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(71) Applicant (for all designated States except US): CHU
SAINTE JUSTINE [CA/CA]; 3175 Côte-ste-Catherine,
Montréal, Québec, H3T 1C5 (CA).

(72) Inventor; and

(75) Inventor/Applicant (for US only): MOREAU, Alain
[CA/CA]; 3489 Jules-Huot, Montréal, Québec, H1A 5T5
(CA).

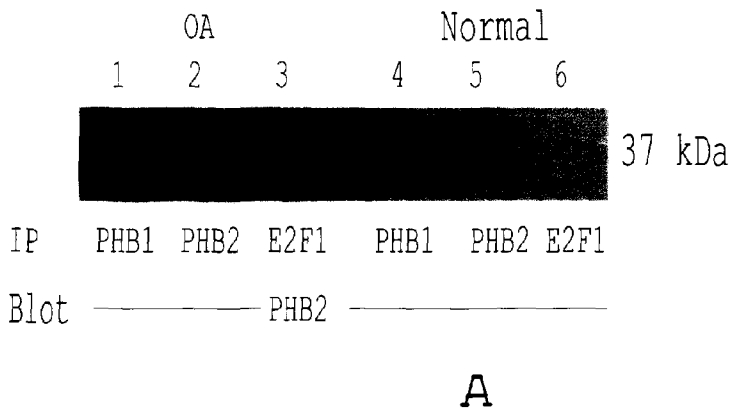
(74) Agents: DUBUC Jean et al.; Goudreau Gage Dubuc,
2200-2000 Mc Gill College Avenue, Montréal, Québec
H3A 3H3 (CA).

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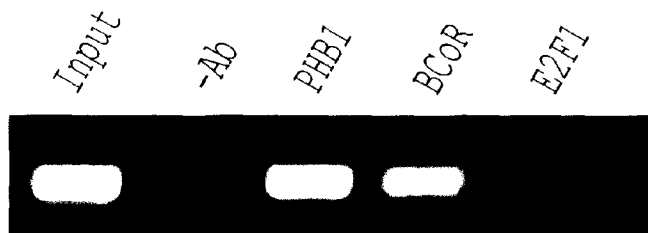
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(54) Title: METHODS OF PROGNOSED OSTEOARTHRITIS, METHODS OF SELECTING COMPOUNDS AND KITS THEREFOR



(57) Abstract: Methods are disclosed for assessing risk of developing osteoarthritis. Said methods comprise determining the cellular localizations, blood or synovial fluid concentrations, sumoylation states and post-translational modifications of pituitary homeobox transcription factor 1 (pitr-1) repressor proteins, identifying mutations in genes which encode said proteins and identifying any mutations or post-translational modifications causing the nuclear accumulation or retention of said proteins. Said proteins include prohibitin (PHB-1), prohibitone (PHB-2) and B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR).



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TITLE OF THE INVENTION

Methods for diagnosing osteoarthritis

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority on U.S. provisional application no. 60/854,077 filed on 25 October 2006. All documents above are herein incorporated by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to methods of prognosing osteoarthritis, methods of selecting compounds and kits therefore.

BACKGROUND OF THE INVENTION

[0003] The etiology of (OA), the most common form of arthritis, remains unclear notwithstanding the multiplicity of factors that have been considered in primary OA (1, 2). At present, it has become increasingly evident that the majority of OA genetic susceptibility loci cannot be attributed only to structural genes or genes regulating bone mass (3-5). These studies have also highlighted the great heterogeneity and differences in the degree of OA heritability between different joint sites (e.g. hand versus knee) and gender. This is also reflected by the multiplicity of loci identified in OA linkage studies and their discrepancies. Moreover, the functional importance of these susceptibility loci has yet to be confirmed and illustrates our incomplete knowledge of the biology of OA.

[0004] Pitx1 (previously called Ptx1) is a homeodomain transcription factor detected initially throughout pituitary development. The Pitx-family contains three related members, Pitx1, Pitx2 and Pitx3, which are members of the paired class of homeodomain proteins. The three Pitx factors have similar transcription

properties (6-8). The *pitx1* gene is highly expressed in mouse hind limb long bones during development (5) and accumulation of high levels of Pitx1 proteins were detected by immunohistochemistry on hind limb long bone sections mainly in the periarticular region, along the perichondrium (including at the hip and knee joints) and also in the nuclei of proliferative chondrocytes (8). Pitx1 expression was also detected in craniofacial structures such as the mandible and at the temporo-mandibular joints. Mice that are homozygous for the *pitx1* deletion are born with the expected Mendelian ratio, but they die soon after birth and phenotypic analysis of *pitx1* mutant newborn mice shows striking craniofacial and hind limb skeletal abnormalities (9). Interestingly, heterozygous mice harboring only one mutated allele were phenotypically normal at birth, but a majority of aging *pitx1*^{+/-} heterozygous mice exhibited gradual degenerative changes of knee joints, showing OA-like lesions at seven months of age. Comparison of histological analysis performed on seven-month old wild-type (Figure 1a) and *pitx1* ^{+/-} mouse (Figure 1b) femurs further confirmed an abnormal thickening of the subchondral, trabecular and cortical bone, a feature commonly found in OA. At higher magnification, histological sections stained with the Goldner method revealed a fibrillation and a marked calcification affecting only the articular cartilage of *pitx1* heterozygous mice (Figure 1d). These abnormalities were reminiscent of clinical changes usually observed in OA patients.

[0005] The prohibitins, prohibitin (also known as PHB-1 or BAP32) and prohibitone (also known as PHB-2, B-cell receptor associated protein or BAP37, REA for Repressor of estrogen receptor) are highly conserved proteins in eukaryotic cells that are present in multiple cellular compartments (30-32). PHB-1 is localized to the mitochondria where it might have a role in the maintenance of mitochondrial function as well as in the nucleus where it facilitates cellular senescence by recruiting specific co-repressors to inhibit E2F target genes (19-21). The role of PHB-1 in cell-cycle regulation is also demonstrated by its physical interaction with the retinoblastoma tumor-suppressor protein families (22, 23) and

through a direct interaction with the highly conserved marked box region of E2Fs (24) preventing E2Fs interaction with their cognate sequence. Nonetheless, the function of PHB-1 in the nucleus is still controversial although its nuclear localization has been found in a variety of cell lines (24).

[0006] To the Applicant's knowledge, there is no molecular test for assessment of the risk of progression and severity of OA.

[0007] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0008] In accordance with an aspect of the present invention, there is provided a method of selecting a compound, said method comprising the steps of (a) contacting a test compound with at least one cell having a mutation within the E2F-like site of the pitx1 promoter; and (b) determining pitx1 transcription and/or expression level; wherein the test compound is selected if pitx1 transcription and/or expression level is increased in the presence of the test compound as compared to that in the absence thereof. In a specific embodiment, the mutation is a mutation corresponding to -3727 C→T within the E2F-like site of the pitx1 promoter. In another specific embodiment, the selected compound is potentially useful in the treatment of primary osteoarthritis in a joint where Pitx1 is normally expressed. In another specific embodiment, the joint where Pitx1 is normally expressed is selected from the group consisting of knee joint, hip joint and temporo-mandibular joint. In another specific embodiment, the joint where Pitx1 is normally expressed is knee joint. In another specific embodiment, the joint where Pitx1 is normally expressed is hip joint. In another specific embodiment, the joint where Pitx1 is normally expressed is temporo-mandibular joint.

[0009] In accordance with another aspect of the present invention, there is provided a method comprising: (a) providing a DNA sample from a subject; and (b) comparing the binding of a pitx1 repressor protein selected from the group consisting of prohibitin (PHB-1), prohibitone (PHB-2) and B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR), to an E2F-like site of the pitx1 promoter in the DNA sample from the subject, with that in a control DNA sample, wherein an increased binding of the pitx1 repressor protein to the E2F-like site of the pitx1 promoter in the subject DNA sample as compared to that in the control DNA sample, is indicative that the subject has or is at risk for developing osteoarthritis. In a specific embodiment, said repressor protein is PHB-1. In another specific embodiment, said repressor protein is PHB-2. In another specific embodiment, said repressor protein is BCoR. In another specific embodiment, said subject was not clinically diagnosed with osteoarthritis.

[0010] In accordance with another aspect of the present invention, there is provided an isolated nucleic acid molecule of no more than 300 nucleotides comprising (a) a sequence of at least 20 contiguous nucleotides of AGTTCCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCTGCTCAAATACATCCTGGCTCT (SEQ ID NO: 1) including nucleotide at position 31 wherein C is replaced by T; or (b) the complement of the sequence in (a). In a specific embodiment, the sequence in (a) is of at least 30 contiguous nucleotides of AGTTCCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCTGCTCAAATACATCCTGGCTCT (SEQ ID NO: 1).

[0011] In accordance with another aspect of the present invention, there is provided an array of nucleic acid molecules attached to a solid support, the array comprising an oligonucleotide hybridizable to the nucleic acid molecule of the present invention, under conditions in which the oligonucleotide will not substantially hybridize to a nucleic acid molecule consisting of AGTTCCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCTGCTCAAATACATC

CTGGCTCT (SEQ ID NO: 1) or its complement.

[0012] In accordance with another aspect of the present invention, there is provided a single-stranded DNA probe of no more than 300 nucleotides hybridizable under high stringency conditions to (a) a nucleic acid molecule consisting of AGTTCCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCTGCTCAAATACATC CTGGCTCT (SEQ ID NO: 1) including nucleotide at position 31 wherein C is replaced by T; or to (b) the complement of the nucleic acid molecule in (a) but not to or to a lesser extent to a nucleic acid molecule consisting of AGTTCCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCTGCTCAAATACATC CTGGCTCT (SEQ ID NO: 1) or to its complement. In a specific embodiment, the probe is detectably labeled.

[0013] In accordance with another aspect of the present invention, there is provided a kit comprising the probe of the present invention and instructions to use the probe to diagnose osteoarthritis in a subject.

[0014] In accordance with another aspect of the present invention, there is provided a kit comprising the probe of the present invention and instructions to use the probe to predict whether a subject is at risk of developing osteoarthritis.

[0015] In a specific embodiment of the kits of the present invention, the kits further comprise a container for a DNA sample from the subject. In another specific embodiment, the kits further comprise an antibody specific to a pitx1 repressor protein.

[0016] In accordance with another aspect of the present invention, there is provided a method comprising: determining the cellular localization of a

pitx1 repressor protein selected from the group consisting of prohibitin (PHB-1) and prohibitone (PHB-2) in a subject cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample. In a specific embodiment, said method further comprises determining whether the repressor protein nuclear concentration is higher in the subject cell sample as compared to that in a control cell sample; wherein a higher repressor protein nuclear concentration in the subject cell sample is indicative that the subject is at risk of developing osteoarthritis.

[0017] In accordance with another aspect of the present invention, there is provided a method comprising: (a) identifying a subject suspected of having osteoarthritis (OA); and (b) detecting the blood concentration of a pitx1 repressor protein in a blood sample of the subject.

[0018] In accordance with another aspect of the present invention, there is provided a method comprising: (a) identifying a subject suspected of having osteoarthritis (OA); and (b) detecting the synovial fluid concentration of a pitx1 repressor protein in a synovial fluid sample of the subject.

[0019] In a specific embodiment of the methods, the osteoarthritis is selected from the group consisting of knee joint arthritis, hip joint arthritis and temporo-mandibular joints arthritis. In another specific embodiment, the osteoarthritis is knee joint arthritis. In another specific embodiment, the osteoarthritis is hip joint arthritis.

[0020] In accordance with another aspect of the present invention, there is provided a method of selecting a compound, said method comprising (a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an

osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and (b) determining a pitx1 repressor protein nuclear localization in the cell sample; wherein the test compound is selected if the pitx1 repressor protein nuclear localization in the cell sample is decreased in the presence of the candidate compound as compared to in the absence thereof. In a specific embodiment, the selected test compound is potentially useful in preventing accumulation or retention of a pitx1 repressor protein in cell nuclei or in promoting a pitx1 repressor protein nuclear export. In another specific embodiment, the selected test compound is potentially useful in the treatment of primary osteoarthritis. In another specific embodiment, the pitx1 repressor protein is PHB-1. In another specific embodiment, the pitx1 repressor protein is PHB-2. In another specific embodiment, the pitx1 repressor protein is BCoR.

[0021] In accordance with another aspect of the present invention, there is provided a method of selecting a compound, said method comprising the steps of (a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and (b) measuring the binding of a pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample, wherein the test compound is selected if the binding of the pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample is decreased in the presence of the test compound as compared to in the absence thereof. In a specific embodiment, the selected test compound is potentially useful in the treatment of primary osteoarthritis. In another specific embodiment, the pitx1 repressor protein or complex comprises PHB-1. In another specific embodiment, the pitx1 repressor protein or complex comprises PHB-2. In another specific embodiment, the pitx1 repressor protein or complex comprises BCoR. In another specific embodiment, said cell sample is from a subject having osteoarthritis. In another specific embodiment, said cell sample is an articular chondrocytes sample. In another specific embodiment, said articular chondrocytes

sample is from a subject having osteoarthritis in a knee joint.

[0022] In accordance with another aspect of the present invention, there is provided a method of identifying a mutation contributing to osteoarthritis, comprising comparing the nucleotide sequence of a gene selected from the group consisting of PHB-1 gene and PHB-2 gene, or of any gene encoding a protein causing the nuclear accumulation or retention of PHB-1 or PHB-2 in articular chondrocytes of a subject having osteoarthritis with that of the corresponding gene in a control subject. In a specific embodiment, said gene is the PHB-1 gene. In another specific embodiment, said gene is the PHB-2 gene. In another specific embodiment, said mutation is one affecting PHB-1 or PHB-2 DNA-binding and/or its cellular localization.

[0023] In accordance with another aspect of the present invention, there is provided a method for diagnosing osteoarthritis comprising detecting in a subject the presence of a mutation directly or indirectly causing the nuclear accumulation or retention of PHB-1 or PHB-2 in cells where PHB-1, PHB-2 or Pitx1 is normally expressed, wherein the presence of the mutation is an indication that the subject has or is at risk of developing osteoarthritis. In a specific embodiment, said method comprises detecting a mutation in CRM-1.

[0024] In accordance with another aspect of the present invention, there is provided a method for diagnosing osteoarthritis comprising detecting in a subject, the presence of at least one post-translational modification directly or indirectly causing the nuclear accumulation or retention of PHB-1 or PHB-2 in cells where PHB-1, PHB-2 or Pitx1 is normally expressed, wherein the presence of the at least one post-translational modification is an indication that the subject has osteoarthritis.

[0025] In accordance with another aspect of the present invention,

there is provided a kit comprising an antibody specific to PHB-1 or to PHB-2 and an antibody specific to BCoR. In a specific embodiment, the kit further comprises instructions to use the antibodies to predicting whether a subject is at risk for developing osteoarthritis.

[0026] In accordance with another aspect of the present invention, there is provided a purified repressor complex comprising BCoR and at least one of PHB-1 and PHB-2. In a specific embodiment, the repressor complex comprises BCoR and PHB-1. In another specific embodiment, the repressor complex comprises BCoR and PHB-2. In another specific embodiment, the repressor complex comprises BCoR, PHB-1 and PHB-2.

[0027] In accordance with another aspect of the present invention, there is provided a method of using the repressor complex of any one of claims 32-34, for binding to a E2F-site. In a specific embodiment, the E2F-site is that of pitx1 promoter.

[0028] In accordance with another aspect of the present invention, there is provided a method comprising: determining the cellular localization of a pitx1 repressor protein or complex selected from the group consisting of prohibitin (PHB-1), prohibitone (PHB-2), B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR), PHB-1-containing complex, PHB-2-containing complex and BCoR-containing complex, in a subject cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample.

[0029] In a specific embodiment, the method further comprises determining whether the repressor protein or complex nuclear concentration is higher in the subject cell sample as compared to that in a control cell sample;

wherein a higher repressor protein or complex nuclear concentration in the subject cell sample is indicative that the subject is at risk of developing osteoarthritis.

[0030] In accordance with another aspect of the present invention, there is provided a method comprising: (a) identifying a subject suspected of having osteoarthritis (OA); and (b) detecting the blood concentration of a pitx1 repressor protein or complex in a blood sample of the subject.

[0031] In accordance with another aspect of the present invention, there is provided a method comprising: (a) identifying a subject suspected of having osteoarthritis (OA); and (b) detecting the synovial fluid concentration of a pitx1 repressor protein or complex in a synovial fluid sample of the subject.

[0032] In a specific embodiment, the osteoarthritis is selected from the group consisting of knee joint arthritis, hip joint arthritis and temporo-mandibular joints arthritis. In another specific embodiment, the osteoarthritis is knee joint arthritis. In another specific embodiment, the osteoarthritis is hip joint arthritis.

[0033] In accordance with another aspect of the present invention, there is provided a method of selecting a compound, said method comprising (a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and (b) determining a pitx1 repressor protein or complex nuclear localization in the cell sample; wherein the test compound is selected if the pitx1 repressor protein or complex nuclear localization in the cell sample is decreased in the presence of the candidate compound as compared to in the absence thereof.

[0034] In accordance with another aspect of the present invention, there is provided a method of selecting a compound, said method comprising (a)

contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and (b) assessing the sumoylation of a pitx1 repressor protein or complex in the cell sample; wherein the test compound is selected if the sumoylation of the pitx1 repressor protein or complex in the cell sample is increased in the presence of the candidate compound as compared to in the absence thereof.

[0035] In a specific embodiment, the selected test compound is potentially useful in preventing accumulation or retention of the pitx1 repressor protein or complex in cell nuclei or in promoting the pitx1 repressor protein or complex nuclear export.

[0036] In accordance with another aspect of the present invention, there is provided a method of selecting a compound, said method comprising the steps of (a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and (b) measuring the binding of a pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample, wherein the test compound is selected if the binding of the pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample is decreased in the presence of the test compound as compared to in the absence thereof.

[0037] In a specific embodiment, the selected test compound is potentially useful in the treatment of primary osteoarthritis. In another specific embodiment, the pitx1 repressor protein or complex comprises prohibitin (PHB-1). In another specific embodiment, the pitx1 repressor protein or complex comprises prohibitone (PHB-2). In another specific embodiment, the pitx1 repressor protein or

complex comprises B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR). In another specific embodiment, the pitx1 repressor protein is prohibitin (PHB-1). In another specific embodiment, the pitx1 repressor protein is prohibitone (PHB-2). In another specific embodiment, the pitx1 repressor protein is B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR). In another specific embodiment, said cell sample is from a subject having osteoarthritis. In another specific embodiment, said cell sample is an articular chondrocytes sample. In another specific embodiment, said articular chondrocytes sample is from a subject having osteoarthritis in a knee joint.

[0038] In accordance with another aspect of the present invention, there is provided a method of identifying a mutation contributing to osteoarthritis, comprising comparing the nucleotide sequence of a gene selected from the group consisting of prohibitin (PHB-1) gene, prohibitone (PHB-2) gene, B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) gene or of any gene encoding a protein causing the nuclear accumulation or retention of PHB-1, PHB-2 or BCoR in articular chondrocytes of a subject having osteoarthritis with that of the corresponding gene in a control subject.

[0039] In a specific embodiment, said gene is the PHB-1 gene. In another specific embodiment, said gene is the PHB-2 gene. In another specific embodiment, said mutation is one affecting PHB-1 or PHB-2 DNA-binding and/or its cellular localization.

[0040] In accordance with another aspect of the present invention, there is provided a method for predicting the risk of developing osteoarthritis comprising detecting in a subject the presence of a mutation directly or indirectly causing the nuclear accumulation or retention of prohibitin (PHB-1), prohibitone (PHB-2) or B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) in cells where PHB-1, PHB-2, BCoR or Pitx1 is normally expressed,

wherein the presence of the mutation is an indication that the subject has or is at risk of developing osteoarthritis.

[0041] In a specific embodiment, said method comprises detecting a mutation in CRM-1.

[0042] In accordance with another aspect of the present invention, there is provided a method of identifying a post-translational modification contributing to osteoarthritis, comprising comparing the post-translational modification of a protein selected from the group consisting of prohibitin (PHB-1) and prohibitone (PHB-2), B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) or of any protein causing the nuclear accumulation or retention of PHB-1, PHB-2 or BCoR in articular chondrocytes of a subject having osteoarthritis with that of the corresponding gene in a control subject.

[0043] In accordance with another aspect of the present invention, there is provided a method for predicting the risk of developing osteoarthritis comprising detecting in a subject, the presence of at least one post-translational modification directly or indirectly causing the nuclear accumulation or retention of prohibitin (PHB-1), prohibitone (PHB-2) or B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) in cells where PHB-1, PHB-2 or Pitx1 is normally expressed, wherein the presence of the at least one post-translational modification is an indication that the subject has or is at risk of developing osteoarthritis.

[0044] In accordance with another aspect of the present invention, there is provided a kit comprising an antibody specific to prohibitin (PHB-1) or to prohibitone (PHB-2) and an antibody specific to B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR).

[0045] In a specific embodiment, the kit of further comprises instructions to use the antibodies to predict whether a subject is at risk for developing osteoarthritis.

[0046] In accordance with another aspect of the present invention, there is provided a purified repressor complex comprising B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) and at least one of prohibitin (PHB-1) and prohibitone (PHB-2).

[0047] In a specific embodiment, the repressor complex comprises BCoR and PHB-1. In another specific embodiment, the repressor complex comprises BCoR and PHB-2. In another specific embodiment, the repressor complex comprises BCoR, PHB-1 and PHB-2.

[0048] In accordance with another aspect of the present invention, there is provided a method of using the repressor complex of the present invention, for binding to a E2F-site. In a specific embodiment, the E2F-site is that of pitx1 promoter.

[0049] The articles "a," "an" and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[0050] The term "including" and "comprising" are used herein to mean, and re used interchangeably with, the phrases "including but not limited to" and "comprising but not limited to".

[0051] The terms "such as" are used herein to mean, and is used interchangeably with, the phrase "such as but not limited to".

[0052] As used herein the term “osteoarthritis” refers to a form of arthritis involving the deterioration of the cartilage that cushions the ends of bones within joints. It is also called degenerative arthritis, degenerative joint disease or hypertrophic arthritis. This term includes early onset of osteoarthritis. Worldwide, osteoarthritis is the most common joint disorder. In western countries, radiographic evidence of this disease is present in the majority of persons by 65 years of age and in about 80 percent of persons more than 75 years of age (33). Approximately 11 percent of persons more than 64 years of age have symptomatic osteoarthritis of the knee (34).

[0053] As used herein the terms “early onset of osteoarthritis” refer to a form of osteoarthritis that either is first diagnosed at 40 years of age or earlier or that leads to knee joint replacement of the subject before he is 55 years old.

