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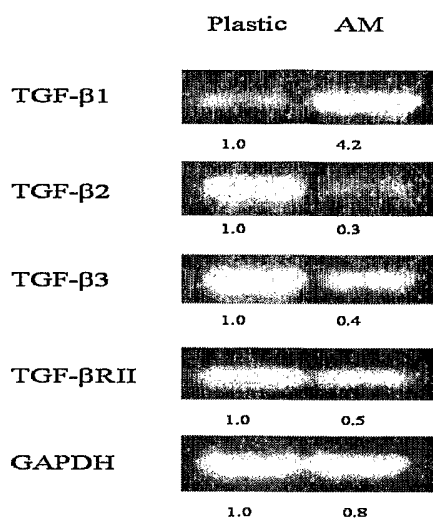
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(54) Title: AMNIOTIC MEMBRANE EXTRACTS, COMPOSITIONS THEREOF, AND METHODS OF USE

RT-PCR (human corneal fibroblast)



(57) Abstract: The present invention relates to the prevention and reduction of scarring and inflammation by amniotic membrane extracts or compositions thereof. The invention also relates to the preparation of soluble and fractionated amniotic membrane extracts and the preparation of gels comprising the amniotic membrane extract for enhanced use and application. The invention further provides an extract composition of amniotic membrane that is effective in the prevention and reduction of scarring and inflammation by suppression of TGF- β activity. The invention further provides a method for reducing scarring and inflammation, and enhancing healing of wounds. The invention also provides purified lumican from human AM for the treatment of scarring and inflammation.

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Description**AMNIOTIC MEMBRANE EXTRACTS, COMPOSITIONS
THEREOF, AND METHODS OF USE**10 **Technical Field**

This invention relates to extracts obtained from amniotic membrane and compositions thereof usable in the prevention and reduction of scarring and inflammation caused by disease and injury.

15 **Disclosure of Invention****Terminology**

Amniotic membrane is the innermost membrane surrounding a fetus in the amniotic cavity. It has two major components: the basement membrane and stroma. The side of the amniotic membrane dominated by the basement membrane is referred to as the "basement membrane side". The side of the amniotic membrane dominated by the stroma is referred to as the "stroma side". An autograft is a tissue transplant from the same recipient. An allograft is a tissue transplant to a recipient from a donor of another individual of the same species.

25 **Previous Clinical Applications:**

The fetal membrane including amnion (amniotic membrane) and chorion has been used in surgeries documented as early as 1910 and has been reviewed by Trelford and Trelford-Sauder in 1979. *See* Trelford, et al., Am. J. Obstet. Gynecol., 134:833 (1979). In the beginning, the fetal membrane was used by Davis in 1910 on burned and ulcerated skin with additional coverage of warm paraffin and dressing. In 1940, De Rötth used live fetal membrane for ophthalmic reconstruction of symblepharon, and noted a success in one of six cases. *See* De Rötth, Archives of Opthamol., 23:522 (1940). In 1952, Douglas thought chorion might be more useful for skin use. Massee and colleagues in 1962 used the fetal membrane in dogs to treat pelvic basins after total exenteration; however, the human trials proved disappointing.

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The isolated amnion alone was first used by Brindeau in 1935 and Burger in 1937 as a graft in forming artificial vaginas. Between 1941 and 1948, Kubanyi used

5 “live” amnion in patients with burns, traumatic skin wounds, and enterocutaneous
fistula secondary to surgery for lysis of adhesions. The isolated preserved amnion,
termed “amnioplastin”, was first reported by Chao and associates in 1940. Chao used
amnioplastin for continual dural repair, peripheral nerve injuries, conjunctival graft and
flexor and tendon repair. In the Russian literature, this technique was also used for
10 flesh trauma by Pikin in 1942.

Mention of “amnioplastin” disappeared from the literature with no real
explanation. No critical reports regarding isolated, non-living amnion with
preservation were found for a thirty-year period. This gap in research ended in 1972
with the research of Trelford and associates, cited above. Trelford, using isolated
15 amnion with an early form of preparation, showed that the orientation with stromal side
down provided more consistent “take.” Robson and colleagues noted in 1972 that,
when used in partial-thickness skin wounds, no “take” occurs, and the amnion peels off.
In 1973, and later, Trelford and associates reported its use as a dressing on full-
thickness skin wounds, to replace pelvic peritoneum, and to cover exposed deep
20 surfaces in pedicle graft procedures, to treat non healing skin wounds in diabetic
patients, as a graft over the surgical defect of total glossectomy, as a biological dressing
in omphalocele, and in the prevention of meningocerebral adhesions following head
injury.

25 Amniotic Membrane Transplantation for Ocular Surface Reconstruction:

Transplantation of amniotic membrane (AM) as a graft has been used to deliver
anti-scarring, anti-inflammatory, anti-angiogenic and growth-promoting effects. *See*
Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004). The use of amniotic membrane
as a surgical graft has been successfully done for ocular surface reconstruction. *See*
30 Tseng, et al., *Hong Kong J. Ophthalmol.*, 2(1):26-34 (1998); Tseng, *Bioscience Rep.*,
21:481-489 (2002); Dua., et al., *Br. J. Ophthalmol.*, 83:748-752 (1999); Dua, et al.,
Surv. Ophthalmol., 49(1):51-77 (2004); and Sippel, et al., *Curr. Opin. Ophthalmol.*,
12:269-281 (2001). Amniotic membranes have been used as permanent and temporary
grafts. As a permanent graft, AM is applied in one or multiple layers for corneal,
35 conjunctival, or entire ocular surface reconstruction. With a permanent graft, the
surrounding host epithelial cells migrate onto the amniotic basement membrane, while
host mesenchymal cells will migrate into the amniotic stromal matrix. As a result,

5 transplanted AM is integrated into the recipient site. As a temporary graft, the host epithelial cells migrate underneath AM, and upon healing, the transplanted AM invariably dissolve.

A number of clinical studies have shown that AM transplantation facilitates epithelial wound healing and reduces inflammation, scarring and angiogenesis.

10

Anti-inflammatory:

The AM contains many factors that may contribute/mediate its anti-inflammatory ability, such as interleukin-10 (IL-10), members of the transforming growth factor-beta (TGF- β) superfamily, protease inhibitors, and IL-1 receptor antagonist (IL-1RA). IL-10 is known to suppress and counteract pro-inflammatory cytokines, such as IL-6, TNF α , and IL-8. *See* Foutunato, et al., *Am. J. Obstet. Gynecol.*, 175:1057-65 (1996); Foutunato, et al., *Am. J. Obstet. Gynecol.*, 177:803-9 (1997); and Foutunato, et al., *Am. J. Obstet. Gynecol.*, 179:794-9 (1998). Activin and inhibin, which are members of the TGF- β superfamily, are produced by the AM.

20 Varying doses of activin give rise to different results. At low doses of activin, production of IL-6, IL-8, and prostaglandin E2 (PGE2) is stimulated, but at high doses, it inhibits production. *See* Petraglia, et al., *J. Clin. Endocrinol. Metab.*, 77:542-8 (1993); Riley, et al., *Hum. Reprod.* 15:578-83 (2000); and Keelan, et al., *Placenta*, 31:38-43 (2000). The AM also contains protease inhibitors such as α 1 anti-trypsin,

25 which may exert an anti-inflammatory effect. *See* Na, et al., *Trophoblast Res.*, 13:459-66 (1999). IL-1RA is an inhibitor of IL-1 and therefore, suppresses the inflammation mediated by IL-1. *See* Romero, et al., *Am. J. Obstet. Gynecol.*, 171:912-21 (1994). The inventors have demonstrated that AM has down-regulates the expression and production of IL-1 and up-regulates IL-1RA. *See* Tseng, et al., *Ocular Surface J.*, 30 2(3):177-187 (2004).

Anti-scarring:

The phenomenon of scarless fetal wound healing has been closely studied to understand how an incision made before the third trimester of pregnancy does not result

35 in scar formation at birth. *See* Mast, et al., *Surg. Gynecol. Obstet.*, 174:441-51 (1992); Adzick, et al., *Ann. Surg.*, 220:10-8 (1994); and Dostal, et al., *Surg. Gynecol. Obstet.*, 176:299-306 (1993). As discussed above, AM contains anti-inflammatory cytokines

5 that inhibit IL-6, which is known to be one of the hallmarks of scarless fetal wound repair.

