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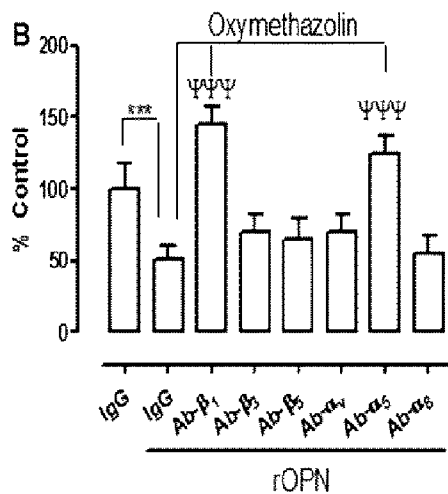
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[Continued on next page]

(54) Title: A METHOD OF INCREASING GIPCR SIGNALIZATION IN THE CELLS OF A SCOLIOTIC SUBJECT

**FIG 5**



(57) Abstract: There is provided a method of increasing GIPCR signalization in the cells of a subject in need thereof comprising administering to the subject an effective amount of an inhibitor of integrin alpha5beta1 expression and/or activity, whereby GiPCR signalization is increased in the cells of the subject. An inhibitor of integrin alpha5beta1 may be, for example, an agent that inhibits the interaction between osteopontin (OPN) and integrin alpha5beta1. Also provided are methods of determining the risk of developing a scoliosis and based on the presence of at least one copy of a CD44 risk allele and methods of stratifying a subject having a scoliosis and kits for performing these methods. In particular, the method of determining risk identifies SNP rs1467558; an isoleucine to threonine mutation at position 230 of CD44.

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## A METHOD OF INCREASING GIPCR SIGNALIZATION IN THE CELLS OF A SCOLIOTIC SUBJECT

**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application is a PCT application Serial No PCT/CA2014/\* filed on June 17, 2014 and published in English under PCT Article 21(2), which itself claims benefit of U.S. provisional application Serial No. 61/835,926, filed on June 17, 2013. All documents above are incorporated herein in their entirety by reference.

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT**

[0002] N.A.

**FIELD OF THE INVENTION**

[0003] The present invention relates to a method of increasing GiPCR signalization in the cells of a scoliotic subject.

**REFERENCE TO SEQUENCE LISTING**

[0004] Pursuant to 37 C.F.R. 1.821(c), a sequence listing is submitted herewith as an ASCII compliant text file named 14033\_121\_ST25.txt, that was created on June 17, 2014 and having a size of 49 kilobytes. The content of the aforementioned file is hereby incorporated by reference in its entirety.

**BACKGROUND OF THE INVENTION**

[0005] Idiopathic Scoliosis is a spine deformity of unknown cause generally defined as a lateral curvature greater than 10 degrees accompanied by a vertebral rotation<sup>1</sup>. Adolescent Idiopathic Scoliosis (AIS) is one of the most frequent childhood deformities worldwide, characterized by a 3D spinal deformity with unknown cause, and represents both an immediate medical challenge and a chronic condition affecting individuals throughout their lives. It is the most common orthopedic condition requiring surgery in adolescents and affects 4% of this population. This condition is most commonly diagnosed between the ages of 9 to 13 years<sup>2,3,4</sup>. The diagnosis is primarily of exclusion and is made only after ruling out other causes of spinal deformity such as vertebral malformation, neuromuscular or syndromic disorders. Traditionally, the trunkal asymmetry is revealed by Adams forward bending test and measured with scoliometer during physical examination. The diagnosis can then be confirmed by radiographic observation of the curve and the angle measurement using the Cobb method.

[0006] Once diagnosed, the primary concern for physicians in managing scoliotic children is whether the curve will progress. Indeed, the curve progression is often unpredictable and is more frequently observed among girls than in boys. If untreated, the curve can progress dramatically, creating significant physical deformity and even cardiopulmonary problems. These manifestations become life threatening when the curve exceeds 70 degrees. The current treatment options to prevent or stop curve progression include bracing and surgery. In general,

bracing is recommended for curves between 25 and 40 degrees, while surgery is reserved for curves greater than 45 degrees or curves that are unresponsive to bracing. Today in the United States there are approximately one million children between ages 10 and 16 with some degree of IS. Approximately, 10 % of children diagnosed with idiopathic scoliosis have curve progression requiring corrective surgery. About 29,000 scoliosis surgeries are done every year in North America, resulting in significant psychological and physical morbidity. (Goldberg MS, Mayo NE, Poitras B et al. The Ste-Justine Adolescent Idiopathic Scoliosis Cohort Study. Part I: Description of the study. *Spine* 1994;19:1551-61; Poitras B, Mayo NE, Goldberg MS et al. The Ste-Justine Adolescent Idiopathic Scoliosis Cohort Study. Part IV: Surgical correction and back pain. *Spine* 1994;19:1582-8).

**[0007]** Currently, there is no proven method or test available to identify subjects at risk of developing IS to predict which affected individuals require treatment to prevent or stop progression of the disease so that appropriate treatment can be early provided and prevent surgical complications and cardiac and/or respiratory problems. (Weinstein SL, Dolan LA, Cheng JC et al. Adolescent idiopathic scoliosis. *Lancet* 2008;371:1527-37).

**[0008]** Therefore, the application of current treatments, such as bracing or surgical correction, is delayed until a significant deformity is detected or until a significant progression is clearly demonstrated, resulting in a delayed, less than optimal treatment and often important psychological sequels (Society SR. Morbidity & Mortality Committee annual Report 1997).

**[0009]** Currently, in order to detect the deformity, diagnosed children are subjected to multiple radiographs over several years, usually until they reach skeletal maturity. It is estimated that the typical patients with scoliosis will have approximately 22 radiological examinations over a 3-year period. There are potential risks in multiple radiographic examinations. For this reason also, alternative approaches that could allow performing the prognosis of idiopathic scoliosis are strongly desirable.

**[0010]** The major limitation in developing prognostic tests that could facilitate treatment choices for patients is the heterogeneous nature of AIS. At the clinical level, the heterogeneity of AIS is clearly illustrated by the variability of curve patterns, localisations and curve magnitude even in families with multiple affected members.

**[0011]** In absence of reliable AIS phenotypes, there is a need to understand better the molecular changes associated with disease onset and spinal deformity progression. Molecular definition of disease is rapidly replacing traditional pathology-based disease descriptions in part because of its utility in identifying the optimal treatment regimen for patients.

**[0012]** To this effect, the existence of a differential melatonin signaling dysfunction was reported among AIS patients leading to their stratification into three functional groups or biological endophenotypes (Moreau et al., 2004); (Azeddine et al., 2007); (Letellier et al., 2008) and WO2003/073102 to Moreau. More particularly, AIS patients were stratified into three functional groups (FG1, FG2 and FG3) representing distinct biological endophenotypes. According to this stratification, scoliotic patients and children more at risk of developing scoliosis are less responsive to Gi protein stimulation when compared with healthy control subjects, and the classification is based on the percentage of degree of reduction relative to the control group. The classification

ranges were fixed between about 10 and 40% of reduction of response relative to control group for FG3, between about 40 and 60% for FG2 and between about 60 and 90% of reduction of response relative to control group for FG1.

[0013] More recently, using the cellular dielectric spectrometry (CDS) technique, which is a label-free method for the functional evaluation of G proteins and endogenous receptors coupled to those proteins (Verdonk et al., 2006), it was found that the cellular response following melatonin receptor stimulation by melatonin was mainly Gi-dependent in normal osteoblasts and was reduced to different extents in osteoblasts derived from AIS patients (Akoume et al., 2010). Approximately 33% of asymptomatic children diagnosed with a defective Gi protein function have developed scoliosis many years later (Akoume et al., 2010).

[0014] Early detection/prognosis of scoliosis is not only critical to successful and less invasive clinical outcomes but broadens the range of treatment options for clinicians. Indeed, improving patients' stratification and disease staging represent key steps to select AIS patients for minimally invasive surgeries before their spinal deformity is too advanced. OPN, a multifunctional cytokine, has been identified as a potentially key pathophysiologic contributor in the development of idiopathic scoliosis. Particularly, increased plasma OPN levels in patients with idiopathic scoliosis and in bipedal mice, a well-established animal model of this disease, were correlated with the disease (see WO 2008/119170 to Moreau).