[0054] As used herein the terms “risk of developing osteoarthritis” refers to a predisposition of a subject of presenting primary OA symptoms and/or more severe primary OA symptoms at a future time. Similarly, the “risk of developing osteoarthritis in a joint where Pitx1 is normally expressed” refers to a risk for a subject of presenting primary OA symptoms, and/or more severe primary OA symptoms at a future time in a joint where Pitx1 is normally expressed.

[0055] As used herein the terms “primary OA” when used to qualify knee/hip joint OA refers to knee/hip joint OA due to a disease or degeneration for instance as opposed to secondary knee/hip joint OA resulting from trauma, joint overuse, obesity, etc.

[0056] As used herein the term “subject” is meant to refer to any mammal including human, mice, rat, dog, cat, pig, monkey, horse, etc. In a particular embodiment, it refers to a human. In another particular embodiment, it refers to a horse and more specifically a racing horse.

[0057] As used herein the terms “control DNA sample” are meant to refer to a genomic DNA that does not come from a subject known to suffer from osteoarthritis (OA) (control subject). In reference to either cartilage sections or articular chondrocytes obtained from OA versus control subjects, these controls subjects are age-matched individuals unaffected by OA as tested at the time of autopsy or at the time of biopsy when knee joints or hip joints are obtained after a trauma.

[0058] As used herein the terms “predisposition for developing a disease or condition” refers to a predisposition of a subject of presenting symptoms of the disease or condition and/or more severe symptoms of the disease or conditions at a future time.

[0059] As used herein the terms “control sample” are meant to refer to a sample that does not come from a subject known to suffer from the disease or disorder or from the subject under scrutiny but before the subject had the disease or disorder. In methods of diagnosing a predisposition of a subject to develop a disease or disorder, the sample may also come from the subject under scrutiny at an earlier stage of the disease or disorder.

[0060] As used herein the terms “subject DNA sample” are meant to refer to any biological sample from the subject from whom genomic DNA can be extracted, namely any subject tissue or cell type including saliva and blood.

[0061] As used herein the terms “cell sample” are meant to refer to a sample containing any type of cell wherein, in a subject affected by OA, PHB-1, PHB-2 and/or BCoR (also known as BCL-6 interacting corepressor, wherein BCL-6 stands for B cell lymphoma-6 transcriptional repressor) pathologically accumulates in the cell nuclei. Without being so limited, it includes articular chondrocytes, growth plate chondrocytes, osteoblasts, skeletal myoblasts and synoviocytes. As

used herein the terms "articular chondrocyte" are meant to refer to chondrocytes found in joints.

[0062] As used herein the terms "not clinically diagnosed with osteoarthritis" are meant to refer to a subject that was never diagnosed with OA using a clinical method such as an imaging method like X-ray, and magnetic resonance imaging (MRI). In particular, for diagnosing hip OA, a current clinical method recommended by the American College of Rheumatology includes hip pain and at least 2 of the following 3 features: ESR < 20 mm/hour; radiographic femoral or acetabular osteophytes; and radiographic joint space narrowing (superior, axial, and/or medial). In particular, for diagnosing knee OA, there are three methods currently recommended by the American College of Rheumatology 1) Clinical and laboratory method: knee pain and at least 5 of the following 9 features: age > 50 years, stiffness < 30 minutes, crepitus, bony tenderness, bony enlargement, no palpable warmth, ESR < 40 mm/hour, RF < 1:40; and SF OA; 2) Clinical and radiographic: knee pain, and at least 2 of the following 3 features, Age > 50 years; stiffness < 30 minutes; crepitus; + osteophytes; and 3) Clinical: knee pain and at least 3 of the following 6 features: age > 50 years, stiffness < 30 minutes, crepitus, bony tenderness, bony enlargement, no palpable warmth.

[0063] As used herein the terminology "purified", "isolated", "purification" or "isolation" in the expressions "purified polypeptide", "isolated polypeptide", "isolated protein", "purified complexes", "isolated complexes" or "tandem affinity purification" means altered "by the hand of man" from its natural state (i.e. if it occurs in nature, it has been changed or removed from its original environment) or it has been synthesized in a non-natural environment (e.g., artificially synthesized). These terms do not require absolute purity (such as a homogeneous preparation) but instead represents an indication that it is relatively more pure than in the natural environment. For example, a protein/peptide naturally present in a living organism is not "purified" or "isolated", but the same

protein separated (about 90-95% pure at least) from the coexisting materials of its natural state is "purified" or "isolated" as this term is employed herein.

[0064] As used herein, the term "antibody" or refers to an antibody that specifically binds to (interacts with) a protein of the present invention and displays no substantial binding to other naturally occurring proteins other than the ones sharing the same antigenic determinants. The term antibody or immunoglobulin is used in the broadest sense, and covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies, and antibody fragments so long as they exhibit the desired biological activity. Antibody fragments comprise a portion of a full length antibody, generally an antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, single domain antibodies (e.g., from camelids), shark NAR single domain antibodies, and multispecific antibodies formed from antibody fragments. Antibody fragments can also refer to binding moieties comprising CDRs or antigen binding domains including, but not limited to, VH regions (VH, VH-VH), anticalins, PepBodies™, antibody-T-cell epitope fusions (Troybodies) or Peptibodies. Additionally, any secondary antibodies, either monoclonal or polyclonal, directed to the first antibodies would also be included within the scope of this invention.

[0065] In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody A Laboratory Manual, CSH Laboratories). The term antibody encompasses herein polyclonal, monoclonal antibodies and antibody variants such as single-chain antibodies, humanized antibodies, chimeric antibodies and immunologically active fragments of antibodies (e.g. Fab and Fab' fragments)

which inhibit or neutralize their respective interaction domains in Hyphen and/or are specific thereto.

Diagnostic or prognostic methods

[0066] A method for diagnosing or screening for the presence of a disease or disorder or a predisposition for developing the disease or disorder in a subject, which disease or disorder is characterized by an aberrant amount, activity, protein composition, intracellular localization and/or formation of a complex, comprising the steps of: (1) comparing the amount of, activity of, protein composition of, intracellular localization of, and/or formation of said complex in a sample from the subject with that in a control sample, wherein a difference in said amount, activity, protein composition of, intracellular localization and/or formation of said complex as compared to that in the control sample is indicative that the subject has the disease or disorder or a predisposition for developing the disease or condition.

[0067] In a specific embodiment, the control sample is selected from a sample from the subject at an earlier stage of the disease or disorder or before the subject had the disease. In another embodiment, the control sample is from a different subject that does not have the disease or disorder or predisposition to develop the disease or condition.

[0068] As used herein the terms "corresponding to" in the expression "a mutation corresponding to -3727 C→T" are meant to reflect the fact that the C→T mutation occurring in the E2F-like site of the human pitx1 promoter may be found in certain subjects at a position that is upstream or downstream from the position -3727. Indeed, because of polymorphism in the pitx1 promoter within the population, the C→T mutation may be at a position other than 3727 nucleotides upstream of the transcription point of pitx1. For instance if, as compared to subjects wherein the C→T mutation occurs at position -3727, a subject has a

deletion of one nucleotide between the transcription point of *pitx1* and the position where the mutation in the E2F-like site of the human *pitx1* promoter occurs, then the position of the mutation will be -3726 in that subject. Similarly as compared to subjects wherein the C→T mutation occurs at position -3727, a subject that possesses an additional nucleotide in the region of interest, the position of the mutation will be -3728 in that subject. The method of the present invention thus encompasses determining whether there is a C→T mutation in the E2F-like site of the *pitx1* promoter at a position that corresponds to that found at position -3727 C→T in the subjects tested in the Examples presented herein. Also as used herein, the terms "corresponding to" in the expression "a mutation corresponding to -3727 C→T" is meant to encompass a G→A mutation found in the strand complementary to that containing the -3727 C-T mutation.

[0069] Other mutations encompass by the present invention include any other mutation within or adjacent the core binding site of the E2F-like site found in human *pitx1* promoter that could prevent the binding of E2Fs or enhance the recruitment and/or stabilization of a repressor complex reducing or abrogating *Pitx1* gene expression.

[0070] As used herein, the terms "joint where *Pitx1* is normally expressed" are meant to refer to, without being so limited, knee joint and hip joint.

[0071] The present invention encompasses methods for identifying a mutation corresponding to -3727 C→T within an E2F-like site in one strand of subject *pitx1* promoter. Such methods include, without being so limited, Wave nucleic acid fragment analysis (dHPLC) and direct sequencing on PCR fragments amplified from genomic DNA isolated from subjects.

[0072] The present invention also relates to methods for the determination of the level of expression of transcripts or translation product of a

single gene such as *pitx1*. The present invention therefore encompasses any known method for such determination including real time PCR and competitive PCR, Northern blots, nuclease protection, plaque hybridization and slot blots.

[0073] The present invention also concerns isolated nucleic acid molecules including probes. In specific embodiments, the isolated nucleic acid molecules have no more than 300, or no more than 200, or no more than 100, or no more than 90, or no more than 80, or no more than 70, or no more than 60, or no more than 50, or no more than 40 or no more than 30 nucleotides. In specific embodiments, the isolated nucleic acid molecules have at least 20, or at least 30, or at least 40 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 300 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 200 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 100 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 90 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 80 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 70 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 60 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 50 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 40 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 20 and no more than 30 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 300 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 200 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 100 nucleotides. In other specific

embodiments, the isolated nucleic acid molecules have at least 30 and no more than 90 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 80 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 70 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 60 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 50 nucleotides. In other specific embodiments, the isolated nucleic acid molecules have at least 30 and no more than 40 nucleotides.

[0074] Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α -nucleotides and the like. Modified sugar-phosphate backbones are generally known (62,63). Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

[0075] The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labeled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

[0076] As used herein the terms "detectably labeled" refer to a marking of a probe in accordance with the present invention that will allow the detection of the mutation of the present invention. Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labeled according to numerous well known methods (64). Non-limiting examples of labels include ^3H ,

¹⁴C, ³²P, and ³⁵S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

[0077] As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ³²P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (e.g. uniformly labeled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

[0078] The present invention also relates to methods of selecting compounds. As used herein the term "compound" is meant to encompass natural, synthetic or semi-synthetic compounds, including without being so limited chemicals, macromolecules, cell or tissue extracts (from plants or animals), nucleic acid molecules, peptides, antibodies and proteins.

[0079] The present invention also relates to arrays. As used herein, an "array" is an intentionally created collection of molecules which can be prepared either synthetically or biosynthetically. The molecules in the array can be identical or different from each other. The array can assume a variety of formats, e.g., libraries of soluble molecules; libraries of compounds tethered to resin beads, silica chips, or other solid supports.

[0080] As used herein "array of nucleic acid molecules" is an intentionally created collection of nucleic acids which can be prepared either

synthetically or biosynthetically in a variety of different formats (e.g., libraries of soluble molecules; and libraries of oligonucleotides tethered to resin beads, silica chips, or other solid supports). Additionally, the term "array" is meant to include those libraries of nucleic acids which can be prepared by spotting nucleic acids of essentially any length (e.g., from 1 to about 1000 nucleotide monomers in length) onto a substrate. The term "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides, deoxyribonucleotides or peptide nucleic acids (PNAs), that comprise purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups, as may typically be found in RNA or DNA, or modified or substituted sugar or phosphate groups. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The sequence of nucleotides may be interrupted by non-nucleotide components. Thus the terms nucleoside, nucleotide, deoxynucleoside and deoxynucleotide generally include analogs such as those described herein. These analogs are those molecules having some structural features in common with a naturally occurring nucleoside or nucleotide such that when incorporated into a nucleic acid or oligonucleotide sequence, they allow hybridization with a naturally occurring nucleic acid sequence in solution. Typically, these analogs are derived from naturally occurring nucleosides and nucleotides by replacing and/or modifying the base, the ribose or the phosphodiester moiety. The changes can be tailor made to stabilize or destabilize hybrid formation or enhance the specificity of hybridization with a complementary nucleic acid sequence as desired.

[0081] As used herein "solid support", "support", and "substrate" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for

example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations.

[0082] Any known nucleic acid arrays can be used in accordance with the present invention. For instance, such arrays include those based on short or longer oligonucleotide probes as well as cDNAs or polymerase chain reaction (PCR) products (52). Other methods include serial analysis of gene expression (SAGE), differential display, (53) as well as subtractive hybridization methods (54), differential screening (DS), RNA arbitrarily primer (RAP)-PCR, restriction endonucleolytic analysis of differentially expressed sequences (READS), amplified restriction fragment-length polymorphisms (AFLP).

[0083] “Stringent hybridization conditions” and “stringent hybridization wash conditions” in the context of nucleic acid hybridization experiments such as Southern and Northern hybridization are sequence dependent, and are different under different environmental parameters. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, 1984; $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, $\%GC$ is the percentage of guanosine and cytosine nucleotides in the DNA, $\% \text{ form}$ is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $>90\%$ identity are sought, the T_m can be decreased 10°C . Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific

sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point T_m ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point T_m ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point T_m . Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequence at a defined ionic strength and pH.

[0084] An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see 64 for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long probes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2X (or higher) than that observed for an unrelated probe in the

particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[0085] Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C.

[0086] Washing with a solution containing tetramethylammonium chloride (TeMAC) could allow the detection of a single mismatch using oligonucleotide hybridization since such mismatch could generate a 10°C difference in the annealing temperature. The formula to determine the washing temperature is $T_m (^{\circ}\text{C}) = -682 (L^{-1}) + 97$ where L represents the length of the oligonucleotide that will be used for the hybridization. When the oligonucleotide of the present invention has a length of 20 nucleotides: 5'-TCACTGGIGGCAGTCCTGCT-3' (SEQ ID NO: 2), underscore indicating the mutation, the hybridization is performed 5°C below the T_m which is calculated using the formula above at 62.9°C. In principle, a single mismatch will generate a 10°C drop in the annealing so that a temperature of 57°C should only detect mutants harbouring the T mutation. Such conditions are high stringency conditions

appropriate to identify a single nucleotide mutation in the 20 nucleotides probes of the present invention (56).

[0087] As used herein the terms “repressor protein” when used in relation to the E2F-like site of the pitx1 promoter region refer to any protein that alone and/or in combination with other proteins lowers or represses expression of Pitx1. Without being so limited, they include BCoR, PHB-1, PHB-2, combination thereof and any interacting partner of PHB-1, PHB-2 and/or BCoR including those disclosed in Table 1 above. As used herein the term “BCoR repressing activity on pitx1” or “PHB-1 repressing activity on pitx1” or “PHB-2 repressing activity on pitx1” is thus meant to refer to the activity of any of these proteins leading to decreased expression of Pitx1.

[0088] As used herein the terms “repressor complex” when used in relation to the E2F-like site of the pitx1 promoter region refers to a combination of repressor proteins that lowers or represses expression of Pitx1.

[0089] The present invention also encompasses arrays to detect and/or quantify the nuclear localization of proteins including PHB-1, PHB-2 and/or BCoR. Such arrays include protein micro- or macroarrays, gel technologies including high-resolution 2D-gel methodologies, possibly coupled with mass spectrometry (55), imaging system at the cellular level such as microscopy combined with a fluorescent labeling system.

[0090] The present invention also includes the use of tissue biopsy to determine the nuclear accumulation of PHB-1, PHB-2 and BCoR within articular chondrocytes, growth plate chondrocytes, osteoblasts, skeletal myoblasts and synoviocytes. For instance, cartilage biopsy could be performed during arthroscopy procedure to assess OA or its progression by immunofluorescence microscopy to determine the nuclear localization of PHB-1, PHB-2 and/or BCoR.

This method could be useful for instance when arthroscopy procedure is required to establish a clinical diagnostic. Alternatively, a muscle biopsy in lower limbs could be used to test whether or not PHB-1 and/or PHB-2 are accumulated in the nuclei of myoblasts. This method would advantageously be less invasive than a regular arthroscopy. The determination of the cellular localization or concentration of a pitx1 repressor protein is typically performed either by a) preparing a nuclear extract of a subject sample and determining concentration of a pitx1 repressor protein; or by (b) determining the localization of the pitx1 repressor protein by immunohistochemistry.

[0091] In accordance with the present invention, an increased pitx1 repressor protein nuclear localization in a subject may be the sign of an increased binding/affinity of the repressor protein to the pitx1's E2F-like site, of an increased nuclear entry/import of the repressor protein or a decreased nuclear export of the repressor protein. The present invention also relates to methods of selecting a compound. Hence, a compound tested with specific embodiments of methods of selecting compounds of the present invention that results in a decreased pitx1 repressor protein nuclear localization may be a compound that decreases binding/affinity of the repressor protein to the pitx1's E2F-like site, that decreases nuclear entry/import of the repressor protein or that increases nuclear export of the repressor protein.

[0092] The present invention also encompasses methods for identifying specific mutation(s) directly or indirectly affecting the function of PHB-1 and/or PHB-2. Without being so limited, mutations of interest include any mutation affecting the transport of these proteins outside the nucleus, or modifying the interactions between PHB-1 and/or PHB-2.

[0093] As used herein, the terms "mutation directly or indirectly causing the nuclear accumulation or retention of PHB-1, PHB-2 or BcoR in cells where

PHB-1, PHB-2, BCoR or Pitx1 is normally expressed" are meant to refer to, without being so limited, a mutation within the NES (nuclear export sequences) of PHB-1, PHB-2 or BCoR; a mutation modulating the formation of homomeric and heteromeric complexes between PHB-1, BCoR and/or PHB-2 resulting in the masking of their respective NES or reducing their accessibility to exportin-1 (also know as CRM-1); a mutation affecting the expression and/or function of exportin-1; a mutation affecting the formation of a ternary complex with CRM-1/exportin 1 and GTP-bound form of Ran in the nucleus; a mutation affecting TGF- β signaling including a mutation affecting binding of TGF- β with asporin, a cartilage extracellular protein elevated in OA cartilage; a mutation affecting the PHB-1 or PHB-2 recruitment of Brg-1/Brm to E2F-responsive promoters; a mutation affecting JNK1's ability to promote PHB-1's association with Brg-1 or Brn on E2F-responsive promoters; a mutation affecting Akt's binding to PHB-1-PHB-2 or Akt's ability to enter the nucleus. It also refers to any mutations in molecules interacting with PHB-1, BCoR and/or PHB-2, which could prevent the nuclear export of PHB-1 and/or PHB-2. The list of known interacting partners of PHB-1 and PHB-2 is indicated in Table 1 but it is not limited to the molecules indicated in this Table 1.

TABLE 1: LIST OF PROTEINS INTERACTING WITH PHB-1 AND/OR PHB-2

PHB-1	PHB-2
AR(37)	Akt (38)
Brg-1 (39)	ER (40)
Brm(39)	HDAC1(41)
CRM-1(42)	HDAC5(41)
E2F1(43)	NR2F1 (41)
ER (44)	NR2F2 (41)
HDAC1 (45)	PHB-1 (46)
HP1 (47)	
JNK1(47)	
MLK2(48)	
N-CoR (45)	
p53(49)	
PHB-2 (46)	
Raf (50)	
Rb (51)	

[0094] As used herein the terms "post-translational modification directly or indirectly causing the nuclear accumulation or retention of PHB-1, PHB-2 or BCoR in cells where PHB-1, PHB-2, BCoR or Pitx1 is normally expressed" includes, without being so limited, a post-translational modification including phosphorylation with kinases such as JNK1, tyrosine kinases such as MTOR, PAK, EGFR and/or JAK, ubiquitinylation or sumoylation modulating the formation of homomeric and heteromeric complexes between PHB-1, PHB-2 and/or BCoR resulting in the masking of their respective NES or reducing their accessibility to exportin-1.

[0095] The present invention relates to a kit for diagnosing OA and/or predicting whether a subject is at risk of developing OA comprising an isolated nucleic acid, a protein or a ligand such as an antibody in accordance with the present invention. For example, a compartmentalized kit in accordance with the present invention includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the subject sample (DNA genomic nucleic acid, cell sample or blood samples), a container which contains in some kits of the present invention, the probes used in the methods of the present invention, containers which contain enzymes, containers which contain wash reagents, and containers which contain the reagents used to detect the extension products. The present invention also relates to a kit comprising the antibodies which are specific to pitx1 repressors. Kits of the present invention may also contain instructions to use these probes and or antibodies to diagnose OA or predict whether a subject is at risk of developing OA.

