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<th>ULTRASOUND MICROBUBBLE MEDIATED GENES DELIVERY SYSTEM</th>
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Title: ULTRASOUND MICROBUBBLE MEDIATED GENES DELIVERY SYSTEM

Abstract:
ULTRASOUND MICROBUBBLE MEDIATED GENES DELIVERY SYSTEM

FIELD OF THE INVENTION

The current invention relates to a method for peritoneal diseases using a local ultrasound-mediated genes/chemicals-bearing gas-filled microbubble system.

Several publications are referenced herein by Arabic numerals within parenthesis. Full citations for these references may be found at the end of the specification immediately preceding the claims. The references cited herein, including the patents and published patent applications, are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Although viral-based vectors have been shown to deliver therapeutic DNA effectively into various tissues including cardiovascular system, lung, kidney, and cancers, major concerns remain over the safety using adenovirus and retrovirus, which may elicit immune responses and have the potential for insertional mutagenesis [1, 2]. To overcome these disadvantages, several non-viral approaches have been reported, including lipid-based vector systems and electroporation. Recently, we and other investigators have reported that ultrasound-microbubbles largely increase gene transfection rate in the kidney, cardiovascular tissues, and cancers [3-12]. However, there is no report the use of ultrasound-microbubble-mediated gene/drug treatment for peritoneal diseases including peritoneal fibrosis, postoperative peritoneal adhesion, peritoneal inflammation and cancers.
Ultrasound itself is believed to be harmless to the body and is widely used clinically for many purposes including physical therapy, diagnosis, guidance for deep organ biopsy, and local drug and genetic material delivery, as described in U.S Patent No. 5,190,766. Most microbubble contrast agents are also safe agents and widely used clinically, as described in U.S. Patents, including gas-filled lipids (U.S. Pat No. 5,580,575), and albumin microbubbles such as Optison [13-16]. Most microbubbles are liquid at room temperature, but they become a gas-filled microbubble with an average of 3 µm in diameter at body temperature. Microbubbles are elastic, compressible, and efficient reflectors of ultrasound.

Microbubbles work by resonating in an ultrasound beam, rapidly contracting and expanding in response to the pressure changes of sound waves. Microbubbles can aid drug delivery by themselves, and as agents to carry drugs or genetic materials for site-specific treatment and gene therapy [13-16].

The precise mechanism of gene transfer with ultrasound-microbubble technique remains largely unknown. It may be associated with the effects of sonoporation, the mechanical index and frequency of ultrasound [15, 16]. The principle of the ultrasound-based strategy is that the use of ultrasound contrast agents lowers the threshold for cavitation by ultrasound energy. Using physical properties of microbubbles and coating materials, genes can be incorporated into ultrasound contrast agents [13, 14].

Gene-bearing microbubbles can be injected intravenously or locally and ultrasound energy applied to the target region. As the microbubbles enter the region of insonation, they cavitate and release DNA locally [13, 14]. Cavitation also likely causes a local shockwave that increases cell permeability and thus improves cellular uptake of DNA [13-16]. Scanning electron microscopy also demonstrates that
ultrasound with microbubble (Optison) causes a transient formation of holes (< 5 μm) in the cell surface, which become undetectable within 24 hours [17].

Peritoneal inflammation/fibrosis is a common disease complication in patients who have end stage renal disease, and who are undergoing maintenance peritoneal dialysis (PD), a most convenient and inexpensive renal replacement therapy. It is a major cause of technical failure of PD, resulting in those PD patients changing to more expensive hemodialysis. In addition, peritoneal adhesions is also a fibrotic process that occurs in a significant proportion of patients undergoing abdominal surgery and contribute to various complications including bowel obstruction, female infertility and chronic abdominal pain, leading to a high morbidity and mortality as well as a high cost of health care. We have shown that the peritoneal fibrosis is mediated by a fibrogenic mediator called transforming growth factor-beta (TGF-β) via its downstream signaling pathway, activation of Smad2/3. The present invention provides ultrasound-triggered gene-bearing microbubbles to locally release an anti-fibrosis and inflammation gene called Smad7 to specifically inhibit the TGF-β/Smad signaling pathway, thereby inhibiting peritoneal fibrosis, as well as peritoneal inflammation, associated with long term peritoneal fibrosis under various disease conditions.

