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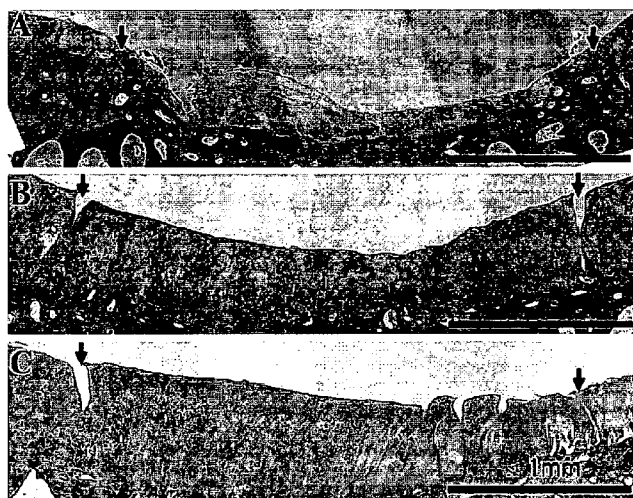
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[Continued on next page]

(54) Title: METHOD AND COMPOSITION FOR INDUCING REGENERATION OF DAMAGED CARTILAGE USING MICROFRACTURE AND HYALURONIC ACID

Figure 3



(57) Abstract: The present invention relates to a method, a composition and use thereof for inducing regeneration of damaged cartilage. Specifically, the invention relates to a method, a composition and use thereof for inducing regeneration of damaged cartilage by applying hyaluronic acid to damaged area of cartilage after microfracture formation. The present method and composition for inducing regeneration of damaged cartilage may regenerate hyaline-like cartilage with a simpler surgery procedure. No involvement of any transplantation step, make the present method more useful.



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NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to non-prejudicial disclosures or exceptions to lack of novelty (Rule 4.17(v))*

Description

METHOD AND COMPOSITION FOR INDUCING RE-GENERATION OF DAMAGED CARTILAGE USING MICRO-FRACTURE AND HYALURONIC ACID

Technical Field

- [1] The present invention relates to a method, a composition and use thereof for inducing regeneration of damaged cartilage. Specifically, the invention relates to a method, a composition and use thereof for inducing regeneration of damaged cartilage by applying hyaluronic acid to damaged area of cartilage after microfracture formation.

Background Art

- [2] Articular cartilage has limited self-healing potential (Buckwalter *et al.* 1997, Hunziker 2002, Prakash *et al.* 2002). Since articular cartilage has neither vascular supply nor easy access to stem cells, repair is often unsuccessful. Partial thickness defects cannot heal spontaneously because stem cells found in the bone marrow cannot be accessed (Buckwalter 1998). Full thickness defects, however, do have access to these cells and thus can heal spontaneously (Wei *et al.* 1997). However, the re-generated cartilage is fibrocartilage rather than hyaline cartilage (Frisbie *et al.* 2003) and, being mechanically weaker than hyaline cartilage, it gradually degenerates over time (Shapiro *et al.* 1993).
- [3] For treating damaged articular cartilage, no promising therapeutic method has been established until recently. Two major methods may be used to regenerate damaged cartilage. Those are microfracture surgery, which stimulates the subchondral bone to induce regeneration of articular cartilage, and transplantation of chondrogenic cells.

Disclosure of Invention

Technical Problem

- [4] Microfracture used to treat articular cartilage injuries can facilitate the intrinsic healing response of stem or progenitor cells recruited by subchondral penetration to the marrow and can stimulate cartilage regeneration. Multiple drilling, arthroplasty, microfracture surgery, or the like may be used in this method. However, microfracture has been shown to result in regeneration of fibrocartilage, with inferior mechanical properties, rather than the desired hyaline cartilage. Microfracture has been failed to regenerate cartilage that maintains its functional integrity of articular cartilage.
- [5] Cartilage transplantation has limitations. Various problems result at the area where the graft has been provided and the method cannot be used when the chondral lesion is too large. Furthermore, margins are generated between the original tissues and healed

regions.