[0015] It is commonly accepted that the development of scoliosis is influenced by a postural mechanism. The bipedal condition, naturally present in humans or experimentally induced in animals seems to play an important role in the manifestation of scoliotic deformities (Machida et al., 1999). Importantly, it has been reported that mice on a C57Bl/6 or C3HHe background develop scoliosis closely similar to human idiopathic scoliosis when they gain bipedal posture for 40 weeks following amputation of their forelimbs and tails (Machida et al., 2006); (Oyama et al., 2006). Hence, to address the question of whether OPN contributes to the development of idiopathic scoliosis we took advantage of the availability of OPN-deficient mice on a C57Bl/6 background.

[0016] 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

[0017] The present invention shows that OPN reduces GiPCR signaling *in vitro* mainly via  $\alpha_5\beta_1$  integrin engagement. Without being bound by such hypothesis, the mechanism by which OPN interferes with signaling of GiPCR is believed to be a depletion of functional Gi proteins necessary for these receptors through the sequestration of a part of Gi proteins by integrin  $\beta_1$  subunit and the inactivation of the remaining Gi proteins following their phosphorylation by various kinases engaged in the signaling cascade of  $\alpha_5\beta_1$  integrin. Furthermore, CD44 levels and/or bioavailability to bind to OPN can modulate the inhibitory effect of OPN on GiPCR signaling. The present invention shows that depletion of CD44 exacerbates the inhibitory effect of OPN and that hyaluronic

acid (HA), a known CD44 ligand, further potentiates OPN's effect in a dose-dependent manner. HA exhibits a higher binding affinity than OPN toward CD44. Therefore, HA is competing against OPN for the liaison of CD44; thereby increasing OPN's bioavailability to bind  $\alpha_5\beta_1$  integrin and to inhibit GiPCR signaling. Finally, the present invention shows that the presence of a mutation in CD44 further decreases GiPCR signaling in osteoblasts of scoliotic subjects.

**[0018]** Accordingly, the present invention concerns a method of increasing GiPCR signalization in the cells of a subject in need thereof comprising administering to a subject in need thereof an effective amount of: (a) an inhibitor of OPN expression and/or activity; (b) an inhibitor of integrin  $\alpha_5\beta_1$ ; or (c) a combination of (a) and (b), whereby GiPCR signalization is increased in the cells of a subject in need thereof.

**[0019]** In accordance with a related aspect, the present invention concerns a method of treating or preventing scoliosis in a subject in need thereof comprising reducing  $\alpha_5\beta_1$  expression or activity (e.g., binding to OPN) in the cells of the subject. In an embodiment, the method comprises administering to the subject an effective amount of an inhibitor of integrin  $\alpha_5\beta_1$  expression or activity.

**[0020]** In a specific embodiment, the inhibitor of OPN activity is an OPN antibody. In another specific embodiment, the inhibitor of OPN expression is a siRNA specific to OPN. In another specific embodiment, the inhibitor of  $\alpha_5\beta_1$  is (a) an antibody that binds specifically to integrin subunit  $\alpha_5$ ; (b) an antibody that binds specifically to integrin subunits  $\beta_1$ ; or (c) a combination of (a) and (b). In a specific embodiment, the inhibitor of  $\alpha_5\beta_1$  is an siRNA specific to  $\alpha_5$  or  $\beta_1$ .

**[0021]** In accordance with another aspect of the present invention, there is provided a method of selecting an agent as a potential candidate for the reduction or prevention of GiPCR signaling inhibition induced by OPN comprising contacting a candidate agent with a cell expressing (i) OPN; (ii) integrin  $\alpha_5\beta_1$ , and/or (iii) CD44/sCD44, wherein (a) when OPN expression or activity is decreased in the presence of the candidate agent as compared to in the absence thereof; (b) when integrin  $\alpha_5\beta_1$  expression or activity (e.g., binding to OPN) is decreased in the presence of the candidate agent as compared to in the absence thereof, (c) when sCD44 and/or CD44 expression (level) or binding to OPN is increased in the presence of the candidate agent as compared to in the absence thereof; or (d) any combination of at least two of (a), (b) and (c), the candidate agent is selected.

**[0022]** In accordance with another aspect of the present invention, there is provided a method of selecting an agent as a potential candidate for the reduction or prevention of GiPCR signalization comprising contacting a candidate agent with a cell expressing (a) OPN; and/or (b) integrin  $\alpha_5\beta_1$ , wherein (a) when OPN expression or activity; and/or (b) when integrin  $\alpha_5\beta_1$  is increased in the presence of the candidate agent as compared to in the absence thereof, the candidate agent is selected.

**[0023]** In accordance with another aspect of the present invention, there is provided (i) an inhibitor of OPN expression or activity; ii) an inhibitor of integrin  $\alpha_5\beta_1$  expression or activity; iii) sCD44 or a stimulator of sCD44/CD44 expression; or iv) any combination of at least two of (i) to (iii) for use in increasing GiPCR

signalization in cells of a subject in need thereof.

**[0024]** In accordance with another aspect of the present invention, there is provided a use of (i) an inhibitor of OPN expression or activity; ii) an inhibitor of integrin  $\alpha_5\beta_1$  expression or activity; iii) sCD44 or a stimulator of sCD44/CD44 expression; or iv) any combination of at least two of (i) to (iii), for the preparation of a medicament for increasing GiPCR signalization in cells of a subject in need thereof.

**[0025]** In accordance with another aspect of the present invention, there is provided a composition for increasing GiPCR signalization in the cells of a subject in need thereof comprising (i) an inhibitor of OPN expression or activity; (ii) an inhibitor of  $\alpha_5\beta_1$  expression or activity; (iii) sCD44 or a stimulator of sCD44/CD44 expression; or (iv) any combination of at least two of (i) to (iii).

**[0026]** In a specific embodiment, the subject in need thereof is a subject diagnosed with a scoliosis or at risk of developing a scoliosis.

**[0027]** In accordance with another aspect of the present invention, there is provided a method of increasing GiPCR signalization in the cells of a subject in need thereof comprising administering to the subject an effective amount of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity, whereby GiPCR signalization is increased in the cells of the subject.

**[0028]** In a specific embodiment, the method further comprises administering to the subject an effective amount of an inhibitor of OPN expression and/ or activity. In another specific embodiment, the inhibitor of OPN activity is an OPN antibody. In another specific embodiment, the inhibitor of OPN expression is a siRNA specific to OPN. In another specific embodiment, the inhibitor of  $\alpha_5\beta_1$  activity is (a) an antibody that binds specifically to integrin subunit  $\alpha_5$ ; (b) an antibody that binds specifically to integrin subunit  $\beta_1$ ; (c) an antibody that binds specifically to integrin subunits  $\alpha_5\beta_1$ ; (d) a molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin; or (e) any combination of at least two of (a) to (d). In another specific embodiment, said molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a RGD peptide or derivative thereof. In another specific embodiment, said molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a peptide fragment of OPN comprising a RGD motif. In another specific embodiment, the peptide fragment of OPN comprises the amino acid sequence GRGDSVYGLRS (SEQ ID NO: 18). In another specific embodiment, the inhibitor of  $\alpha_5\beta_1$  expression is an siRNA specific to  $\alpha_5$  or  $\beta_1$ . In a specific embodiment, the method further comprises administering to the subject i) an effective amount of sCD44 or a fragment thereof which specifically binds to OPN; or ii) a stimulator of sCD44 and/or CD44 expression.

**[0029]** In accordance with another aspect of the present invention, there is provided a use of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity in the preparation of a medicament for inhibiting GiPCR signalization in cells of a subject in need thereof.

**[0030]** In accordance with another aspect of the present invention, there is provided a use of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity for inhibiting GiPCR signalization in cells of a subject in need thereof.

**[0031]** In accordance with another aspect of the present invention, there is provided an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity for use in inhibiting GiPCR signalization in cells of a subject in need thereof.

**[0032]** In a specific embodiment of the use or the inhibitor, said inhibitor of  $\alpha_5\beta_1$  activity is (a) an antibody that binds specifically to integrin subunit  $\alpha_5$ ; (b) an antibody that binds specifically to integrin subunit  $\beta_1$ ; (c) an antibody that binds specifically to integrin subunits  $\alpha_5\beta_1$ ; (d) a molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin; or (e) any combination of at least two of (a) to (d). In another specific embodiment of the use or the inhibitor, the molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a RGD peptide or derivative thereof. In another specific embodiment, the molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a peptide fragment of OPN comprising a RGD motif. In another specific embodiment, the peptide fragment of OPN comprises the amino acid sequence GRGDSVVYGLRS (SEQ ID NO: 18). In another specific embodiment, the inhibitor of integrin  $\alpha_5\beta_1$  expression is an siRNA specific to  $\alpha_5$  or  $\beta_1$ .