**SUMMARY OF THE INVENTION**

The invention provides a method for delivering one or more genes, DNA molecules or plasmids to a patient’s peritoneal region for treatment of peritoneal disease therein, comprising providing a source of microbubbles containing one or more genes, DNA molecules, or plasmids for treatment of peritoneal disease; perfusing the peritoneal region of the patient with the microbubbles; providing
ultrasonic energy to the abdominal region sufficient to cause transfection of the one or more genes, DNA molecules or plasmids from the microbubbles into the peritoneal region to penetrate peritoneal tissue found therein.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Further features and advantages of the invention will become apparent upon review of the following detailed description of the preferred embodiments, taken together with the drawings in which:

**Figure 1** shows photomicrographs and functional data showing the safety of ultrasound microbubble treatment on peritoneal tissues in accordance with the present invention.

**Figure 2** shows the efficiency of ultrasound microbubble mediated Smad7 transgene expression in peritoneal tissues as demonstrated by anti-flag-m2Smad7 immunostaining and RT-PCR (B), where U denotes uremia, PD denotes peritoneal dialysis, and CV denotes control vector.

**Figure 3** demonstrates the mechanism by which ultrasound microbubble mediated Smad7 gene therapy blocks activation, but not expression, of peritoneal TGF-Smad signaling (p-Smad2/3) by Western blot and RT-PCR.

**Figure 4** reveals the therapeutic efficacy of ultrasound-microbubble-mediated Smad7 gene therapy on improving peritoneal functions during the peritoneal dialysis.
**Figure 5** shows photomicrographs of histology and immunohistochemistry, showing that ultrasound-microbubble-mediated Smad7 gene therapy inhibits peritoneal fibrosis associated with peritoneal dialysis in uremia rats by Mason Trichrome (Blue) staining and collagen I immunostaining (Brown).

**Figure 6** is semi-quantitative data showing that ultrasound microbubble mediated Smad7 gene therapy blocks peritoneal fibrosis as determined by inhibiting collagen I and III mRNA and protein expression during peritoneal dialysis.

**Figure 7** is semi-quantitative data showing that ultrasound-microbubble-mediated Smad7 gene therapy inhibits peritoneal fibrosis by blocking α-SMA + myofibroblast transition and fibronectin expression during peritoneal dialysis.

**Figure 8** shows photomicrographs of histology and immunohistochemistry, after ultrasound-microbubble-mediated Smad7 gene therapy. The therapy inhibits peritoneal fibrosis associated with postoperative peritoneal adhesion demonstrated by Mason Trichrome (Blue) staining and collagen I immunostaining (Brown).

**Figure 9** is quantitative real-time PCR data showing that ultrasound-microbubble-mediated Smad7 gene therapy inhibits peritoneal fibrosis associated with postoperative adhesion by blocking collagen I, III, α-SMA, and fibronectin gene expression.
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides a method for delivering one or more genes, DNA molecules or plasmids to a patient's peritoneal region for treatment of peritoneal disease in the patient, comprising providing a source of microbubbles containing the one or more genes, DNA molecules, or plasmids useful for treating peritoneal disease; perfusing the microbubbles into the peritoneal region of the patient; and administering ultrasonic energy to the peritoneal region sufficient to cause disruption of the one or more genes, DNA molecules or plasmids microbubbles allowing the to penetrate peritoneal tissue found therein.

Preferably, the microbubbles are a plurality of filmogenic protein-encapsulated insoluble microbubbles, and are filled with an insoluble perfluorocarbon gas, such as (without limitation) perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, or perfluoropentane. In one embodiment, the microbubbles are about 1 to about 5 microns in diameter.