[6] Autologous chondrocyte transplantation, wherein chondrocytes that were harvested and *in vitro* cultured beforehand are implanted in a chondral lesion. This method involves two serial surgeries, chondral harvest and cell implantation, which necessitate chondrocyte-harvest site morbidity and also burdensome in terms of time, cost and medical aspects.

[7] There also have been developed methods of chondral regeneration using bone marrow- or adipose tissue-derived stem cell. In these methods, *in vitro* cultured stem cells are differentiated to chondrocytes and implanted in a chondral lesion. Dexamethasone which is added to the cell culture may be toxic to cells. The possibility of contamination during *in vitro* cultivation and the duration of time that is required to culture cells before they can be implanted to treat a patient are also raised as main problems.

Technical Solution

[8] The present inventors, surprisingly, found that application of hyaluronic acid to the area including a microfractured site for treating damaged cartilage resulted in regeneration of hyaline-like cartilage rather than fibrocartilage by the stem or progenitor cells recruited by subchondral penetration to the marrow.

[9] The present invention will be hereinafter described in detail.

[10] In an embodiment, the present invention provides a method for inducing regeneration of cartilage in a mammal comprising:

[11] forming one or more microfractures on a damaged area of cartilage;

[12] applying biomaterial containing hyaluronic acid to said damaged area of cartilage including the formed microfracture(s); and

[13] allowing said damaged area regenerate cartilage.

[14] Hyaluronic acid is a type of complex polysaccharide composed of N-acetyl glucosamine and glucuronic acid.

[15] In an embodiment, the present invention provides the present method for inducing regeneration of cartilage is provided, wherein the hyaluronic acid being in a cross-linked form or the carboxyl group or alcohol group of the hyaluronic acid being chemically modified.

[16] In another embodiment, the present invention provides the present method for inducing regeneration of cartilage, wherein bone marrow-derived stem cells flowing over the damaged area of cartilage as a result of the formation of microfracture(s). Preferably, when carrying out the present method for inducing regeneration of cartilage, one forms one or more microfractures and confirms that bone marrow-derived stem cells flow over the damaged area of cartilage before applying biomaterial

containing hyaluronic acid.

- [17] 'Biomaterial', as used herein, means material which is generally allowed to be implanted, injected or applied to human and whose biocompatibility is verified. Biomaterial may comprise pharmaceutically acceptable buffer, injection or the like including saline solution. The biomaterial may also comprise one or more carrier, vehicle, excipient for efficient delivery of an effective component.
- [18] In the method of the present invention, any type of biomaterial containing hyaluronic acid may be used as long as that can be applied to a damaged area of cartilage including microfracture(s). Preferable, the biomaterial containing hyaluronic acid is gel-type.
- [19] In another embodiment, the method for inducing regeneration of cartilage of the present invention does not comprise transplantation of cells including chondrocytes.
- [20] The method for inducing regeneration of cartilage of the present invention may be used in combination with other method for inducing cartilage regeneration. The method that can be used with the present method include any surgical or drug administering methods known in the art.
- [21] In an embodiment, the present invention provides a composition for applying to a microfractured site for inducing regeneration of damaged cartilage comprising hyaluronic acid. Preferably, the molecular weight of hyaluronic acid is greater than 10,000 Da.
- [22] In another embodiment, the present composition for applying to a microfractured site for inducing regeneration of damaged cartilage further comprises one or more inducers of chondrogenic differentiation. Preferable inducers of chondrogenic differentiation may be selected from the group consisting of transforming growth factor- β (TGF- β), BMPs, IGF, Wnt proteins. More preferably, the inducer of chondrogenic differentiation is TGF- β or BMP-2.
- [23] In an embodiment, the present invention provides a use of hyaluronic acid for applying to a microfractured site for inducing regeneration of damaged cartilage.

