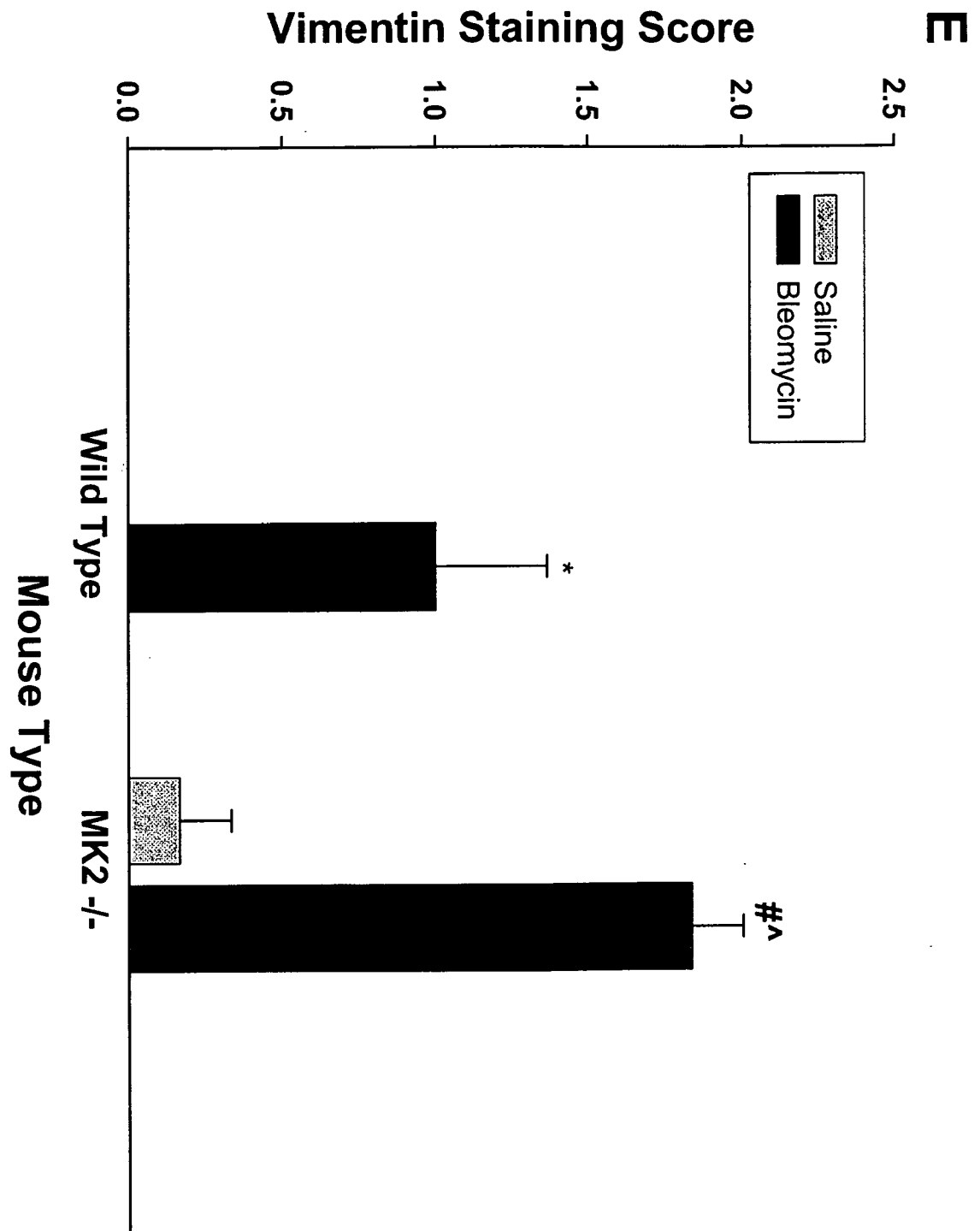
E