The ultrasound energy should preferably be administered to the peritoneal region at a frequency of about 0.5-to about 5MHz, and should be sufficient to cause rupture of the microbubbles within the patient's peritoneal cavity, which includes abdominal wall and mesentery areas. As a person of ordinary skill will appreciate the amount of ultrasonic energy applied should be adjusted so that it is sufficient to degrade, burst, disrupt or break apart the microbubbles without causing damage to peritoneal tissues or the DNA enclosed in the microbubbles. The DNA should be freed from the microbubbles into adjacent peritoneal tissues, where it can be absorbed by diseased and other cells, and the DNA can incorporate or itself into or transfect the host cell genome.
The peritoneal diseases treatable using the present invention include inflammation, fibrosis, postoperative peritoneal adhesion, or cancer in the peritoneal cavity. The peritoneal disease may be caused by or related to peritoneal infusion/dialysis, surgery, trauma, infection, genetics, or a systemic disease.

Importantly, the one or more DNA molecules, or plasmids include oligonucleotides, DNA, DNA plasmids, siRNA, shiRNA, and micro-RNA. In one embodiment, the DNA molecule is a SMAD7 transgene, while in a preferred embodiment, the DNA molecule is a SMAD7 cDNA.

The present invention thus provides a new strategy for prevention and treatment of peritoneal disease such as peritoneal inflammation/fibrosis and postoperative peritoneal adhesion using the ultrasound-mediated, involving local release of a target gene from the gas-filled microbubbles into the peritoneal tissues. This invention has several advantages.

Ultrasound-microbubble-mediated gene therapy for peritoneal fibrosis is safe because it does not cause either detectable histological and functional damage or cytotoxicity in normal peritoneal tissues. A small DNA molecule with contrast agent is injected into peritoneal cavity followed by transcutaneous ultrasound at the physical therapy levels with 1 MHz at 2 W/cm². Furthermore, it is also feasible to avoid ultrasound-induced heat damage to the tissues by controlling the temperature around 37°C during ultrasound exposure. We found that energy output at 2 W/cm² with interval exposure time 30 sec for total of 4-6 mins is safe for direct ultrasound exposure to the skin.

An important advantage of the present invention is that ultrasound-mediated gene transfer into the peritoneal tissues is temporary or transient. The transfected Smad7 DNA will be degraded gradually within the peritoneal tissues within 3-4 weeks.
and repeated gene transfer at day 14 to maintain high levels of Smad7 is feasible. This suggests that, unlike viral-based techniques which mediate a stable transgene expression, ultrasound might not introduce the targeted gene into genome. This may explain why ultrasound mediates a temporary transgene expression. Again, transient expression of Smad7 transgene within the diseased tissues suggests that ultrasound-mediated gene transfer is safer than the virus-based method in terms of the potential for insertional mutagenesis as previously reported [1, 2]. High gene transfection rate is the second advantage of ultrasound microbubble mediated gene transfer. Indeed, we found that more than 80% of peritoneal cells being transfected with Smad7 gene, which is consistent with our previous report that ultrasound substantially increases gene transfection rate in different cell types of normal and disease rat kidney by about 1000-fold [10-12].

Controlling the transfected gene expression within the diseased tissues using the inducible gene therapy as stated in this invention at the therapeutic level without causing side effects is another important advantage of the use of the present invention in gene therapy. For example, while overexpression of Smad7 is able to block TGF-β/Smad signaling and tissue scarring, it is also noted that intensive expression of Smad7 in the kidney induced by higher concentrations of doxycycline results in a massive apoptosis and acute renal injury. Thus, it is critical to control the level of Smad7 transgene expression at the therapeutic level while minimizing side effects when attempting to overexpress Smad7.

Local therapy by direct injection of a mixture of Smad7 gene and microbubble contrast agent into the peritoneal cavity followed by direct ultrasound local treatment via the abdominal skin is a further aspect of the present invention. This largely
enhances the local therapeutic effects while it minimizes the side effects due to systemic administration of drugs or genes through an intravenous route.