**[0033]** In accordance with another aspect of the present invention, there is provided a composition comprising the inhibitor of the present invention, and a pharmaceutical carrier.

**[0034]** In another specific embodiment, the composition is for increasing GiPCR signaling in cells of a subject in need thereof.

**[0035]** In specific embodiments of the method, the use, the inhibitor, or the composition of the present invention, the subject is a subject diagnosed with a scoliosis or at risk of developing a scoliosis. In another specific embodiment, the scoliosis is an idiopathic scoliosis. In another specific embodiment, the scoliosis is adolescent idiopathic scoliosis (AIS).

**[0036]** In accordance with another aspect of the present invention, there is provided a method of determining the risk of developing a scoliosis in a subject comprising: (i) providing a cell sample isolated from the subject; (ii) detecting, in the cell sample from the subject, the presence of at least one copy of a CD44 risk allele which introduces a mutation at amino acid position 230 of CD44 or a marker in linkage disequilibrium therewith; and (iii) determining that the subject is at risk of developing a scoliosis when at least one copy of the risk allele or marker in linkage disequilibrium therewith is detected in the cell sample from the subject.

**[0037]** In a specific embodiment, the risk allele comprises SNP rs1467558 and the mutation changes an isoleucine at position 230 of CD44 to a threonine.

**[0038]** In accordance with another aspect of the present invention, there is provided a method of stratifying a subject having scoliosis (e.g., idiopathic scoliosis such as adolescent idiopathic scoliosis (AIS)) comprising: (i) providing a cell sample isolated from the subject; (ii) detecting, in the cell sample from the subject, the presence of at least one copy of a CD44 risk allele which introduces a mutation at amino acid position 230 of CD44 or a marker in linkage disequilibrium therewith; and (iii) (a) stratifying the subject into a first AIS subclass when at least one copy of the CD44 risk allele or marker in linkage disequilibrium therewith is detected in the cell sample from the subject; or (b) stratifying the subject into a second AIS subclass when the CD44 risk allele is not

detected in the cell sample from the subject.

[0039] In a specific embodiment, the mutation changes an isoleucine at position 230 of CD44 to a threonine. In another specific embodiment, the at least one copy of the CD44 risk allele consists of two copies of the CD44 risk allele and the subject is homozygote for the mutation. In another specific embodiment, the sample is a nucleic acid sample. In another specific embodiment, said sample is a protein sample.

[0040] In accordance with another aspect of the present invention, there is provided a kit for performing the method as defined in any one of claims 24 to 30 comprising: (i) a nucleic acid probe or primer for detecting a CD44 risk allele comprising a mutation in a codon encoding amino acid 230 of CD44; and/or (ii) a protein ligand which specifically detects a mutation at amino acid 230 of CD44.

[0041] In a specific embodiment, the nucleic acid probe or primer comprises a nucleic acid sequence which specifically hybridizes to a nucleic acid encoding a threonine at amino acid position 230 of CD44. In another specific embodiment, the protein ligand is an antibody specific for a CD44 protein comprising a threonine at amino acid position 230.

[0042] 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

[0043] In the appended drawings:

[0044] **Figure 1 shows that genetic deletion of OPN protects bipedal C57BL/6 from scoliosis by improving Gi protein-mediated receptor signal transduction.** (A-D) Female C57Bl/6 wild-type (WT) and OPN knockout (OPN<sup>-/-</sup>) mice were amputated from forelimbs and tails at 1 month of age and subjected to bipedal ambulation for 36 weeks to induce scoliosis. X-ray radiographs of the spine as taken at the end of the experiment period. OPN<sup>-/-</sup> mice did not develop scoliosis in the quadrupedal or even bipedal while WT developed it in the bipedal mice. (E) Plasma OPN was detected in C57Bl/6 (WT) quadrupedal and bipedal mice, and the bipedal mice showed higher plasma OPN than quadrupedal WT mice. (F-I) GiPCR signaling was checked in osteoblasts from WT and OPN<sup>-/-</sup> mice using DAMGO, somatostatin, oxymethazolin and apelin. The response was greater in OPN<sup>-/-</sup> than WT osteoblasts. On the other hand, bipedal WT mice showed lower response than the quadrupedal WT. (J-M) The pre-treatment of osteoblasts from WT and OPN<sup>-/-</sup> mice with PTX blocked the Gi coupling for their cognate receptors in these cells, response to each of the previous agonists. This is indicating that these compounds evoked typical CDS response profiles of GiPCR in WT and OPN<sup>-/-</sup> osteoblasts. (N-O) Isoproterenol and desmopressin were used to activate Gs while bradykinin and endothelin-1 were used to activate Gq through their cognate receptors. Osteoblasts from OPN<sup>-/-</sup> mice were less responsive to Gs stimulation than those from WT mice, whereas no difference in the Gq stimulation elicited in WT and OPN<sup>-/-</sup> osteoblasts.

[0045] **Figure 2 shows that extracellular OPN causes Gi protein-coupled receptor signaling**

**dysfunction.** (A) OPN was knockdown by siRNA in MC3T3-E1 osteoblastic cell line and the capacity of GiPCR ligands to induce cell signaling as measured by CDS. Cellular response to DAMGO and oxymethazolin was significantly greater in cells depleted of OPN. (B) OPN, blocked by OPN specific antibody, in MC3T3-E1 osteoblastic cell line gave the same results as in (A). (C-D) MC3T3-E1 osteoblastic cells were treated with exogenous recombinant OPN (rOPN) prior to DAMGO and oxymethazolin stimulation. In each case, rOPN caused a decrease in the integrated response in a concentration-dependent manner, which was prevented by OPN antibody.

**[0046] Figure 3 shows that CD44 is not involved in the inhibition of GiPCR signaling caused by extracellular OPN but can modulate OPN's effect.** (A) The blockage of CD44 by CD44 specific antibody in MC3T3-E1 osteoblastic cell line and cells treated with rOPN 0.5µg/ml further aggravated GiPCR signaling defect induced by OPN and did not reduce the GiPCR signaling defect cause by OPN. DAMGO and oxymethazolin were used as agonist for GiPCR. (B-C) To validate the activity of the anti-CD44 antibody, the cells pre-treated with IgG control or CD44 antibody were stimulated with increasing concentrations of hyaluronic acid (HA), a high affinity ligand of CD44. The integrated response induced by HA was completely abrogated by pre-treatment with the CD44 antibody. Moreover, the effect of the CD44 antibody on response to HA stimulation was concentration-dependent. (D-E) CD44 was knockdown in MC3T3-E1 osteoblastic cell line by siRNA. The efficiency of siRNA transfection and inhibition of CD44 expression was demonstrated by qPCR (D) and Western blot analysis (E). (F) The knockdown of CD44 led to similar results as (A) and CD44 knockdown did not reduce the GiPCR signaling defect cause by OPN but further aggravated the defect. (G-H) rOPN treatment caused a concentration-dependent decrease in response to both DAMGO and oxymethazolin in osteoblasts from bipedal CD44 knockout (CD44<sup>-/-</sup>) mice. (I-J) Osteoblasts cells from bipedal (CD44<sup>-/-</sup>) mice were less responsive to DAMGO or oxymethazolin when compared with osteoblasts from quadrupedal (CD44<sup>-/-</sup>) mice. (K-P) CD44 inhibition and HA intensify the effect of OPN on GiPCR signaling in a dose-dependent manner. (K) In response to LPA stimulation on GiPCR signaling, CD44<sup>-/-</sup> osteoblasts are less responsive and OPN<sup>-/-</sup> osteoblasts are more responsive compared to the wild type cells in a dose dependent manner. (L) Treatment with HA, the natural agonist for CD44 receptor, exacerbated the GiPCR signaling defects in wild type osteoblasts, compared to the control. Inhibition of CD44 using HA or an antibody (CD44 fab) in presence of recombinant OPN (rOPN) also exacerbate the signaling defect. The response is most reduced when CD44 is inhibited in both ways (HA+CD44 Fab). (M) The same experiment using CD44<sup>-/-</sup> osteoblast does not show any sensitivity to HA or anti-CD44 antibody. (N, O and P). Removing the effect of CD44 by using CD44<sup>-/-</sup> osteoblasts or inhibiting the CD44 receptor by treating the wild type cells with HA has the same effect on GiPCR signaling response in the presence of OPN.