Advantageous Effects

- [24] The composition of the present invention may be applied to an damaged area of cartilage after microfracture formation in that area. Bone marrow-derived stem cells flowed over the damaged area of cartilage facilitate regeneration of hyaline-like cartilage, which is more natural, rather than fibrocartilage thereby improve remedial results.
- [25] Furthermore, no additional surgery for harvesting bone marrow or adipose tissue is needed to obtain bone marrow- or adipose tissue-derived stem cells. This single-stage surgical procedure requires minimal surgical time, equipment and supply cost.
- [26] Even further, since no cell transplantation is involved, there are no need for

chondrocyte cultivation and no fear of contamination or infection, which makes the method much safe. A patient can be treated immediately and does not need to wait until cells are cultured and ready to use, which generally takes more than one month. This may be very advantageous for clinical perspectives.

Brief Description of the Drawings

- [27] Fig. 1 shows photographs of rabbit knee articular cartilage defects immediately after creation (A) and after microfracture surgery (B). (A) A full-thickness cartilage defect (2.5 x 5 mm, depth 1-2 mm) was created on the patellar groove. The dotted line indicates the defect. (B) Four holes of subchondral penetration (arrows) were made in the defects.
- [28] Fig. 2. shows photographs of rabbit knee articular cartilage defects three months after treatments. (A) Treatment with microfracture only (group A). (B) Treatment with microfracture and hyaluronic gel (group B). (C) Treatment with microfracture and Hyaluronic gel containing TGF- β (group C). The dotted lines indicate the defects.
- [29] Fig. 3 illustrates H&E-stained histological sections of defects three months after treatments. (A) Treatment with microfracture only (group A). (B) Treatment with microfracture and HA gel (group B). (C) Treatment with microfracture and HA gel containing TGF- β (group C). The arrows indicate defect edge. The scale bars indicate 1 mm.
- [30] Fig. 4 illustrates Alcian blue-stained histological sections of defects three months after treatments. (A) Treatment with microfracture only (group A). (B) Treatment with microfracture and HA gel (group B). (C) Treatment with microfracture and HA gel containing TGF- β (group C). The arrows indicate defect edge. The scale bars indicate 1 mm.

Mode for the Invention

- [31] Certain embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one with ordinary skill in the art. It is intended that the specification including the examples be considered exemplary, with the scope of the invention only being indicated by the claims.

[32] Examples

[33] Example 1: Preparation of Hyaluronic acid solution

- [34] Hyaluronic acid (molecular weight=4,000,000 Da, LG life Sciences Ltd. Seoul, Korea) was dissolved in saline solution or DMEM culture medium (Invitrogen, USA) containing 100 U/ml of Penicillin and 100 μ g/ml of Streptomycin to make 4% hyaluronic acid solution. To test the therapeutic effect of the inducers of chondrogenic differentiation, TGF- β (5 μ g/ml) were added to saline solution or DMEM medium to

prepare 4% hyaluronic acid solution.

[35] Example 2: Microfracture surgery on animal model for cartilage defects

[36] Three-month-old, skeletally mature, male New Zealand white rabbits (3.5 ± 0.5 kg, Halim Animal Laboratory Co. Seoul, Korea) were anesthetized with an intramuscular injection of 250 mg ketamine hydrochloride (Yuhan Co., Seoul, Korea), 35 mg xylazine hydrochloride (Bayer, Seoul, Korea), and 5 mg of acepromizine (Yuhan Co., Seoul, Korea) Guidelines for the care and use of laboratory animals published by National Institute of Health (NIH publication No. 85-23, revised 1985) were observed during the animal experiments. A longitudinal skin incision was made over the anterior aspect of the knee joint, and a medial parapatellar arthrotomy was performed. The patella was reflected laterally and the entire distal femur was exposed. A full-thickness cartilage defect (2.5 x 5 mm, depth 1-2 mm) was created on the patellar groove by controlled drilling without violation of the subchondral bone (Figure 1A). Using a fine 0.03-inch Kirschner wire (Sanatmetal Co., EGER, Hungary), meticulous subchondral penetration (microfracture method) was introduced to the defects (Figure 1B). Four holes were made in each defect without violation of neighboring holes. The debris was completely removed and hemostasis was achieved.