Ultrasound mediated gene transfer into the peritoneal tissues from the gene-bearing microbubbles locally is a safe and effective therapy for peritoneal diseases. Gas filled microbubbles (3μm in diameter) either in lipid or albumin forms are able to carry drugs/genes to become drug/gene-bearing microbubbles [13-16]. Microbubbles are elastic, compressible, and efficient reflectors of ultrasound. Microbubbles work by resonating in an ultrasound beam, rapidly contracting and expanding in response to the pressure changes of the sound wave, resulting in lowering of the threshold for cavitation by ultrasound energy [13-16]. Gene-bearing microbubbles can be injected intravenously or locally and ultrasound energy applied to the target region. As the microbubbles enter the region of insonation, they cavitate, locally releasing DNA and drug materials. Cavitation also likely causes a local shockwave that increases cell permeability and thus improves cellular uptake of DNA [13-16].

In the present study, a mouse Smad7 cDNA with a flag tag (m2) at its NH2 terminus in pcDNA3 was subcloned into a tetracycline-inducible vector, pTRE, to obtain pTRE-m2Smad7. To achieve doxycycline (a tetracycline derivative)–induced Smad7 transgene expression, pTRE-m2Smad7 and an improved pTet-on vector, pEFpurop-Tet-on were co-transfected into the peritoneal cavity. In the pilot study, we found that the peak of gene expression of exogenous Smad7 in peritoneal tissues was observed on the second day of transfection and the expression of transgene decreased in a time-dependent manner. To ensure the effectiveness of transfection, exogenous Smad7 was administered into peritoneal cavity on the first day and 14th day of PD in uremic rats. The uremic PD rats receiving empty vectors without Smad7
insert were taken as the treatment controls. The procedures of transfection were as follows. The rats were anesthetized by inhalation of isoflurane. The mixture of plasmids and microbubbles (Optison, Amersham Health Inc., Princeton, NJ, USA or SonoVue, Bracco International B.V., Amsterdam, Netherlands) was prepared with 1:1 vol/vol ratio. Then 4 ml of the mixed solution containing 100 μg of plasmids were immediately injected into the abdominal cavity. Ultrasound mediating gel was then applied on the shaved abdominal skin. The ultrasound transducer (Sonitron 2000, Rich-Mar Corp., Inola, Oklahoma, USA) was applied directly onto the abdominal wall with 1-MHz input frequency, 2 W/cm² output intensity and 20% duty cycle for a total of 4 mins with 30s intervals. After the gene transfer procedures, one milliliter of doxycycline (500 μg/ml, Sigma) was injected into the peritoneal cavity to induce Smad7 transgene expression, followed by 200 μg/ml of doxycycline in drinking water to maintain the induction of transgene expression.

Safety with ultrasound microbubble mediated gene therapy for peritoneal fibrosis is the most important aspect of this invention. Ultrasound microbubble mediated gene therapy for peritoneal fibrosis is safe because it is noninvasive and does not cause either detectable histological and functional damage or cytotoxicity in normal peritoneal tissues by injecting a small DNA with contrast agent into peritoneal cavity followed by 4 minutes transcutaneous ultrasound at the physical therapy levels with 1 MHz at 2 W/cm². Ultrasound itself is harmless to the body and is widely used clinically for many purposes including physical therapy, diagnosis, guidance for deep organ biopsy, and local drug and genetic material delivery [13-16]. The microbubble contrast agents are also safe and widely used clinically. It has been well documented that microbubbles can aid drug delivery by themselves and as agents to carry drugs or genetic materials for site specific treatment and gene therapy [13-16]. In addition,
injection of small DNA into the peritoneal cavity is also safe. It is also feasible to avoid ultrasound-induced heat damage to the tissues by controlling the temperature around 37°C during ultrasound exposure. We found that energy output at 2 W/cm² with interval exposure time 30 sec for up to 6 mins is safe for direct ultrasound exposure to the skin. As demonstrated in Figure 1, there is no detectable histological or functional damage associated with ultrasound microbubble treatment. Most importantly, we also found that ultrasound mediated gene transfer into the peritoneal tissues is temporary, which mimics the clinical therapies. The transfected Smad7 DNA will be degraded gradually within the peritoneal tissues within 3-4 weeks and repeated gene transfer at day 14 to maintain high levels of Smad7 is feasible since it is practical and no invasive. This suggests that, unlike viral-based techniques which mediate a stable transgene expression, ultrasound might not introduce the targeted gene into the genome. This may explain why ultrasound mediates a temporary transgene expression. Again, transient expression of Smad7 transgene within the diseased tissues suggests that ultrasound-mediated gene transfer is safer than the virus-based method in terms of the potential for insertional mutagenesis [1,2]. Taken together, the ultrasound microbubble gene therapy method is safe.