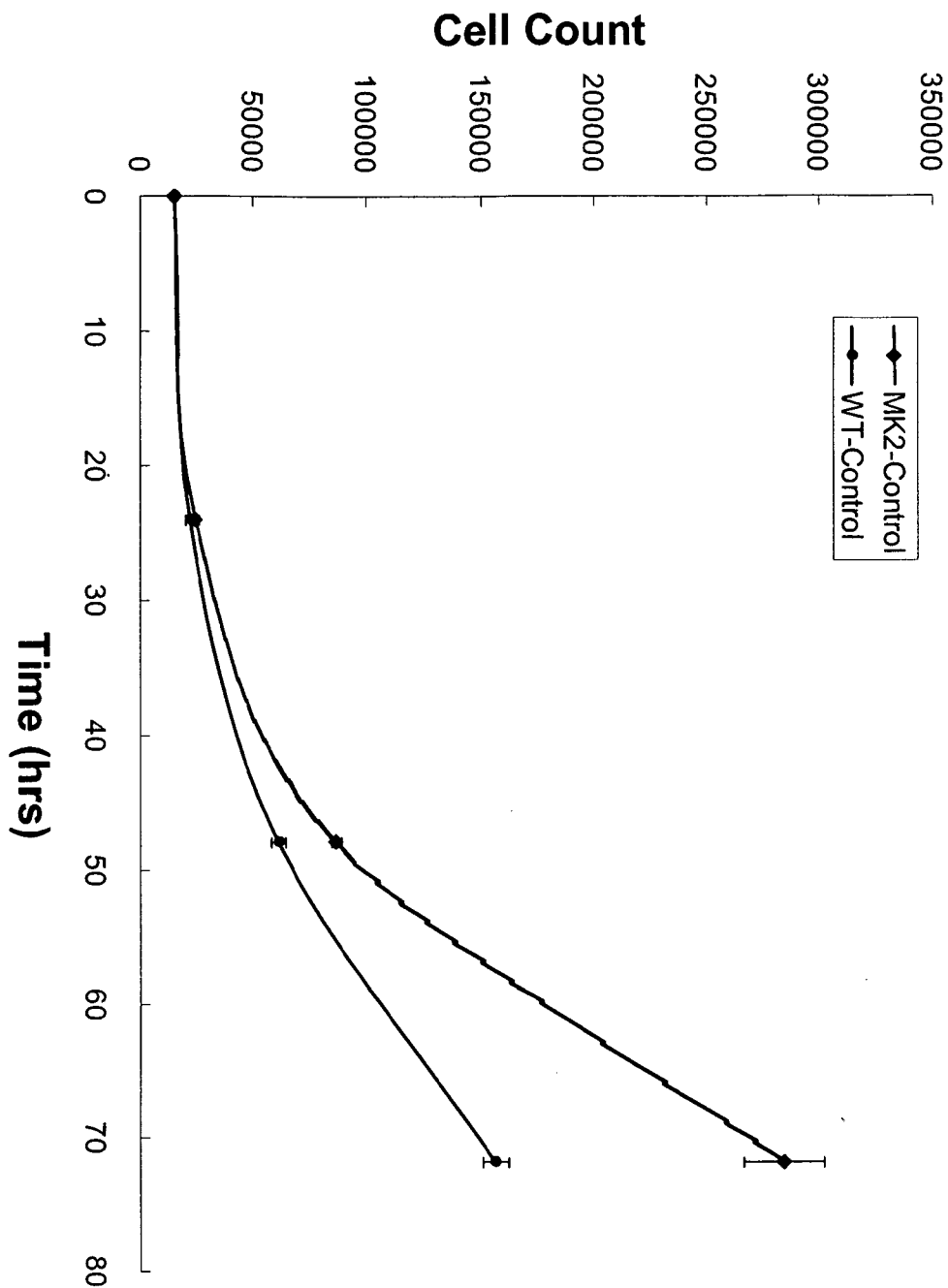
7/15

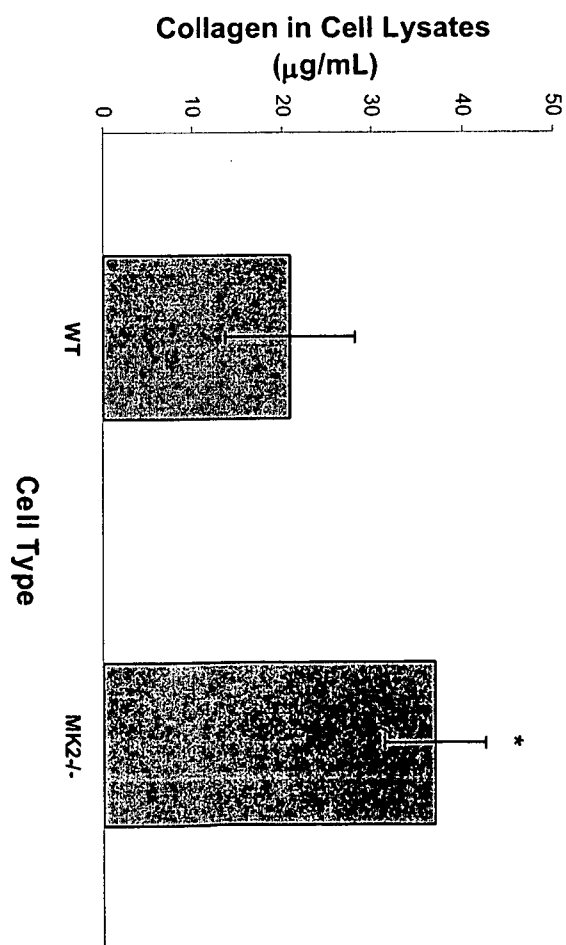