Amniotic epithelial cells, although not innervated, synthesize various neurotransmitters, neuropeptides, and neurotrophins. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); Sakuragawa, et al., *Jpn. J. Pharmacol.*, 85, 20-3 (2001); and
10 Uchida, et al., *J. Neurosci. Res.*, 62:585-90 (2000). The production of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), is significant because these factors control the growth and targeting of sensory and autonomic nerves to the peripheral tissues. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); and Lewin, et al., *Annu. Rev. Neurosci.*,
15 19:289-317 (1996). The inventors have previously shown that cryopreserved AM contains abundant amounts of NGF and permits the expression of NGF receptors by the limbal epithelial cells when grown over the membrane culture. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); and Touhami, et al., *Invest. Ophthalmol. Vis. Sci.*, 43:987-94 (2002). This supports the concept that the AM plays an active role in
20 supporting the target tissue to express and respond to neurotrophins such as NGF. This finding may explain, in part, why AM transplantation is effective in treating neurotrophic corneal ulcers, which are caused by corneal denervation. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); Lee, et al., *Am. J. Ophthalmol.*, 123:303-12 (1997); Chen, et al., *Br. J. Ophthalmol.*, 84:826-33 (2000); and Solomon, et al.,
25 *Comprehensive Ophthalmol. Update*, 3:165-74 (2000).

The inventors have also previously studied whether the AM exerts a direct anti-scarring action on ocular surface fibroblasts. When human AM was transplanted into the rabbit corneal stromal pocket, the epithelial-induced differentiation of corneal stromal fibroblasts (keratocytes) into myofibroblasts was inhibited. *See* Tseng, et al.,
30 *Ocular Surface J.*, 2(3):177-187 (2004); and Choi, et al., *Cornea*, 20:197-204 (2001). To confirm that such an in vivo anti-scarring action is not caused indirectly by suppressing inflammation via inflammatory cells or epithelial cells, the expression of TGF- β genes was investigated by ocular surface fibroblasts cultured directly on AM stromal side. The investigation demonstrated that AM exerts a direct, potent, anti-
35 scarring action on ocular surface fibroblasts by suppressing the TGF- β signaling at the transcription level. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004). It has also been shown that transcript expression of TGF- β 2 and TGF- β 3, but not TGF- β 1, as

5 well as transcript expression of TGF- β RI, TGF- β RII and TGF- β RIII, are markedly down-regulated in fibroblasts derived from normal human cornea, limbus and conjunctiva and in abnormal fibroblasts derived from patients with pterygium, cultured in either a serum-containing or serum-free medium. *See* Tseng, et al., Ocular Surface J., 2(3):177-187 (2004). Furthermore, such transcript suppression is followed by
10 marked suppression of both transcripts and proteins of downstream TGF- β target genes encoding α -SMA, EDA-containing Fn, and integrin $\alpha 5\beta 1$. *See* Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Tseng, et al., J. Cell. Physiol., 179:325-35 (1999); and Lee, et al., Curr. Eye Res., 20:325-34 (2000).

15 Wounds and Scarring:

Wounds are internal and external bodily injuries or lesions caused by physical means, such mechanical, chemical, viral, bacterial, fungal and other pathogenic organisms, or thermal means, which disrupt the normal continuity of tissue structures. Wounds may be caused by accident, surgery, pathological organisms, or by surgical
20 procedures.

As is well known, the healing of wounds in tissue such as skin generally involves, at least in adult humans and other mammals, a process of extra-cellular matrix (ECM) biosynthesis, turnover, and organization, which commonly leads to the production of fibrous, connective tissue scars and consequential loss of normal tissue
25 function. A scar is the mark left in the skin or internal organ by new connective tissue that replaces tissue which has been injured by, e.g., burns, ulcers, abrasions, incisions, etc.

Wound healing consists of a series of processes whereby injured tissue is repaired, specialized tissue is regenerated, and new tissue is reorganized. Wound
30 healing consists of three major phases: a) an inflammation stage, b) proliferation stage, and c) a remodeling phase.

During the inflammation phase, platelet aggregation and clotting form a matrix which traps the plasma proteins and blood cells to induce the influx of various types of cells. During the cellular proliferation phase, new connective or granulation tissue and
35 blood vessels are formed. During the remodeling phase, granulation tissue is replaced by a network of collagen and elastin fibers leading to the formation of scar tissue.

5 In adult humans and other mammalian vertebrates, wound healing in tissue such
as skin is generally a reparative process, in contrast to a regenerative process which
appears to take place in healing of fetal and embryonic tissue. The outcome of a wound
repair process appears to be influenced by a number of different factors, including both
10 dressings. There is, however, considerable evidence indicating that the overall process
of healing and repair of wound damaged tissue, including the necessary intercellular
communication, is regulated in a coordinate manner in adult humans and other
mammals by a number of specific soluble growth factors which are released within the
wound environment and which, among other things, appear to induce
15 neovascularization, leukocyte chemotaxis, fibroblast proliferation, migration, and
deposition of collagen and other extra-cellular matrix molecules within the wounds.
Such growth factors that have been identified and isolated are generally specialized
soluble proteins or polypeptides and include transforming growth factor alpha (TGF- α),
transforming growth factor beta (TGF- β 1, TGF- β 2, TGF- β 3, etc.), platelet derived
20 growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factors I
and II (IGF1 and IGF2) and acidic and basic fibroblast growth factors (acidic FGF and
basic FGF).

TGF- β :

25 TGF- β is the prototypic cytokine that is involved in wound healing and scar
formation and modulation of tissue inflammation. *See* Border, et al., J. Clin. Invest.,
90:1-7 (1992); Grande, Proc. Soc. Exp. Biol. Med., 214:27-40 (1997); Jester, et al.,
Prog. Retin. Eye Res., 18(3):311-356 (1999); and Marek, et al., Med. Sci. Monit.,
8(7):RA145-151 (2002). Mammalian cells express three different TGF- β s, i.e. TGF-
30 β 1, TGF- β 2, and TGF- β 3. TGF- β turns out to be the most potent cytokine promoting
myofibroblast differentiation by up-regulating expression of α -SMA, integrin α 5 β 1, and
EDA domain-containing fibronectin (Fn) in a number of cell types, including
fibroblasts. *See* Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Ronnov-Jessen,
et al., Lab. Invest., 68:696-707 (1993); Verbeek, et al., Am. J. Pathol., 144:372-82
35 (1994); Hales, et al., Curr. Eye Res., 13:885-90 (1994); Jester, et al., Cornea, 15:505-16
(1996); Serini, et al., J. Cell. Biol., 142:873-81 (1998); Grande, Proc. Soc. Exp. Biol.
Med., 214(1):27-40 (1997); and Jester, et al., Prog. Retin. Eye. Res., 18:311-56 (1999).

5 TGF- β also up-regulates the expression of such matrix components as collagens and proteoglycans, down-regulates proteinase and matrix metalloproteinases, and up-regulates their inhibitors. Collectively, these actions result in increased cell-matrix interactions and adhesiveness, as well as deposition and formation of scar tissue. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); Grande, *Proc. Soc. Exp. Biol. Med.*, 214(1):27-40 (1997); Jester, et al., *Prog. Retin. Eye. Res.*, 18:311-56 (1999); and
10 Lawrence, *Eur. Cytokine Netw.*, 7:363-74 (1996).

TGF- β s exert their actions via binding with TGF-beta receptors (TGF- β R) on the cell membrane. In human cells, there are three TGF- β R, namely TGF- β R type I (TGF- β RI), type II (TGF- β RII), and type III (TGF- β RIII). TGF- β s, serving as ligands,
15 bind with a serine, threonine kinase receptor complex made of TGF- β RI and TGF- β RII; such a binding is facilitated by TGF- β RIII, which is not a serine, threonine kinase receptor. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); and Massague, et al., *Genes and Development.*, 14:627-44 (2000). Binding with TGF- β RII activates TGF- β RI, which is responsible for direct phosphorylation of a family of effector
20 proteins known as Smads, which modulate transcription of a number of target genes, including those described above, participating in scar formation. *See* Tseng, et al., *Ocular Surface J.*, 2(3):177-187 (2004); Massague, et al., *Genes and Development.*, 14:627-44 (2000); and Derynck, et al., *Biochem. Biophys. Acta.*, 1333:F105-F150 (1997).