High gene transfection rate mediated by ultrasound-microbubble system is another distinct advantage of this invention. It has long been noted that low gene transfection rate is a major disadvantage when using non-viral-based gene delivery systems such as naked DNA and liposome. Using ultrasound-triggered gene released from microbubbles, a high gene transfection rate was evident by the finding that more than 80% of total peritoneal cells on the surface mesothelial cell layer and submesothelial cells are positive for flag-M2 Smad7 transgene (Figure 2A), resulting in a marked upregulation of Smad7 (Figure 2B), which is a key mechanism of
anti-peritoneal fibrosis since overexpression of Smad7 is able to block activation of TGF-β/Smad signaling by inhibiting Smad2/3 phosphorylation (Figure 3C).

The effectiveness of prevention and treatment of peritoneal fibrosis using ultrasound microbubble technique is a key aspect of this invention. Although peritoneal fibrosis is a major cause and common feature of technique failure of peritoneal dialysis, it is not yet available for specific and effective therapies for preventing and treating this disease. This invention has shown that ultrasound mediated release of Smad7 gene from albumin-type microbubbles is able to substantially inhibit the development of peritoneal fibrosis associated with peritoneal dialysis in uremia rats that have clinical features of end stage renal disease. As demonstrated in Figures 1-7, overexpression of peritoneal Smad7 by ultrasound-microbubble-mediated gene therapy results in substantial inhibition of Smad2/3 activation (Figure 3C), thereby blocking peritoneal dialysis related peritoneal fibrosis, as illustrated by preserving peritoneal function (Fig.4), attenuating peritoneal fibrotic thickening (Fig.5), and preventing peritoneal fibrosis through inhibition of collagen I, III, fibronectin, and α-SMA expression (Figs 6,7).

In addition, this invention also demonstrates that ultrasound-mediated gene expression of Smad7 is able to block peritoneal fibrosis associated with the postoperative peritoneal adhesion/fibrosis in rats (Fig.8). Indeed, after four weeks of surgical abrasion, the rats developed significant peritoneal adhesions including overexpression of α-SMA, collagen I, III, and fibronectin. The enhanced expression of TGF-β and activation of TGF-β/Smad signaling are blocked by ultrasound-microbubble-mediated Smad7 transfection (Figs. 8, 9). Thus, blockade of TGF-β/Smad signaling pathway via an ultrasound-microbubble-mediated system
represents a safe and novel therapeutic approach for preventing postsurgical peritoneal adhesions.

Controlling the transfected gene expression within the diseased tissues using inducible gene therapy, as set forth herein, at the therapeutic level but without causing side effects is another important aspect of the present invention, and has application to gene therapy. For example, while overexpression of Smad7 is able to block TGF-β/Smad signaling and tissue scarring, our previous finding also shows that intensive expression of Smad7 in the kidney induced by higher concentrations of doxycycline results in a massive apoptosis and acute renal injury [10]. Thus, it is critical to control the level of Smad7 transgene expression at the therapeutic level while minimizing side effects when attempting to overexpress Smad7.