[0096] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0097] In the appended drawings:

[0098] Figure 1 shows a bone histology from a distal end of right femur of 7-month old normal mouse (wt) and pitx1+/- (ht). Goldner staining shows in bone and calcified tissues as green and cartilage and bone marrow cells as red. A representative section of subchondral, cortical and trabecular bone thickening observed in pitx1+/- mice (b) as compared to wild-type ones (a) At higher magnification, a substantial increase in fibrillation and calcification is observed in the articular cartilage of heterozygous mice (d) as compared to wild-type ones (c). Note that original magnification of panels (a) and (b) is $\times 5$ and panels (c) and (d) is $\times 20$;

[0099] Figure 2 compares Pitx1 expression in articular chondrocytes from OA patients and matched control subjects. (a) Reverse transcription-polymerase chain reaction for pitx1 gene expression in human articular chondrocytes derived from knee cartilage of control subjects (N, n=4) and patients with osteoarthritis (OA, n=7). Pitx1 specific mRNA transcripts were detected in all control tissues (N1-N4). Loss of the pitx1 gene expression was observed in all examined OA samples (OA1-OA7) and β -actin expression was used as internal control. Immunodetection of Pitx1 proteins in human control (b) and OA (c) cartilage tissues was performed using a specific antibody against human Pitx1 protein. In control cartilage sections (n=8), specific immunoreactivity was demonstrated by intense brown staining in nucleus of chondrocytes of the

superficial and deep zones (arrow). In OA cartilage (n=8), only a few cells stained specifically for Pitx1. These were located mainly in the superficial layer (arrow). The specificity of staining was evaluated by omission of the primary antibody and by substitution of the primary antibody with non-immune IgG (Nordic Immunology, Tilburg, The Netherlands) following the same experimental protocol. No staining was observed. (Original magnification $\times 20$.) (d) Cell scores for Pitx1-positive chondrocytes, indicating significant differences between OA and normal (control) cartilage and between the deep zones for each cartilage type, by Mann-Whitney U test. Values are the mean + SEM of 8 normal and 8 OA specimens;

[00100] Figure 3 shows an alignment of the human pitx1 E2F-like DNA-binding site with known E2F sites found in mammalian cell cycle regulation promoters. Alignment of different E2F binding sites (E2F core) and conserved adjacent cyclin homology region (CHR) found in promoter of genes involved in the regulation of cell cycle. The presented E2F sites are DNA Pol α (SEQ ID NO: 3); p107 (SEQ ID NO: 4); Cdc2 (SEQ ID NO: 5); Adeno E2a (SEQ ID NO: 6); E2F-1 (SEQ ID NO: 7); H2A (SEQ ID NO: 8); DHFR (SEQ ID NO: 9); HsOrc1 (SEQ ID NO: 10); c-Myc (SEQ ID NO: 11); CycA (SEQ ID NO: 12); Cdc25 (SEQ ID NO: 13); TK (SEQ ID NO: 14); B-Myb (SEQ ID NO: 15); Hpitx1 (SEQ ID NO: 16). Positions are indicated and numbered in function of the transcriptional start site while Act and Rep indicate an activating and a repressing site respectively. Note that E2F element found in human pitx1 promoter is more distal than usual cell cycle genes;

[00101] Figure 4 shows an electrophoretic mobility shift assay (EMSA) of nuclear extracts prepared with OA articular chondrocytes. Representative EMSA using the wild type probe (a) or the mutant probe (b). Note that attempts to supershift the bound complexes were performed with different antibodies and addition of anti-BCoR antibodies disrupted partially the main complex and generated an additional band suggesting the presence of BCoR in the bound complex. (c) Representative off-rate autoradiograph showing the effect of addition

of unlabeled double-stranded oligonucleotide corresponding to E2F-like site found in human *pitx1* promoter as a competitor on the E2F complex binding to radiolabeled wild-type E2F site (wild-type probe) versus mutant E2F site found in OA patients (mutant probe). Competitor double-stranded oligonucleotide (70-fold molar excess) was added ($t=0$), and aliquots of gel mobility shift reaction mixtures were removed at the indicated times after the addition. (d) The dissociation rate of wild-type E2F and the mutant (OA) site were compared by directly counting the radioactivity of each bound E2F complex by cutting the corresponding bands on the dried gel. The half-life of binding for off-rate experiments was computed by plotting the disappearance of 50% when compared to the initial binding of E2F complex. The graph depicts the average half-life value for the E2F complex;

[00102] Figure 5 shows *in vitro* and *in vivo* characterization of PHB-1, PHB-2 and BCoR interactions in primary OA articular chondrocytes. (a) Co-immunoprecipitation were performed with anti-PHB-1, anti-PHB-2 (as internal control) and anti-E2F1 in OA and normal nuclear extracts obtained from primary human articular chondrocytes cultures. Western blot performed with anti-PHB-2 shows that PHB-2 interacts physically with PHB-1 and E2F1 only in OA nuclear extracts. (b) Chromatin immunoprecipitation (ChIP) assays were performed and showed that PHB-1 and BCoR co-localise *in vivo* with the distal E2F site identified in the human *pitx1* promoter. Amplification of a 372 bp PCR fragment were obtained with input (positive control) and after immunoprecipitation with anti-PHB-1 and anti-BCoR antibodies. Immunoprecipitation with anti-E2F1 did not generate a positive PCR amplification indicating that E2F1 does not co-localise on the *pitx1* promoter with PHB-1 and BCoR in spite of the fact that PHB-2 can interact in solution with E2F1 in OA nuclear extracts. The second lane represents a negative control generated by omission of the primary antibody during the immunoprecipitation (-Ab);

[00103] Figure 6 shows immunodetection of PHB-1 proteins in human

articular cartilage. Panels a) and b) represent cartilage section obtained from control and OA subjects respectively immunostained with anti- human PHB-1 protein (cat #RB-292-PO, Lab Vision Corp., Fremont CA, USA). Nuclear signal for PHB-1 was increased significantly in superficial and deep zones (arrow) only in OA cartilage (10X and 40X magnifications). A non specific immunostaining was detected also with the ECM in both groups (normal and OA). (c) Cell scores for PHB-1-nuclear chondrocytes, indicating significant differences between OA and normal (control) cartilage and between the zones for each cartilage type, by Mann-Whitney U test. Values are the mean \pm SEM of normal (n=3) and OA (n=3) specimens;

[00104] Figure 7 (Table S2) shows peptides (SEQ ID NOs: 17-27) sequencing results identifying PHB-1, PHB-2 and BCoR as part of the repressing complex that binds to the pitx1 E2F-like site;

[00105] Figure 8 shows EMSA using total nuclear extract (alone or with PHB-1 antibody (Prohibitin Ab-2 (RB-292-P0) Lab Vision Corp., Fremont, CA)) and PHB-1-depleted nuclear extract. The use of a PHB-1 antibody did not allow any supershift but depletion of PHB-1 in OA nuclear extract abrogated all the complexes formation bound to the wild-type radiolabeled probe;

[00106] Figure 9 shows an expression analysis by semi-quantitative RT-PCR of PHB2, PHB-1 and BCoR in human articular chondrocytes (normal vs. OA);

[00107] Figure 10 shows the nuclear localization of PHB-1 in an OA patients by immunohistochemistry using an anti-PHB-1 (Santa Cruz, sc-18196);

[00108] Figure 11 upper left panel shows a western blot of PHB-1 expressed in cytoplasm (c) and nucleus (n) of normal and OA human articular chondrocytes. Upper right panel shows two putative sumoylation sites at proximity

of a nuclear export sequence (SEQ ID NO: 68) found in human PHB-1 protein. Lower panel shows the two putative sumoylation sites at positions 202 (SEQ ID NO: 69) and 204 (SEQ ID NO: 70);

[00109] Figure 12 presents the nuclear (n) and cytoplasmic (c) protein expression of PHB-1 and Pan-SUMO in articular chondrocytes of OA patients (C73 and C74) and control patient (C75) analyzed by western blot. 3 markers are presented: Lamine (nucleus), GAPDH (cytoplasm) and ATP-Synthase (mitochondria);

[00110] Figure 13 graphically presents the repression of mir20a promoter by corepressors BCoR, PHB-1, PHB-2 and combinations thereof;

[00111] Figure 14 graphically presents the repression of human PITX1 gene promoter promoter by corepressors BCoR, PHB-1, PHB-2 and combinations thereof;

[00112] Figure 15 shows the sequence of a 10 kb pitx1 promoter region (SEQ ID NO: 28) and polymorphisms in that pitx1 promoter region between human subjects. The primers used to cover the different amplicons to cover the 10 kb regions are provided in Table 2 below;

[00113] Figure 16 shows human PHB-1 mRNA nucleotide (obtained from [gi|6031190|ref|NM_002634.2](#)) (SEQ ID NO: 29) and amino acid sequence (obtained from [gi|4505773|ref|NP_002625.1](#)) (SEQ ID NO: 30);

[00114] Figure 17 shows human PHB-2 mRNA nucleotide (obtained from [>gi|31543548|ref|NM_007273.3](#)) (SEQ ID NO: 31) and amino acid sequence (obtained from [gi|6005854|ref|NP_009204.1](#))(SEQ ID NO: 32); and

[00115] Figure 18 shows a) a sequence comprising 30 bp upstream and 30 bp downstream of the C to T mutation found in the human pitx1 promoter (SEQ ID NO: 1); b) nucleotide fragment showing restriction sites surrounding wild-type pitx1 E2F-like site (SEQ ID NO: 16) and (SEQ ID NO: 33); and c) nucleotide fragment showing restriction sites surrounding OA mutated human pitx1 E2F-like site (SEQ ID NO: 2) and (SEQ ID NO: 34).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[00116] The Applicant observed a correlation between accumulation of PHB-1, PHB-2 and BCoR in the nuclei of articular chondrocytes and OA.

[00117] The present invention is illustrated in further details by the following non-limiting examples.

Mice

[00118] Pitx1 +/- mice were prepared as previously reported (65).

Human Specimens

[00119] Human tissues were collected with the consent of patients. The Institutional Ethics Committee Board of Sainte Justine and Notre Dame Hospitals in Montreal, Canada approved the study protocol. All OA (n=58), RA (n=39) and control subjects (n=18) were evaluated by a certified rheumatologist based on the American College of Rheumatology Diagnostic Subcommittee for OA criteria (25). Of those, 93 patients (43 OA, 39 RA and 11 healthy controls) were used for the genetic study. Other patients (OA and controls) were used either for EMSA analysis, expression analysis or immunohistochemistry studies using cartilage sections.

Articular chondrocyte cultures

[00120] Cartilage was sectioned from the tibial plateaus, rinsed, finely chopped and cells released by enzymatic digestion performed as previously described (26).

[00121] The cells were seeded in Falcon culture flasks at high density (10^8 cells per 175 cm^2 flask) and grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) containing 10% heated-inactivated foetal calf serum (FCS; Hyclone, Logan, UT) and 1% penicillin/streptomycin (Gibco BRL). Only first passage cultured cells were used. Expression analysis was performed using RNA prepared from articular cartilage isolated from OA patients and age- and gender-matched control subjects.

Total RNA isolation and RT-PCR

[00122] Total RNA was prepared by phenol/chloroform extraction. For RT-PCR, 2 μg of total RNA was reversed transcribed using ThermoScriptTM reverse transcriptase (Invitrogen), and the equivalent of 0.1 μg of reverse-transcribed RNA used for PCR reactions. These were carried out in a final volume of 25 μl containing 200 micromolar dNTPs, 1,5 mM MgCl_2 , 10 pM of each primer, and 1U Pfx DNA-polymerase (Invitrogen). PCR reactions were performed using the following primers and conditions: human pitx1 (960-bp PCR product), forward primer 5'-CCCACCTCCATGGACGCCTT-3' (SEQ ID NO: 35); reverse primer 5'-GTCAGCTGTTGTACTIONGGCAGCGC-3' (SEQ ID NO: 36) (35 cycles: 94°C/45 seconds, 65°C/45 seconds, 68°C/1 minute), human β -actin (233-bp PCR product), forward primer 5'-GGAAATCGTGCGTGACAT-3' (SEQ ID NO: 37), reverse primer 5'-TCATGATGGAGTTGAATGT AGTT-3' (SEQ ID NO: 38) (32 cycles: 94°C/1 minute, 55°C/1 minute, 72°C/1 minute), human PHB2 (730-bp PCR product), forward primer 5'-GCCCAGAACTTGAAGGACTT-3' (SEQ ID NO: 39); reverse primer 5'-TCTTGCTCAGTGCTTCTCCA-3' (SEQ ID NO: 40) (30 cycles: 94°C/45 seconds, 66°C/45 seconds, 72°C/1minute), human PHB-1 (546-bp PCR product), forward primer 5'-AGTATGTGTGGTTGGGGAAT-3' (SEQ ID NO: 41); reverse primer 5'-

GCTCGCTCTGTAAGGTCGTC-3' (SEQ ID NO: 42) (30 cycles: 94°C/45 seconds, 65°C/45 seconds, 72°C/1 minute), and human BCoR (640-bp PCR product), forward primer 5'-*AAAGAGCCGGATCGCAGG-3' (SEQ ID NO: 43); reverse primer 5'-CACCATTGATGTTGAGAGGGC-3' (SEQ ID NO: 44) (35 cycles: 94°C/45 seconds, 72°C/45 seconds, 72°C/1 minute). For quantitative and semi-quantitative analysis, all amplifications were normalized against that of the housekeeping gene β -actin. PCR amplified product were separated on 1.5% agarose gel (1.0% for semi-quantitative analysis) and visualized by ethidium bromide staining.

Pitx1 promoter sequencing

[00123] One hundred (100) ng of genomic DNA was mixed in a final volume of 25 μ l containing 200 micromolar dNTPs, 1,5 mM MgCl₂, 10 pM of each primer (see Table 2 below for full list of primers used), and 1U Pfx DNA-polymerase (Invitrogen). PCR reactions were performed using the following conditions (35 cycles : 94°C/30 seconds, 60°C/30 seconds, 68°C/1 minute 20 seconds) and primers:

TABLE 2: PITX PROMOTER PRIMERS

PP1 (962 bp)	forward primer 5'-CTGTTTGCTCAAGACGCTGA-3' (SEQ ID NO: 45) reverse primer 5'-CTCGGCCTCACAAAAGAAAC-3' (SEQ ID NO: 46)
PP2 (966 bp)	forward primer 5'-TGTCTGCATT CAGGCTG TTC-3' (SEQ ID NO: 47) reverse primer 5'-GATTCCCTCCTCGAGTCCTT-3' (SEQ ID NO: 48)
PP3 (1039 bp)	forward primer 5'-CAAGTGAGCTGGATGCTGAA-3' (SEQ ID NO: 49) reverse primer 5'-AGGGAGTGTCCCTTCACAGA-3' (SEQ ID NO: 50)
PP4 (1085 bp)	forward primer 5'-GCTCAGCCATTCTCAGGAAC-3'; (SEQ ID NO: 51) reverse primer 5'-GCCATTGTCCCAGTCAAGAT-3' (SEQ ID

	NO: 52)
PP5 (1011 bp)	forward primer 5'-TCGCGTCAAGAGGGTATTTT-3' (SEQ ID NO: 53) reverse primer 5'-TAGGACCCATGGCTCTACCC-3' (SEQ ID NO: 54)
PP6 (1098 bp)	forward primer 5'-CACGAGTCAGGTGGGAAACT-3' (SEQ ID NO: 55) reverse primer 5'-GACGTCTGCTGCTTTTCTGC-3' (SEQ ID NO: 56)
PP7 (963 bp)	forward primer 5'-AGGCACGGACTAGCAGGAC-3' (SEQ ID NO: 57) reverse primer 5'-ATGCGGACGAAGCCAGAG-3' (SEQ ID NO: 58)
PP8 (986 bp)	forward primer 5'-TTAGCATTCAGCCCCTCTGT-3' (SEQ ID NO: 59) reverse primer 5'-TTCATGAGATGCAGTCAGCAG-3' (SEQ ID NO: 60)
PP9 (951 bp)	forward primer 5'-ACAACCTGGTAGGGGCAACAG-3' (SEQ ID NO: 61) reverse primer 5'-TGTGTGGCTTTGGCAAATAA-3' (SEQ ID NO: 62)
PP10 (990 bp)	forward primer 5'-GCACTGTGCTCCAACCTGTGT-3' (SEQ ID NO: 63) reverse primer 5'-GGGGGAGTGTTCTTTTCCTT-3' (SEQ ID NO: 64)

Immunohistochemistry assays

[00124] IHC assays for Pitx1 were performed using cartilage sections obtained from 8 OA patients (2 males and 6 females, 59 ± 10 years, mean \pm SEM) undergoing total knee replacement, and from 8 donors without a known history of joint disorders. These tissues were obtained post-mortem from knee condyles within 24 h of death (2 males and six females aged 67 ± 8 years, mean \pm SEM). For IHC assay with PHB-1 antibody, cartilage was obtained from 3 OA patients (61 ± 8 years, mean \pm SEM) and 3 control patients (57 ± 28 years, mean \pm SEM). Tissue specimens were embedded in paraffin, sectioned and examined by IHC for Pitx1 or PHB-1 (and counterstained with hematoxylin for PHB-1 experiments). OA

severity was previously evaluated on adjacent sections using the Mankin's histological/histochemical scale (27).

Nuclear protein extraction and EMSA

[00125] For nuclear protein extraction, cells were rinsed with cold 1X PBS and scraped in a buffer containing 10 mM hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1% NP40, 0.5 mM DTT, 1mM PMSF, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin and 10 µg/ml of pepstatin. They were left on ice for 10-20 minute while mixed regularly using a vortex. After a 3 minute centrifugation at 3000 rpm, the pellet was resuspended in a buffer containing 20 mM hepes (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 2 mM PMSF, 10 µg/ml of aprotinin, 10 µg/ml of leupeptin and 10 µg/ml of pepstatin. After a 20 minute incubation on ice and a 2 minute centrifugation at 12500 rpm, the supernatant was collected.

[00126] For the analysis of E2F DNA complexes formed in the presence of OA articular chondrocytes, gel mobility shift reactions were performed essentially as previously described (28). Nuclear extracts (5 µg of proteins) were incubated for 30 min at a temperature of between about 20-25°C in 20 µl of a solution containing 20 mM HEPES (pH 7.5), 40 mM KCl, 5% glycerol, 5 mM spermidine, and 100 ng of poly(dA-dT) and 4 µg of anti-BCoR antibodies (Abcam, ab5276) were added to specific samples (with a + sign). The following probes were then added and incubated for another 30 min at room T°: wild-type 5'-CTGTGCCTCACTGGCGGCAGTCCTGCTCAA-3' (SEQ ID NO: 65); and mutant 5'-CTGTGCCTCACTGGTGGCAGTCCTGCTCAA-3' (SEQ ID NO: 66). Samples were then separated on 7% native polyacrylamide gels (acrylamide:bis 29:1 in 0.5 x Tris-borate EDTA). Gels were run at 180 V for 3 h with a recirculation pump for the buffer and a cooling tank.

[00127] In the presence of radiolabeled probe (14ng/100,000cpm) a cold

competitor was added as indicated. For supershift analysis, the nuclear extracts were pre-incubated 30 min on ice then incubated in presence of 0,2 µg of E2Fs antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; listed in Table 3 below) and incubated on ice for 1 h.

TABLE 3 : LIST OF ANTIBODIES USED

Antibodies used for results presented in Figure 4:	
E2F1	(KH95, sc-251 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F3	(C-18, sc-878 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F4	(A20, sc-1082 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F5	(C-20, sc-1083) X Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F6	(K-20, sc-8176 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
DP1	(K-20, sc-610 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sp1	(1C6, sc-420 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sp3	(D-20, sc-644 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
BCoR	(ab5276) Abcam Inc., Cambridge, MA, USA
RXR α	(D-20, sc-553 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
Others not shown:	
E2F1	(H-137, sc-22820 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F2	(L-20, sc-632 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F3	(N-20, sc-879 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
E2F8	(H00079733-M01) Abnova Corporation, Taipei, Taiwan
DP-2	(C-20, sc-829 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sp1	(H-225, sc-14027 X) Santa Cruz Biotechnology, Santa Cruz, CA, USA

[00128] The sequences of the oligonucleotide probes were: E2F-like (wild-type) 5'-CTGTGCCTCACTGGCGGCAGTCCTGCTCAA-3' (SEQ ID NO: 65); and E2F-like mutant (OA) site, 5'- CTGTGCCTCACTGGTGGCAGTCCTGCTCAA-3' (SEQ ID NO: 66). Samples were then separated on 7% native polyacrylamide

gels (acrylamide:bis 29:1 in 0.5 x Tris-borate EDTA). Gels were run at 180 V for 3 h with a recirculation pump for the buffer and a cooling tank. For analysis of dissociation rate (off-rate), a 70-fold excess of unlabeled competitor oligonucleotide corresponding to the wild-type E2F site found in human pitx1 promoter was added after 30 min of incubation on ice with either labelled probe. Aliquots from the same binding mixture were taken at different times and frozen on dry ice to stop the reaction prior loading on gel.

Nuclear complexes precipitation by DNA pull down

[00129] Nuclear extracts (300 mg of protein) prepared from cultured human OA articular chondrocytes from knee joints were initially incubated in presence of 25 ml of streptavidin-magnetic beads slurry (BioClone Inc. San Diego, CA, USA) in order to remove unspecific protein interactions. Following this, pre-cleared nuclear extract were incubated with 5 mg of biotinylated ds probe (5'-CTGTGCCTCACTGGTGGCAGTCCTG CTCAA-3' (SEQ ID NO: 66)) for 2 h at 4°C with slow agitation in 1X EMSA binding buffer. 50 ml of streptavidin-magnetic beads slurry was added and after 1 h of incubation at 4°C the bound complexes were recovered using a magnetic stand and several washes according to the manufacturer's specifications. The complexes were then detached from the beads in a boiling mixture of NuPAGE™ 4x LDS loading buffer (Invitrogen) and β -mercaptoethanol. After centrifugation, the supernatant was loaded on a SDS-PAGE gel and visualized by Coomassie blue staining.

Co-immunoprecipitation

[00130] Nuclear and cytoplasmic extracts obtained from chondrocytes of normal or OA patients were incubated overnight with goat anti-PHB-1 antibodies (Santa Cruz, sc-18196). Immunoprecipitated proteins were collected using protein A-sepharose beads. 50 μ g of proteins were loaded and run on SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane at 100V for 70 minutes. For blotting, rabbit anti-PHB-1 antibodies (Santa Cruz, sc-28259) were

used in a 15:10000 dilution.

Transfection

[00131] The Lipofectamine™ 2000 protocol from Invitrogen (www.invitrogen.com) was followed: All amounts and volumes are given on a per well basis. A DNA (µg) to Lipofectamine™ 2000 (µl) ratio of 1:2 to 1:3 was used. Cells were transfected at high cell density for high efficiency, high expression levels, and to minimize cytotoxicity. One day before transfection, $0.5-2 \times 10^5$ cells were plated in 500 µl of growth medium without antibiotics so that cells were 90-95% confluent at the time of transfection. For each transfection sample, complexes were prepared as follows: a. DNA was diluted in 50 µl of Opti-MEM® I Reduced Serum Medium without serum and mixed gently. Lipofectamine™ 2000 was gently mixed before used, then the appropriate amount was diluted in 50 µl of Opti-MEM® I Medium and incubated for 5 minutes at room temperature. After the 5 minute incubation, the diluted DNA was combined with diluted Lipofectamine™ 2000 (total volume = 100 µl), mixed gently and incubated for 20 minutes at room temperature. The 100 µl of complexes were added to each well containing cells and medium. The plate was mixed gently by rocking back and forth. The cells were incubated at 37°C in a CO₂ incubator for 18-48 hours prior to testing for transgene expression.

Peptide sequencing analysis

[00132] Bands containing proteins from the DNA pulldown assay and EMSA were cut, washed, sliced, dried and rehydrated in ammonium bicarbonate buffer. Proteins were digested with trypsin and extracted from the gel pieces using 50% acetonitrile and 0.1% formic acid. Samples were evaporated to dryness and resuspended in 3% acetonitrile-0.1% formic acid in a final volume of 20 µl.

Mass spectroscopy analysis

[00133] Samples (10 μ l volume) were analyzed using a LC-MS/MS system consisting of Agilent™ 1100 Series nanoflow liquid chromatography system and 1100 Series LC MSD SL ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Peptides were enriched on a Zorbax™ 300SB-C18 trap column (5 μ m, 5 x 0.3 mm) and separated by reversed phase on a Zorbax™ 300SB-C18 analytical column (3.5 μ m, 150 x 0.075 mm, Agilent) with a gradient of 5-90% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. The column eluent was sprayed directly into the mass spectrometer. Spectra were interpreted using Spectrum Mill™ software (Agilent) and NCBI NR mammalian database.

Extraction parameters with Spectrum Mill™ software

[00134] Cysteine modification: carbamidomethylation; sequence tag length >1; mass range of precursor ions: 600.0 to 4000.0 Da; scan time range: 0 to 300 minutes; scans were merged for same precursor m/z: +/- 15 seconds; +/- 1.4 m/z.