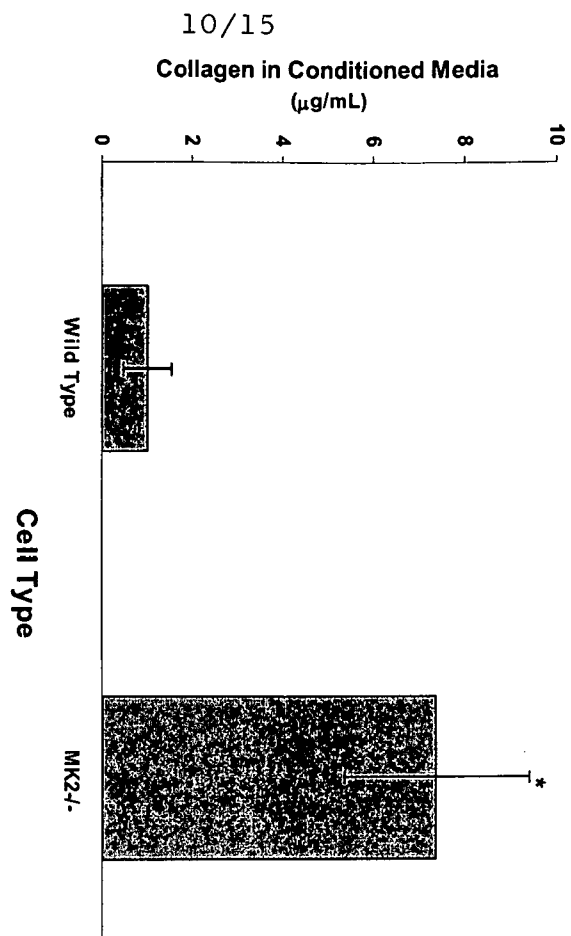