Local therapy using the direct peritoneal injection of Smad7-bearing microbubbles followed by local ultrasound application over the abdominal skin is the most appreciative one. This avoids the use of systemic treatment. Indeed, traditional treatment for peritoneal diseases relies on the systemic administration of drugs. This approach is usually not effective and may also cause undesirable side effects. The invention has overcome this disadvantage and largely improved the effectiveness of local expression of negative TGF-β signaling molecule Smad7, resulting in inhibition of TGF-β/Smad-mediated peritoneal fibrosis.
The following references are incorporated by reference herein:


*Circulation* 2002; 105:1233-1239.
WHAT IS CLAIMED IS:

1. A method for delivering one or more genes, DNA molecules or plasmids useful for treatment of a peritoneal disease to a patient’s peritoneal region for treatment of a peritoneal disease therein, comprising:
   - providing a source of microbubbles containing the one or more genes, DNA molecules, or plasmids for treating peritoneal disease;
   - perfusing the peritoneal region of the patient with the microbubbles; and
   - administering ultrasonic energy to the abdominal or peritoneal region sufficient to cause transfection of the one or more genes, DNA molecules or plasmids into the abdominal or peritoneal tissue of the patient.

2. The method according to claim 1, wherein the microbubbles comprise a plurality of filmogenic protein-encapsulated insoluble microbubbles.

3. The method of claim 1, wherein the microbubbles are filled with an insoluble perfluorocarbon gas.

4. The method of claim 3, wherein the perfluorocarbon gas is perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, or perfluoropentane.

5. The method of claim 1, wherein the microbubbles are 1 to 5 microns in diameter.

6. The method according to claim 3, wherein the ultrasound energy is administered at a frequency of about 0.5 to about 5MHz.
7. The method of claim 6, wherein the ultrasound energy causes disruption of the microbubbles in the peritoneal tissues.

8. The method according to claim 1, wherein the peritoneal tissues include those tissues within the patient’s peritoneal cavity, abdominal wall and mesentery areas.

9. The method according to Claim 1, wherein the peritoneal disease includes inflammation, fibrosis, postoperative peritoneal adhesion, or cancer in the peritoneal cavity.

10. The method according to claim 2, wherein the one or more genes, DNA molecules, or plasmids include oligonucleotides, DNA, DNA plasmids, siRNA, shiRNA, and micro-RNA.

11. The method according to claim 1, wherein the peritoneal disease is caused by or related to peritoneal infusion/dialysis, surgery, trauma, infection, genetics, or a systemic disease.

12. The method according to claim 1, wherein the DNA molecule is a SMAD7 transgene including Smad7 peptides and protein.

13. The method according to claim 1, wherein the DNA molecule is a SMAD7 cDNA, Smad7 ODN, Smad7 siRNA, and Smad7 micro-RNA.
Figure 1. Ultrasound-microbubble-mediated Smad7 gene therapy does not cause detectable abnormalities to peritoneal tissues histologically (A) and functionally (B, C).

(A) Peritoneal histology

Normal  Smad7  Smad7+Ultrasound

(B) Peritoneal permeability of BUN

(C) Peritoneal absorption of glucose
Figure 2. Ultrasound-microbubble largely enhances Smad7 transgene expression in peritoneal tissues as demonstrated by anti-flag-m2Smad7 immunostaining (A, arrows) and RT-PCR (B).

CV= control vectors. *p<0.05, **p<0.01 compared to normal control and Smad7 groups.
Figure 3. Ultrasound-microbubble-mediated Smad7 gene therapy blocks TGF-β-Smad signaling at the level of activation as demonstrated by virtually suppression of Smad2/3 phosphorylation (p-Smad2/3, C), but not in the levels of Smad2 mRNA and protein expression. (A, B). * p<0.05, ** p<0.01, *** p<0.001 vs normal controls; Δ p<0.05, ΔΔ p<0.01 vs Smad7 treatment.
Figure 4. Ultrasound-microbubble-mediated Smad7 gene therapy improves peritoneal functions on week 4 demonstrated by reduced permeability of glucose, BUN, and total protein during the dwell time. * p<0.05, ** p<0.01, *** p<0.001 vs normal controls; ΔΔ p<0.01, ΔΔΔ Δ p<0.001 vs Smad7 treatment.
Figure 5. Ultrasound-microbubble-mediated Smad7 gene therapy inhibits peritoneal fibrosis associated PD in uremia rats on week 4 demonstrated by Mason Trichrome (Blue) staining and collagen I immunostaining (Brown).