**[0047] Figure 4 shows that RGD-dependent integrins mediate the inhibitory effect of OPN on GiPCR signaling.** (A- B & C) OPN binds to integrins through RGD motif and this was clear when we inhibit the SVVYGLR binding motif by bio1211, which selectively inhibits  $\alpha_4\beta_1$  integrin, the only SVVYGLR-containing integrin present in osteoblasts. Bio1211 did not affect the OPN signaling while coincubation of cells with rOPN and RDG peptide completely prevented the inhibitory effect of rOPN on response to DAMGO and oxymethazolin. (D-

E) Incubation of osteoblasts from WT mice with high concentrations of RGD demonstrated significant increase of response to DAMGO or oxymethazolin while no change in osteoblasts from OPN<sup>-/-</sup> mice was observed.

**[0048] Figure 5 shows the identification of integrins involved in the inhibition of GiPCR signaling by OPN.** (A, B) Blockage of the different integrins using specific antibodies and (C) knockdown of these integrins in MC3T3-E1 osteoblastic cells. Only inhibition of  $\alpha_5$  and  $\beta_1$  integrins in cells reversed the inhibitory action of OPN on GiPCR-signaling. (D-E-F) Knockdown of  $\alpha_5$  and  $\beta_1$  integrins in C57Bl/6 bipedal WT and CD44<sup>-/-</sup> mice reversed the inhibitory effect of rOPN on response to both DAMGO and oxymethazolin.

**[0049] Figure 6 shows that OPN reduces the availability of Gi proteins for their cognate receptors.** (A, B, C) MC3T3-E1 osteoblastic cells were treated with PBS or rOPN. Cell lysates were analyzed by Western blot for the expression of three different GPCRs: mu-opioid (MO), lysophosphatidic acid type 1 (LPA1) and melatonin type 2 (MT2) receptors (A) while Gi<sub>1</sub>, Gi<sub>2</sub> and Gi<sub>3</sub> proteins isoforms expression was detected by Western blot (B) and qPCR (C). There was no significant effect of rOPN treatment on the quantity of Gi proteins or mRNAs (B, C) or GiPCR proteins (A). (D-E-F) Cells lysates were immunoprecipitated with antibodies against MO, MT2 or  $\beta_1$  integrin receptors, and the presence of Gi proteins in each precipitate was examined by Western blot using antibodies specific for Gi<sub>1</sub>, Gi<sub>2</sub> or Gi<sub>3</sub> isoform. The amount of each of these Gi protein isoforms was elevated in the  $\beta_1$  integrin precipitates following rOPN treatment.

**[0050] Figure 7 shows that OPN enhances the phosphorylation of Gi proteins.** (A) MC3T3-E1 cells were treated with rOPN, or pre-treated with antibody against  $\alpha_5\beta_1$  integrin prior to rOPN treatment and then immunoprecipitation (IP) was performed on cell lysates using antibodies against Gi<sub>1</sub>, Gi<sub>2</sub> or Gi<sub>3</sub> protein isoforms, and the presence of phosphorylation revealed by Western blot by anti-phospho serine/threonine or anti-tyrosine antibodies. (B) IP of osteoblasts cell lysates from scoliotic bipedal WT or CD44<sup>-/-</sup> mice and quadrupedal control mice by antibodies against Gi<sub>1</sub>, Gi<sub>2</sub> and Gi<sub>3</sub> proteins followed by Western blot using antibodies against anti-phospho-serine/threonine or an anti-phospho-tyrosine. (C-E) Cell extracts prepared from MC3T3-E1 cells treated with rOPN in the presence or absence of kinases inhibitors followed by IP with antibodies against Gi<sub>1</sub>, Gi<sub>2</sub> or Gi<sub>3</sub> protein isoforms, and Western blot using anti-phospho serine/threonine or anti-tyrosine antibodies.

**[0051] Figure 8 shows the effect of OPN on GsPCR proteins.** (A) MC3T3-E1 cells were treated with rOPN or PBS (A) stimulated by isoproterenol or desmopressin or (B) IP by antibodies against Gs followed by Western blot with antibodies against anti-phospho-serine/threonine or (C-D) IP by antibodies against MOR or MT2R followed by Western blot with antibodies against Gs. (E) MC3T3-E1 cells were treated with PTX to mimic disruption action of rOPN and compared to cells treated with rOPN alone or in combination with Src inhibitor (PP2) to prevent tyrosine phosphorylation.

**[0052] Figure 9 shows that the CT SNP in CD44 exacerbates OPN's inhibitory effect on GiPCR signaling.** (A) Osteoblasts from AIS subjects carrying the CT SNP and from healthy subjects were treated with OPN and the effect on GiPCR signaling was assessed following stimulation with LPA. (B) Same as in (A) except that the cell samples were treated with increasing concentrations of OPN. The presence of the CT SNP reduces

by about 50% GiPCR signaling.

[0053] Figure 10 shows a schematic representation of the proposed mechanism by which OPN reduces GiPCR signaling and contributes to the development of spinal deformity. Following OPN binding,  $\alpha_5\beta_1$  integrin recruits a part of Gi proteins from the common pool, then promotes different signaling pathways leading to the activation of various kinases, which directly or indirectly phosphorylate a part of the remaining Gi protein in the common pool, leading to reduced GiPCR signaling.

#### DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0054] As used herein the terms "risk of developing scoliosis" refer to a genetic or metabolic predisposition of a subject to develop a scoliosis (i.e. spinal deformity) and/or to develop a more severe scoliosis at a future time (i.e. curve progression). For instance, an increase of the Cobb's angle of a subject (e.g. from 40° to 50°, or from 18° to 25°) is a "development" of scoliosis.

[0055] In an embodiment, the above-mentioned subject is a likely candidate for developing a scoliosis, such as idiopathic scoliosis (e.g., Infantile Idiopathic Scoliosis, Juvenile Idiopathic Scoliosis or Adolescent Idiopathic Scoliosis (AIS)). As used herein the terms "likely candidate for developing scoliosis" include subjects (e.g., children) of which at least one parent has a scoliosis (e.g., adolescent idiopathic scoliosis). Among other factors, age (adolescence), gender and other family antecedent are factors that are known to contribute to the risk of developing a scoliosis and are used to a certain degree to assess the risk of developing a scoliosis. In certain subjects, scoliosis develops rapidly over a short period of time to the point of requiring a corrective surgery (often when the deformity reaches a Cobb's angle  $\geq 50^\circ$ ). Current courses of action available from the moment a scoliosis such as AIS is diagnosed (when scoliosis is apparent) include observation (when Cobb's angle is around 10-25°), orthopedic devices (when Cobb's angle is around 25-30°), and surgery (over 45°). A more reliable determination of the risk of progression could enable to 1) select an appropriate diet to remove certain food products identified as contributors to scoliosis; 2) select the best therapeutic agent; and/or 3) select the least invasive available treatment such as postural exercises, orthopedic device, or less invasive surgeries or surgeries without fusions (a surgery that does not fuse vertebra and preserves column mobility). The present invention encompasses selecting the most efficient and least invasive known preventive actions or treatments in view of the determined risk of developing scoliosis.

[0056] As used herein the term "subject" is meant to refer to any mammal including human, mouse, rat, dog, chicken, cat, pig, monkey, horse, etc. In a particular embodiment, it refers to a human.

[0057] A "subject in need thereof" or a "patient" in the context of the present invention is intended to include any subject that will benefit or that is likely to benefit from the modulation (increase or decrease) of OPN's effect on GiPCR signal transduction. In an embodiment, the subject in need thereof is a subject that will benefit or is likely to benefit from an increase in GiPCR signal transduction and thus from (a) an antagonist (inhibitor) of

OPN expression and/or activity; (b) an antagonist (inhibitor) of  $\alpha_5$  (e.g., Gene ID 3678, NP\_002196) and/or  $\beta_1$  expression and/or activity or (c) an increase in sCD44 or CD44 expression. In a specific embodiment, the subject in need thereof is a subject having high levels of OPN as compared to a control subject. In a specific embodiment, the high level of OPN corresponds to a level  $\geq$  to about 600 ng/ml,  $\geq$  to about 700 ng/ml,  $\geq$  to about 800 ng/ml,  $\geq$  to about 900 ng/ml or  $\geq$  to about 1000 ng/ml of OPN as measured in a blood sample from the subject. In an embodiment, a subject in need thereof is a subject diagnosed with a scoliosis (e.g., AIS). In another embodiment, the subject in need thereof is a subject is likely to develop a scoliosis (e.g., AIS).