Chromatin immunoprecipitation assays

[00135] ChIP experiment was carried out with the ChIP-IT™ kit (Active Motif, Carlsbad, CA, USA) ChIP-IT™ kit. Briefly, cells were cross-linked with 1% formaldehyde and collected in cell scraping solution. Nuclear extraction was then performed using lysis buffer and total DNA was sheared to an approximate length of 0.3-1.5 kb (Branson Sonifier™ 450). Chromatin was pre-cleared with Protein G beads and separated as follows: total input, negative control without antibody, and ChIP with either anti-PHB-1 (Santa Cruz), anti-BCoR (Abcam) or anti-E2F1 antibodies (Santa Cruz). After overnight incubation with the appropriate antibody, Protein G beads were added and several washes were carefully done. Chromatin was eluted from the beads using ChIP elution buffer. Cross-linking reversal was

done by heating samples at 65°C for 4h, and DNA was purified using columns provided in the kit. PCR analysis was performed using the following primers and conditions: human pitx1 promoter harbouring the E2F site (372 bp PCR product), forward primer 5'-GCTCAGCCATTCTCAGGAAC-3' (SEQ ID NO: 51); reverse primer 5'-CCACCTACCTCTTTCTGCCT-3' (SEQ ID NO: 67) (35 cycles: 94°C/45 seconds, 68°C/45 seconds, 68°C/1 minute).

Statistical analysis

[00136] The 95% confidence intervals for sensitivity, specificity, positive predictive value and negative predictive value presented in Table 4 were calculated according to Deeks and Altman (29). The association between the presence of mutation and diagnosis was assessed using the Fisher's Exact Test (two-tailed test). A p value < 0.05 was considered statistically significant.

EXAMPLE 1

Comparison of Pitx1 expression in articular chondrocytes of OA subjects with that of in articular chondrocytes matched controls

[00137] To determine whether pitx1 plays a role in the genetic control of OA onset, an expression analysis of pitx1 gene using RNA prepared from articular chondrocyte cultures derived from knee cartilage of OA patients (n=7) and age- and gender-matched control subjects (n=4) was performed. Pitx1 expression was detected only in articular chondrocytes derived from matched controls, while in OA articular chondrocytes, Pitx1 expression was abrogated or barely detectable by RT-PCR (Figure 2a). Analysis of Pitx1 protein levels and distribution in human knee joint sections showed Pitx1 proteins only in control cartilages (n=8), while Pitx1 proteins were hardly detected in OA cartilage sections (n=8) (Figure 2bcd).

EXAMPLE 2

Identification of pitx1 promoter mutation

[00138] To examine the mechanisms turning off pitx1 gene expression

in OA patients, the 5' regulatory region of human *pitx1* gene was examined for specific mutations leading to a progressive loss of Pitx1 expression during adulthood. Sequencing analysis of genomic DNA obtained from OA, rheumatoid arthritis (RA) and matched control subjects revealed, along a 10 kb promoter region of human *pitx1* gene, a single homozygous mutation (-3727 C→T) (position corresponds to distance from transcription point) affecting only OA patients (11/43) with a high frequency (25%) while none of the RA patients (0/29) and matched control subjects (0/11) had the homozygous mutation. The specificity, the positive predictive values and negative predictive values of the mutation were calculated for each group as reported in Table 4 below. A statistically significant association between the mutation and diagnosis was calculated (two-tailed test) by comparing OA versus RA patients ($p=0.002$) or by combining RA and control subjects ($p<0.001$). Heterozygous mutation was present in OA patients (3/43), RA subjects (6/29) and control subjects (3/11) although the association between the heterozygous mutation and diagnosis was not statistically significant ($p=0.14$ for OA versus RA and $p=0.09$ for OA versus control).

TABLE 4: SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE* (PPV) AND NEGATIVE PREDICTIVE VALUE (NPV) OF PITX1 mutation (-3727 C→T) for OA**

Mutation	Sensitivity	Specificity	PPV	NPV
Homozygous				
OA-Control	25.6 (13.8-39.5)	100.0 (71.5-100.0)	100.0 (71.5-100.0)	25.6 (13.8-
OA-RA		100.0 (88.1-100.0)	100.0 (71.5-100.0)	39.5)
OA-(Control & RA)		100.0 (97.6-100.0)	100.0 (71.5-100.0)	47.5 (35.2-60.0)
				55.6 (44.0-66.8)
Heterozygous				
OA-Control	7.0 (1.4-16.4)	72.7 (39.0-94.0)		
OA-RA		79.3 (60.3-92.0)		
OA-(Control & RA)		77.5 (63.4-88.9)		

[00139] Promoter sequence analysis performed with the ALGGEN PROMO™ search program (using TRANSFAC™ version 8.3; (35, 36) revealed that the homozygous mutation was localized within the core of an E2F-like site (10, 11). The sequence encompassing the E2F-like site found upstream of the human pitx1 gene is more distal although it shows an overlap with several functional E2F sites and also with an adjacent region termed cyclin homology region (CHR) found in promoter of genes involved in the regulation of cell cycle 12 (Figure 3). The transcriptional relationship between pitx1 and E2Fs is further strengthened by the works of Muller et al. showing that E2F1, E2F2 and E2F3 up regulate Pitx1 expression by several fold in osteosarcoma cell line U2OS (13). To date, eight different mammalian E2Fs have been cloned; each of them can heterodimerize with either DP1 or DP2/3 proteins and bind with similar affinity to the same collection of target sites (14). These factors could be broadly divided into two classes: activators (E2F1-3) and repressors (E2F4-8) of transcription (15), which could explain why E2F sites on a promoter do not always indicate that it is induced by E2F but, on the contrary, that it can be repressed through those sites as well (16-18).

EXAMPLE 3

Determination of functional consequences of mutation in the E2F-like site on complex binding

[00140] To determine the functional consequences of the homozygous mutation found in OA patients, it was investigated whether E2Fs were able to bind this E2F-like site using nuclear extracts prepared with OA articular chondrocytes as described above. EMSA analysis using both radiolabeled E2F-like sites (wild-type Figure 4a versus mutant Figure 4b) showed no supershift of the bound complex with any antibodies against E2Fs, or their dimerization partners DP-1 or DP-2 (E2F2, E2F8 and DP2 data not shown). The Sp1 and Sp3 transcription factors were also analysed since they bind GC-rich regions such as the E2F-like site found in the human Pitx1 promoter. Unfortunately, there was no supershift with

either anti-Sp1 or anti-Sp3 antibodies. Addition of BCoR antibodies generated the binding of an additional lower complex bound in presence of either probes although the binding was increased with the mutant one as is mostly apparent by comparing lanes 4 to 6. Functional analysis by EMSA revealed a slower dissociation rate when the complex was bound to the mutant E2F probe indicating that C→T mutation increased the stability of the bound complex.

EXAMPLE 4

Identification of BCoR as a member of the pitx1 repressor complex

[00141] In order to characterize the nature of the complex identified in Example 3, bands corresponding to the bound complex were cut and extracted from an EMSA wet gel to perform a peptide sequencing analysis combined with tandem mass spectrometry.

[00142] This experiment led to the identification of peptides corresponding to BCoR, a known co-repressor (see Figure 7). The presence of BCoR in the bound complex was confirmed also by the detection of an additional lower band in EMSA when BCoR antibodies were added (see Example 3 and Figure 4a, 4b).

EXAMPLE 5

Identification of PHB-1 and PHB-2 as members of the pitx1 repressor complex

[00143] A DNA-pull down method as described above was used with a biotinylated double-stranded oligonucleotide harboring the mutant E2F-like site to allow the identification of peptides corresponding to prohibitin (PHB-1) and prohibitone (PHB-2) (Figure 7). To confirm the presence of PHB-1 in the bound complex, an immuno-depletion of PHB-1 in nuclear extracts prepared from OA articular chondrocytes was performed and no binding was observed in EMSA with PHB-1 depleted nuclear extracts using either radiolabeled probes (Figure 8).

EXAMPLE 6**Identification of the PHB-1 and PHB-2 nucleus localization in OA and normal articular chondrocytes**

[00144] The cellular localization of PHB-1 was compared in normal and OA articular chondrocytes by IHC assays using anti-PHB-1 antibodies. As may be seen in Figure 6, an increased nuclear localization of PHB-1 was observed in OA cartilages when compared to age-matched control cartilages. See also Figure 10 for a larger magnification using different anti-PHB-1 antibodies. The cellular localization of PHB-2 is also compared in normal and OA articular chondrocytes by IHC assays using anti-PHB-2 antibodies.

EXAMPLE 7**Co-immunoprecipitation assay of PHB-1, PHB-2 and E2F1**

[00145] Co-immunoprecipitation assay of PHB-1, PHB-2 and E2F1 were performed as described above. Physical interactions between PHB-1, PHB-2 and E2F1 were detected in nuclear extracts prepared from human OA articular chondrocytes while controls did not show any interaction with PHB-2 (Figure 5a).

EXAMPLE 8**Chip assay to demonstrate the functionality of the EF2 site in OA chondrocytes and co-occupancy of PHB-1 and BCoR**

[00146] Chromatin immunoprecipitation (ChIP) assays were performed as described above with primary OA articular chondrocytes cultures to examine the co-occupancy of PHB-1, BCoR and E2F1 on the E2F-responsive element identified in human pitx1 promoter. PHB-1 and BCoR co-localised *in vivo* only in OA chondrocytes confirming also the functionality and specificity of this E2F site although E2F1 was not detected (Figure 5b). The interaction is however not dependent on the -3727 C→T mutation since wild-type also interacts with the repressor complex.

EXAMPLE 9

Identification of the -3727 C to T homozygous mutation in a subject sample

[00147] Genomic DNA is isolated (directly using available commercial kits or after extraction of lymphocytes) from the subject saliva or blood. The human pitx1 promoter region harbouring the -3727 C→T homozygous mutation is then amplified by PCR using specific primers. Amplified products are identified by direct sequencing using genomic DNA extracted from lymphocytes. They may also be digested with either restriction enzyme Acil or Fnu4H to detect the presence of the mutation. Because replacement of C by T in OA patients abrogates Acil and Fnu4HI restriction sites normally present in the wild-type sequence, the presence of the mutation are assessed by absence of relevant restriction fragments.

EXAMPLE 10**Assay to identify blood and synovial concentration levels of PHB-1, PHB-2 or native peptide agonists to cell surface PHB-1 in subjects**

[00148] PHB-1 and PHB-2 are also found in the bloodstream (Mishra et al. 2006). Diet-induced obesity was successfully reversed in mice through subcutaneous injection of a chimeric proapoptotic peptide that binds to PHB-1 at the cell surface of white fat vasculature (57). The peptide cut off the blood supply to fat tissue leading to its reabsorption. Most of the OA patients are obese. Dosage of PHB-1 in biological fluids like plasma/serum, urine or synovial fluids are performed using an ELISA method (see for instance 58).

EXAMPLE 11**Assay to identify RNA levels of PHB-1, PHB-2 and BCoR between normal and OA human articular chondrocytes**

[00149] Expression analysis was performed as described above by semi-quantitative RT-PCR of PHB-2, PHB-1 and BCoR in human articular chondrocytes (normal vs. OA). RNA was extracted from 4 normal patients and 10 OA patients. No significant change was observed between normal and OA articular chondrocytes as may be seen in Figure 9.

EXAMPLE 12**Detection of PHB-1 immunoreactive bands in nuclear and cytoplasm of human articular chondrocyte fractions from OA subjects**

[00150] Co-immunoprecipitation of PHB-1 was performed as described above.

[00151] The detection using an anti-PHB-1 antibody revealed (upper left panel of Figure 11) a major PHB-1 immunoreactive band of 32kDa, which corresponds to the molecular weight of the PHB-1 alone. PHB-1 immunoreactive bands of higher molecular weight suggest post-translational modifications of PHB-1 modifying its weight.

[00152] On Figure 11, a higher molecular weight bands pattern is detected mostly in the nuclear fraction of OA patients. This pattern is not found in the nuclear fraction of the control subject.

EXAMPLE 13**Comparison of sumoylation of PHB-1 between normal and OA human articular chondrocytes**

[00153] Sumoylation is the binding of one or more small proteins of 12kDa, designated SUMO, to another protein. SUMO proteins will lead to a laddering profile of the protein to which they are binding in a western blot. The hypothesis that the laddering of PHB-1 immunoreactive bands observed in Figure 11, was caused by sumoylation was thus tested.

[00154] SUMOsp (SUMOsp: a web server for sumoylation site prediction. Yu Xue, Fengfeng Zhou, Chuanhai Fu, Ying Xu, and Xuebiao Yao. Nucl Acids Res 34: W254-W257, 2006.) predicted that PHB-1 contains two putative sumoylation sites at proximity of a nuclear export sequence (NES), namely at positions 204 and 240. The upper right panel of Figure 11 presents the position of

these putative sites and the sequence of the NES (SEQ ID NO: 68). The table in the lower panel of Figure 11 presents the sequences of these sites, namely 202 (SEQ ID NO: 69) and 240 (SEQ ID NO: 70).

[00155] Total proteins of nuclear and cytoplasmic fractions from articular chondrocytes of OA patients (C73 and C74) and age-matched control patient (C75) were then analyzed by western blot against PHB-1 and Pan-SUMO. They were loaded and run on SDS-PAGE. Proteins were then transferred to a PVDF membrane at 100V for 70 minutes. The 3 markers used, namely lamine (nucleus), GAPDH (cytoplasm) and ATP-Synthase (mitochondria), show that the samples have been separated efficiently into a nuclear fraction (X-N) and a cytoplasmic fraction (Cyto). These antibodies were used for blotting: anti-PHB-1 (Santa Cruz, sc-28259), Pan SUMO antibodies (ABGENT, AP1299a), anti-Lamin A/C (Cell Signaling, #2032), anti-GAPDH (Santa Cruz, sc-20357) and anti-F1-ATPase (Santa Cruz, sc-16689).

[00156] Pan-SUMO immunoreactive bands in Figure 12 reveal proteins (different proteins or a single protein) modified by the binding of one or more SUMO proteins, as reflected by bands with increasing molecular weights.

[00157] Figure 12 shows that the two OA subjects have the same sumoylation pattern in the nuclear fraction, and that this pattern is absent from the control subject. Arrows (except for the 32kDa bands) point to bands that are immunoreactive to both PHB-1 and PAN SUMO, and are detected in the nuclear fraction of OA subjects but not in the control nuclear fraction. These results suggest that sumoylation contributes to PHB-1 nuclear localization in OA patients.

[00158] Clinically, this suggests that increased sumoylation is the primary event leading to PHB-1 nuclear accumulation and repression of Pitx1 in primary OA. The biological consequences of sumoylation include the increase of

protein stability, increase targeting of proteins (including transcription factors) from the cytoplasm to the nucleus, regulates transcriptional activities of proteins, mediates the binding of the protein to other proteins and increases the repressor activity of certain transcription factors. Recent studies linked sumoylation of several proteins to important diseases (neurodegenerative diseases, acute promyelocytic leukemia, type I diabetes and other disorders). The regulation of these postranslational modifications may provide new targets for therapeutic intervention in several human diseases.

[00159] PHB-1 is a molecule having a molecular weight of less than 50kDa and can passively penetrate the cell nucleus. In normal subjects, PHB-1 is quickly exported out of the cell nucleus through the exportin system by the recognition of a nucleus export signal (NES). Without being bound by such hypothesis, the proximity of the 2 sumoylation sites to the NES, could contribute to the nuclear retention of PHB-1 in OA subjects by hiding the NES and thereby prevent the recognition PHB-1 by exportin.

EXAMPLE 14

Determination of repression of mir20a promoter by co-repressors PHB-1, PHB-2, BCoR and combinations thereof

[00160] MG-63 cells were transfected as described above with Lipofectamine™ 2000 during 6 hours. After the transfection, fresh media was added to cells during 24 hours and 10^{-7} M 4-OH-Tamoxifen was added to cells during 24 hours.

[00161] The luciferase assay was then performed. Results are presented in Figure 13. Results are expressed by the fold induction of luciferase signal of reporter construct compared to that of a control. The control (CTRL) luciferase activity was that measured in osteoblastic MG-63 cells co-transfected

with mir20A reporter promoter, known to be regulated by E2Fs, from Sylvestre Y et al. 2007, pBAPE-ER and pLPC NEP-Flag vectors. The E2F1 was the condition where cells were co-transfected with E2F1-ER over-expressing vector. All luciferase signal was normalized by a beta-gal reporter vector. The X-axis represents the names of each repressor co-overexpressed in the conditions tested. This transient transfection assay shows that BCoR alone or in combinations with PHB-1 (designated PHB in Figure 13) and/or PHB-2 is sufficient to repress the induction of an E2F reporter construct like mir20a even in the presence of the mir20a agonist E2F1.

EXAMPLE 15

Determination of repression of PITX1 promoter by corepressors PHB-1, PHB-2, BCoR and combinations thereof

[00162] MG-63 cells were transfected as described above with a vector containing 3000 or 2000bp of PITX1 gene promoter upstream of a firefly luciferase gene. Cells were synchronised 16h by serum starvation. Fresh serum was added during 48h and the luciferase assay was performed. The luciferase signal was normalised against beta-gal. Results are expressed by the fold induction of luciferase signal compared to a luciferase signal from an empty vector. Results are presented in Figure 14. These results show the repression driven when BCoR, PHB-1 and PHB2 are combined together using a fragment of human pitx1 promoter (fragment -3000bp). This demonstrates the repressor activity of this complex. Without being bound by this hypothesis, it is suggested that more than one E2F-like sites may be present on the PITX1 gene.

EXAMPLE 16

Analysis of Prohibitin-CRM-1 interaction in normal and OA articular chondrocytes

[00163] PHB-1 physical interaction with CRM-1 is measured by co-IP assays as described above, while Western blot with anti-CRM-1 antibodies (Santa Cruz) are performed with nuclear extracts prepared with normal and OA articular

chondrocytes to assess changes in CRM-1 levels in normal versus OA cartilage cells. In parallel, functionality of CRM-1 is tested by an immunostaining method. Cells are either untreated or treated with 30 μ M camptothecin (Sigma) for 4 h, a topoisomerase I inhibitor that stimulates the nuclear export of PHB-1 through a CRM-1-dependent mechanism. Subcellular localisation of p53 is used as internal control since p53 normally co-localises with PHB-1 and migrate to the cytoplasm upon camptothecin treatment. Cells are fixed in 4% PFA for 5 min and blocked in 5% BSA in PBS buffer at RT for 1 h, followed by primary antibody incubations overnight at 4°C. After washing, secondary antibody incubation is performed with goat anti anti-mouse IgG Alexa™ Fluor-488 (green) and goat anti-rabbit IgG Alexa™ Fluor-546 (red) for 30 min at room temperature. Cells are visualised with a Zeiss™ LSM 510 confocal microscope. Finally, the contribution of TGF- β signalling to PHB-1 nuclear export is investigated since it was reported that TGF- β 1 mediates the nuclear export of PHB-1 in a prostate cancer cell line.

EXAMPLE 17

OA genetic association studies

[00164] Genotyping of the (-3727 C→T) found in the promoter of human pitx1 gene is performed in a large cohort that currently comprises 1,400 cases (510 males and 890 females) and 750 controls (350 females and 400 males) well characterised. The cases have each undergone elective joint-replacement surgery (hip or knee) due to severe, end-stage primary OA. The controls have no symptoms of OA or of any other joint or musculoskeletal disease. The cases and controls are all aged 45 or over, they are unrelated to each other and are of UK Caucasian origin. The DNA from the cases and the controls are arrayed on to 96-well microtitre plates at concentrations of 100ng/ μ l and 10ng/ μ l. Results are analysed in function of differences in genotype or allele frequencies between OA cases and controls. The cases are stratified by sex, age, by joint replaced or by sex combined with joint replaced or age combined with joint replaced.

[00165] Genetic association, and Hardy-Weinberg equilibrium for the distribution of genotypes, are tested by χ^2 analysis with Yates's correction. Odds ratios are calculated with 95 percent confidence intervals. The pair wise linkage disequilibrium coefficient $(r^2)^2$ is calculated using the GOLD™ program (59). Haplotype frequencies between variants showing evidence of linkage disequilibrium at $r^2 > 0.2$ is estimated using the EH-PLUS™ program (60). Haplotype frequency differences are then compared using χ^2 .

EXAMPLE 13

Characterisation of nuclear factors known to interact with PHB-1 and/or PHB-2

[00166] Additional partners of PHB-1 and PHB-2 are expected to exist since there are no indications in the literature that either molecule can bind directly to the DNA to exert their transcriptional repression. It was shown that prohibitin recruits Brg-1/Brm to E2F-responsive promoters, and that this recruitment is required for the repression of E2F-mediated transcription by PHB-1. Although PHB-1 associates with, and recruits, Brg-1 and Brm independently of pRb, prohibitin/Brg-1/Brm-mediated transcriptional repression requires pRb. PHB-1 and PHB-2 mediated transcriptional repression required also histone-deacetylase activity (HDAC1), but unlike pRb, additional co-repressors like N-CoR are also involved (24,41). In addition, PHB-2 also associates with the class II histone deacetylase HDAC5. Finally, it was reported that PHB-2 specifically interacts with the chicken ovalbumin upstream binding transcription factors I and II (COUP-TFI and COUP-TF-II). The nuclear receptor chicken ovalbumin upstream binding transcription factor I was found to cooperate with PHB-2 and histone deacetylases in the repression of target genes. PHB-1 and PHB-2 thus appear to repress E2F-mediated transcription utilising different molecular mediators and facilitate channelling of specific signalling pathways to the cell cycle machinery.

[00167] Whether pitx1 repression is mediated by recruitment of Brg-1/Brm to this E2F site is investigated. The contribution of specific histone

deacetylases (HDACs) is also investigated. To test whether Brg-1, Brm and prohibitin physically interact in OA nuclear extracts, co-immunoprecipitation studies are carried out using nuclear extracts from OA articular chondrocytes, which contain PHB-1 and PHB-2 endogenously. Immunoprecipitation are then performed with anti-cmyc (negative control), anti-Brg-1 or anti-Brm antibodies, and the precipitated proteins are then immunoblotted for PHB-1 or PHB-2. Normal articular chondrocytes obtained from control subjects or purchased (PromoCell) are tested in parallel as negative controls. Positive controls are generated with the use of anti-E2F1, anti-E2F2 and anti-E2F3 antibodies since E2F1 and E2F3 mRNA were detected in normal and OA articular chondrocytes.

[00168] The same approach is used to determine whether additional known partners of PHB-1 and PHB-2 are present in the repressor complex detected in OA patients (the candidate proteins are indicated in Table 1 above). Notably, the presence of pRb or of one of its family members (p107 and p130) are investigated since prohibitin/Brg-1/Brm-mediated transcriptional repression was shown to require pRb. To assess whether PHB-1 co-localises *in vivo* with Brg-1 and Brm to the E2F-responsive element found in the human pitx1 promoter, chromatin immunoprecipitation (ChIP) assays, using anti-Brg-1 and anti-Brm antibodies are performed. ChIP assays are performed with OA articular chondrocytes derived from at least ten distinct OA patients according to the manufacturer's specifications (ActiveMotif, Carlsbad CA, USA). Articular chondrocyte cultures are obtained from distinct OA patients in order to investigate whether these factors and repressing mechanism are predominantly found only in a specific subset of OA patients or they are conserved among all primary OA patients (excluding secondary knee joint OA). Presence of the mutation is determined by direct sequencing of the promoter region harbouring the E2F-like site for each OA and control subjects to assess whether the mutation seen in human pitx1 promoter has effects in the recruitment of different nuclear interacting partners. Whether pRb or one of its family members (p107 and p-130) is also

recruited *in vivo* with Brg-1 and Brm is investigated. Control experiments is carried out in parallel with normal human articular chondrocytes using anti-E2F1, anti E2F-2 and anti-E2F3 antibodies as positive controls to demonstrate that this E2F site is also subjected to a positive transcriptional regulation by E2Fs in normal cartilage cells. Negative controls will be provided by anti-PHB-1 and anti-PHB-2 antibodies.