8/15



9/15





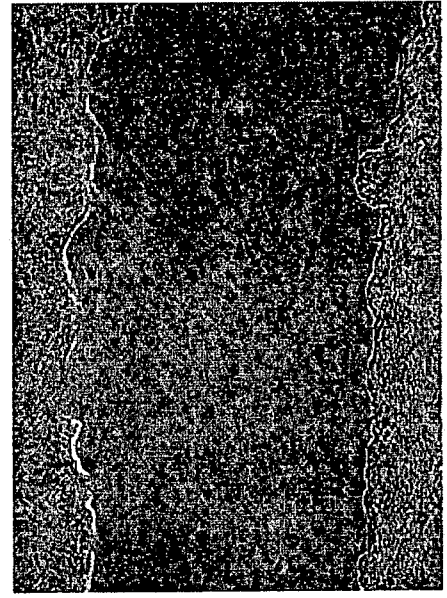
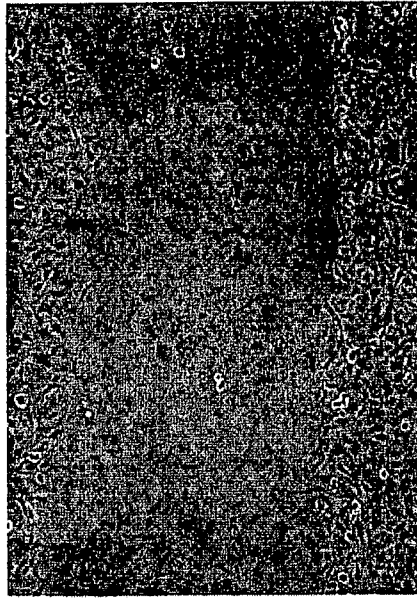
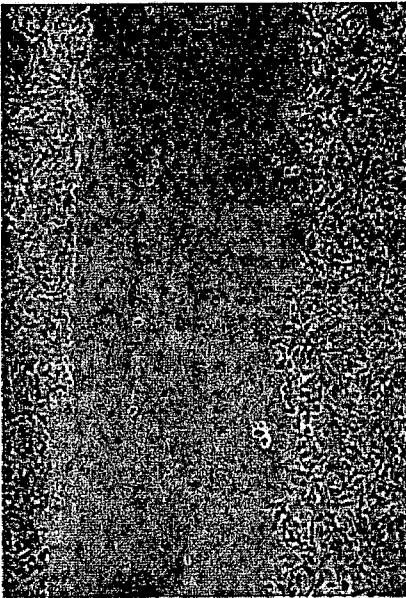
11/15

pmHSP27-
MK2^{-/-}

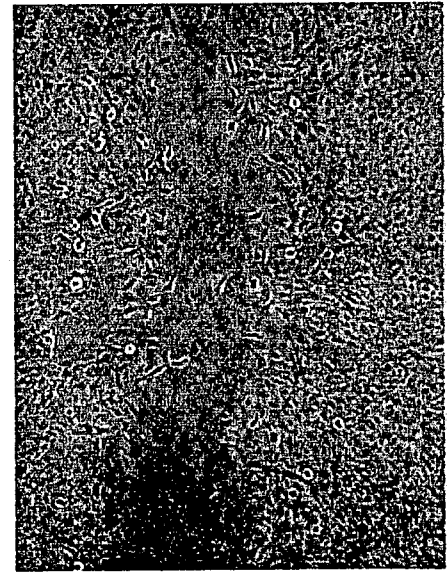
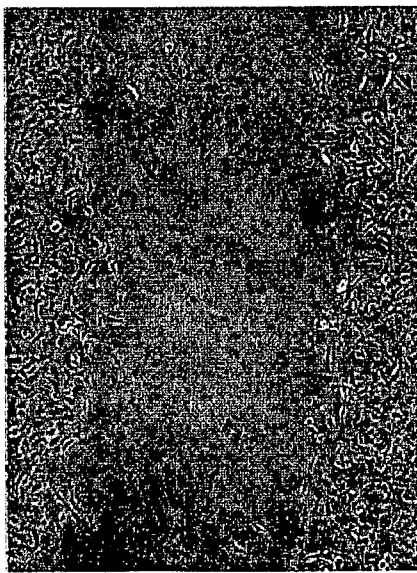
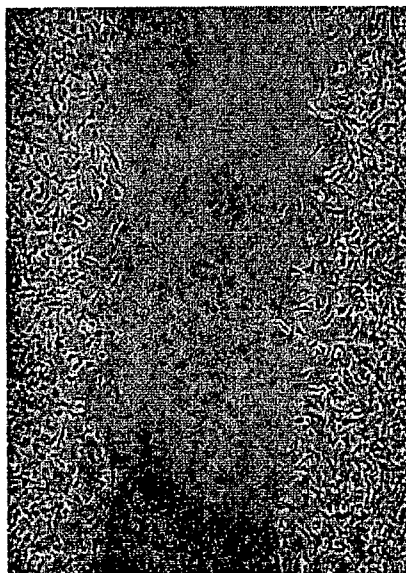
MK2^{-/-}