**[0058]** As used herein the terminology "biological sample" refers to any solid or liquid sample isolated from a living being. In a particular embodiment, it refers to any solid or liquid sample isolated from a human. Without being so limited it includes a biopsy material, blood, tears, saliva, maternal milk, synovial fluid, urine, ear fluid, amniotic fluid and cerebrospinal fluid. In a specific embodiment it refers to a blood sample.

**[0059]** As used herein the terminology "blood sample" is meant to refer to blood, plasma or serum.

**[0060]** As used herein the terminology "control sample" is meant to refer to a sample that does not come from a subject known to have scoliosis or known to be a likely candidate for developing a scoliosis. In methods for determining the risk of developing scoliosis in a subject that is pre-diagnosed with scoliosis, the sample may however also come from the subject under scrutiny at an earlier stage of the disease or disorder. In a specific embodiment, the control sample can come from another subject diagnosed with scoliosis and belonging to the same functional group (e.g., FG1, FG2 or FG3) at an earlier (or later stage) of the disease or disorder.

**[0061]** As used herein the terminology "control" is meant to encompass "control sample". In certain embodiments, the term "control" also refers to the average or median value obtained following determination of GiPCR signalization in a plurality of samples (e.g., samples obtained from several subjects not known to have scoliosis and not known to be a likely candidate for developing scoliosis).

**[0062]** As used herein the term "treating" or "treatment" in reference to scoliosis is meant to refer to at least one of a reduction of Cobb's angle in a preexisting spinal deformity, improvement of column mobility, preservation/maintenance of column mobility, improvement of equilibrium and balance in a specific plan; maintenance/preservation of equilibrium and balance in a specific plan; improvement of functionality in a specific plan, preservation/maintenance of functionality in a specific plan, cosmetic improvement, and combinations of any of the above.

**[0063]** As used herein the term "preventing" or "prevention" in reference to scoliosis is meant to refer to a at least one of a reduction in the progression of a Cobb's angle in a patient having a scoliosis or in an asymptomatic patient, a complete prevention of apparition of a spinal deformity, including changes affecting the rib cage and pelvis in 3D, and a combination of any of the above.

**[0064]** The terms "suppressor" "inhibitor" and "antagonist" are well known in the art and are used herein interchangeably. They include intracellular as well as extracellular inhibitors.

**[0065]** The terms “inhibitor of OPN” or “OPN antagonist” include any compound able to negatively affect (i.e., reduce totally or partially) the expression and/or activity of OPN (e.g., Gene ID 6696, NP\_001035147.1 (SEQ ID NO: 25) and NM\_001040058 (SEQ ID NO:26)) in cells. In a specific embodiment, the activity of OPN in cells is a reduction in GiPCR signaling. Similarly, the terms “inhibitor of  $\alpha_5\beta_1$ ”, “inhibitor of  $\alpha_5$ ” or “inhibitor of  $\beta_1$ ” and the like include any compound able to negatively affect the expression and/or activity of  $\alpha_5$  (e.g., Gene ID 3678, NP\_002196 (SEQ ID NO:23) and NM\_002205.2 (SEQ ID NO: 24)) and/or  $\beta_1$  (Gene ID 3688, NP\_002202 (SEQ ID NO: 21) and NM\_002211.3 (SEQ ID NO:22)) in cells. In a particular embodiment, the “activity” of  $\alpha_5$  and/or  $\beta_1$  in cells is the transduction of the signal leading to the OPN-dependent inhibition of GiPCR signaling.

**[0066]** The terms “inhibitor of OPN expression” or “inhibitor of  $\alpha_5\beta_1$  expression” include any compound able to negatively affect OPN’s,  $\alpha_5$ ’s and/or  $\beta_1$ ’s expression (i.e., at the transcriptional and/or translational level) i.e. the level of OPN/  $\alpha_5$  or  $\beta_1$  mRNA and/or protein or the stability of the protein. Without being so limited, such inhibitors include RNA interference agents (siRNA, shRNA, miRNA), antisense molecules, and ribozymes. Such RNA interference agents are design to specifically hybridize with their target nucleic acid under suitable conditions and are thus substantially complementary their target nucleic acid.

**[0067]** The terms “inhibitor of OPN activity” or “inhibitor of  $\alpha_5\beta_1$  activity” refers to any molecules that is able to reduce or block the effect of OPN or  $\alpha_5\beta_1$  on Gi-mediated signaling. These molecules increase GiPCR signaling in cells by blocking/reducing totally or partially the inhibitory effect induced by OPN and/or  $\alpha_5\beta_1$  activity. Non-limiting examples of inhibitors of OPN’s activity include proteins (e.g., dominant negative, inactive variants), peptides, small molecules, anti-OPN antibodies (neutralizing antibodies), antibody fragments, inactive fragments of  $\alpha_5$  and/or  $\beta_1$  integrins etc. Non-limiting examples of inhibitors of  $\alpha_5\beta_1$  activity include proteins (e.g., dominant negative, inactive variants), peptides (RGD peptides or RGD peptide-derivatives), small molecules, anti  $\alpha_5$  and/or  $\beta_1$  antibodies (e.g., neutralizing antibodies such as Volociximab™ M200), antibody fragments, etc. In an embodiment, the RGD peptide is a peptide fragment of OPN comprising a RGD motif comprising the amino acid sequence GRGDSVYGLRS corresponding to amino acid 158 to 169 of OPN (SEQ ID NO:18). In an embodiment, the OPN fragment comprising the RGD motif comprises amino acids 158 to 162, 158 to 165, 158 to 167, 158 to 170, 158 to 175, 158 to 180, 158 to 185, 158 to 190, 158 to 195, or 158 to 200 of OPN (e.g., SEQ ID NO: 25). In an embodiment, peptide fragment of OPN comprising a RGD motif comprises amino acids 158 to 161, 156 to 161, 154 to 161, 152 to 162, 150 to 162, 148 to 162, 146 to 162, 144 to 162, 140 to 162, 159 to 163, 159 to 164, 159 to 162, 159 to 166, 159 to 167, or 159 to 169 of OPN (e.g., SEQ ID NO: 25).

**[0068]** In an embodiment, the “inhibitor of OPN’s activity” is a neutralizing antibody directed against (or specifically binding to) a human OPN polypeptide which inhibits its binding to  $\alpha_5\beta_1$  (i.e. binding to  $\alpha_5$  and/or  $\beta_1$  integrin) In an embodiment, the “inhibitor of  $\alpha_5\beta_1$  activity” is a neutralizing antibody directed against (or specifically binding to) a human  $\alpha_5\beta_1$  ( $\alpha_5$  and/or  $\beta_1$ ) polypeptide which inhibits the binding of OPN to  $\alpha_5\beta_1$  (i.e. binding to  $\alpha_5$  and/or  $\beta_1$  integrin). In an embodiment, the antibody binds to the RGD domain of OPN. In an embodiment, the antibody is directed against amino acids 159 to 162, 158 to 162, 158 to 165, 158 to 167, 158 to 170, 158 to 175,

158 to 180, 158 to 185, 158 to 190, 158 to 195, or 158 to 200 of OPN (e.g., SEQ ID NO: 25). In an embodiment, the antibody is directed against amino acids 158 to 161, 156 to 161, 154 to 161, 152 to 162, 150 to 162, 148 to 162, 146 to 162, 144 to 162, 140 to 162, 159 to 163, 159 to 164, 159 to 162, 159 to 166, 159 to 167, or 159 to 169 of OPN (e.g., SEQ ID NO: 25).

**[0069]** The term “stimulator” or “enhancer” of sCD44/CD44 expression (e.g., Gene ID 960, NM\_000610 (SEQ ID NO: 19), NP\_000601.3, (SEQ ID NO: 20)) refers to an agent able to increase the level or expression of CD44, an agent able to increase CD44 secretion. In an embodiment, the stimulator of sCD44/CD44 is an agent able to increase CD44 affinity toward OPN. Without being so limited, the agent can be a protein, a peptide, a small molecule or a nucleotide.

**[0070]** One possible way of decreasing OPN's effect on GiPCR-signaling is to reduce OPN's interaction with the  $\alpha_5\beta_1$  integrin by, for example, increasing or favoring OPN's binding to CD44. Hyaluronic acid (HA) is known to bind to CD44. As shown herein HA potentiates the effect of OPN, probably by increasing OPN's bioavailability to  $\alpha_5\beta_1$  (e.g., by sequestering CD44 which is no longer available for binding to OPN). Accordingly, subjects which will benefit from OPN inhibition and increase in GiPCR-signalization should not take HA supplements and/or should decrease their food intake of HA by for example avoiding or decreasing his/her consumption of foods that are particularly rich in HA or that are known to increase HA synthesis. Non-limiting examples of such food include meat and meat organs (e.g., veal, lamb, beef and gizzards, livers, hearts and kidneys), fish, poultry (including meat fish and poultry broths), soy (including soy milk), root vegetables containing starch including potatoes and sweet potatoes. Sweet potatoes particularly rich in HA include satoimo and imoji, two Japanese sweet potatoes.