EXAMPLE 18

Determination of mechanisms modulating prohibitins interactions with Brg-1/Brm in OA

[00169] Several reports showed that JNK1 promotes the association of PHB-1 with Brg-1 or Brm on E2F-responsive promoters and represses the transcriptional activity of E2F. The finding that JNK1 phosphorylates PHB-1 (*in vitro*) and regulates prohibitin/Brg1/Brm associations *in vivo* now adds a testable potential mechanistic link to this pathway. Furthermore, PHB-1 interacts with MLK2, a binding partner of, activator of, and substrate for JNK1. To determine whether JNK1 could affect the physical association between PHB-1 and Brg-1/Brm, co-IP methods are used with normal and OA articular chondrocyte cultures untreated or treated with 100 μ M of SP600125, a specific inhibitor of JNK1 for two hours (Stressgene Bioreagents, Ann Arbor, MI, USA). The effect of JNK1 on the recruitment of PHB-1, PHB-2, Brg-1 and Brm to the endogenous and mutant E2F site in the human pitx1 promoter is then tested by ChIP assays.

[00170] Akt subcellular localisation is compared in normal and OA chondrocytes by IHC methods as well as by Western blot analysis with corresponding cytoplasmic and nuclear extracts. Detection of Akt and phospho-Akt (pAkt) is preformed with specific antibodies (Santa Cruz). In the event that cytoplasmic Akt is predominantly detected in OA cells, Akt phosphorylation is stimulated by adding physiological doses of 17- β -estradiol (10^{-10} M, Sigma) since estrogenic stimulation was reported to promote accumulation of activated Akt in the nucleus (61).

[00171] Additional PHB-1 and PHB-2 interacting partners such as HDAC1 and HDAC5 are tested while others are determined by DNA-pull down method. The analysis of the proteins complexed is carried-out by peptide sequencing coupled to mass spectrometry as described above. The role of estrogens in Akt activation and nuclear localisation is of interest in OA pathogenesis because postmenopausal women are more affected by OA than males after 65 years.

EXAMPLE 19

Determination of post-translational modifications masking/interfering with NES of PHB-1

[00172] There is evidence that PHB-1 and PHB-2 can undergo post-translational modification such as phosphorylation and ubiquitination, but the role of post-translational modifications in modulating the various functions of the prohibitins has yet to be determined. *In-silico* analysis revealed that PHB-1 NES can be phosphorylated by tyrosine kinases MTOR, PAK, EGFR and JAK. The effect of individual kinases is tested by adding specific kinase inhibitors to the cells prior to analysing PHB-1 interaction with CRM-1 as described above.

[00173] Whether the NES of PHB-1 is a substrate for an unknown kinase or any other enzymatic action reducing its interaction with CRM-1 is also tested. A peptide corresponding to the NES of PHB-1 is synthesized and conjugated to a carrier peptide, penetratin™ (Q-Biogene), for delivery into the cells (cytoplasm and nucleus). The effect of delivering this peptide on the nuclear export of PHB-1 is assessed by double immunofluorescence experiments. Conformation changes in PHB-1-PHB-2 heterodimers or homodimers are also examined by cross-linkage analyses and Western blot.

[00174] Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from

the spirit and nature of the subject invention as defined in the appended claims.

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CLAIMS:

1. A method comprising:
determining the cellular localization of a pitx1 repressor protein or complex selected from the group consisting of prohibitin (PHB-1), prohibitone (PHB-2), B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR), PHB-1-containing complex, PHB-2-containing complex and BCoR-containing complex, in a subject cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample.
2. The method of claim 1, wherein said method further comprises determining whether the repressor protein or complex nuclear concentration is higher in the subject cell sample as compared to that in a control cell sample;
wherein a higher repressor protein or complex nuclear concentration in the subject cell sample is indicative that the subject is at risk of developing osteoarthritis.
3. A method comprising:
 - (a) identifying a subject suspected of having osteoarthritis (OA); and
 - (b) detecting the blood concentration of a pitx1 repressor protein or complex in a blood sample of the subject.
4. A method comprising:
 - (a) identifying a subject suspected of having osteoarthritis (OA); and
 - (b) detecting the synovial fluid concentration of a pitx1 repressor protein or complex in a synovial fluid sample of the subject.
5. The method of any one of claims 1 to 4, wherein the osteoarthritis is selected from the group consisting of knee joint arthritis, hip joint

arthritis and temporo-mandibular joints arthritis.

6. The method of any one of claims 1 to 4, wherein the osteoarthritis is knee joint arthritis.

7. The method of any one of claims 1 to 4, wherein the osteoarthritis is hip joint arthritis.

8. A method of selecting a compound, said method comprising
(a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and

(b) determining a pitx1 repressor protein or complex nuclear localization in the cell sample;

wherein the test compound is selected if the pitx1 repressor protein or complex nuclear localization in the cell sample is decreased in the presence of the candidate compound as compared to in the absence thereof.

9. A method of selecting a compound, said method comprising
(a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and

(b) assessing the sumoylation of a pitx1 repressor protein or complex in the cell sample;

wherein the test compound is selected if the sumoylation of the pitx1 repressor protein or complex in the cell sample is increased in the presence of the candidate compound as compared to in the absence thereof.

10. The method of claim 8 or 9, wherein the selected test

compound is potentially useful in preventing accumulation or retention of the pitx1 repressor protein or complex in cell nuclei or in promoting the pitx1 repressor protein or complex nuclear export.

11. A method of selecting a compound, said method comprising the steps of

(a) contacting a test compound with a cell sample selected from the group consisting of an articular chondrocytes sample, a growth plate chondrocytes sample, an osteoblasts sample, a skeletal myoblasts sample and a synoviocytes sample; and

(b) measuring the binding of a pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample,

wherein the test compound is selected if the binding of the pitx1 repressor protein or complex on pitx1's E2F-like site in the cell sample is decreased in the presence of the test compound as compared to in the absence thereof.

12. The method of claim 9 or 11, wherein the selected test compound is potentially useful in the treatment of primary osteoarthritis.

13. The method of any one of claims 1-12, wherein the pitx1 repressor protein or complex comprises prohibitin (PHB-1).

14. The method of any one of claims 1-12, wherein the pitx1 repressor protein or complex comprises prohibitone (PHB-2).

15. The method of any one of claims 1-12, wherein the pitx1 repressor protein or complex comprises B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR).

16. The method of any one of claims 1-12, wherein the pitx1

repressor protein is prohibitin (PHB-1).

17. The method of any one of claims 1-12, wherein the pitx1 repressor protein is prohibitone (PHB-2).

18. The method of any one of claims 1-12, wherein the pitx1 repressor protein is B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR).

19. The method of any one of claims 1-2 and 8-18, wherein said cell sample is from a subject having osteoarthritis.

20. The method of any one of claims 1-2 and 8-19, wherein said cell sample is an articular chondrocytes sample.

21. The method of claim 20, wherein said articular chondrocytes sample is from a subject having osteoarthritis in a knee joint.

22. A method of identifying a mutation contributing to osteoarthritis, comprising comparing the nucleotide sequence of a gene selected from the group consisting of prohibitin (PHB-1) gene, prohibitone (PHB-2) gene, B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) gene or of any gene encoding a protein causing the nuclear accumulation or retention of PHB-1, PHB-2 or BCoR in articular chondrocytes of a subject having osteoarthritis with that of the corresponding gene in a control subject.

23. The method of claim 22, wherein said gene is the PHB-1 gene.

24. The method of claim 22, wherein said gene is the PHB-2 gene.

25. The method of claim 23, wherein said mutation is one

affecting PHB-1 or PHB-2 DNA-binding and/or its cellular localization.

26. A method for predicting the risk of developing osteoarthritis comprising detecting in a subject the presence of a mutation directly or indirectly causing the nuclear accumulation or retention of prohibitin (PHB-1), prohibitone (PHB-2) or B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) in cells where PHB-1, PHB-2, BCoR or Pitx1 is normally expressed, wherein the presence of the mutation is an indication that the subject has or is at risk of developing osteoarthritis.

27. The method of claim 26, wherein said method comprises detecting a mutation in CRM-1.

28. A method of identifying a post-translational modification contributing to osteoarthritis, comprising comparing the post-translational modification of a protein selected from the group consisting of prohibitin (PHB-1) and prohibitone (PHB-2), B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) or of any protein causing the nuclear accumulation or retention of PHB-1, PHB-2 or BCoR in articular chondrocytes of a subject having osteoarthritis with that of the corresponding gene in a control subject.

29. A method for predicting the risk of developing osteoarthritis comprising detecting in a subject, the presence of at least one post-translational modification directly or indirectly causing the nuclear accumulation or retention of prohibitin (PHB-1), prohibitone (PHB-2) or B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) in cells where PHB-1, PHB-2 or Pitx1 is normally expressed, wherein the presence of the at least one post-translational modification is an indication that the subject has or is at risk of developing osteoarthritis.

30. A kit comprising an antibody specific to prohibitin (PHB-1) or to prohibitone (PHB-2) and an antibody specific to B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR).

31. The kit of claim 30 further comprising instructions to use the antibodies to predict whether a subject is at risk for developing osteoarthritis.

32. A purified repressor complex comprising B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR) and at least one of prohibitin (PHB-1) and prohibitone (PHB-2).

33. The repressor complex of claim 32 comprising BCoR and PHB-1.

34. The repressor complex of claim 32 comprising BCoR and PHB-2.

35. The repressor complex of claim 32 comprising BCoR, PHB-1 and PHB-2.

36. A method of using the repressor complex of any one of claims 32-35, for binding to a E2F-site.

37. The method of claim 36, wherein the E2F-site is that of pitx1 promoter.



Pitx1 +/+

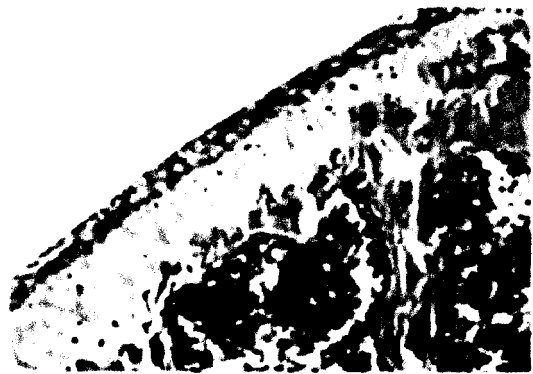


FIG. 1A

FIG. 1B



Pitx1 +/-

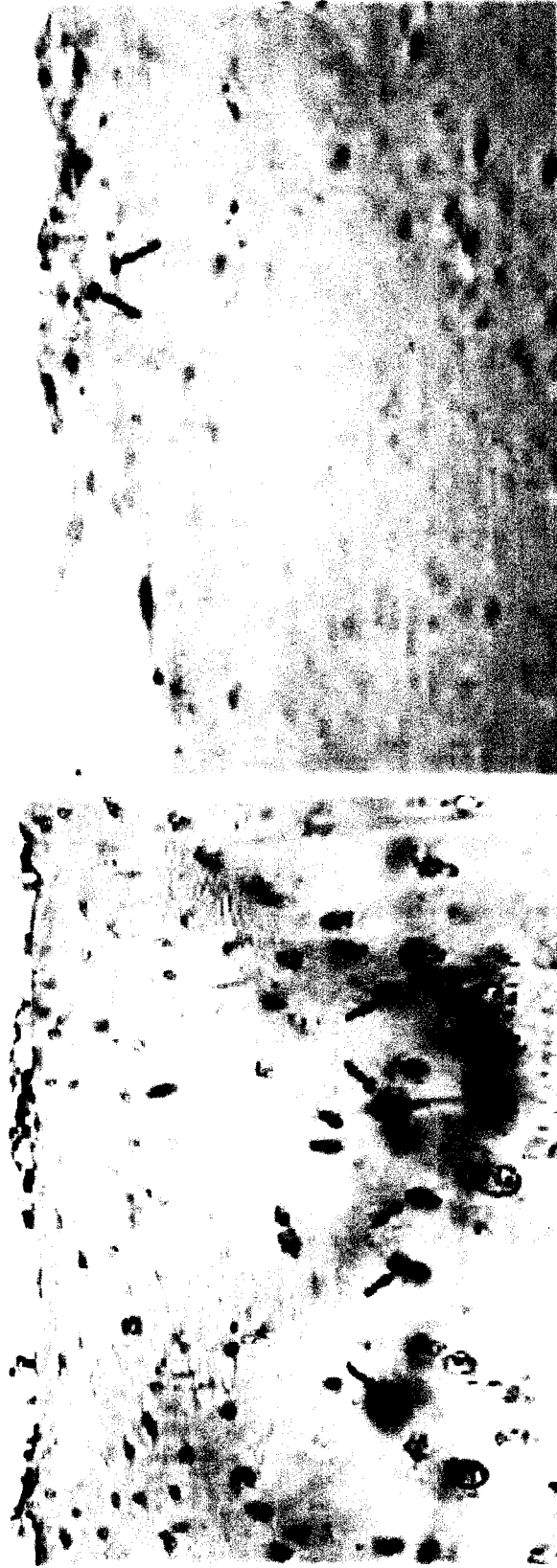


FIG. 1C

FIG. 1D



FE10-2A



FE10-2B

FE10-2C

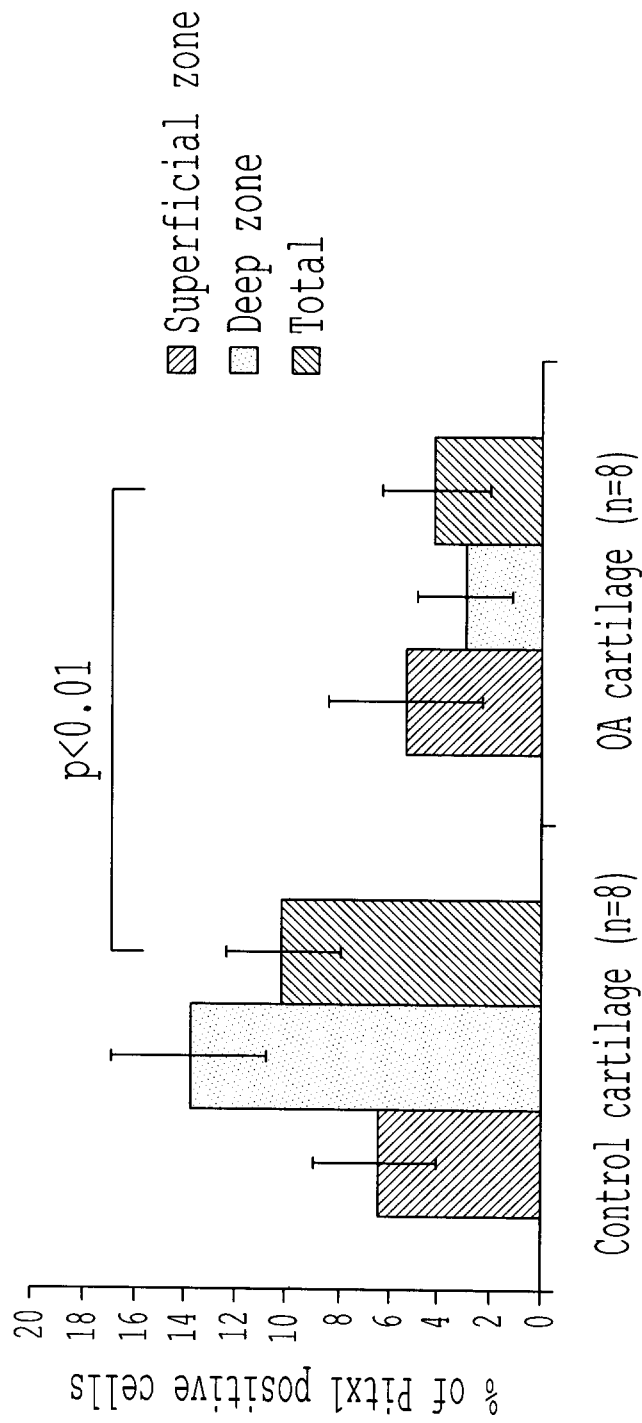


FIG. 20

	E2F core	CHR	
DNA Pol α	CGTTTGGCGGCC-----TGTGAT		-143 Act
p107	ATTTTCGGCGGC-----TTTGGC		-1 Rep
Cdc2	TTTAGCGCGGT--GAGTTTGAA		-8 Rep
Adeno E2a	GTTTTCGGCGCT-TAAATTTGAG		-49 Act
E2F-1	GATTTGGCGCG-TAAAAGTGGC		+5 Rep
H2A	TTTTTCGGCGCCAA-TAGTGT		-31 Act
DHFR	AATTTTCGGCGC--AAACTTGGG		+18 Act
HsOrcl	AACTTCGGCGC-----AATCGGC		-14 Rep
c-Myc	TTTTTCCCGCCAAGCCTCTGAG		-77 Act
CycA	ATAGTCGGCG--GATACCTGAA		-22 Rep
Cdc25	GGGCTGGCGGA--AGGTTTGAA		-2 Rep
TK	GACCTGGCGG--AGATTTGGC		-106 Act
B-Myb	CACCTGGCGG--AGATAGGAA		-32 Rep
hPitx1	TCACCTGGCGGC--AGTCCTGCT		-3715 Act/Rep
	* **		*

FIG. 3

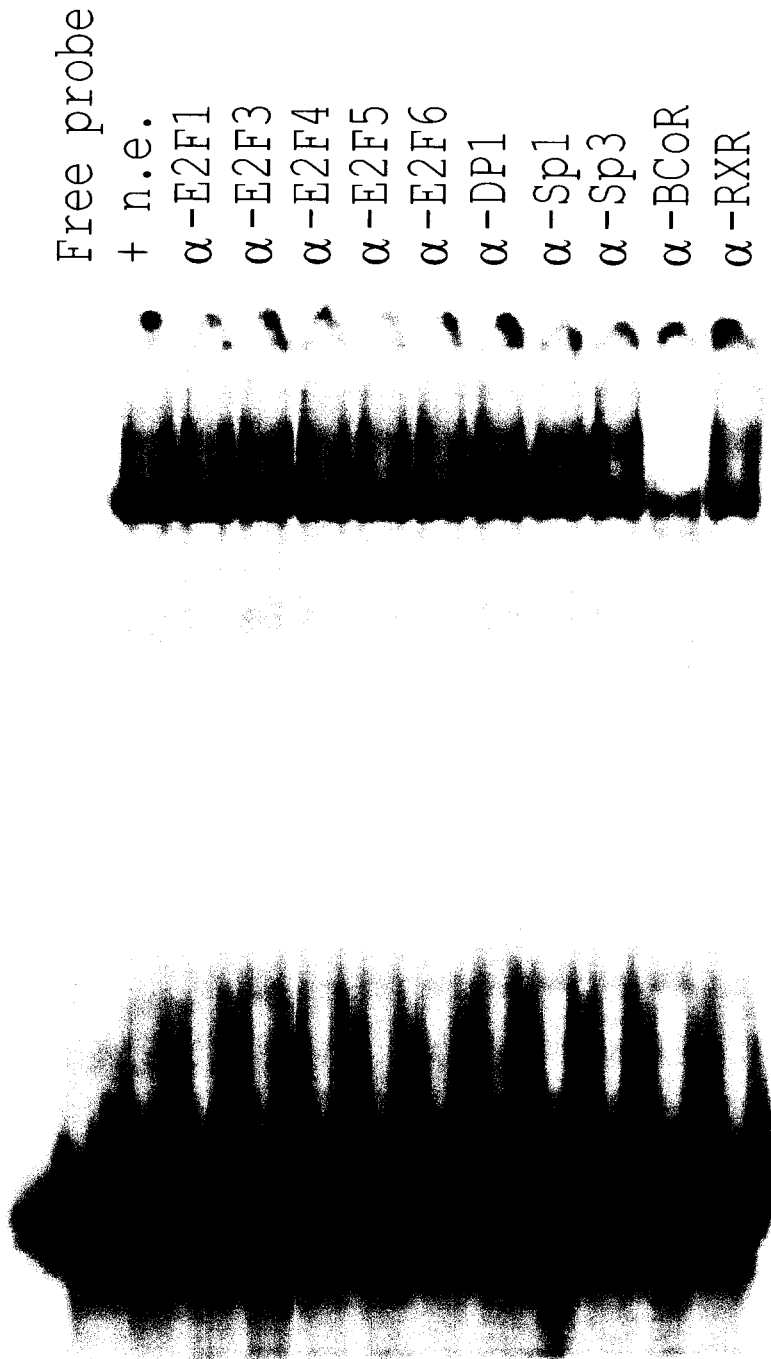


FIG. 4A

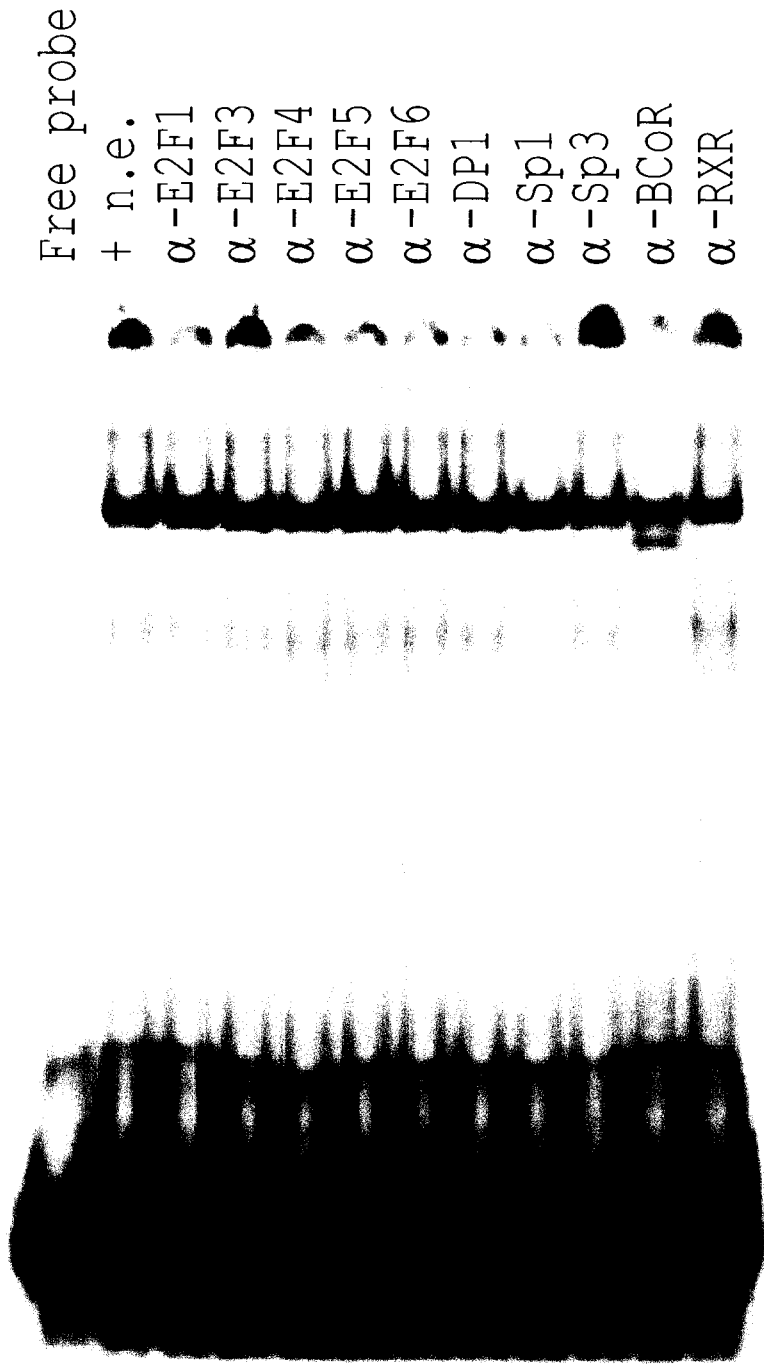


FIG. 4B

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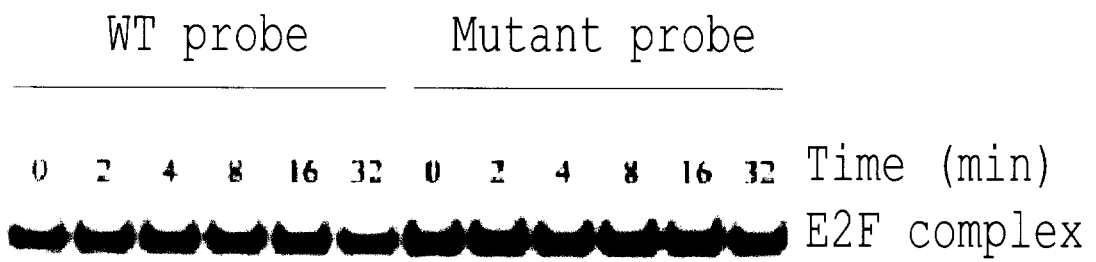