25 Suppression of TGF- β can be achieved by neutralizing antibodies to TGF- β and agents that intercede the signaling mediated by TGF- β such as decorin. *See* Shahi, et al., *Lancet*, 339:213-214 (1992); Petroll, et al., *Curr. Eye Res.*, 1739:736-747 (1998); Yamaguchi, et al., *Nature*, 346(6281):281-284 (1990); and Logan, et al., *Exp. Neurol.*, 159:504-510 (1999). Most of the literature has shown suppression of TGF- β being
30 achieved at the level of modulating the TGF- β activation, binding with its receptor, or its signal transduction. It has been shown that amniotic membrane can achieve such an inhibition at the level of transcription, i.e., to turn off transcription of TGF- β genes. In particular, amniotic membrane has been shown to suppress TGF- β signaling in human corneal and limbal fibroblasts, and human conjunctival and pterygium body fibroblasts.
35 *See* Tseng, et al., *J Cell Physiol.*, 179:325-335 (1999); and Lee, et al., *Curr. Eye Res.*, 20(4):325-334 (2000). The present invention circumvents the need for using amniotic

- 5 membrane as a membrane or graft by providing a method and composition that are useful in the prevention and reduction of scarring and inflammation.

Lumican:

- Lumican, a small leucine-rich proteoglycan (SLRP), is one of the major
10 extracellular components in interstitial collagenous matrices of the corneal stroma, aorta, skin, skeletal muscle, lung, kidney, bone, cartilage, and intervertebral discs. *See* Yeh, et al., Invest. Ophthalmol. & Vis. Sci., 46(2):479-486. In the cornea, lumican contains keratan sulfate chains. However, it is present as a low or nonsulfated glycoprotein (50-57 kDa) in noncorneal tissues. *See* Yeh, et al., Invest. Ophthalmol.
15 Vis. Sci., 46(2):479-486; Funderburgh, et al., J. Biol.Chem. 266:24773-24777 (1991); Funderburgh, et al., J. Biol. Chem. 262:11634-11640 (1987); Grover, et al., J. Biol. Chem., 270:21942-21949 (1995); Corpuz, et al., J. Bio. Chem., 271:9759-9763 (1996); and Funderburgh, et al., Biochem. Soc. Trans., 19:871-876 (1991). Its wide distribution implies that it has multiple functions in tissue morphogenesis and
20 maintenance of tissue homeostasis. This is best illustrated by the multiple clinical manifestations observed in Lumican-knockout ($lum^{-/-}$) mice that exhibit corneal opacity, skin and tendon fragility, delayed wound healing, and low fertility. Lumican has been shown to play essential roles in corneal transparency by regulating collagen fibrillogenesis in wound healing, by modulating epithelial cell migration, and in the
25 epithelium-mesenchyme transition of the injured lens. *See* Chakravarti, et al., J. Biol.Chem., 141: 1277-1286 (1998); Saika, et al., J. Biol. Chem., 275:2607-2612 (2000); and Saika, et al., Invest. Ophthalmol. Vis. Sci., 44:2094-2102 (2003).

The present invention provides an AM purified lumican, which facilitates proliferation and migration of corneal epithelial cells during wound healing.

30

Summary of the Invention

The present invention provides a method of preparing an extract from amniotic membrane that is effective in the prevention and reduction of scarring and inflammation.

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The present invention also provides an extract composition of amniotic membrane that is effective in the prevention and reduction of scarring and inflammation by suppression of TGF- β activity. The amniotic membrane extracts of

5 the present invention can be incorporated into a gel composed of such components as collagen and/or hyaluronic acid, allowing for enhanced use and application.

It is an object of the invention to provide a method for preparing a placenta to advantageously provide enhanced activity for amniotic membrane tissue (used, for example, for ocular grafts) or extracts prepared from amniotic membrane tissue.

10 Another object of the subject invention includes preparing soluble and fractionated amniotic membrane extract.

It is another object of the invention to provide compositions comprising amniotic membrane extract, such as solutions, drops, suspensions, gels, etc., for enhanced application and use.

15 A further object of the invention is to provide a method of reducing scarring and inflammation, and enhancing healing of wounds.

A further object of the invention is to provide a TGF-promoter assay for rapid and easy screening of TGF- β activity.

20 A further object of the invention is to provide AM purified lumican as well as methods of treating scarring and inflammation, and enhancing healing of wounds with AM purified lumican.

Brief Description of the Drawings

25 Fig. 1 illustrates the transcription of TGF- β 1, 2, 3, and RII in human corneal fibroblast and that it is suppressed by AM.

Fig. 2 illustrates the transcription of TGF- β 1, 2, 3, and RII in human limbal epithelium and that it is suppressed by AM.

Figs. 3A & 3B illustrates that TGF- β 1 and 3 promoter activity is suppressed by AM extract.

30 Fig. 4 illustrates that TGF- β 1 promoter activity is suppressed by collagen-AM extract gels.

Figs. 5A & 5B illustrate that TGF- β 1 promoter activity is suppressed by hyaluronan-AM extract gels.

Detailed Description of the Preferred Embodiments

35 One of the consequences of surgery, disease, or injury can be scarring and inflammation. Scarring can cause not only psychological problems, but also physical

5 problems. Scarring results from many contributing factors and among them is TGF- β . TGF- β is a very active growth factor that is involved in the organization of collagen leading to scarring. By preventing or reducing the expression of TGF- β , scarring and inflammation, and their effects, can be minimized. It has been shown through the present invention that soluble or fractionated extract of amniotic membrane reduces the
10 activity of TGF- β . Application of the extract to areas of potential scarring and inflammation suppresses the expression of the growth factor, thereby reducing its normal activity.

The extracts can also be combined with a delivery vehicle to create compositions such as solutions, drops, suspensions, or gels to deliver the anti-scarring
15 and anti-inflammatory actions. Delivery vehicles include such components as collagen, hyaluronic acid, or other vehicles being soluble as polymers or gels. The gel can then be applied to areas where scarring and inflammation may pose a problem; or when promotion of healing is desired. Collagen is a major structural protein found in the body. It provides support for tissues, connects tissue to bone, and provides the
20 structure of the body. When the body is in the healing process, collagen plays a role in helping to build a cellular structure. Hyaluronic acid is a natural sugar found in the synovial joint fluid, the vitreous humor of the eye, the cartilage, blood vessels, extra-cellular matrix, skin, and umbilical cord. As known in the art, both of these components, as well as others, have been used in such things as, implantable material,
25 making of gels, and medical devices.

In the present invention, soluble extracts were obtained from amniotic membrane and tested for their ability to reduce the effects of scarring and inflammation. The present invention shows the remarkable ability of the extracts to suppress TGF- β . When human corneal epithelium and fibroblast were cultured on
30 intact or denuded AM, expression of TGF- β in those cells was down-regulated compared with cells cultured on collagen gels or plastic.

The gel composition provides for enhanced use and application during surgery or surgical procedures. The applicable use of the invention is widespread and includes, but is not limited to all types of surgery, such as plastic, spinal cord, or caesarian
35 section; disease, such as cancer, congestive heart failure, and kidney disease; and conditions as a result of burns, acne, or other injuries. The present invention can be used by physicians to deliver anti-scarring and anti-inflammatory actions in

5 reconstructive or plastic surgeries. The present invention can also be applied topically on the body surface or tissue planes to achieve short-term and long-term therapeutic effects.

It has been unexpectedly discovered that the initial preparation of the placenta can provide enhanced activity to certain compositions extracted therefrom, and
10 particularly compositions extracted from the amnion prepared in accordance with the subject extraction methods. Specifically, subjecting a freshly obtained placenta to freezing can provide such advantageous result. After the placenta is procured from the delivery room, e.g., via caesarean section, the placenta is preferably stored and shipped to an amnion extraction site under frozen conditions (below 0 degrees Celsius), for
15 example in dry ice. Once received at the extraction site, the frozen placenta can be stored frozen, but now preferably at conditions of - 80 degree Celsius. Duration of frozen storage can vary from hours up to more than one year. Thawing of the stored, frozen placenta is preferably performed gradually by transferring from the frozen conditions to refrigerated conditions (4 to 8 degree Celsius) for one or two days before
20 moving it to the room temperature with or without addition of normal saline solution. After 1 to 8 hours following refrigeration, the amnion can be separated from the chorion for subsequent extraction procedures as described herein.

Placenta prepared by the procedures as described herein, e.g., immediate freezing for storage and shipping, and/or slow thawing prior to manipulation for tissue
25 preparation or extraction of particular biological components from the amniotic membrane can advantageously provide biological activity for the resultant tissue or extract that is enhanced as compared to tissue or membrane extract from placentas that have not been frozen. Amniotic tissue preparation useful for grafts is described, for example, in US Patent Nos. 6,152,142 and 6,326, 019.