[37] Twenty-four rabbit knee joints were divided into three groups: A, B, and C. The cartilage defects in the knee joints of group A (n=8) were not treated with HA(hyaluronic acid) and served as controls. The cartilage defects in the knee joints of group B (n=8) were filled with commercially available, medical grade 4% (w/v) HA in phosphate buffered saline (50 μ l/site, Hyruan plus, LG Pharmacological Co., Chunbuk, Korea). The cartilage defects in the knee joints of group C (n=8) were filled with 4% HA gel mixed with TGF- β (5 μ g/ml, 250 ng/site). After confirmation of the stability of HA in the cartilage defects, the surgical wound was closed in layers. Antibiotics (gentamycin) were given for 3 days, postoperatively. All rabbits were sacrificed at three months for analysis.

[38] Example 3: eye observation

[39] Three months after treatment, all the experimental animals were sacrificed and the joint and synovium from each were examined grossly. The entire distal femur was exposed and resected without any soft tissue attachments. Femoral and tibial condyles from each knee were examined macroscopically and photographed.

[40] The articular surfaces appeared normal in all groups with no evidence of synovial hypertrophy. The defects treated with microfracture and HA with or without TGF- β (groups B and C) appeared to be filled to a greater extent than the lesions without HA (group A). The defects treated without HA are partially filled and the surface was less smooth (Fig. 2).

[41] Example 4: histological analysis

- [42] For histological analyses, specimens were fixed in 10 % (v/v) buffered formalin, decalcified with decalcifying agent (Decal Rapid™, National Diagnostics, Georgia, USA), dehydrated with a series of graded alcohol, and embedded in paraffin. Tissue sections (4 µm thick) were stained with hematoxylin and eosin (H&E) for morphologic analysis and with alcian blue staining for glucosaminoglycan production.
- [43] Histological analyses showed that the quality of the regenerated tissues differed depending on group. H&E staining showed that cartilaginous tissues were regenerated in the defects in all groups (Figure 3) but that the thickness of the regenerated cartilaginous tissue in groups B and C was much thicker than that in group A. Furthermore, alcian blue staining appeared to be more intense in the regenerated cartilaginous tissues in groups B and C than in group A. This indicates the presence of greater amounts of glucosaminoglycan in groups B and C and suggests regeneration of a more hyaline-like cartilage (Figure 4).

Claims

- [1] A method for inducing regeneration of cartilage in a mammal comprising:
 - (a) forming one or more microfractures on a damaged area of cartilage;
 - (b) applying biomaterial containing hyaluronic acid to said damaged area of cartilage including the formed microfracture(s); and
 - (c) allowing said damaged area regenerate cartilage.
- [2] The method according to claim 1, wherein as a result of step (a), bone marrow-derived stem cells flow over the damaged area of cartilage.
- [3] The method according to claim 1 or 2, wherein the biomaterial being gel-type.
- [4] The method according to claim 1 or 2, wherein the hyaluronic acid being in a cross-linked form or the carboxyl group or alcohol group of the hyaluronic acid being chemically modified.
- [5] The method according to claim 1 or 2, wherein the method does not comprise transplantation of cells including chondrocytes.
- [6] A composition for applying to a microfractured site for inducing regeneration of damaged cartilage comprising hyaluronic acid.
- [7] The composition of claim 6, wherein the molecular weight of hyaluronic acid is greater than 10,000 Da.
- [8] The composition of claim 6 or 7, wherein the composition further comprises one or more inducers of chondrogenic differentiation selected from the group consisting of transforming growth factor - β (TGF- β), BMPs, IGF, Wnt proteins.
- [9] The composition of claim 8, wherein the inducer of chondrogenic differentiation is TGF- β or BMP-2.
- [10] The composition of claim 6 or 7, wherein the hyaluronic acid being in a cross-linked form or the carboxyl group or alcohol group of the hyaluronic acid being chemically modified.
- [11] Use of hyaluronic acid for applying to a microfractured site for inducing re-generation of damaged cartilage.