**[0071]** The present invention also relates to methods for the determination of the level of expression (i.e. transcript (RNA) or translation product (protein)) of OPN,  $\alpha_5\beta_1$  integrin and/or CD44. As used herein the term  $\alpha_5\beta_1$  is used herein to refer to  $\alpha_5\beta_1$  integrin. In specific embodiments, it also includes a method that comprises the determination of the level of expression of one or more other scoliosis markers (see for example WO 2008/119170 to Moreau). The present invention therefore encompasses any known method for such determination including ELISA (Enzyme Linked Immunosorbent Assay), RIA (Radioimmunoassay), immunofluorescence, real time PCR and competitive PCR, Northern blots, nuclease protection, plaque hybridization and slot blots.

**[0072]** The present invention also concerns isolated nucleic acid molecules including probes and primers to detect OPN,  $\alpha_5\beta_1$  and sCD44/CD44 (and optionally other scoliosis markers). 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 17, or at least 18, or at least 19, or 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 17 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 17 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. It should be understood that in real-time PCR, primers also constitute probe without the traditional meaning of this term. Primers or probes appropriate to detect OPN and/or  $\alpha_5\beta_1$  in the methods of the present invention can be designed with known methods using sequences distributed across their respective nucleotide sequence. The probes and/or primers of the present invention are designed to specifically hybridize with their target nucleic acid ( $\alpha_5$  (SEQ ID NO:24),  $\beta_1$  (SEQ ID NO:22), CD44 wild type (SEQ ID NO:19) or CD44 risk allele (e.g., comprising 230I→T (SNP (CT)) variant) and/or OPN (SEQ ID NO:26)). In an embodiment, the primers and probes of the present invention are substantially complementary to their target nucleic acid.

**[0073]** Substantially complementary nucleic acids are nucleic acids in which the complement of one molecule is substantially identical to the other molecule. Two nucleic acid or protein sequences are considered substantially identical if, when optimally aligned, they share at least about 70% sequence identity. In alternative embodiments, sequence identity may for example be at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 98% or at least 99%. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, Adv. Appl. Math 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, J. Mol. Biol. 48:443, the search for similarity method of Pearson and Lipman, and the computerized implementations of these

algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be determined using the BLAST algorithm, described in Altschul et al. 1990 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information (through the internet at <http://www.ncbi.nlm.nih.gov/>). The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length  $W$  in the query sequence that either match or satisfy some positive-valued threshold score  $T$  when aligned with a word of the same length in a database sequence.  $T$  is referred to as the neighborhood word score threshold. Initial neighborhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity  $X$  from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters  $W$ ,  $T$  and  $X$  determine the sensitivity and speed of the alignment. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability ( $P(N)$ ), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

**[0074]** An alternative indication that two nucleic acid sequences are substantially complementary is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C. Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M NaHPO<sub>4</sub>, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C. Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

**[0075]** Probes of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and  $\alpha$ -nucleotides and the like. Modified sugar-phosphate backbones are generally known. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

**[0076]** 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.

**[0077]** As used herein the terms "detectably labeled" refer to a marking of a probe or an antibody in accordance with the present invention that will allow the detection of OPN and/or  $\alpha_5\beta_1$  in accordance with 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. Non-limiting examples of labels include  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ , and  $^{35}\text{S}$ . 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.

**[0078]** 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  $^{32}\text{P}$  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.

**[0079]** 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.

**[0080]** 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.

**[0081]** 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.

**[0082]** 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.

**[0083]** 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. Other methods include serial analysis of gene expression (SAGE), differential display, as well as subtractive hybridization methods, differential screening (DS), RNA arbitrarily primer (RAP)-PCR, restriction endonucleolytic analysis of differentially expressed sequences (READS), amplified restriction fragment-length polymorphisms (AFLP).

#### Antibodies

**[0084]** The present invention encompasses using antibodies for detecting or determining OPN and/or  $\alpha_5\beta_1$  levels for instance in the samples of a subject and for including in kits of the present invention. Antibodies that specifically bind to these biological markers can be produced routinely with methods further described below. The present invention also encompasses using antibodies commercially available. Without being so limited antibodies that specifically bind to OPN and/or  $\alpha_5\beta_1$  include those listed in Table 1 below.

Table 1: Non-limiting examples of commercially available human OPN Elisa kits.

Company	Kit name	Catalogue number	Sensitivity
IBL Hambourg	Human Osteopontin ELISA	JP 171 58	3,33ng/ml
IBL America	Human Osteopontin N-Half Assay Kit - IBL	27258	3,90 pmol/L
IBL-America	Human Osteopontin Assay Kit - IBL	27158	3,33ng/ml
Assay designs	Osteopontin (human) EIA Kit	900-142	0,11ng/ml
American Research Products, Inc.	Osteopontin, human kit	17158	?
R&D Systems	Human Osteopontin (OPN) ELISA Kit	DOST00	0.024 ng/mL
Promokine	Human Osteopontin ELISA	PK-EL-KA4231	3,8ng/ml
.Usnclife	Human Osteopontin,OPN ELISA Kit	E0899h	?

Table 2: Non-limiting examples of commercially available antibodies for OPN (Human, Unconjugated)

Company Name	Catalogue Number	Host
EMD Millipore	AB10910	rabbit
Boster Immunoleader	PA1431	
LifeSpan BioSciences	LS-C63082-100	mouse
LifeSpan BioSciences	LS-B5940-50	mouse
LifeSpan BioSciences	LS-C137501-100	mouse
LifeSpan BioSciences	LS-C31763-100	rabbit
LifeSpan BioSciences	LS-C99283-400	rabbit
LifeSpan BioSciences	LS-C9410-100	rabbit
LifeSpan BioSciences	LS-C122259-20	rabbit
LifeSpan BioSciences	LS-C88774-0.1	rabbit
LifeSpan BioSciences	LS-C136850-100	rabbit

LifeSpan BioSciences	LS-C96393-500	rabbit
LifeSpan BioSciences	LS-C193595-200	mouse
LifeSpan BioSciences	LS-C193596-100	mouse
LifeSpan BioSciences	LS-C63081-100	mouse
LifeSpan BioSciences	LS-C193597-100	mouse
LifeSpan BioSciences	LS-C169155-100	mouse
LifeSpan BioSciences	LS-C189569-1000	mouse
LifeSpan BioSciences	LS-C189635-1000	mouse
LifeSpan BioSciences	LS-C189636-1000	mouse
LifeSpan BioSciences	LS-C189634-1000	mouse
LifeSpan BioSciences	LS-C73947-500	mouse
LifeSpan BioSciences	LS-C189134-50	rabbit
LifeSpan BioSciences	LS-B5272-250	rabbit
LifeSpan BioSciences	LS-C176152-100	rabbit
LifeSpan BioSciences	LS-C194024-100	rabbit
LifeSpan BioSciences	LS-B5626-50	rabbit
LifeSpan BioSciences	LS-C131159-20	rabbit
LifeSpan BioSciences	LS-B9287-200	rabbit
LifeSpan BioSciences	LS-C73949-200	rabbit
LifeSpan BioSciences	LS-C182368-50	rabbit
LifeSpan BioSciences	LS-B2411-50	goat
LifeSpan BioSciences	LS-B8326-100	mouse
LifeSpan BioSciences	LS-B7193-50	rabbit
LifeSpan BioSciences	LS-B425-50	rabbit
LifeSpan BioSciences	LS-C9413-100	rabbit
LifeSpan BioSciences	LS-B7193-50	rabbit
LifeSpan BioSciences	LS-C9413-100	rabbit
LifeSpan BioSciences	LS-B9080-100	rabbit
LifeSpan BioSciences	LS-C201116-100	rabbit