30

The following examples illustrate the successful use of the soluble AM extracts. By measuring luciferase activity driven by the TGF- β promoter, the following examples demonstrate that: (1) intact AM suppresses the activity of TGF- β ; (2) soluble or fractionated AM extract suppresses the activity of TGF- β ; (3) collagen-AM extract
35 gels enhance the suppression; and (4) HA-AM extract coating enhances the suppression.

5 The present invention also provides soluble lumican purified from human AM for the treatment of scarring and inflammation. The present invention shows that lumican facilitates proliferation and migration of cells during wound healing. Further, application of purified lumican alone or combined with a delivery vehicle to create a composition such as solutions, drops, suspensions, or gels to deliver the anti-scarring and anti-inflammatory actions, can be applied to areas where scarring and inflammation may pose a problem; or when promotion of healing is desired. The purified lumican or composition containing lumican can be applied topically, as well as to other areas or parts of the body. The applicable use of the invention is widespread and includes, but is not limited to all types of surgery, such as plastic, spinal cord, or caesarian section; disease, such as cancer, congestive heart failure, and kidney disease; and conditions as a result of burns, acne, or other injuries.

 The references cited herein are hereby incorporated by reference in their entirety. The following examples are provided to illustrate the invention and are in no way limiting.

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Example 1: Demonstration of suppression of TGF- β and its receptor by AM.

 Total RNAs were extracted from keratocytes and RT-PCR was done with respective primers for human TGF- β 1, TGF- β 2, TGF- β 3, TGF- β RII, and GADPH. PCR products were electrophoresed on a 1.5% agarose gel. Gels were scanned and the intensity of each band was quantitated. The relative numbers were given. The results showed that AM down-regulated the expression of TGF- β 2, TGF- β 3, TGF- β RII in both human corneal keratocytes and human limbal epithelia. The expression of TGF- β 1 in human limbal epithelia was also down-regulated but was up-regulated in the human corneal keratocytes. Collectively, the data demonstrated the TGF- β signaling was diminished in both cell types by AM. See Fig. 1 and 2.

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Example 2: Placental storage and preparation

 After the placenta is procured from the delivery room via caesarean section, it is frozen below 0 degrees Celsius. Preferably, the placenta can be initially frozen by being placed in a container with dry ice, for storage at the collection site and/or shipping to a site for later extractions of the amniotic membrane. After this initial freezing, storage at the amniotic membrane extraction site, e.g., laboratory, is done at -

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5 80 degrees Celsius, such as a commercially available -80 degree C deep freezer. Such storage conditions are preferred only and can be at the any temperature below 0 degrees C. Duration of storage can vary and can be up to more than one year. It is further preferred that the frozen storage be continuous until thawing occurs just before amniotic membrane extraction.

10 The thawing of the placenta is performed by retrieving it from frozen storage, and transferring the placenta to an environment of about 4 to 8 degrees Celsius, e.g., conventional refrigeration. The placenta is stored under the refrigeration conditions for one or two days before moving it to the room temperature with or without addition of normal saline solution. After 1 to 8 hours of storage at room temperature, the amnion is
15 separated from the chorion as has been described.

Example 3: Preparation of soluble AM extract.

Human AM (provided by Biotissue, Inc., Miami, FL) was washed in HBSS (Invitrogen, Cat# 14175) to remove storage medium containing glycerol. After
20 measuring the surface area of the AM, the AM was placed into a sterile centrifuge tube and centrifuged at 4 °C for 5 minutes at 5000 xg. The fluid was removed and the AM was weighed. The AM was then transferred to one or more 100 mm sterile petri dishes. The dish(es) was sealed with paraform and it was placed into the air phase of a liquid nitrogen container for 20 minutes. The dish was taken out and the AM was cut into
25 small pieces. The freezing and cutting was repeated once. The small pieces of AM were transferred into a 50 ml sterile tube, and 5 ml Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, 21063)/20 g wet AM (without phenol red but with proteinase inhibitor and phosphatase inhibitors) was added to the tube. The tube was placed on ice and the AM was homogenized (Tissue Tearor from Biospec Products, Inc.) at speed
30 5 for 2 minutes. The procedure was repeated 4 times with 2 minute intervals to cool the AM. The AM homogenate was mixed at 4 °C for 1 hour and centrifuged at 4 °C at 12,000 xg for 15 minutes. The supernatant (soluble AM extract) was transferred to a sterile tube, mixed, aliquoted into 0.5 or 1.0 ml per tube, and stored at -80 °C for later use. The pellet was also saved at -80 °C. The frozen soluble extract can be lyophilized
35 for storage and subsequently reconstituted for use.

-14-

- 5 **Example 4:** Demonstration of AM suppression of the activity of TGF- β promoter in human corneal fibroblast (HCF) cultured in the stromal matrix of intact AM.

Infection control was done by infecting cells on plastic for 4 hours, then trypsinized, harvested, and seeded on plastic wells or on the stromal side of intact AM inserts. The cell density was $\sim 1 \times 10^5$ cells/6-well. The multiplicity of infection (MOI) equaled 30 (#1 of TGF- β 1 and #4 of TGF- β 3).

When HCF (P4), cultured on 2 x 100 mm plastic dishes in DMEM/10% fetal bovine serum (FBS), reached 80% confluency ($\sim 8 \times 10^5$ cells/dish), the medium was removed, and the cells were washed twice with DMEM/10% FBS (5 ml each). Two ml of the fresh DMEM/10% FBS was added to each dish and 30 μ l of adeno-TGF- β 1 promoter-luciferase (#1, MOI=37.5) or 30 μ l of adeno-TGF- β 3 promoter-luciferase (#4, MOI= $\sim$ 30) was mixed with the medium. After 1 hour incubation with adenoviruses, 8 ml of the fresh medium was added to each dish. The incubation continued for 2.5 hours, at this time the medium containing adenoviruses were removed. Cells were trypsinized for 5 minutes using 4 ml/100 mm dish prewarmed trypsin/EDTA.

20 Trypsin/EDTA activity was neutralized with 8 ml/100 mm dish DMEM/10% FBS. Cells were collected into a 15 ml tube and centrifuged at 1000 rpm ($\sim$ 600xg). The medium was carefully removed and cells resuspended into 15 ml DMEM/10% FBS ($\sim 5 \times 10^4$ /ml). Two ml of cell suspension was added to each 6-well or AM inserts. Cells were further cultured for 44 hours before being lysed for measuring luciferase activity.

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1. Adeno-TGF- β 1 (#1) promoter-luciferase

P $\sim 1.0 \times 10^5$ cells MOI=37.5	P $\sim 1.0 \times 10^5$ cells MOI=37.5	P $\sim 1.0 \times 10^5$ cells MOI=37.5
AM $\sim 1.0 \times 10^5$ cells MOI=37.5	AM $\sim 1.0 \times 10^5$ cells MOI=37.5	AM $\sim 1.0 \times 10^5$ cells MOI=37.5

1. Adeno-TGF- β 3 (#4) promoter-luciferase

P $\sim 1.0 \times 10^5$ cells MOI=37.5	P $\sim 1.0 \times 10^5$ cells MOI=37.5	P $\sim 1.0 \times 10^5$ cells MOI=37.5
AM $\sim 1.0 \times 10^5$ cells MOI=37.5	AM $\sim 1.0 \times 10^5$ cells MOI=37.5	AM $\sim 1.0 \times 10^5$ cells MOI=37.5

5 Results:

Table 1: A comparison of inhibition of TGF- β 1 and 3 promoter activity with AM versus plastic.

	Relative Luciferase Units (TGF- β 1 promoter)	Relative Luciferase Units (TGF- β 3 promoter)
Before cells seeding (infection for 4 hours)	0	0
Plastic	3754 ± 188	1362 ± 54
AM	304 ± 18	261 ± 14
AM/plastic (residual promoter activity)	8%	19%
P value	0.00031	0.00036

P values of both TGF- β 1 and 3 promoter activity on AM vs. on plastic are less than 0.05. Data presented as means $\pm$ SEM (at least 3 replicates). Statistical analysis was performed by the Student unpaired test. P values less than 0.05 were considered statistically significant. The results showed that TGF- β 1 or 3 promoter activity was significantly suppressed on AM, and that TGF- β 1 promoter activity was suppressed more than that of TGF- β 3 (8% vs. 19%) on AM.

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Example 5: Demonstration of suppression of TGF- β activity by soluble AM extract.