Figure 1

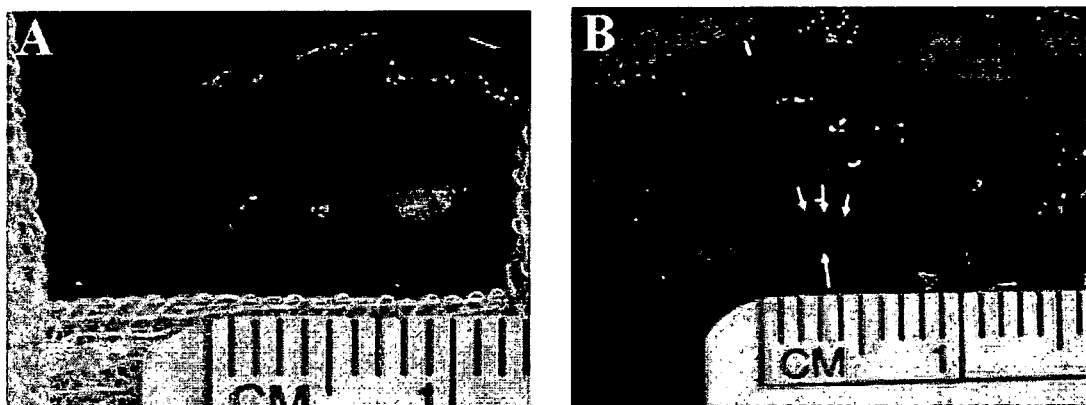


Figure 2

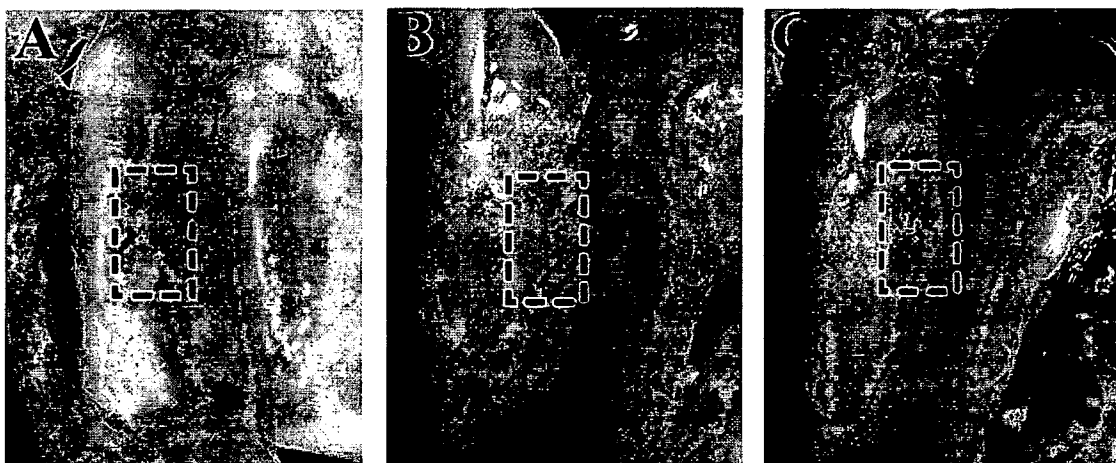


Figure 3

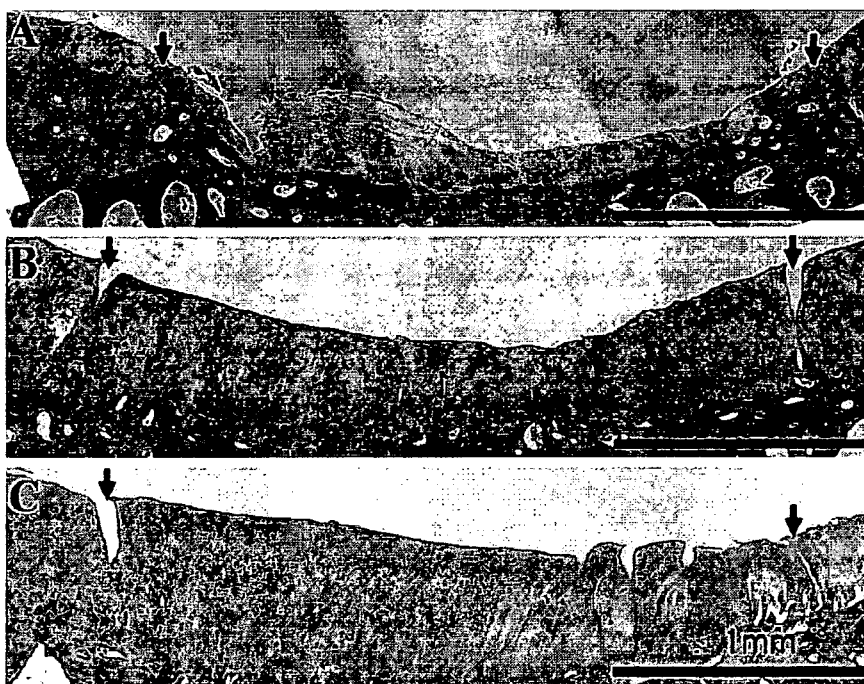
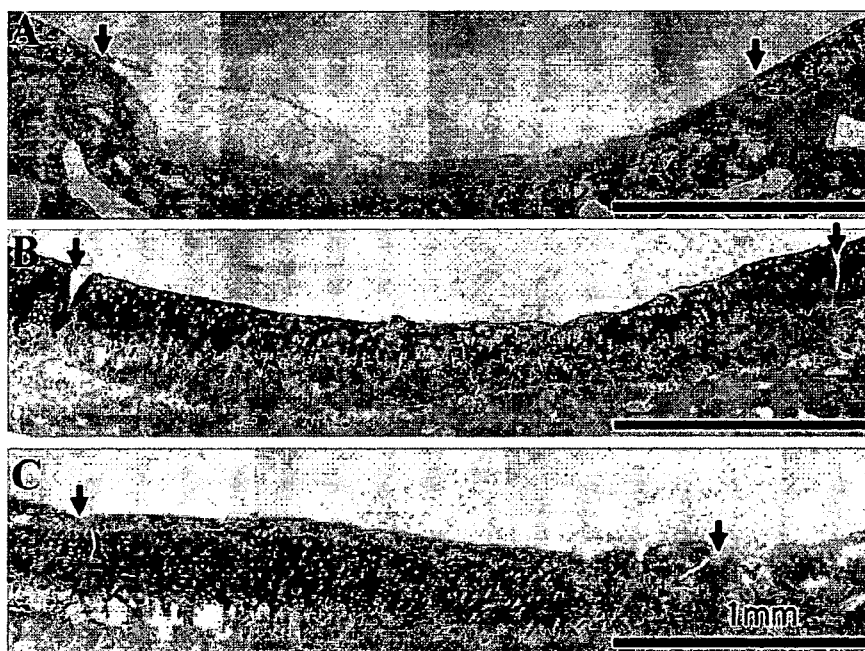


Figure 4



A. CLASSIFICATION OF SUBJECT MATTER***A61K 31/728(2006.01)i, A61P 19/00(2006.01)i***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K 31/728, 31/715, 31/726, 38/18, C12N 5/06, A61P 19/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), PubMed, JPO, USPTO, Google

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sun-Woong Kang et al. 'Articular cartilage regeneration with microfracture and hyaluronic acid.' Biotechnol Lett. online. October 31, 2007. See Abstract, Pages 436-439, Fig. 2	6-11
X	US 6,645,945 B1 (Michael Radomsky and Mohammad A. Heidaran) 11 November 2003 See Abstract, Column 5-6(Example), Claims 1 and 4	6-11
X	JP 2008-37770 A (Eto Tadashi) 21 February 2008 See Abstract, Page 2, Claims 1-2	6-11
A	WO 2005/073365 A1 (Harukazu Tohyama and Tomoo Okuizumi) 11 August 2005 See Abstract, Claims 1 and 5	6-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 DECEMBER 2008 (24.12.2008)

Date of mailing of the international search report

24 DECEMBER 2008 (24.12.2008)

Name and mailing address of the ISA/KR

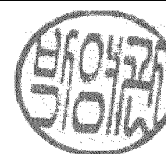
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PARK, Yeong-Gwan

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2008/002417**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-5 pertain to methods for treatment of the human or animal body by therapy, as well as diagnostic methods, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/002417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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