(A) Normal  (B) Urea (U)  (C) PD

Mason Trichrome

Collagen I

(D) UPD  (E) UPD+Smad7  (F) UPD + Control Vectors

Mason Trichrome

Collagen I
Figure 6. Ultrasound-microbubble-mediated Smad7 gene therapy blocks peritoneal fibrosis associated with PD in uremia rats on week 4 demonstrated by inhibition of collagen 1 mRNA (A) and protein (B), and collagen III mRNA (C) and protein (D) expression. * p<0.05, ** p<0.01, *** p<0.001 vs normal controls; ΔΔ p<0.01, ΔΔΔ p<0.001 vs Smad7 treatment.
Figure 7. Ultrasound-microbubble-mediated Smad7 gene therapy blocks peritoneal fibrosis associated with PD in uremia rats on week 4 as demonstrated by inhibition of α-SMA mRNA (A) and protein (B), and fibronectin mRNA (C) and protein expression (D). * p<0.05, ** p<0.01, *** p<0.001 vs normal controls; △△ p<0.01, △△△ p<0.001 vs Smad7 treatment.
Figure 8. Ultrasound-microbubble-mediated Smad7 gene therapy blocks peritoneal adhesion induced by surgical abrasion on week 4 as demonstrated by inhibition of thickening of peritoneal (A) and collagen 1 and α-SMA expression (B). Note that TGF-β expression is also inhibited by Smad7 treatment (B).

(A) Histology (Masson Trichrome staining)

(B) Immunohistochemistry

Col.1

α-SMA

TGF-β
Figure 9. Ultrasound-microbubble-mediated Smad7 gene therapy blocks postoperative peritoneal adhesion/fibrosis as demonstrated by inhibition of collagen 1 (A,B), collagen III (C, D), fibronectin (E, F), and α-SMA (G,H) mRNA expression. Horizontal lines represent the median levels of the factors assayed. * p<0.05, ** p<0.01, *** p<0.001 vs normal controls; # p<0.05, ## p<0.01 vs adhesion receiving no treatment.
PATENT COOPERATION TREATY

PCT

DECLARATION OF NON—ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT
(PCT Article 17(2)(a), Rules 13 ter.1(c) and 39 )

Applicant's agent's reference
FPCH07160075

INTERNATIONAL DECLARATION

Date of mailing (day/month/year)

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A61P31/00 (2008.01)i, A61P41/00 (2008.01)i, A61P55/00 (2008.01)i, A61P43/00 (2008.01)i

Applicant
THE UNIVERSITY OF HONG KONG et al.

This International Searching Authority hereby declares, according to Article 17(2)(a), that no international search report will be established on the international application for the reasons indicated below.

1. ☑ The subject matter of the international application relates to:
   a. ☑ scientific theories
   b. ☑ mathematical theories
   c. ☑ plant varieties
   d. ☑ animal varieties
   e. ☑ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
   f. ☐ schemes, rules or methods of doing business
   g. ☐ schemes, rules or methods of performing purely mental acts
   h. ☐ schemes, rules or methods of playing games
   i. ☑ methods for treatment of the human body by surgery or therapy
   j. ☑ methods for treatment of the animal body by surgery or therapy
   k. ☐ diagnostic methods practised on the human or animal body
   l. ☐ mere presentations of information
   m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art

2. ☑ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
   ☑ the description
   ☑ the claims
   ☐ the drawings

3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

4. ☐ A meaningful search could not be carried out without the tables related to the sequence listings; the applicant did not, within the prescribed time limit, furnish such tables in electronic form complying with the technical requirements provided for in Annex C-bis of the Administrative Instructions, and such tables were not available to the International Searching Authority in a form and manner acceptable to it.

5. Further comments:

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