Boster Immunoleader	PA1431	
antibodies-online	ABIN933617	mouse
antibodies-online	ABIN1381708	Chicken
BACHEM	T-4816.0400	Rabbit
BACHEM	T-4815.0050	Rabbit
Biorbyt	orb12414	mouse
Biorbyt	orb128774	Rabbit
Biorbyt	orb12506	mouse
Biorbyt	orb94522	Rabbit
Biorbyt	orb13123	Rabbit
Biorbyt	orb88187	goat
Biorbyt	orb94961	mouse
Biorbyt	orb86662	rabbit
Biorbyt	orb170816	mouse
Biorbyt	orb175965	mouse
Biorbyt	orb19047	goat
Biorbyt	orb43142	rabbit
Biorbyt	orb120032	rabbit
Biorbyt	orb11192	rabbit
Biorbyt	orb11191	rabbit
antibodies-online	ABIN933617	mouse
BioVision	5426-100	mouse
BioVision	5422-100	mouse
BioVision	5424-100	mouse
BioVision	5423-100	mouse
BioVision	5425-100	mouse
BioVision	5421-100	mouse
Merck Millipore	04-970	mouse
Merck Millipore	MAB3055	rabbit
Merck Millipore	AB1870	Rabbit
Merck Millipore	AB10910	Rabbit
GenWay Biotech, Inc.	GWB-T00561	mouse
GenWay Biotech, Inc.	GWB-T00557	mouse
GenWay Biotech, Inc.	GWB-T00558	mouse
GenWay Biotech, Inc.	GWB-T00559	mouse
GenWay Biotech, Inc.	GWB-T00560	mouse
GenWay Biotech, Inc.	GWB-3A2E99	goat
GenWay Biotech, Inc.	GWB-23C38D	rabbit
GenWay Biotech, Inc.	GWB-295359	Rabbit
GenWay Biotech, Inc.	GWB-806785	Goat
Enzo Life Sciences, Inc.	ADI-905-629-100	mouse
Enzo Life Sciences, Inc.	ADI-905-630-100	mouse

Enzo Life Sciences, Inc.	ADI-905-500-1	Rabbit
Enzo Life Sciences, Inc.	ALX-210-309-R100	Rabbit
GeneTex	GTX28448	Rabbit
GeneTex	GTX37500	rabbit
GeneTex	GTX15489	rabbit
GeneTex	GTX89519	goat
Spring Bioscience	E3282	rabbit
Spring Bioscience	E3280	rabbit
Spring Bioscience	E3281	rabbit
Spring Bioscience	E3284	rabbit
Abbotec	251924	rabbit
Abbotec	250801	rabbit
MBL International	CY-P1035	
Rockland Immunochemicals, Inc.	100-401-404	Rabbit
Bioss Inc.	bs-0026R	Rabbit
Bioss Inc.	bs-0019R	Rabbit
Proteintech Group Inc	22952-1-AP	Rabbit

Table 4: Non-limiting examples of commercially available ELISA Kits for integrin  $\alpha_5$  (ITGA5, Human)

Company Name	Catalogue number	Range	Sensitivity
antibodies-online	ABIN417612	0.156-10 ng/mL	0.054ng/mL
antibodies-online	ABIN365741	na	na
DLdevelop	DL-ITGa5-Hu	0.156-10ng/mL	0.054ng/mL
MyBioSource.com	MBS814027	na	na
R&D Systems	DYC3230-2	312-20,000 pg/mL	na
Biomatik	E91287Hu	0.156-10ng/mL	0.054ng/mL

Table 5: Non-limiting examples of commercially available Antibodies for  $\alpha_5$  (ITGA5, human)

Company Name	Catalogue Number	Host
Novus Biologicals	NBP1-84576	rabbit
Biorbyt	orb69201	mouse
Abcam	ab72663	rabbit
Acris Antibodies GmbH	BM4033	mouse
Aviva Systems Biology	OAAF05375	rabbit
St John's Laboratory	STJ32097	mouse
GeneTex	GTX86915	rabbit

GeneTex	GTX86905	rabbit
OriGene Technologies	TA311966	rabbit
OriGene Technologies	TA310024	rat
Abbexa	abx15590	rabbit
Abbexa	abx15591	rabbit
Abbiotec	252937	mouse
Abnova Corporation	MAB10703	mouse
Abnova Corporation	MAB5267	mouse
Bioss Inc.	bs-0567R	rabbit
Cell Signaling Technology	4705S	rabbit
Atlas Antibodies	HPA002642	rabbit
GenWay Biotech, Inc.	GWB-MX190A	rabbit
GenWay Biotech, Inc.	GWB-D9743E	mouse
antibodies-online	ABIN656138	rabbit
antibodies-online	ABIN219716	rabbit
Novus Biologicals	NBP1-71421-0.1mg	rabbit
Novus Biologicals	NBP1-71421-0.05mg	rabbit
BioLegend	328009	mouse
BD Biosciences	610634	mouse
BioLegend	328002	mouse
BD Biosciences	610634	mouse
Abcam	ab72665	rabbit
Abcam	ab55988	rabbit
Bioworld Technology	BS7053	rabbit
Santa Cruz Biotechnology, Inc.	sc-166665	mouse
Bioworld Technology	BS7052	rabbit
R&D Systems	AF1864	goat
R&D Systems	FAB1864A	mouse
Thermo Scientific Pierce Antibodies	MA5-15568	mouse
Thermo Scientific Pierce Antibodies	MA1-81134	mouse
AbD Serotec (Bio-Rad)	MCA1187	mouse
AbD Serotec (Bio-Rad)	MCA1187T	mouse
Life Technologies	132600	mouse
Proteintech Group Inc	10569-1-AP	rabbit
Raybiotech, Inc.	119-14178	mouse
Creative Biomart	CAB-3671MH	mouse
Merck Millipore	CBL497	mouse
Fitzgerald Industries International	10R-1984	mouse
EMD Millipore	AB1921	rabbit
EMD Millipore	AB1949	rabbit

Table 6: Non-limiting examples of commercially available ELISA Kits for  $\beta$ 1 (ITGB1,

human)

Company Name	Catalogue number	Range	Sensitivity
antibodies-online	ABIN833710	na	na
Merck Millipore	ECM470	na	na
DLdevelop	DL-ITGb1-Hu	1.56-100ng/mL	na
Biomatik	E91042Hu	1.56-100ng/mL	0.64ng/mL

Table 7: Non-limiting examples of commercially available antibodies for  $\beta_1$ ITGB1 (Human, Unconjugated)

Company Name	Catalogue number	Host
Abgent	AM2241b	mouse
Biorbyt	orb86390	rabbit
LifeSpan BioSciences	LS-C84969-100	mouse
Novus Biologicals	NB110-55545	rabbit
Abbexa	abx12778	rabbit
Bethyl Laboratories, Inc.	A303-735A	rabbit
Abcam	ab5189	Rabbit
St John's Laboratory	STJ60344	Rabbit
Cell Signaling Technology	4706S	Rabbit
Bioss Inc.	bs-0486R	Rabbit
Antigenix America Inc.	MA290020	
GenWay Biotech, Inc.	GWB-312F4D	mouse
Fitzgerald Industries International	20R-2722	rabbit
GeneTex	GTX50784	rabbit
Thermo Scientific Pierce Antibodies	MA1-80764	mouse
R&D Systems	AF1778	goat
Abbiotec	251162	rabbit
Bioworld Technology	BS1817	rabbit
Abgent	AM2241b	mouse
Enzo Life Sciences, Inc.	BML-IG6060-0100	mouse
BIOCARE MEDICAL	CME 386 A	rabbit
ProSci, Inc	48-392	Rabbit
eBioscience	14-0299-82	mouse

**[0085]** Both monoclonal and polyclonal antibodies directed to OPN,  $\alpha_5$  and/or  $\beta_1$  are included within the scope of this invention as they can be produced by well established procedures known to those of skill in the art. Additionally, any secondary antibodies, either monoclonal or polyclonal, directed to the first antibodies would also

be included within the scope of this invention.

**[0086]** As used herein, the terms “anti-OPN antibody”, “anti- $\alpha_5$  antibody”, “anti- $\beta_1$  antibody” and “anti- $\alpha_5\beta_1$  antibody” or “immunologically specific anti-OPN antibody”, “immunologically specific anti- $\alpha_5$  antibody”, “immunologically specific anti- $\beta_1$  antibody” or “immunologically specific anti- $\alpha_5\beta_1$  antibody” refers to an antibody that specifically binds to (interacts with) an OPN,  $\alpha_5$ ,  $\beta_1$  or  $\alpha_5\beta_1$  protein, respectively and displays no substantial binding to other naturally occurring proteins other than the ones sharing the same antigenic determinants as the OPN,  $\alpha_5$ ,  $\beta_1$  or  $\alpha_5\beta_1$  protein, respectively. 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')<sub>2</sub>, 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 (V<sub>H</sub>, V<sub>H</sub>-V<sub>H</sub>), 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.