Wild Type

0 Hours



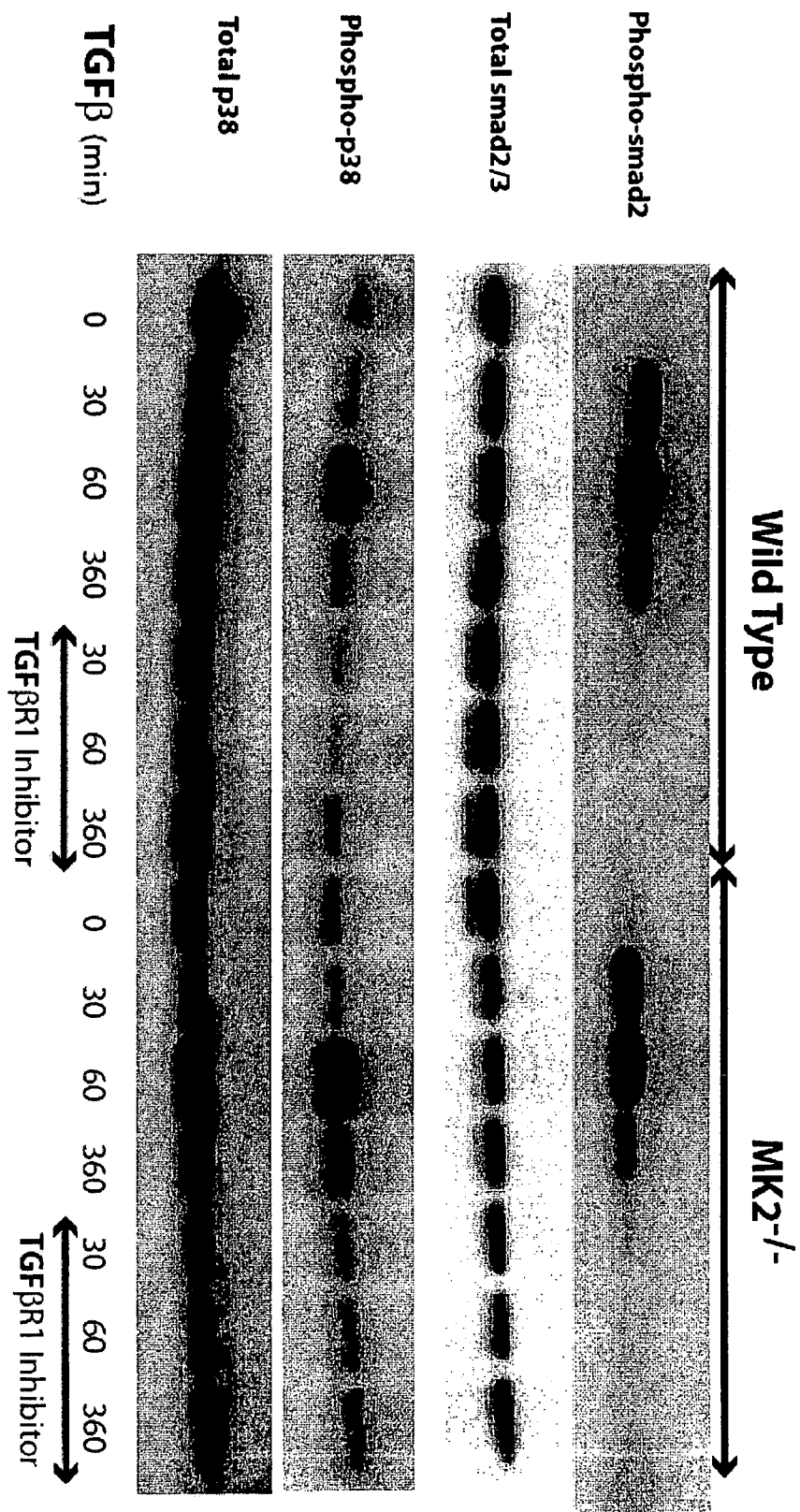
24 Hours