Fig. 3A and 3B show the TGF- β 1, 3 promoter activity with respect to soluble AM extracts versus plastic. The TGF- β 1 activity was significantly reduced with the soluble AM extract when compared to plastic. The TGF- β 3 activity was also reduced with the soluble AM extract when compared to plastic. The TGF- β 1 activity with application of soluble AM was 19.2% of the activity on plastic ($P=0.0005$ (TGF- β 1 AM vs. P)). The TGF- β 3 activity with application of soluble AM was 31% of the activity on plastic ($P=0.0007$ (TGF- β 3 AM vs. P)). Thus, the results illustrate the suppression of TGF- β activity by AM extract.

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TGF- β 1 or 3 promoter activity was significantly suppressed by the soluble AM extract, and the major TGF- β inhibition activity is in the soluble fraction.

5 **Example 6:** Demonstration of suppression enhancement with collagen gels.

HCF in collagen or in collagen-AM extract gels were created as test samples. The positive control was HCF on plastic and the negative control was HCF on plastic with AM extract. The cells used were human corneal fibroblast (HCF, Passage 4). The adenoviruses used were TGF- β 1-luciferase, MOI=30, which the cells were infected
 10 with for 4 hours, then trypsinized, harvested, and seeded at 1.5×10^4 /each for the 24 wells.

P + DMEM	P+AM/DMEM 300 μ g/ml	Collagen +DMEM	Collagen+AM/DMEM 300 μ g/ml
P + DMEM	P+AM/DMEM 300 μ g/ml	Collagen +DMEM	Collagen+AM/DMEM 300 μ g/ml
P + DMEM	P+AM/DMEM 300 μ g/ml	Collagen +DMEM	Collagen+AM/DMEM 300 μ g/ml

Soluble AM extract was utilized (extracted in DMEM, 2.34 mg/ml) in this
 15 example. HCF (P4) was cultured on a 1 x 100 mm plastic dish in DMEM/10% FBS to 80% confluency ($\sim 8 \times 10^5$ cells). HCF was infected with adenoviruses-TGF- β 1-luciferase (MOI=30) for 4 hours.

The medium was removed, and the cells were washed twice with DMEM/10% FBS (5 ml each). Ten ml of the fresh DMEM/10% FBS with 8×10^7 adenoviruses-TGF- β 1 promoter-luciferase (#1, assuming 1×10^9 , add 24 μ l, MOI=30) was added to
 20 the dish. The incubation continued for 4 hours, followed by the removal of the medium containing adenoviruses. Cells were trypsinized for 5 minutes using a 3 ml/100 mm dish with pre-warmed trypsin/EDTA. Trypsin/EDTA activity was neutralized with 9 ml/100 mm dish DMEM/10% FBS. Cells were collected into a 15 ml tube and
 25 centrifuged at 1000 rpm ($\sim 600 \times g$) for 5 minutes. The medium was carefully removed and cells resuspended into 0.2 ml DMEM/10% FBS ($\sim 4.0 \times 10^6$ /ml). 30 μ l of cell suspension was added to 3.462 ml DMEM/10%. 436 μ l was transferred to each of the 24 wells. 46 μ l of either DMEM (P) or AM extract (2.34mg/ml) (AM) was added to the above wells. For the collagen gel matrix, 15 μ l of cell suspension was mixed with
 30 the collagen solution (CL) or AM extract (CL-AM) (see below: preparation of collagen

5 gel) and 200 μ l was added to each of the 24 wells. After gelation, (~1 hour at 37 °C) 500 μ l DMEM/10% FBS was added to each well. Cells were further cultured for 44 hours and harvested either by directly adding 50 μ l 1 x lysis buffer (P and AM) or digested with collagenase at 37 °C for 1 hour, collected by centrifugation, and lyzed into 50 μ l 1 x lysis buffer.

10 Type I collagen solution was made by the following protocol. (Rat tail tendon, BD Biosciences, 354236). Rat tail collagen, sterile 20X DMEM (20X DMEM), sterile dH₂O, and sterile 1N NaOH, were placed on ice. A final volume of 200 μ l of collagen solution and the desired final collagen concentration of 2.4 mg/ml were determined. A sterile tube, of sufficient capacity to contain the final volume of collagen, was placed
15 on ice.

The following techniques were used using aseptic techniques in a Class 100 Hood. First, 40 μ l of 20X DMEM (to make 4 x 20 μ l, collagen gels) was added. Next, the volume of collagen was calculated to be 480 μ l 1 stock collagen (1.92 mg/ 4 mg/ml) and set aside to be added later. Next, 11 μ l of sterile ice cold 1N NaOH was added
20 followed by 13 μ l of sterile ice cold H₂O. The contents of the tube were mixed and held on ice. The calculated volume of collagen (480 μ l) was added and mixed. Next, 256 μ l /6.0 x 10⁴ cell was added and mixed. 250 μ l of the mixture was added to each of the 24 wells. The mixture was allowed to gel at 37 °C for 60 minutes. Next, 500 μ l DMEM/10% FBS was added to the top of the collagen gel and allowed to culture for 44
25 hours. The cells were then lyzed by adding 50 μ l buffer or digested with 500 μ l of 1 mg/ml collagenase A/DMEM for each collagen gel at 37 °C for 60 minutes. The cells were collected by centrifugation for 5 minutes at 5000 rpm at 4 °C. The cells were then washed once with PBS and lyzed by adding 50 μ l lysis buffer. Finally, the luciferase was assayed.

30 Preparation of Cell Lysates for Luciferase Assay:

Four volumes of distilled water were added to 1 volume of 5x lysis buffer. The 1 x lysis buffer was equilibrated to room temperature before use.

Table 2

Tissue culture dish	1 x PBS wash (ml)	Lysis buffer (μ l)
100 mm	10	900
60 mm	5	400

35 mm (6-well)	2	200
24-well	0,5	50

5

The growth medium from the cells to be assayed was carefully removed. The cells were rinsed with PBS (Mg^{2+} - and Ca^{2+} -free, see table 2 for volume added), being careful not to dislodge attached cells and the PBS rinse was removed. Enough 1 x lysis buffer (cell culture lysis reagent, see table 2 for volume added) was added to cover the cells. The culture dishes were rocked several times to ensure complete coverage of the cells with lysis buffer. The attached cells were then scraped from the dish and transferred (with all of the liquid) to a 1.5 ml microcentrifuge tube and placed on ice.

Luciferase Assay:

The luciferase assay reagent was prepared by adding luciferase assay buffer to the vial containing the lyophilized luciferase assay substrate, avoiding exposure of the luciferase assay reagent to multiple freeze-thaw cycles by dispensing the reconstituted reagent into working aliquots. Any unused luciferase assay reagent was stored at $-70^{\circ}C$. The luciferase assay reagent was equilibrated to room temperature before each use. Each reaction required 100 μ l of the luciferase assay reagent to initiate enzyme activity. 1 x cell culture was prepared by diluting 1 volume of 5 x lysis buffer with 4 volumes of distilled water. One mg/ml of acetylated BSA was added to the 1 x lysis buffer if used for diluting purified luciferase. The 1 x lysis buffer was equilibrated to room temperature before each use. 100 μ l of the luciferase assay reagent was dispensed into one microtiter well. The Analyzer was programmed to perform a 10-second measurement read for luciferase activity. 20 μ l of the cell lysate was added to the luciferase assay reagent and mixed by pipeting 3 times. The plate was then placed into the Analyzer and the reading initiated.

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Results:

Matrix	RLUs (TGF- β 1)	% residual TGF- β 1 activity	P value
PL	3438 $\pm$ 280.4	100	
PL+ AM extract	1508 $\pm$ 3.6	44	0.007(PL vs. PL+AM)

-19-

Collagen	1785±113.1	100(52)	
Collagen + AM extract	1270±115.2	71(37)	0.005(collagen vs. collagen+AM)

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The soluble AM extract (the control) consistently showed significant inhibition of TGF- β 1 promoter activity ($P=0.007$). The soluble AM extract in collagen gels also significantly suppressed TGF- β promoter activity (from 100% to 71%, $p=0.005$). Collagen also showed suppressed TGF- β promoter activity compared with plastic (from 100% to 52%, $p=0.004$), but this suppression could be due to the difference of harvested cells, that is, more cells were harvested from plastic dishes than from collagen gel dishes.

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Example 7: Demonstration of enhanced suppression of TGF- β promoter activity with HA-AM extract coating.

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HCF test samples on HA-AM extract gel were created. The positive controls were HCF on plastic and the negative controls were HCF on plastic with AM extract. The cells used were human corneal fibroblast (HCF, Passage 4). The adenoviruses used were TGF- β 1-luciferase (MOI=30). The cells were infected for 4 hours, then trypsinized, harvested, and seeded at 1.5×10^4 /each for the 24 wells.