FIG. 4C

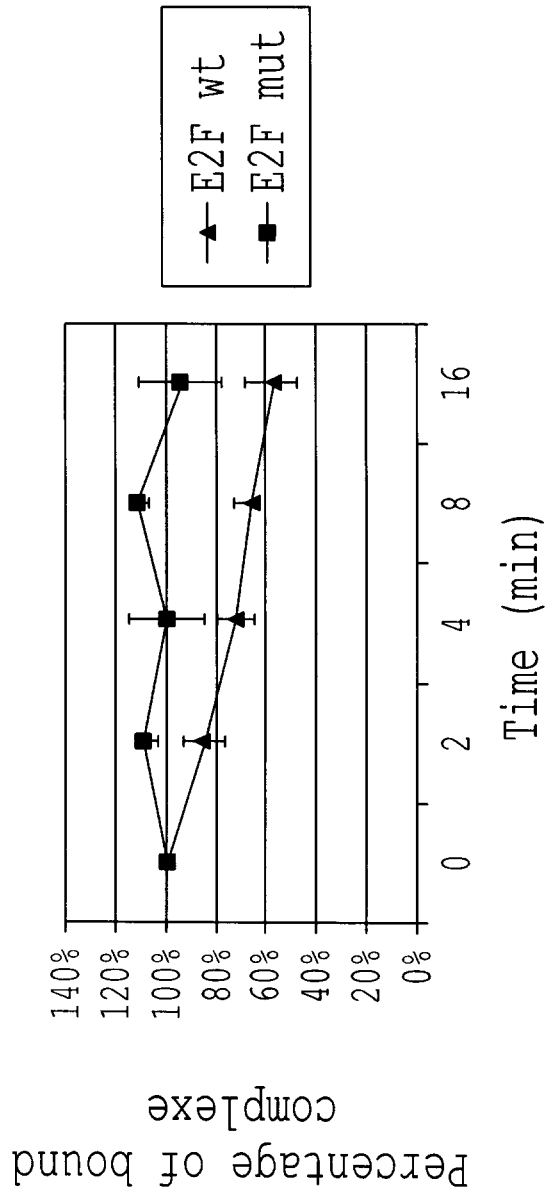


FIG. 4D

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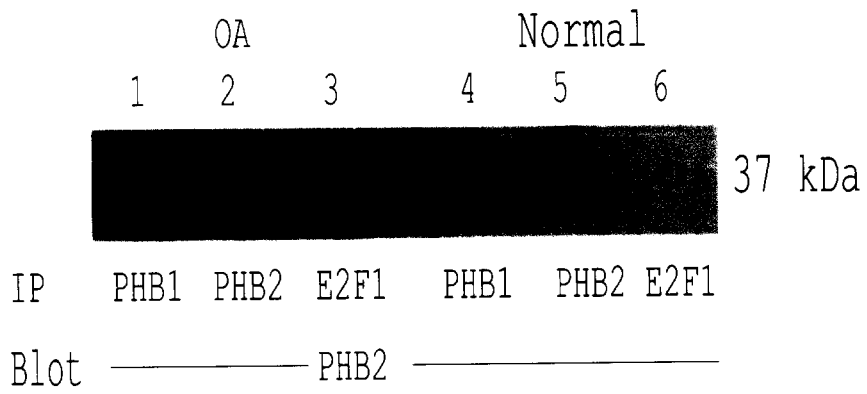


FIG. 5A

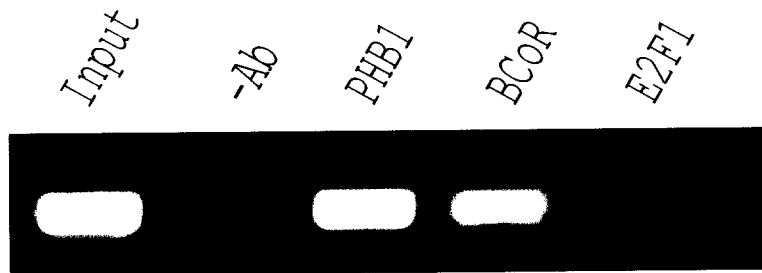


FIG. 5B

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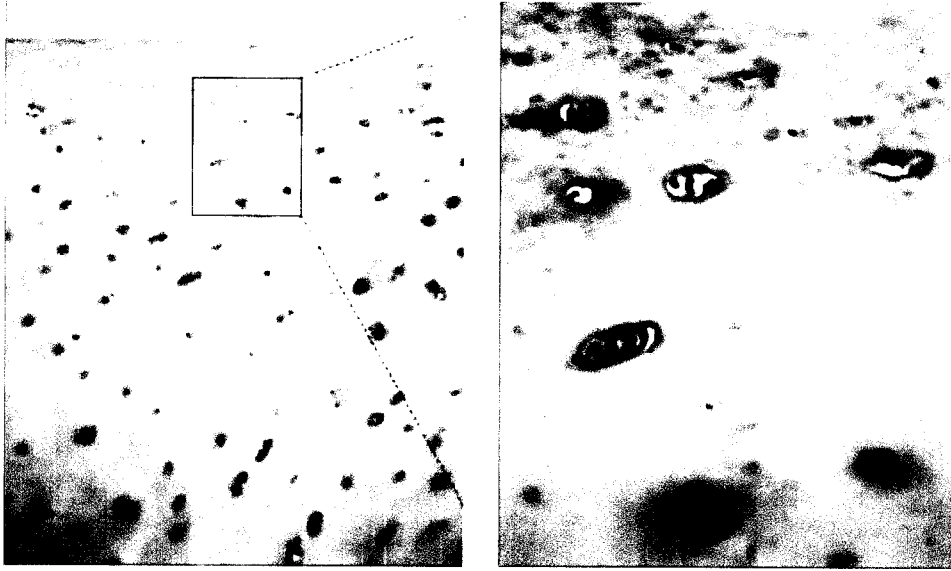


FIG. 6A

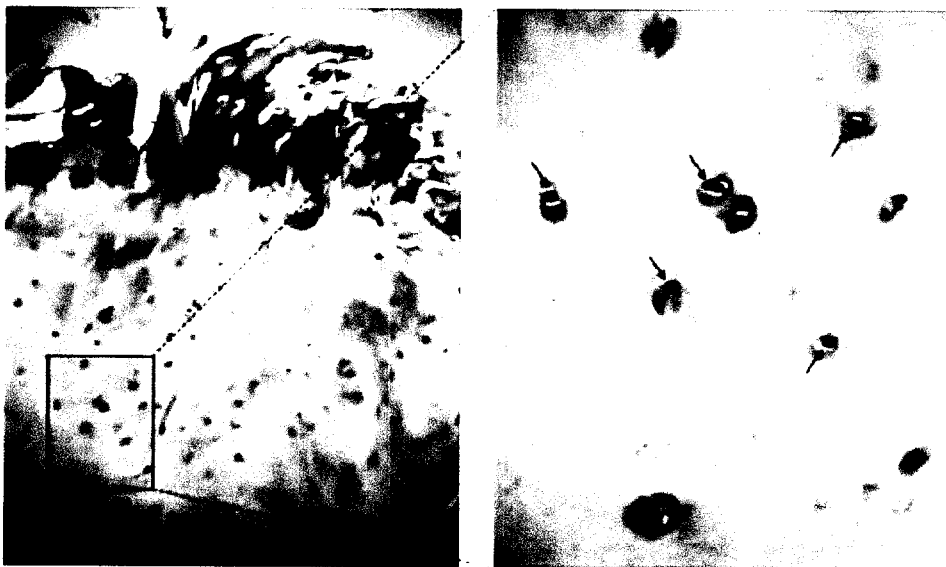


FIG. 6B

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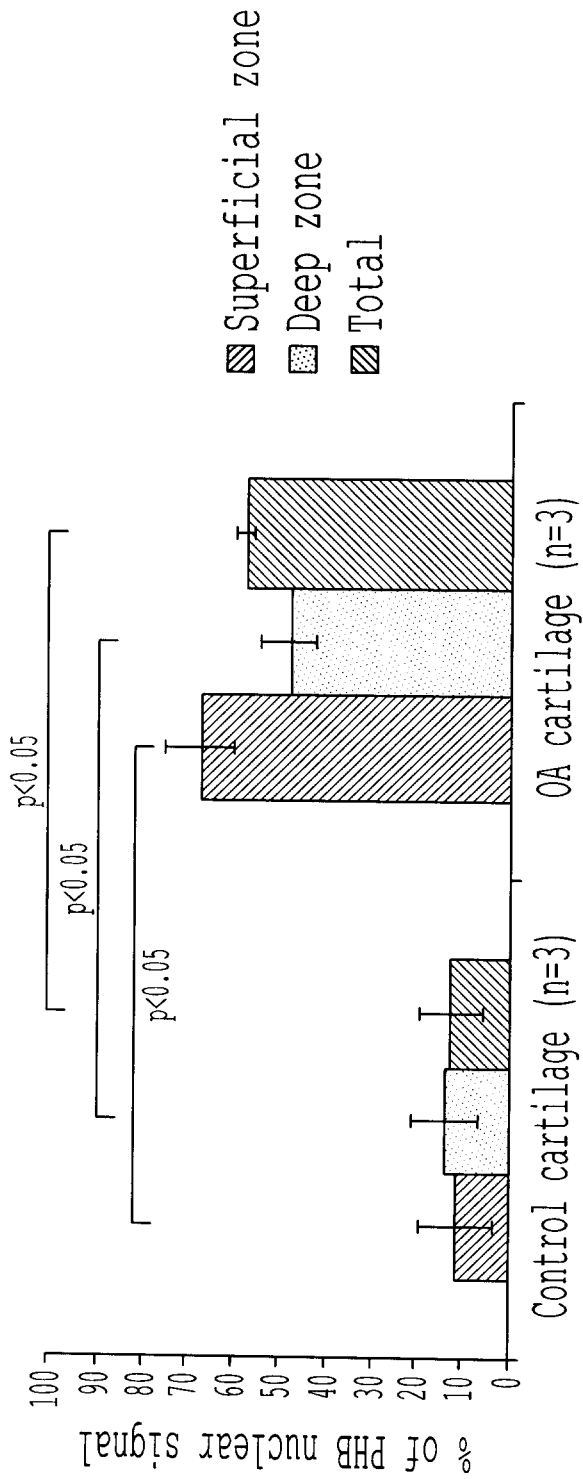


FIG. 6C

Table S2. Identification of peptides corresponding to nuclear factors interacting with the mutant E2F site found in human Pitx1 promoter in OA articular Chondrocytes.

Group	Spectra	Distinct	Distinct	%AA	Mean	Dababase	
(#)	(#)	Peptides	Summed	Coverage	Peptide	Accession #	Protein Name
		(#)	MS/MS		Spectral	Species	
			Search		Intensity		
1	1	1	9.35	2	1.41e+009	Homo sapiens	20149302 BCL- interacting corepressor isoform 2
1	1	1	9.35	1	1.41e+009	Homo sapiens	21071037 BCL- interaction corepressor isoform 1
1	1	1	9.35	1	1.41e+009	HUMAN	57012588 BCoR protein (BCL-6 corepressor)

#	Filename	Z	Score	MH* Matched (Da)	Spectrum Intensity	Sequence
1	1 GelShift_0273.0437.3	3	9.35	2781.563	1.41e-009	(R) VPSAKAVTSGIPGSTALLPPSPR(V) SEQ ID NO: 17

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7

Group	Spectra	Distinct	Distinct	%AA	Mean	Dabatase
(#)	(#)	Peptides	Summed	Coverage	Peptide	Accession #
		(#)	MS/MS		Spectral	Protein Name
			Search		Intensity	
			Score		Homo sapiens	
1	5	5	65.87	21	3.73e+006	46360168 prohibitin
1	5	5	65.87	21	3.73e+006	49456373 PHB
#	Filename	Z	Score	MH* Matched (Da)	Spectrum Intensity	Sequence
1	180306000010.0625.0635.2	2	16.01	1149.590	4.59e+006	(R)FDAGELIYTOR(E) SEQ ID NO: 18
2	180306000010.0990.0993.2	2	14.96	1855.033	5.95e+006	(R)NITVLPAGQSVLLQLPQ(-) SEQ ID NO: 19
3	180306000010.0613.0617.0	2	13.46	1185.659	3.13e+006	(K)DLQNVNITLR(I) SEQ ID NO: 20
4	180306000010.0925.0929.0	3	13.44	2098.166	4.00e+006	(R)SRNITVLPAGQSVLLQLPQ(-) SEQ ID NO: 21
5	180306000010.1023.1023.0	2	8.00	1998.087	9.55e+005	(K)AELIANSIATAGGGLIEIR(K) SEQ ID NO: 22

FIG. 7 (continued)

Group	Spectra	Distinct	Distinct	%AA	Mean	Database
(#)	(#)	Peptides	Summed	Coverage	Peptide	Accession #
		(#)	MS/MS		Spectral	Protein Name
			Search		Intensity	
			Score		Intensity	
			42.96	26	1.76e+006	Homo sapiens
			5	5		1673514
						B-cell receptor-associated protein

#	Filename	Z	Score	MH*	Matched	Intensity	Sequence
				(Da)			
1	180306000012.0390.0401.0	3	9.23	1602.856	1.34e+005	(R)AQNISKITATSONR(I) SEQ ID NO: 23	
2	180306000012.0382.0385.2	2	9.15	1471.776	4.74e+005	(R)QKIVQEGEFAFAK(M) SEQ ID NO: 24	
3	180306000012.0498.0501.0	2	8.96	994.485	5.02e+006	(R)IGDYVER(V) SEQ ID NO: 25	
4	180306000012.0621.0626.0	2	8.16	899.568	2.24e+006	(R)AOVSLIR(R) SEQ ID NO: 26	
5	180306000012.0890.0890.0	2	7.46	1210.741	9.33e+005	(R)VLPSTVNEVLK(S) SEQ ID NO: 27	

FIG. 7 (continued)

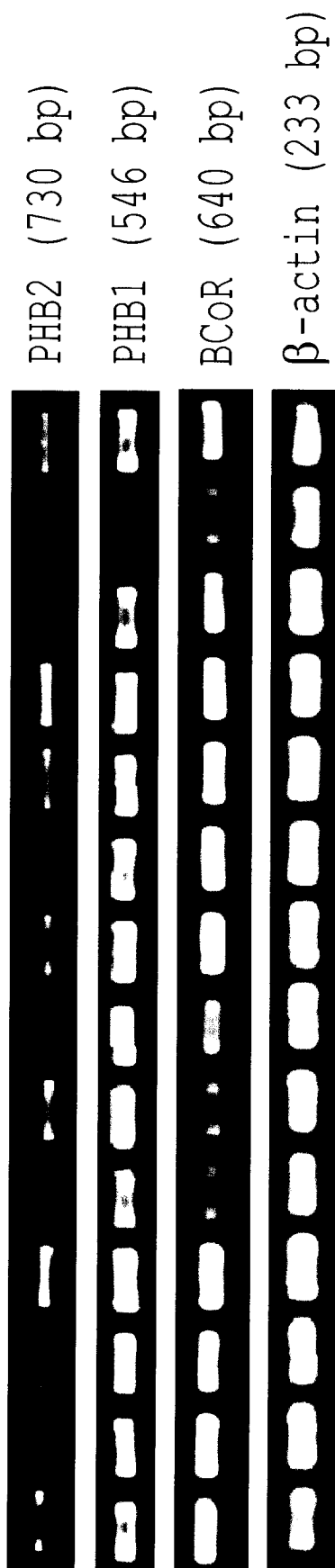
15/36



FE10-8

Normal

OA



FE10-8

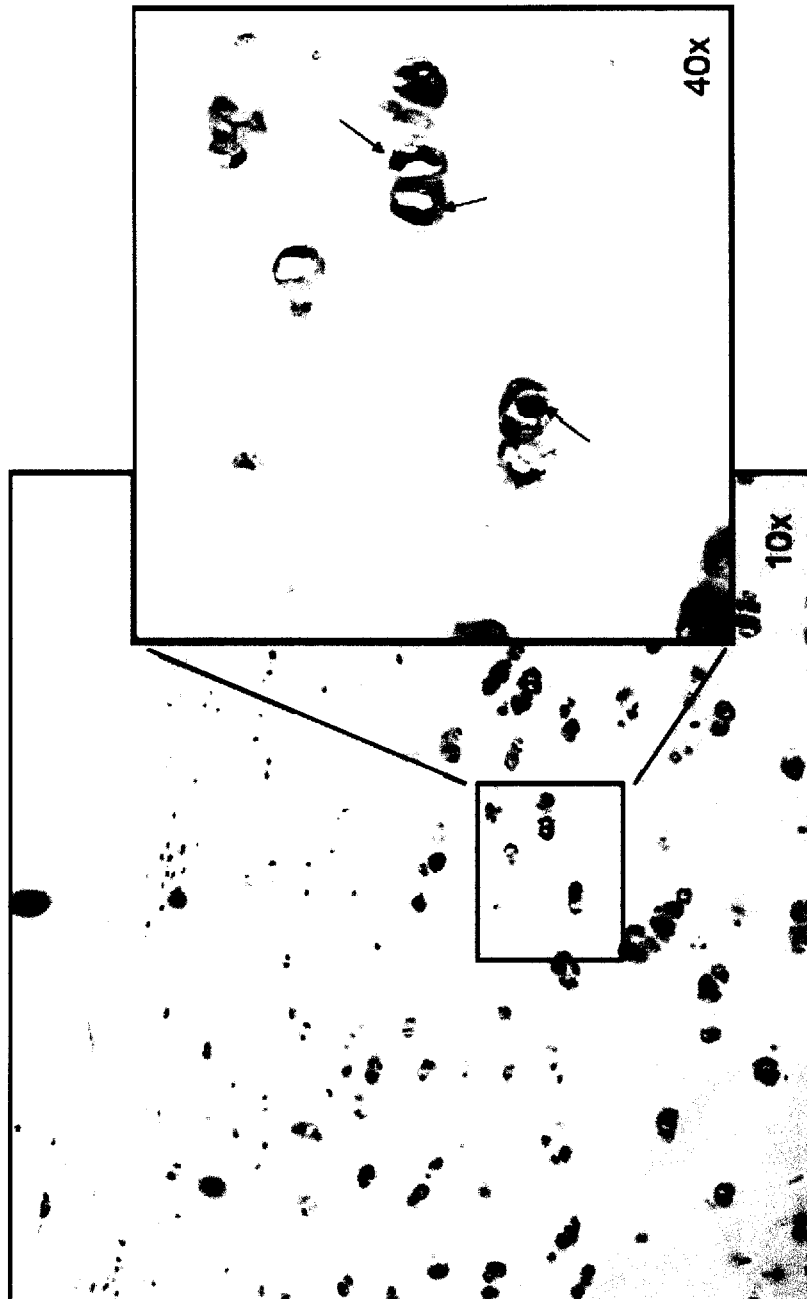
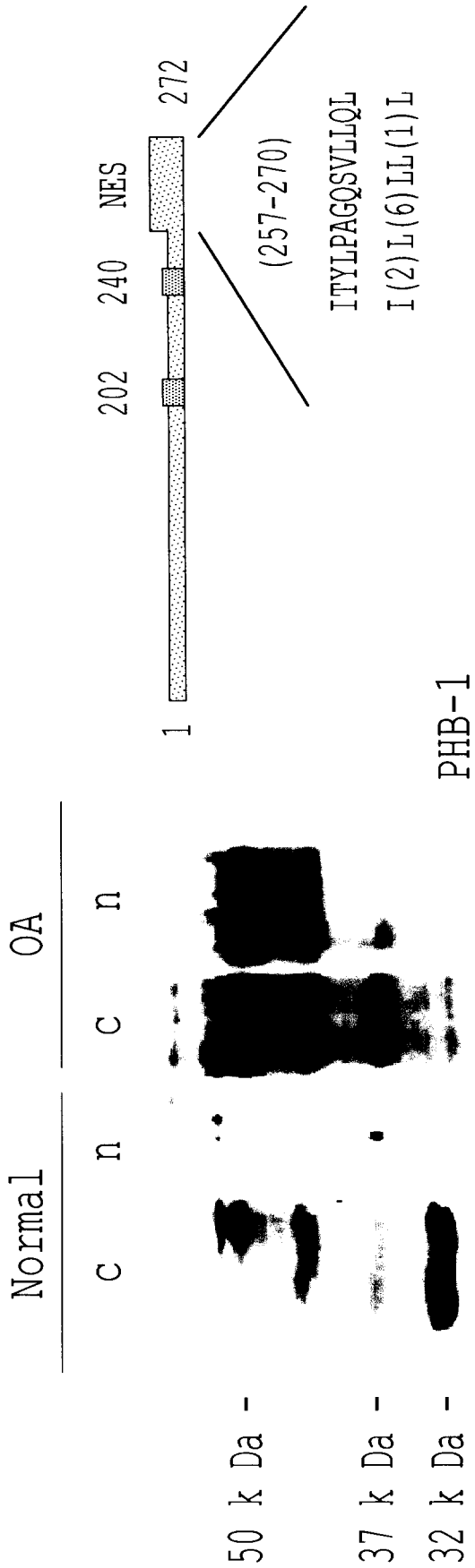