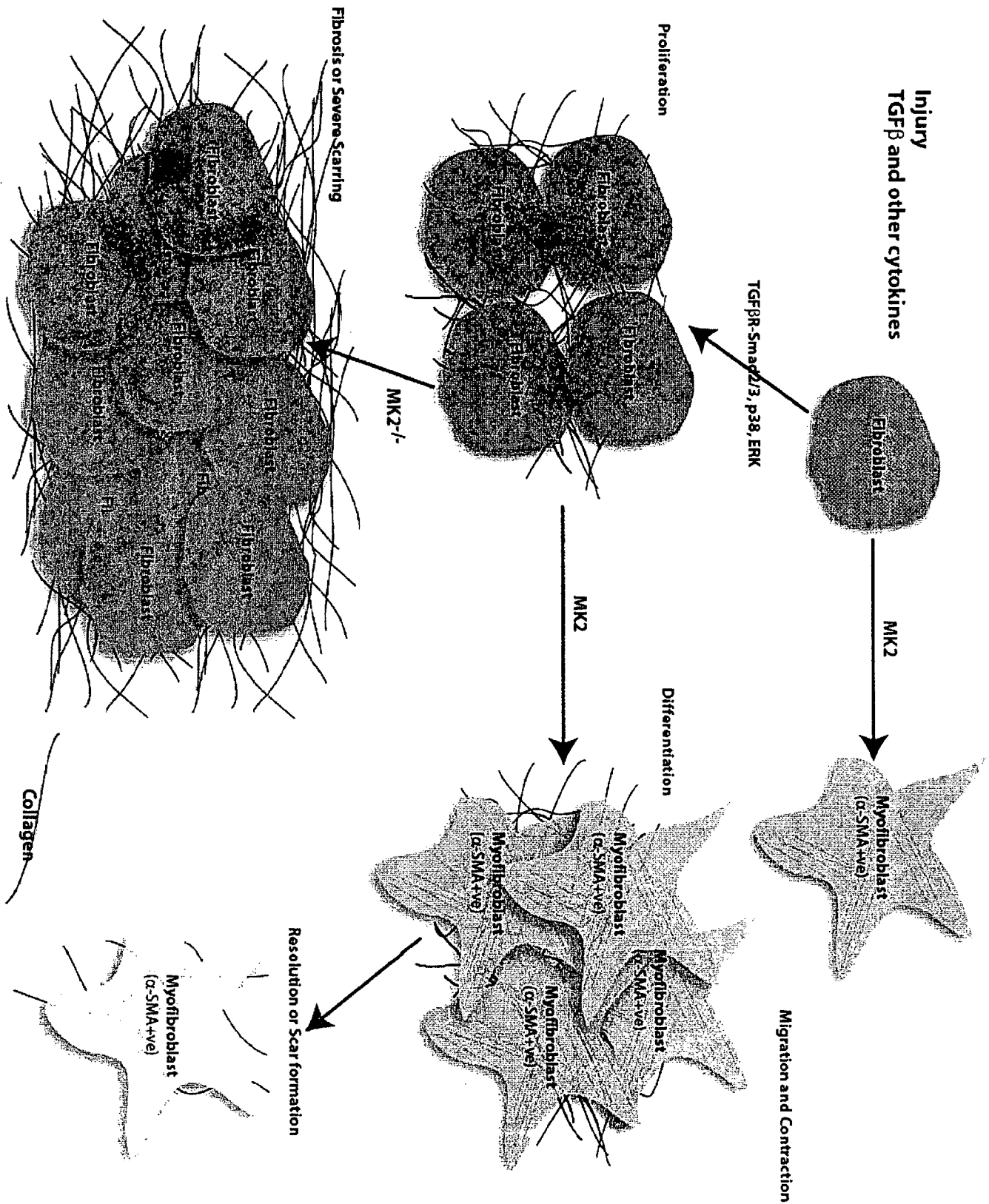


13/15

B



14/15



15/15