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P DMEM	P BSA/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml ON	HA+BSA/DMEM Coated 300 μ g/ml O/N	HA+AM/DMEM Coated 300 μ g/ml ON
P DMEM	P BSA/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml ON	HA+BSA/DMEM Coated 300 μ g/ml O/N	HA+AM/DMEM Coated 300 μ g/ml ON
P DMEM	P BSA/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml	P AM/DMEM 300 μ g/ml ON	HA+BSA/DMEM Coated 300 μ g/ml O/N	HA+AM/DMEM Coated 300 μ g/ml ON

- 5 This example utilizes soluble AM extract (extracted in DMEM, 2.34 mg/ml). HCF (P4) was cultured on a 1 x 100 mm plastic dish in DMEM/10% FBS to 80% confluency ($\sim 8 \times 10^5$ cells). A 24-well dish was coated with HA or HA-AM extract mixture.

10 **Preparation of cells in HA-BSA (control) gels and HA-AM extract gels**

Components	HA-AM mixture	X3.2	HA-BSA mixture	X 3.2
HA (Healon) 10 mg/ml	300 μ g/30 μ l	96 μ l	300 ug/30 μ l	96 μ l
BSA 10 mg/ml	---	-	300 ug/30 μ l	96 μ l
AM extract 2.34 mg/ml in DMEM	150 μ g/64 μ l	204.8 μ l	--	
DMEM #11965 (Invitr.	55.1 μ l	176.3 μ l	89.1 μ l	285.1 μ l
Calcium (100 mM)	0.9 μ l (final conc. to 2.0 mM)	2.9 μ l	0.9 μ l (final conc. to 2.0 mM)	2.9 μ l
Total volume	150 μ l	480 μ l	150 μ l	480 μ l

Note: calcium conc. in DMEM (cat# 11965, invitrogen) is 1.8 mM (200 mg CaCl_2/L)

- Aliquots of 150 μ l of the above mixture were added to each of the wells of the 24-well dish and incubated at 37 °C for 24 hours. After that, the remaining fluid was removed and 500 μ l of cell resuspension (1.5×10^4) was added to each well. HCF was infected with adenovirus-TGF- β 1-luciferase (MOI=30) and adenovirus CMV- β -gal for 4 hours.

- The medium was removed, and the cells were washed twice with DMEM/10% FBS (5 ml each). Ten ml of the fresh DMEM/10% FBS with 2.4×10^7 adenoviruses-TGF- β 1 promoter-luciferase (#1, assuming $1 \times 10^9/\text{ml}$, add 24 μ l, MOI=30) and 8.0×10^5 Adeno-CMV- β -gal ($1 \times 10^{12}/\text{ml}$) were added to the dish. The incubation continued for 4 hours, followed by the removal of the medium containing adenoviruses. Cells were trypsinized for 5 minutes using 3 ml/100 mm dish prewarmed trypsin/EDTA. Trypsin/EDTA activity was neutralized with 9 ml/100 mm dish DMEM/10% FBS. Cells were collected into 15 ml tubes and centrifuged at 1000 rpm ($\sim 600\text{xg}$) for 5 minutes. The medium was carefully removed and cells were resuspended into 2 ml DMEM/10% FBS ($\sim 4.0 \times 10^5/\text{ml}$). Cell resuspension was further diluted to $3.0 \times 10^4/\text{ml}$ with DMEM/10% FBS or plus 300 μ g/ml AM extract or BSA.

5 **Preparation of cells in culture medium with DMEM, BSA (control) and AM extract**

	DMEM	AM extract	BSA
Cell resuspension (-4.0×10^5 /ml)	0.60 ml	0.131 ml	0.131 ml
DMEM/ 10% FBS	6.376 ml	1.395 ml	1.357 ml
DMEM	1.024 ml		0.210 ml
AM extract 2.34 m /ml	---	0.224 ml	
BSA 10 mg/ml	---		0.053 ml
Total volume	8 ml	1.75 ml	1.75 ml

500 μ l was transferred to each of the 24 wells. Cells were further cultured for 44 hours and harvested. The cell lysate was used to measure luciferase activity.

10 **Results:**

Matrix	RLUs (TGF- β 1)	% residual TGF- β 1 activity	P value
PL	662 $\pm$ 32.7	88.6	0.31
PL+ BSA	747.3 $\pm$ 109.7	100	
PL + AM Ext	378.3 $\pm$ 62.4	50.6	0.013 (vs BSA)
HA-BSA coat	823 $\pm$ 34.6	100	
AM Ext coat	405.7 $\pm$ 26.6	49.3	0.00014
HA-AM Ext coat	326.3 $\pm$ 43.1	39.7	0.00012

The addition of BSA (300 μ g/ml) to the culture medium did not decrease, but rather, increased non-significantly ($p=0.31$) TGF- β 1 promoter activity. The addition of AM extract (300 μ g/ml) to the culture medium for 44 hours did significantly ($P=0.013$) down-regulate TGF- β 1 promoter activity. It was determined that the HA-BSA coat up-regulated TGF- β 1 promoter activity significantly ($P=0.004$) and the AM extract coat alone also down-regulated TGF- β 1 promoter activity, but HA-AM extract coating was even more inhibited (61% vs. 51%). See Fig. 5A & 5B.

20 **Example 8:** Therapeutic application of amniotic membrane extract and/or compositions containing amniotic membrane extract.

5 In eyes with persistent epithelial defect and ulceration, after retrobulbar anesthetic injection, the base of the ulcer is debrided with surgical sponges and forceps, and the poorly adherent epithelium adjacent to the edge of the ulcer is removed up to the area where the epithelium becomes more adherent. The amniotic membrane extract alone or in a gel type composition is applied to the recipient eye. The extract and/or
10 composition should fill the ulcer bed. The treated area then should be properly bandaged to protect the area and allow for the extract and/or composition to take affect. Repeat the application of the extract and/or composition as needed.

 The amniotic membrane extract and compositions of the present invention can also be used for application to areas of the body that have sustained burns. The extract
15 and/or composition is applied to the burn area at an appropriate stage for healing. Following application, the area should be bandaged to protect the treated area and allow for the extract and/or composition to take effect. Repeat the application as needed.

Example 9: Purification of Lumican from Human AM.

20 A kit (IgG Plus Orientation kit; Pierce) was used to prepare an affinity column for purifying native lumican from human AM. The anti-lumican antibody was bound to immobilized recombinant protein A through the Fc region. Then, the homobifunctional NHS-ester crosslinker, disuccinimidyl suberate (DDS), was used to form the covalent bond between the antibody and protein A. A piece of human AM
25 (5.75 g wet weight, ~100 cm²) was subjected to homogenization with 50 ml lysis-250. The soluble fraction was then dialyzed against PBS at 4°C for 16 hours, yielding 128.62 mg AM extract. Approximately 10 mg of the AM extract was loaded on the anti-lumican antibody column and incubated overnight at 4°C. Unbound AM proteins were washed from the affinity column by PBS until the eluant reached a baseline
30 absorbance at OD_{280nm}. The bound lumican was dissociated from the column by an elution buffer (0.1 M glycine HCl, pH 3.0) and neutralized with 1 M Tris-HCl (pH 9.5). After the absorbance was assayed at OD_{280nm}, fractions, each of 100 µl volume, were pooled and concentrated. In the affinity-column chromatograph, 10 mg of lysis-250 AM-soluble extract that passed through the column constantly yielded 100 µg lumican
35 (1.08% ± 0.03%, n = 11). Therefore, approximately 0.2 mg soluble lumican per human AM wet weight (g) was obtained.

5 Preparation of an Epitope-Specific, Polyclonal, Anti-Lumican Antibody:

Rabbit anti-lumican peptide antiserum was obtained from EvoQuest Custom Antibody Services (Invitrogen Corp., Carlsbad, CA). An oligopeptide with the sequence of SLPPDMYECLRVANE (SEQ ID NO:1), corresponding to the C-terminal amino acid residues deduced from lumican cDNA (GenBank U18728; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) was synthesized and coupled to a maleimide-activated carrier protein, KLH. The KLH-conjugated peptide was then used to raise antiserum in rabbit. To purify the anti-lumican antibody further, the SLPPDMYECLRVANE (SEQ ID NO:1) oligopeptide was immobilized by covalent reaction with iodoacetyl groups on a gel (Sulfolink; Pierce, Rockford, IL). The rabbit antiserum was loaded onto a peptide-conjugated gel column, according to the manufacturer's instruction. Fractions containing purified anti-lumican antibody were pooled and concentrated. The protein concentration was measured by spectrophotometry at OD_{280nm}. The specificity of the anti-lumican antibody was evaluated by Western blot analysis.