FIG. 10



Position	Flanking peptide	GPS Score	Cut-off	Matching Motifx?
202	RARFVVEAEQKKKA	4.00	18	Matched
240	DGLIELRLEAAEDI	1.52	18	Matched

~~FIG - 11~~

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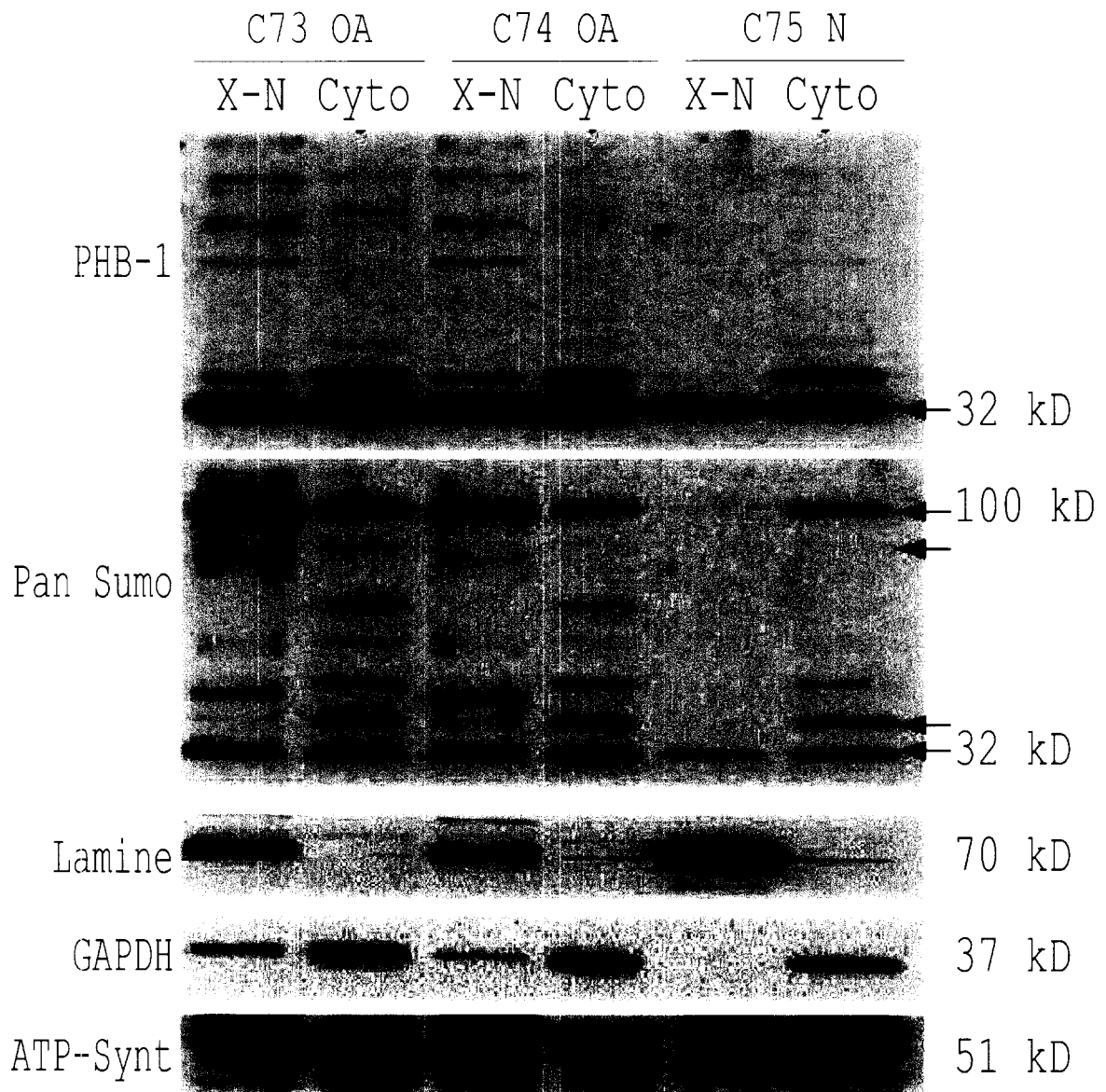


FIG. 12

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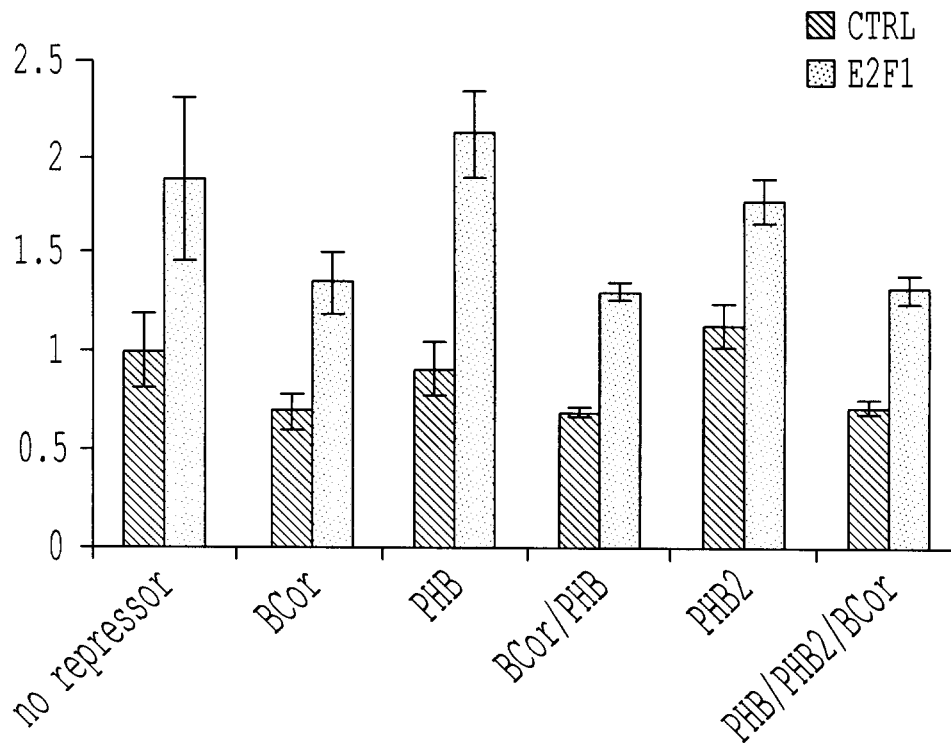


FIG. 13

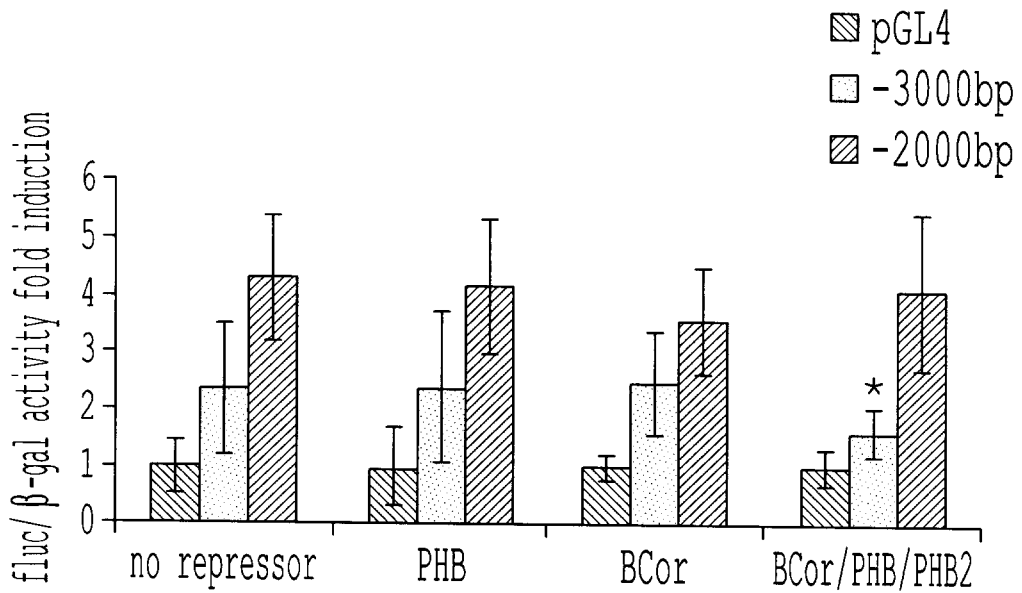


FIG. 14

_____ CCCAAATTGTCCTATCTGTGATAGTGGCTGTGCCCTTGGGGCCCTGAGCACCCCTGTGTCTCTGTGCA
 -10006
 GCAGTCAGATACTGGAGGGAGACTGAGGCACTGGCTGCAGAGCTTGTGATCATGAGAGAGACTCACTAGG
 -9940
 ACTACAGATGGGTAAACTGAGGCCCTTCGAGGGGGCAGCTCCAGAAAGGCAGGGCCATAAATGTCTCACCTT
 -9869
 CATATTCCCGTGCCAAAGCTGTGGCCCTTCTGCATTTCATGGCAGATGAGTGGACAAGGCTGATGGACTGAT
 -9798
 GGAGAAACAAGGATAGATGGAGCAGCTGGCAGCTCAGCAAAATGATGCTGCAATGATCTGCTTCCAAC
 -9727 PP11
 CACCTCAAATCCATCCTTCTCTCCAGGCAGAGTGGCTTTAAGATACACATCTGGCCAGGTCTCTCAC
 -9656
 TGTTCAAACCCCTTCATCTGCTCCTTTTGGCCCTCAGGATAACA**A**TCCCACCCCTCCTATCAAGGACTATGGAG
 -9585 (-9543) G
 CCTGTGGATCTGGTCCCACCTTGATTCCCAACCTTCCCTCTTCCCCTATGCCCTGCCCTTCTCATCTGTT
 -9514
 CAGTCTATTATGAAGCCACACGTTCTTCTTATATCAAGCATACCACAGTTTATCTCACCTCAGAGGC
 -9443
 TTTGCACAGTATATTTCTTAGGGAGGGTCCCAGGTGGTAGAAAAC**GG**TACAGCCAACCTCCTCCAATG
 -9372 (-9323) T
 TGTCACTAAGACCCCTTCAACAGCAGGCTGCAGATTCTCTCCA**GCAC**TGTCTC**CAACT**GTGTAATGGA
 -9301

FIG. 15

TTTCTGTGTCCTGCTGACTCAACCCAAATGAACAAGAGCCATCTATCTCTGTATCTCTGCAATCACA
 -9230
 GGCACAAAATAGGTGCTCTACATTTTTCACACCTGAGAGGCCATTCTAGAAGGGTCTCAGGCCACGGT
 -9159
 TCTGTCAGTATTCATGCAGATGCTGACAGGACTGCAATTAATAAAAAATACTTGAGATGCCCAAAATGCCCA
 -9088
 AATAGCTTCTCATTTTGCTTGGACTACCAATAATTGCACAGTGCAATA**G**AATAATGCTCAAAATACATTAAC
 -9017
 ATCTTACTTGATCCTAGGGGTCCTCTACTTTTAAAGCCTCAAACTTCTCCCTCTCACAGGTGAAAAG
 -8946
 GGGAGTACAAAATACATTCCTCCCTTGCTCGGGATCCATTCCCTACAGGTAGTCAAGACTCTGAGCTTCC
 -8875
 CCTCTGACTTTCGGCAGTGCTTACCCTCTCCCCACAGATGAGTGCAGGAACAATTTAAACAGACTTCAGA
 -8804
 CTCTTCCAACAGAGCCAATCCCTCCCATCACGTTGGAGTGGACTTGCTACCCACACCCATCAACAGGCCCC
 -8733
 TGAGAACTTCAAGGCAGGGGCTTCTTTGGTAAACCAACCTTTCCTTCCACCAGCTCAGAGAAGTTG
 -8662
 TTCCAGATAGATGCCAGGATTCTTTGGAGGAGCATGGTGATTCTGGGGTGGAGCCTCTGACCCCTGGCCAACC
 -8591

PP10

FIG. 15 (continued)

AAAGGCCGTGAGCCACTCTTCCCCCACAAGTGA TTGGCCGAGGAGTGGCCATGTAAGCTGGACCCAGCCAA
-8520
TCAGCATACCCAAATCCCCTTGCCCCACCCTGGGAGGGTCCCTATAGTTGTTGCCAAGAGAGGCTCACCCCTG
-8449
TTCTCAAGAAGCTTACAACTGGTAGGGCAACAGGTTTAAATCTATCATTACACAAAATATTTAATTTCAAT
-8378
ACTGTTGCAATAAGAGCTATGAGGAAAGAA GAACA CTCCCCG TACTTTTACTTGGTGTAAAGATTTGAAGAA
-8307
GAAAAA AAAAACA CTGCCTGAAGGATTGTTATGGCCCTCTATAATAATAGTGGCTGCAGACATTTGCCCA
-8236
TTATGTTCAGCATGAACCCATGTGACAAAATTCATCAAAGCGTTTTGCAC TAGGGAGAAAAATTTGTATTAG
-8165
AGGAAGCACAGCAGTTTGGACTGAAAGACAAAGAAAAATTCAGCCAATTCCTGCTGATCTTTTGTGATGGGC
-8094
ACCTGGAAGCTGAAAGCTAAAGTGGTACTCAGGAACAGGACTGCTACTTCTGTTCCTGGTGAATCCTGCC
-8023
CCAAACCTCCTCTCTGATTCCTGATTCACCTGTGCCAGTGGAAATATAATGCTCCCCAAGATGTCAAA
-7952
ACTAAAAGGGAAAATTGCCAAAAAATAATACATAATATTTT**AGAGAG**AAAAATAAGATTATAAAAAATGTGTTT
-7881
(-7842) +ACTG +ACTG (-7839)

PP9


FIG - 15 (continued)

TGTACCCCCAAGTTTCACCTAAGAAC**TT**CCTGACTTCCAGGCCCTGGTTGTGCCCCACGCACCAGCCTGCC
-7810 (-7784) C
CAGCTTTCCTGGACCAAACTTCCTAGCACCTAAGCAGGGGATGAGGGCAGATAAACTAAATCAGAAAAGGG
-7739
ATCTGTTCCCTAGACTCAACCAACATGACCACCGTGGGAAAGAACAACAAAGAGCAAAC**TCT**
-7668
CTTAAAGAGCAGCCTGGCAGCTATCACCA**TTAGCA**TT**CAGCCCTCTG**TTCCACAGGACTCAGGACCA**AA**CC
-7597
CCTCACCTTCACATCC**CA**TCCGTTTCCCAAGAAGCAGAAATACTTATTTCCACATTTCCACAGATGGGGAA
-7526 (-7509) T
GCTGAGGCTAGGAGAGGTTATG**TTATTTGCCAAAGCCACACA**ACTAGTAAAGCCACTGACAAGAT**TCTGG**
-7455
CTCAGGCCATCAGGTGCCAGAGGCAGCATTTTTGGCCACCACAGGCCCTGCCCTGGGAACAAGAGCATGCAG
-7384
AAAATCTACAAGAGATGGGAACAATAATTGGAAAATTGCTAGCGTGCAGGGGGGAAGGTGTGATTT
-7313
CCTGCTACAGACGCCAGAGTAAAGCCACCCAGGAGTGCCCTGTGCAGCCCTCCATAGTAAGTCCAGCGG
-7242
CTGCATTTATGCCCAAGATGCCCTGGTGGTGGAGTGGAGTGGAGGAAGAATTCCAGAGACAAGATTAGAAACT
-7171

PP8

FIG - 15 (continued)

TCTCAGCTTAGCAGCTCTAGGGCTGGACCCGCCAACCAAGCCATTTTACACATAAAGCAGTCAATGGGAGGG
-7100
GGTAGACGTAGGGGGCTAAACTCCCCACAGCACAGGGTCCAAGTTGGTAGACTGCACCTTCTCCAGGGCGCA
-7029
GGTCCGCTAGTCCCGGCAICGGGGACTCGTTATCTTAACTTGGACCCCTGGGTGCACAGCCCTGCACAC
-6958
ACCACTGGAGAGGGTTCCTGCTGTCGAGGGTTGAGAGGAGGGTATGGAGTCCCTGGAACAGCACGACAGG
-6887
GTCCAGAGGCCACCTGGCAGGGCCCTGAACACCGAGGCCCTCTGTGAGCTTGGGTCTGGGCCGGCTTCCCGCTT
-6816
CGGAGGTTGGGAGGGGTCGTGGTCTCTGCGTTCACAGGCCAAGCGGCCCTGGAGGCACGGACTAGCAG
-6745
GACGCCGAGGTGGCGGGGTCGGGCCCTCTCCCGCAGCAGCTGTCCGGGAGAACCAAGCAGGAGGGCGCCG
-6674
CTGCTGACTGCATCTCATGAAAGATTACAGGCCCGGCTGCCGCTGCCATCTCCCGCACCTTGGCCCGGA
-6603
AACGGTCCGCTCTGGAGCCCCTGGCCGTCGGCGGCAGGCTTAGCCGCTCCAGTCCCTGAGAAAGCAGGCC
-6532
ACAGCCGACCTGCCCTGTGGTCCCATCCCATAATCCCAACAGCAAGCAGGCTCAGGCTGGCCTTCGGG
-6461
GTACCAGGAGTAGGTTCCGGCCAACACTGGTTCCACCATGAGGCTTCGGCCACAGGGTTATCTGGCCACCGA
-6390
(-6345) C
(-6322) G

 - 15 (continued)

PP7

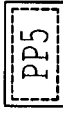
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-6319
CTCCAGCGGACAGCCAGGAACGGCCTGCGGGCCACAGGGGTGGAGGCGTCAACGTTCCGACGGCCCCG
-6248
CAGCAGGATGGTCGCTGGGGATGTGCAGGCATAGGGGTGGACAAAGGGCCCCAGAAAGTGTGTCTCTGA
-6177
GGGTGGTGTGCCCTTTCCCTCACCGCCAGCCCCGTGAGGAGGGAAGGCAATCCCCCAACCAGG
-6106
GCAGGTCGGGGGTTGCCCAACCTAACCCCCCTACCCCCAAACAGAAAACCTGGGGTCTGTCTCCCAA
-6035
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-5964
GCTGAGCAGAAAGAAGGTCCTCAAAACCCCAACTCGACCACCCCGCTCTAAACGGAAG
-5893
TAGGGCCAGCCCCCTCACGAGTCAGGTGGAAACTGGGCCCAGGGAGAGAAAGTCCCCAGGGCCAGGCTGG
-5822
GACCCGCTCTGGCTTCGGCATGGGCAGGGCCCCCTCCACGGAGTCCCAGGGGGGCTCCCCGGCCCTC
-5751
GAGGCCGGCCAGCCGGGACCCAGCCTACGCCCCGAGGGAGGCCAGCCCTAGCCGGGGGA
-5680
CTGGCGCCGCCCTCTCCCGCAGGTCCTGGGACACCTAGCTTCCCTCCCCCACTTCCCCGCTC
-5609 (-5591)A

~~FE~~-15 (continued)

CCGGCCAGTGTCCCGCCCTTCCCGCGGGCCAGCGGGCGGGCGGGAGGAGCGGGCCGAGCCGAGGAA
 -538
 GCCCGGCTCGCGCGCTGGGATGTAGCGAACAGCGGGCCGAAGAACCCTGCAGTCCAGAGCCAGAG
 -5467
 CTGGATCCGGGCCCCAGCCGGAGCCGAAACCTGAGCCAGAGTCCGGCGGGCCGAGCCCGGAGCCACGA
 -5396 (-5330) G
 GCCGCAGCGCAGCGCTGCCACAGGTGGGTAAAGACGCTGGGCTAGGGCCGACGGTCTCCGCGGTGGAG
 -5325
 GGGCGCAGGAGGTGGCGCCGAGTCCTGCCAGTTTGCTCCTGGCGTGTGGGTCCACCCGGCGGCGCG
 -5254 (-5185) +G
 GGACAGCGCAAGCGCGGAAGTCAAGGCTTCGAGGCAGCGCGGAGGAGTCTGTTCCGCGCCAGGGCA
 -5183
 CAGTCATAGCCCGCTCACCGGTGCTACCTCACCCAACCGGGGATCAACCTCTGCTTTGGCTCCGGG
 -5112
 CACCTCAAGAGGTAGCAGCCTCGGGGCACGGCCACGGCCCCCGGAAGGCACAACCTGAGAAGCCCGT
 -5041
 GGCAGCCCTCGCAGCGTGGGTGACACAGGCTCCCCCACCCAGGAGAAGTGGGCAGGAGAGGGCC
 -4970
 GCCCGTGTCCCGCTGCGTCCAGGATGGAGGGCCCCACCCATGGAAATTGCTGGCCCCCTCTGCGTG
 -4899 T (-4889)
 GCCCGGACTTCAGCCGTGGCTTCGGCTCAAGAGGGTATTTTCCTAAACGAACCGCTTCGTTCTCGTTCGT

PP6

~~FIG - 15~~ (continued)