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Affinity Purification of Human AM Lumican:

Human lumican was eluted as a single peak, measured by spectrophotometry at OD_{280nm}. The purified lumican appeared as a single band in an SDS-polyacrylamide gel followed by silver staining. Two-dimensional SDS-PAGE revealed that purified AM lumican was moderately glycosylated, as evidenced by a band smearing between pH 3.0 and 6.0 and by its conversion to a single spot at pH 6.0 (molecular mass of 50 kDa), after preincubation with endo- β -galactosidase to remove the sugar moiety.

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Example 10: Mouse Model of Corneal Wound Healing

C57BL/6 mice, age 6 to 8 weeks, obtained from Jackson Laboratories (Bar Harbor, ME), were anesthetized by intraperitoneal injections of ketamine hydrochloride (2 mg/g body weight) and xylazine (0.4 mg/g body weight). After topical application of 1 drop of proparacaine (Alcaine; Alcon Laboratories, Inc., Fort Worth, TX) to each eye, the central cornea was marked by a trephine 2 mm in diameter, and the epithelium was debrided by a corneal rust ring remover with a 0.5-mm burr (Algerbrush IITM; Alger Equipment Co., Inc., Lago Vista, TX) under a stereomicroscope (SV11; Carl Zeiss Meditec, Dublin, CA). The animal was killed, and the eyeballs were enucleated

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5 and cultured in DMEM containing 1% FBS and 50 µg/ml gentamicin, with or without
10 µg/ml purified lumican, in a humidified atmosphere of 5% CO₂ at 37°C. To label
the proliferating cells, 40 µg/ml of BrdU (Sigma-Aldrich) was added to the cultured
eyeballs for 1 hour before 6, 12, 18, and 24 hours of cultivation, respectively. After
incubation, the extent of corneal wound closure was examined by fluorescein staining
10 and photographed with a digital camera (Axiovision4; Carl Zeiss Meditec). The
circumference of the wound margin of each mouse eye, as projected onto the
photograph, was traced on a digitizer, and the remaining defect area was determined by
image-analysis software (Image Beta 4.02; Scion Corp., Frederick, MD). All
measurements were counted in a masked fashion, and the size of epithelial defect was
15 expressed as a percentage of the total corneal area. The eyeballs were then fixed in 4%
paraformaldehyde in PBS and embedded in paraffin.

**Effect of Exogenous Human AM Lumican on Corneal Epithelial Wound Healing
in Wild-Type and *lum*^{-/-} Eyes:**

Exogenous purified human AM lumican was administered in cultured wild-type
20 mouse eyes to determine if lumican facilitates corneal re-epithelialization. One
milligram per milliliter and 10 µg/ml purified AM lumican were used. The dosage of
AM lumican of 10 µg/ml showed a better effect than 1 µg/ml. Exogenous human AM
lumican (10 µg/ml) accelerated healing of the epithelium when compared with the
control eyes receiving the vehicle (Fig. 7). Twelve and 24 hours after corneal epithelial
25 lesions, the lumican-treated eyes showed a reduction of the wounded area: 30% ± 6%
and 9% ± 8% of the originally debrided area, respectively, whereas the control eyes still
had larger wounded area (45% ± 17% and 20% ± 11%, respectively). Forty-eight
hours after debridement, both lumican-treated and control eyes were completely healed.
Furthermore, the effect of exogenous lumican was even more dramatic in the *lum*^{-/-} eye
30 (Fig. 8). Twenty-four hours after the corneal epithelial lesion, lumican-treated eyes
showed a 3.4 –fold reduction in the wound area compared with the control eyes (17% ±
11% vs. 61% ± 19%). The statistical difference was confirmed by Student's *t*-test
(*P*<0.05).

35 **Effect of Exogenous Lumican on Cell Proliferation during Corneal Epithelial
Wound Healing:**

5 To monitor the cell proliferation index, proliferating cells were labeled with the thymidine analogue BrdU at different times during wound healing. Figure 9 shows the temporal spatial patterns of cell proliferation during wound healing. Six hours after epithelial debridement, very few BrdU-labeled epithelial cells were detected in either the control (Figs. 9A1-9A3) or the lumican-treated group (Figs. 9B1-9B3). At 12 hours
10 after debridement, the number of BrdU-labeled cells was significantly more at central and peripheral zones in the lumican-treated group (3.5 ± 2.9 and 5.5 ± 2.1 cells, respectively, $n = 6$) compared with the control group (0.4 ± 0.9 and 0.8 ± 1.1 cells, respectively, $n = 5$ $P < 0.05$; Figs. 9A4-9A6, 9B4-9B6, 9C). There was no difference between both groups in the midperipheral zone (Figs. 9A5, 9B5, 9C). There were
15 many more BrdU-labeled cells in the central area of the lumican-treated group at 18 hours after debridement than in the control group, which had very few BrdU-labeled cells (18.9 ± 5.6 vs. 0.3 ± 0.8 cells, $n = 7$ $P < 0.05$; Figs. 9A9, 9B9, 9C). Twenty-four hours after debridement, many BrdU-labeled cells were still present in the central (16.4 ± 2.0 cells, $n = 5$) and midperipheral zones (10.2 ± 5.4 cells, $n = 5$) of the lumican-
20 treated groups (Figs. 9B11-9B12, 9C). However, fewer BrdU-labeled cells were found in these two zones of the control group: 2.3 ± 2.0 cells in the central ($n = 7$) and 2 ± 1.6 cells in the midperipheral zones ($n = 7$) (Figs. 9A11-9A12, 9C). It was obvious that lumican-treated eyes contained more BrdU-labeled cells than did the control eyes at 12, 18, and 24 hours after corneal debridement. A similar stimulation of cell proliferation
25 by lumican was also seen when lumican was added to the *lum*^{-/-} eyes (Fig. 10A). Twenty-four hours after debridement, many more BrdU-labeled cells were found in all three zones of the lumican-treated *lum*^{-/-} eyes than in those zones in the control eyes: 12.6 ± 2.8 , central; 14.6 ± 2.6 , midperipheral; and 15.8 ± 1.92 , peripheral versus 2.8 ± 0.84 , central; 5.2 ± 0.8 , midperipheral; and 8.2 ± 3.0 , peripheral ($P < 0.05$; $n = 21$; Fig.
30 10B).

5

Claims

1. An amniotic membrane extract prepared by the process comprising:
 - a. obtaining amniotic membrane;
 - b. freezing said amniotic membrane; and
 - 10 c. homogenizing said frozen amniotic membrane to provide an amniotic membrane homogenate comprising soluble amniotic membrane extract and insoluble amniotic membrane extract.
2. The amniotic membrane extract of claim 1 wherein said process further
15 comprises centrifuging said amniotic membrane homogenate to separate soluble amniotic membrane extract in liquid phase and insoluble amniotic membrane extract in solid pellet phase.
3. The amniotic membrane extract of claim 1 wherein said process further
20 comprises separately collecting said soluble amniotic membrane extract and said insoluble membrane extract.
4. The amniotic membrane extract of claim 1 wherein said preparation further
25 comprises repeating said freezing step and said homogenization step.
5. The amniotic membrane of claim 1 wherein said membrane is obtained from frozen placenta.
6. The amniotic membrane of claim 5 wherein said frozen placenta is thawed to
30 separate amnion from chorion.
7. The amniotic membrane of claim 5 wherein said frozen placenta is thawed under refrigeration conditions, then under room temperature conditions.
- 35 8. The amniotic membrane of claim 1, wherein said extract is frozen and subjected to lyophilization.

- 5 9. A method of preparing amniotic membrane extract, said method comprising:
- a. obtaining amniotic membrane;
 - b. freezing said amniotic membrane;
 - c. homogenizing said frozen amniotic membrane to provide an
- 10 amniotic membrane homogenate comprising ;
- soluble amniotic membrane extract and insoluble amniotic membrane
- extract.
10. The method of claim 9 wherein said method further comprises
- centrifuging said amniotic membrane homogenate to separate soluble amniotic
- 15 membrane extract in liquid phase from insoluble amniotic membrane extract in
- solid pellet phase.
11. The method of claim 9 said method further comprising
- separately collecting said soluble amniotic membrane extract and said insoluble
- 20 membrane extract.
12. The method of claim 9 wherein said membrane is obtained from frozen
- placenta.
- 25 13. The method of claim 9 wherein said frozen placenta is thawed to separate
- amnion from chorion.
14. The method of claim 9 wherein said frozen placenta is thawed under
- refrigeration conditions, then under room temperature conditions.
- 30
15. A composition for the prevention or reduction of scarring or inflammation in an
- animal, said composition comprising soluble amniotic membrane extract prepared by
- the method of claim 9.
- 35 16. The composition of claim 15 wherein said composition further comprises a
- delivery vehicle.

5 17. The composition of claim 16 wherein said delivery vehicle is selected from the group consisting of collagen, hyaluronic acid, soluble polymer, and soluble gel.

18. A method for preventing or reducing scarring or inflammation in an animal, said method comprising

10 applying to the animal at an area susceptible to scarring or inflammation a soluble amniotic membrane extract prepared by the method of claim 9.

19. A method for modulating TGF- β activity in an animal, said method comprising
15 applying to the animal a soluble amniotic membrane extract prepared by the method of claim 9.

20. The method of claim 19 wherein said TGF- β modulation is TGF- β suppression.

21. The method of claim 19 wherein TGF- β suppression is enhanced by a
20 composition comprising the soluble amniotic membrane extract and a vehicle selected from the group consisting of collagen and hyaluronic acid.

22. The method of claim 9, wherein said extract is frozen and subjected to lyophilization.

RT-PCR (human corneal fibroblast)

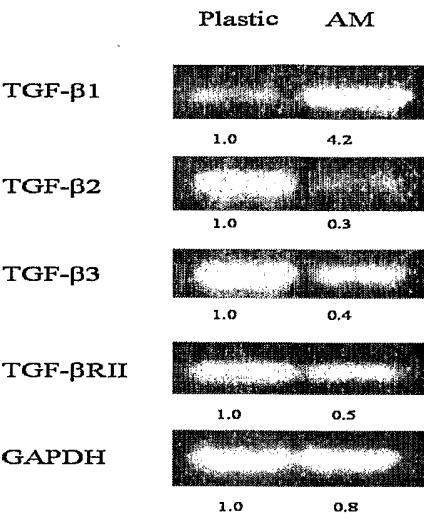


FIG. 1

RT-PCR of TGF-beta 1,2 3, and RII
(Human corneal epithelium)

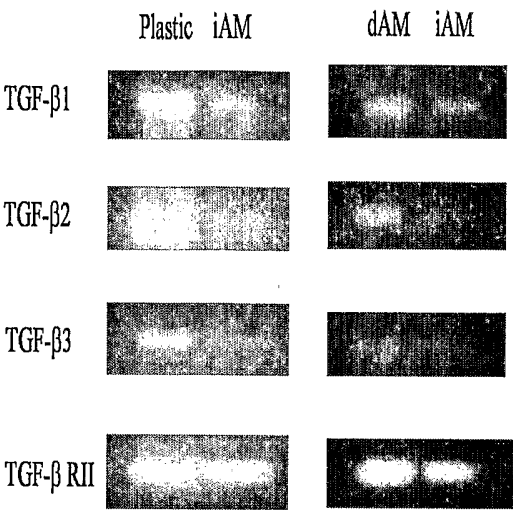
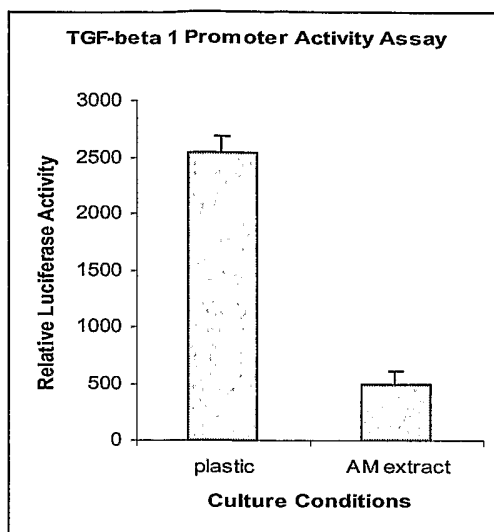
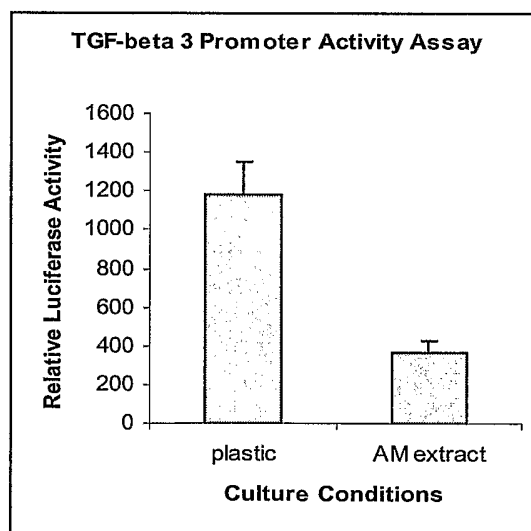
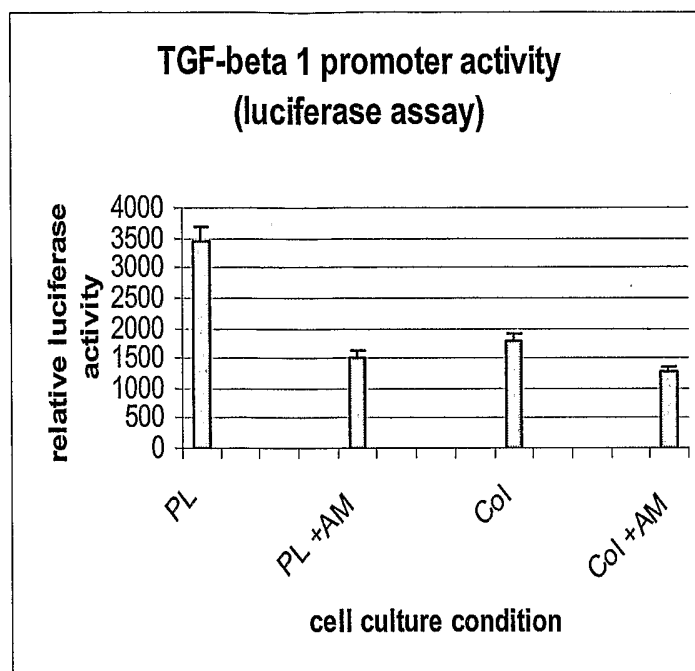
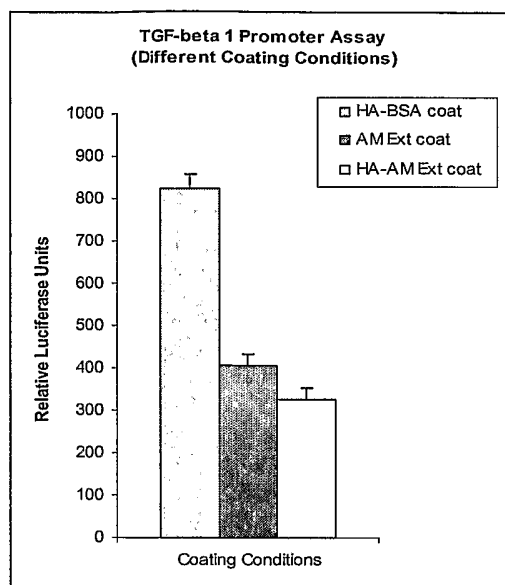
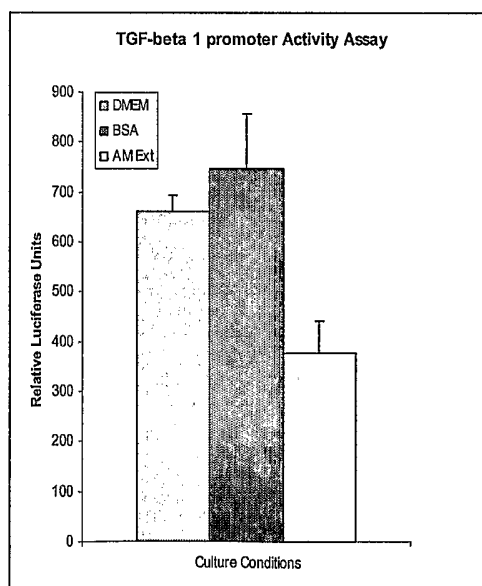


FIG. 2

**FIG. 3A****FIG. 3B**

**FIG. 4**

**FIG. 5A****FIG. 5B**