TCGTTTCGTTTCGGCCAGCAATGCCGCA~~GA~~AAAGCAGCAGACGTCGGTCCGGCCCTGGCTCTCTTCGC
-4757 (-4719)A (-4692)T
CCCGACCCGACGTTCCCGCCGACGCTCGGAGTGC~~CC~~CCAGCCCAAGCAGCCTGCTCTCGCCGGCAC
-4686 (-4626)G
AGGTCGGCTTTTCTTCCAGGAGAAACCCCAATTCCCTTCGTAACGTCCAATAAAGACATTC~~CC~~CGG
-4615
GCTTCTCCAGGTTTGGTTGACGCAGGTTCCCGGACCGCAGTCGCTTCTCAAGAACCGGGTCTCGG
-4544
ATTCTGAAATGACCAGCTTCGTAATGGAGCCTATTCTCCCGGCAAGCAGGGCCCAAGCCGG
-4473
GATCGCAGTAATGGGAACCCAGGCTGGAATCCGGTCCCAAGCTTTCCGATTTAGGAATTC~~CC~~CGAATC
-4402
TACAAATAATTAGTCCACTTTCTGAAAAACTAAATTCGAAAAACACAAAATTCCTTGACATCC~~CC~~TGTGA
-4331 T(-4329)
CCTCTGAAAGCCACAGGGCCAGAGGAGGAAATCCAGGTTGCTGTCCA~~CT~~GGGGGAGGATTCAGGTCTA
-4260 (-4238)C (-4211)G
GGGTTCAGGTTACGGTAGTCAGGGCAAAAGCTACAGGCAGGGCAGGCACAGGAGACTTGTGT~~CC~~CC
-4189 (-4120)G

GTGCCCTTCCCGGGCTGCTTTCGGCCCTCCCGCATCTCTCCAGGAAAGAAAGAGGTGGGCTGGGGC
 -4118
 TTGGAGACCAGGCTGTCTGGACTCTAGGATGCAGAGGCCCTCCAGACAGGCTCAGGGTCTCTTCTCCCAATG
 -4047 G(-4039)
 AAAGCAGCCGCTGGAGGAGGAGGCTATGGTGCCATCCATAAGTTGCCCTCTGCTCCCCAGTTGTGGGACC
 -3976 A(-3966)
 AGCTGCTACCTCCTTCCCTAGTCTTCTTCCCCACAGCTCAGCCATCTCAGGAACCAGACAGGCTCCATGGA
 -3905
 CTTAGGTGAGAGATGGCCGGGTAGAGCCATGGGTCCTACCAGCCGCTGACTGAGCGGCCACGGCCACAGA
 -3834
 GTCCTGAGTCCATACTCCCATCTGTGCCCTCACTGGGGCAGTCCCTGCTCAAAATACATCCCTGGCTCTCCCC
 -3763 (-3727)T
 GGGACAGGCTGGGGATCCCCATTTGGCAGGAAGCCCTCAGACTGGGGTCCCAGGAAGCCCTAAGGAGCCAGT
 -3692
 GAGGCTTCCAGCCCCCTACCTGAGCACCCCTCCCTCCACCTTACCCAGTAATTGCTGTATTCAAAGAAACG
 -3621
 GGAGCTTTATTGGGAGGGGTGTAGATCAGGCAGAAAGAGGTAGGTGGTCCAAACCTGCACTCCCCAAA
 -3550
 ACAGGGTTTCAAGTTGAACCTTCTCCACGGACTAAGAGGCTTAGGGCTGGAAATGTCCTCCAGAGAGTCAATGG
 -3479 (-3415)ACT
 ATAGCCCTGGTGGCAGGCCATGGCACATTCCTTCTTTTCCCTAAATAACCTTGATTCCTGGGAGCAAGGAT
 -3408

PP5

PP4

~~FIG. 15~~ (continued)

TAGGGCACGGTGCCCCCGTGGGTAGAAAGGATGCCCCCCCACTGAGAGCCTTCCAACCCACCCCTTCCCA
 -3337
 AATTACATTACTAAACCAATCTTTGGGCACAGGGTGTTTTAGTGAGCCAGGCTTCAGGAAGGGTCCCTCATG
 -3266
 GTGACTACTTCAACCCACACAAGCCAAAGCTCTTCTGCTCAGCCCAGCCAAAGACCCATAAACTCCAAAATT
 -3195
 CTTGAAAATCAGAGAAATCATTTGCTGGCTTTGTGTGGTCAAGGAGGGGTGGGGAACAGGGCACATGGTTCCA
 -3124
 GCTCCACTAAGCCCCCTTCCCTCCTCTCTTCTGTTGCCATCAGCAAGTGGATGCTGAAGGCAGCAGG
 -3053
 CAGAGTCCGGTGTGGACATGGGAACAGGACAGTGCAGATCAAGCCTTAACCTTGAGGGAAACACAGG
 -2982
 TCACATAGCACAGCTGGGGAACACAAAGCCCTCTGCTTACTCCTGAAGAGTGCTGTTTCTGTCTGTAT
 -2911
 GTGTGACCTGTGTGAGCGTGCAAGAAGCCCCCTATCTTGACTGGGACAAATGGCCAGTGAGTGTAGCTGGG
 -2840 T (-2833)
 GAAGAATTGAGAGCATGTCCAGGTCCCTTCCCCAGCCCAAGATCAGGCCACAGCCTCCTCACAAT
 -2769
 CAATTGCCCTCCTCACTCCTTGATCACTCAGTGTGCTGCCAGGCCAGCAGAACAGACTCTGCCAGCAGGCC
 G (-2697)
 CACTAGCCCCAGCTCCTTTGGGTCTCAGGTCCCCCTGAGGATATGGGGCTTACCTGAAATGGTCTGAGG
 -2627

FIG - 15 (continued)

PP3

GCTTTTCCTTCTACACAGCAGGCAACAAGATCACCAAATAAAGGGACTATTGTGCCCTGCCCTGGAGGCCCTGC
-2556
CAGAGGTTGGGCCAGAGGGCCACACAGCAGGTGCTCAATAACTGCATTAAATGCACATAACAGTGAGGAA
-2485
ACAGCCCTCAGACTAAGCAGTGAGTGCTGCACAGAAATAGTCCCATTGGGGGATGGCCCAAAGAGTC
G (-2412)
ACTTTGGTCCCTCTGGGAAGTGAGAAGGCAAGTGAGAAGGCTGTGAGTCTTAACCTCCTCTAGAGGCCCCAC
-2343
AGACAGACCATTCATTTCTAAGTCTCTACCCAGAGACGCACGTGTGCTTCCCACCTTGGCCCTGACATGTGGC
-2272
AGGTTAGAACACACCTCCTATCCCCTGCCAGCCCGGTTCAAGCAAGTAGCACATAATATGCCATAAACTC
-2201
AGCACTTCCATAGTGCAGTGAATACATGTGTGTACAGCATCTCCGCAATGGATGTACAGGATGTGTGTGT
-2130
GTGTGCGTGCCCCATGCTGTCTGCATTCAGGCTGTCTTTTGGTAAGACAGCTAAAAAAGAATGGTCT
-2059
GTGAAGGACACTCCCTAGCACGCTGCAACACCTGAATATCTCCTTGAAGGAGGGATCTTCTACTGCAGG
-1988
AGACTCGTGGTAAAGGTGGCCAAAGAAACATGGCAACGGTGGGGCTGAGGGCAAATGCTGGGCAACTGTGCT
-1917
(-1882)A

~~FIG. 15~~ (continued)

AATTGTTGAGCAAAATCCTTCGACAAACTTCACCTACGTGGAAGGACTCGAGGAGGGAATCACTCTTAGGA
 -1136
 GTGGAGAGTAATGTCCTTGCCCTGTGCCAGTGAAGGCCCATTTGGAGCTGCAGCTCAGCTACCCACTGTGTG
 -1065
 GGAGAGAAGCTGGAAGACTGAGGGCTTCCCTGGGCTGCTGGCCCAGGGTTGGGAGACAGCAGTCACCTGGCT
 -994
 TACCAGCCCTATGCCCTGAAGCCCTGGGAAGCCAGGACGCAGGCCCCAGGCTGGGACAAAGCTACCCCTGAAG
 -923
 GAGGCAAAAGGCTGCCAAGGCCAACCCCATGCCCTGCCAAGGCCAGGCCCTGGCCCATTTGGCCAAGGCCCTAA
 -852
 GGTGTAACAAGGGGAGAGGTACAAGAGGCTGTGGGCTCTGGCTGGGATCCTTGGGGTCTTCCCTTCTGCA
 -781
 TTCTCCAAACGCCCTAGAGCCAGCAAAACGTTTCGTCTGATTAGAAGCCATCATTTCTATCCCAATCCCGG
 -710
 AAAATTGACTGCCGTGCAGAGAGGGAGGCCCTGAGAAGCAGCCGTAGGGGAGAAAGGTCCAAGCTAATTAGGA
 -639

PP1

FIG - 15 (continued)

AGTATGTGTGGTTGGGGAATTCATGTGGAGGTCAGAGTGAAGCAGGTGTGAGAGGGTCCAGCAGAAGG
 AAACATGGCTGCCAAAGTGTGAGTCCATTGGCAAGTTTGGCCTGGCCTTAGCTGTTGCAGGAGGCCGT
 GGTGAACTCTGCCTTATATAATGTGGATGCTGGGCACAGAGCTGTCACTTTGACCGATTCCGTGGAGT
 GCAGGACATTGTGGTAGGGGAAGGGACTCATTTTCTCATCCCGTGGGTACAGAAACCAATTATCTTTGA
 CTGCCGTTCTCGACCACGTAATGTGCCAGTCATCACTGGTAGCAAAGATTTACAGAATGTCAACATCAC
 ACTGCGCATCCTCTCCGGCCTGTGCCAGCCAGCTTCTCGCATCTTACCAGCATCGGAGAGGACTA
 TGATGAGCGTGTGCTGCCGTCCATCACAAGTGAATCCTCAAGTCAGTGGTGGCTCGCTTTGATGCTGG
 AGAACTAATCACCCAGAGAGAGCTGGTCTCCAGGCAGGTGAGCGACGACCTTACAGAGCGAGCCGCCAC
 CTTTGGGCTCATCCTGGATGACGTGTCTTGACACATCTGACCTTCGGGAAGGAGTTCACAGAAGCGGT
 GGAAGCCAAACAGGTGGCTCAGCAGGAAGCAGAGAGGGCCAGATTTGTGGTGGAAAAGGCTGAGCAACA
 GAAAAGGCGGCCATCATCTGCTGAGGGCGACTCCAAGGCAGCTGAGCTGATTGCCAACTCACTGGC
 CACTGCAGGGGATGGCCTGATCGAGCTGCGCAAGCTGGAAGCTGCAGAGGACATCGCGTACCAGCTCTC
 ACGCTCTCGGAACATCACCTACCTGCCAGCGGGGCAGTCCGTGCTCCTCCAGCTGCCCCAGTGAGGGCC
 CACCCTGCCTGCACCTCCGCGGGCTGACTGGGCCACAGCCCCGATGATTCTAACACAGCCTTCTTCT
 GCTCCCACCCAGAAATCACTGTGAAATTTGATGATTGGCTTAAAGTGAAGGAAATAAAGGTAATAATCA
 CTTCAGATCTCTAATTAGTCTATCAAATGAAACTCTTTCATTCTTCTCACATCCATCTACTTTTTTATC
 CACCTCCCTACCAAAAATTTGCCAAGTGCCTATGCAAAACAGCTTTAGGTCCCAATTCGGGGCCTGCTGG
 AGTTCGGCCTGGGCACCAGCATTGGCCAGCAGCAGCGGGGCAGTATGTGATGACTGGGGAGCACA
 GGTGTCTGCCTAGATCCACGTGTGGCTCCGTCTGTCACTGATGGAAGGTTTGGCGATGAGGGCATGT
 GCGGCTGAACTGAGAAGGCAGGCCTCCGTCTTCCCAGCGGTTCTGTGCAGATGCTGCTGAAGAGAGGT
 GCCGGGGAGGGGCAGAGAGGAAGTGGTCTGTCTGTTACCATAAGTCTGATTCTCTTTAACTGTGTGACC
 AGCGGAAACAGGTGTGTGTAAGTGGGCACAGATTTGAAGAATCTGCCCTGTTGAGGTGGGTGGGCCTG
 ACTGTTGCCCCCAGGGTCTTAAACTTGGATGGACTTGTATAGTGAAGAGAGGAGGCCTGGACCCAGAT
 GTGAGTCTGTTGAAGACTTCTCTTACCCCCACCTTGGTCCCTCTCAGATACCCAGTGAATTTCCA
 ACTTGAAGGATTCATCCTGCTGGGGCTGAACATGCCTGCCAAAGACGTGTCCGACCTACGTTCTTCTGCC
 CCCCTCGTTCAGAGACTGCCCTTCTCAGGGCTCTATGCCTGCACTGGGAAGGAAACAAATGTGTATAA
 ACTGCTGTCAATAAATGACACCCAGACCTTCC

FIG. 16A

MAAKVFESIGKFLALAVAGGVNSALYNVDAGHRAVIFDRFRGVQDIVVGEETHFLIPWVQKPIIFDC
 RSRPRNVPVITGSKDLQNVNITLRLIFRPVASQLPRIFTSIGEDYDERVLPSTITTEILKSVVARFDAGE
 LITQRELVSRQVSDDLTERAATFGLILDDVSLHLTFGKEFTEAVEAKQVAQQAERARFVVEKAEQOK
 KAAIISAEGDSKAAELIANSLATAGDGLIELRKLEAAEDIAYQLSRSRNITYLPAGQSVLLQLPQ

FIG. 16B

AAGTTCGGGTCCGTAGTGGGCTAAGGGGAGGGTTTTCAAAGGGAGCGCACTTCCGCTGCCCTTTCTTTC
 GCCAGCCTTACGGGCCCCGAACCCCTCGTGTGAAGGGTGCAGTACCTAAGCCGGAGCGGGGTAGAGGCGGG
 CCGGCACCCCTTCTGACCTCCAGTCCCGCCGGCTCAAGATCAGACATGGCCCAGAACTTGAAGGACT
 TGGCGGGACGGCTGCCCGCCGGGCCCCGGGGCATGGGCACGGCCCTGAAGCTGTTGCTGGGGCCGGCG
 CCGTGGCCTACGGTGTGCGGAATCTGTGTTCCACCGTGAAGGCGGGCACAGAGCCATCTTCTTCAATC
 GGATCGGTGGAGTGCAGCAGGACACTATCCTGGCCGAGGGCCTTCACTTCAGGATCCCTTGGTCCAGT
 ACCCCATTATCTATGACATTCGGGCCAGACCTCGAAAAATCTCCTCCCCTACAGGCTCCAAGACCTAC
 AGATGGTGAATATCTCCCTGCGAGTGTGTCTCGACCCAATGCTCAGGAGCTTCCTAGCATGTACCAGC
 GCCTAGGGCTGGACTACGAGGAACGAGTGTGCGCTCCATTTGTCAACGAGGTGCTCAAGAGTGTGGTGG
 CCAAGTTC AATGCCTCACAGCTGATCAGCCAGCGGGCCAGGTATCCCTGTTGATCCGCCGGGAGCTGA
 CAGAGAGGGCCAAGGACTTCAGCCTCATCCTGGATGATGTGGCCATCACAGAGCTGAGCTTTAGCCGAG
 AGTACACAGCTGCTGTAGAAGCCAACAAGTGGCCAGCAGGAGGCCAGCGGGCCCAATCTTGGTAG
 AAAAAGCAAAGCAGGAACAGCGGCAGAAAATTTGTGCAGGCCGAGGGTGAGGCCGAGGCTGCCAAGATGC
 TTGGAGAAGCACTGAGCAAGAACCCTGGCTACATCAAACCTTCGCAAGATTCGAGCAGCCCAGAATATCT
 CCAAGACGATCGCCACATCACAGAATCGTATCTATCTCACAGCTGACAACCTTGTGCTGAACCTACAGG
 ATGAAAGTTTACCAGGGGAAGTGACAGCCTCATCAAGGGTAAGAAATGAGCCTAGTCACCAAGAACTC
 CACCCCCAGAGGAAGTGGATCTGCTTCTCCAGTTTTTGGAGGCCAGCCAGGGGTCCAGCACAGCCCTA
 CCCC GCCCAGTATCATGCGATGGTCCCCACACCGGTTCCCTGAACCCCTCTTGGATTAAGGAAGACT
 GAAGACTAGCCCCTTTTCTGGGAAATTACTTTCCTCCTCCCTGTGTTAACTGGGGCTGTTGGGGACAGT
 GCGTGATTTCTCAGTGATTTCTTACAGTGTGTTCCCTCCCTCAAGGCTGGGAGGAGATAAACCCAAC
 CCAGGAATCTCAATAAATTTTTATTACTTAACCTG

FIG. 17A

MAQNLKDLAGRLPAGPRGMGTALKLLLGAGAVAYGVRESVFTVEGGHRAIFFNRIIGGVQDDTILAEGLH
 FRIPWFAQYPIIYDIRARPRKISSPTGSKDLQMVNISLRVLSRPNAQELPSMYQRLGLDYEERVLPSIVN
 EVLKSVVAKFNASQLITQRAQVSLIRRELTERAKDFSLILDDVAITELSFREYTAAVEAKQVAQQA
 QRAQFLVEKAKQEQRQKIVQAEGEAEAAKMLGEALSKNPGYIKLRKIRAAQNI SKTIATSQNRIYLTAD
 NLVNLQDESFTRGSDSLIKGKK

FIG. 17B

AGTTCATACTCCCATCTGTGCCTCACTGGCGGCAGTCCCTGCTCAAATACATCCCTGGCTCT

T

-3757

-3727

-3697

FIG-1BA

*AciI
*Fnu4HI
BsrI

5' ... TCACTGGCGGCAGTCCCTGCT ... 3' 5' ... TCACTGGTGGCAGTCCCTGCT ... 3'
+1 | +10 | +20 +1 | +10 | +20
3' ... AGTGACCCGGTCAGGACCGA ... 5' 3' ... AGTGACCCCGTCAGGACCGA ... 5'

36/36

BsrI

BsrI

BsrI
*Fnu4HI
*AciI

FIG-1BB

FIG-1BC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2007/001901

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C12Q 1/68* (2006.01), *C07K 14/47* (2006.01), *C12Q 1/00* (2006.01), *C12Q 1/02* (2006.01),
G01N 33/53 (2006.01), *G01N 33/68* (2006.01), *C12N 15/12* (2006.01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C12Q 1/68* (2006.01), *C07K 14/47* (2006.01), *C12Q 1/00* (2006.01), *C12Q 1/02* (2006.01),
G01N 33/53 (2006.01), *G01N 33/68* (2006.01), *C12N 15/12* (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms
Electronic databases: Delphion, PubMed, Scopus and CPlus; Search terms: pituitary homeobox transcription factor 1 (pitx1), pitx1 repressor protein, prohibitin (PHB-1), prohibitone (PHB-2), B cell lymphoma-6 transcriptional repressor interacting co-repressor (BCoR), osteoarthritis, chondrocyte, osteoblast, myoblast, synoviocyte, blood, synovial fluid, sumoylation, E2F, nuclear localization and complex.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	PICARD, C. ET AL.: "New emerging role of Pitx1 transcription factor in osteoarthritis pathogenesis" CLINICAL ORTHOPAEDICS AND RELATED RESEARCH, September, 2007 No. 462, pages 59-66, ISSN:0009-921X	1-37
A	MARCIL, A. ET AL.: "Pitx1 and Pitx2 are required for development of hindlimb buds" DEVELOPMENT, January, 2003 vol. 130, no. 1, pages 45-55, ISSN:0950-1991	1-37
A	MISHRA, S. ET AL.: "The prohibitins: emerging roles in diverse functions" JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, April-June, 2006 vol. 10, no. 2, pages 353-363, ISSN:1582-1838	1-37

Further documents are listed in the continuation of Box See patent family annex.

* Special categories of cited documents :	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search 6 December 2007 (06-12-2007)	Date of mailing of the international search report 15 February 2008 (15-02-2008)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Sandra Hurley 819- 934-7934

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2007/001901**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. Claim Nos. : 1-21
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 :

Claims 1-21 are directed to methods having no clearly defined purpose. An essential technical feature of any method claim is the purpose of the method, without which the method has no utility. Thus, present claims 1-21 so lack clarity (CONTINUED ON PAGE 7)

3. Claim Nos. :
because they are dependant 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 :

The claims are directed *a priori* to the following groups of inventions which are not so linked as to form a single inventive concept under Rule 13.1 of the Regulations under the PCT:

Group A - Claims 1-29 and 31 are directed to methods involving pitx1 repressor proteins in the diagnosis of osteoarthritis (OA) and in the development of compounds to treat OA; and

Group B - Claims 30 and 32-37 are directed to a repressor complex comprising prohibitin (PHB-1) or prohibitone (PHB-2) and B cell lymphoma-6 transcriptional interacting co-repressor (BCoR), a kit comprising antibodies to PHB-1 or PHB-2 and BCoR and a method using said complex.

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 additional fees, this Authority did not invite payment of additional fees.

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 claim 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 claim 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

International application No.
PCT/CA2007/001901

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant	Relevant to claim No.
A	<p>RASTOGI, S. ET AL.: "Camptothecin induces nuclear export of prohibitin preferentially in transformed cells through a CRM-1-dependent mechanism"</p> <p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, 3 February 2006 (03-02-2006); available online 30 November 2005 (30-11-2005) vol. 281, no. 5, pages 2951-2959, ISSN:0021-9258</p>	1-37
A	<p>KASASHIMA, K. ET AL.: "Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2"</p> <p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, 24 November 2006 (24-11-2006); available online 28 September 2006 (28-09-2006) vol. 281, no. 47, pages 36401-36410, ISSN:0021-9258</p>	1-37
A	<p>GEARHART, M. ET AL.: "Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCL6 targets"</p> <p>MOLECULAR AND CELLULAR BIOLOGY, September, 2006 vol. 26, no. 18, pages 6880-6889, ISSN:0270-7306</p>	1-37

专利名称(译)	诊断骨关节炎的方法		
公开(公告)号	EP2089546A1	公开(公告)日	2009-08-19
申请号	EP2007816051	申请日	2007-10-25
申请(专利权)人(译)	褚SAINTE-JUSTINE		
当前申请(专利权)人(译)	褚SAINTE-JUSTINE		
[标]发明人	MOREAU ALAIN		
发明人	MOREAU, ALAIN		
IPC分类号	C12Q1/68 C07K14/47 C12Q1/00 C12Q1/02 G01N33/53 G01N33/68 C12N15/12		
CPC分类号	G01N33/6893 C07K14/4703 C12Q1/6883 C12Q2600/136 C12Q2600/156 C12Q2600/158 G01N2333/47 G01N2333/4704 G01N2333/4706 G01N2800/105 G01N2800/50		
优先权	60/854077 2006-10-25 US		
其他公开文献	EP2089546A4 EP2089546B1		
外部链接	Espacenet		

摘要(译)

公开了用于评估发生骨关节炎的风险的方法。所述方法包括确定垂体同源框转录因子1 (pitx-1) 阻遏蛋白的细胞定位，血液或滑液浓度，SUMO化状态和翻译后修饰，鉴定编码所述蛋白质的基因中的突变并鉴定任何突变或后导致所述蛋白质的核积累或保留的翻译修饰。所述蛋白质包括抑制素 (PHB-1)，抑制素 (PHB-2) 和B细胞淋巴瘤-6转录抑制因子相互作用共抑制因子 (BCoR)。