**[0087]** 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.

**[0088]** Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc), intravenous (iv) or intraperitoneal (ip) injections of the relevant antigen with or without an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl<sub>2</sub>, or R<sup>1</sup>N=C=NR, where R and R<sup>1</sup> are different alkyl groups.

**[0089]** Animals may be immunized against the antigen, immunogenic conjugates, or derivatives by combining the antigen or conjugate (e.g., 100  $\mu$ g for rabbits or 5  $\mu$ g for mice) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals

are boosted with the antigen or conjugate (e.g., with 1/5 to 1/10 of the original amount used to immunize) in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, for conjugate immunizations, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

**[0090]** Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, Nature, 256: 495 (1975), or may be made by recombinant DNA methods (e.g., U.S. Patent No. 6,204,023). Monoclonal antibodies may also be made using the techniques described in U.S. Patent Nos. 6,025,155 and 6,077,677 as well as U.S. Patent Application Publication Nos. 2002/0160970 and 2003/0083293.

**[0091]** In the hybridoma method, a mouse or other appropriate host animal, such as a rat, hamster or monkey, is immunized (e.g., as hereinabove described) to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell.

**[0092]** The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

**[0093]** As used herein, the term "purified" in the expression "purified antibody" is simply meant to distinguish man-made antibody from an antibody that may naturally be produced by an animal against its own antigens. Hence, raw serum and hybridoma culture medium containing anti-OPN antibody are "purified antibodies" within the meaning of the present invention.

**[0094]** The present invention also encompasses arrays to detect and/or quantify the translation products of OPN and  $\alpha_5\beta_1$ . Such arrays include protein micro- or macroarrays, gel technologies including high-resolution 2D-gel methodologies, possibly coupled with mass spectrometry imaging system at the cellular level such as microscopy combined with a fluorescent labeling system.

**[0095]** The present invention also encompasses methods to screen/select for potential useful therapeutic agents using whole cells assays, the therapeutic compound being able to increase the transcription and/or synthesis of OPN and/or  $\alpha_5$  and/or  $\beta_1$  or to decrease the transcription or synthesis of CD44/sCD44. Cells for use in such methods includes cells of any source (including in house or commercially available cell lines) and type (any tissue). In house cell lines could be made for instance by immortalizing cells from AIS subjects. In

specific embodiments, methods of screening of the invention seek to identify agents that inhibit OPN and/or  $\alpha_5\beta_1$  expression and/or activity. Useful cell lines for these embodiments include those producing high levels of OPN and/or  $\alpha_5\beta_1$  or low levels of CD44/sCD44. Non-limiting examples of useful cells include MC3T3-E1 cells and PBMCs.

**[0096]** In a particular embodiment, it includes cells of any cell type derived from a scoliotic patient. (whole cell assay). In specific embodiments, it includes osteoblasts, chondrocytes, myoblasts or blood cells including PBMCs including lymphocytes. As used herein, the term "cell derived from a scoliotic patient" refers to cells isolated directly from scoliotic patients, or immortalized cell lines originating from cells isolated directly from scoliotic patients. In specific embodiments, the cells are paraspinal muscle cells. Such cells may be isolated by a subject through needle biopsies for instance.

**[0097]** The present invention also concerns pharmaceutical compositions for modulating OPN-mediated GiPCR cell signalling and/or for treating or preventing scoliosis in a subject in need thereof (e.g., idiopathic scoliosis or AIS). Such compositions include agents for increasing or decreasing OPN-mediated GiPCR signalling inhibition in a subject in need thereof. For instance, pharmaceutical compositions of the present invention may comprise OPN,  $\alpha_5$ ,  $\beta_1$ , and/or  $\alpha_5\beta_1$  antibodies (i.e., neutralizing antibodies), RGD peptides or antisense/siRNAs against OPN,  $\alpha_5$ ,  $\beta_1$ , and/or  $\alpha_5\beta_1$  to decrease OPN and/or  $\alpha_5\beta_1$  activity. In an embodiment, the pharmaceutical compositions may comprise sCD44 or a stimulator/enhancer of sCD44/CD44 expression. Pharmaceutical compositions can be administered by any suitable routes such as nasally, intravenously, intramuscularly, subcutaneously, sublingually, intrathecally, or intradermally. The route of administration can depend on a variety of factors, such as the environment and therapeutic goals.

#### Dosage

**[0098]** Any suitable amount of a pharmaceutical composition can be administered to a subject. The dosages will depend on many factors including the mode of administration. Typically, the amount of anti-scoliosis composition (e.g., agent that decreases OPN and/or  $\alpha_5\beta_1$ ) contained within a single dose will be an amount that effectively prevents, delays or reduces scoliosis without inducing significant toxicity "therapeutically effective amount".

**[0099]** The effective amount of the agent that decreases OPN and/or  $\alpha_5\beta_1$  may also be measured directly. The effective amount may be given daily or weekly or fractions thereof. Typically, a pharmaceutical and/or nutraceutical and/or dietary supplement composition of the invention can be administered in an amount from about 0.001 mg up to about 500 mg per kg of body weight per day (e.g., 10 mg, 50 mg, 100 mg, or 250 mg). Dosages may be provided in either a single or multiple dosage regimen. For example, in some embodiments the effective amount is a dose that ranges from about 1 mg to about 25 grams of the anti-scoliosis preparation per day, about 50 mg to about 10 grams of the anti-scoliosis preparation per day, from about 100 mg to about 5 grams of the anti-scoliosis preparation per day, about 1 gram of the anti-scoliosis preparation per day, about 1 mg to about 25 grams of the anti-scoliosis preparation per week, about 50 mg to about 10 grams of the anti-scoliosis preparation per week, about 100 mg to about 5 grams of the anti-scoliosis preparation every other day,

and about 1 gram of the anti- scoliosis preparation once a week.

**[00100]** By way of example, a pharmaceutical (e.g., containing an agent that decreases OPN and/or  $\alpha_5\beta_1$ ) composition of the invention can be in the form of a liquid, solution, suspension, pill, capsule, tablet, gelcap, powder, gel, ointment, cream, nebulae, mist, atomized vapor, aerosol, or phytosome. For oral administration, tablets or capsules can be prepared by conventional means with at least one pharmaceutically acceptable excipient such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets can be coated by methods known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspension, or they can be presented as a dry product for constitution with saline or other suitable liquid vehicle before use. Preparations for oral administration also can be suitably formulated to give controlled release of the active ingredients.

**[00101]** In addition, a pharmaceutical (e.g., containing an agent that decreases OPN and/or  $\alpha_5\beta_1$ ) composition of the invention can contain a pharmaceutically acceptable carrier for administration to a mammal, including, without limitation, sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include, without limitation, propylene glycol, polyethylene glycol, vegetable oils, and injectable organic esters. Aqueous carriers include, without limitation, water, alcohol, saline, and buffered solutions. Pharmaceutically acceptable carriers also can include physiologically acceptable aqueous vehicles (e.g., physiological saline) or other known carriers appropriate to specific routes of administration.

**[00102]** An agent that decreases OPN and/or  $\alpha_5$  and/or  $\beta_1$  expression or activity or an agent that increases sCD44/CD44 level (e.g., binding to OPN) may be incorporated into dosage forms in conjunction with any of the vehicles which are commonly employed in pharmaceutical preparations, e.g. talc, gum arabic, lactose, starch, magnesium stearate, cocoa butter, aqueous or non-aqueous solvents, oils, paraffin derivatives or glycols. Emulsions such as those described in U.S. Pat. No. 5,434,183, may also be used in which vegetable oil (e.g., soybean oil or safflower oil), emulsifying agent (e.g., egg yolk phospholipid) and water are combined with glycerol. Methods for preparing appropriate formulations are well known in the art (see e.g., Remington's Pharmaceutical Sciences, 16th Ed., 1980, A. Oslo Ed., Easton, Pa.).

**[00103]** In cases where parenteral administration is elected as the route of administration, preparations containing agent that decreases OPN and/or  $\alpha_5\beta_1$  may be provided to patients in combination with pharmaceutically acceptable sterile aqueous or non-aqueous solvents, suspensions or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose, or fixed oils. Intravenous vehicles may include fluid and nutrient replenishers, electrolyte replenishers, such as those based upon Ringer's dextrose, and the like.

**[00104]** These are simply guidelines since the actual dose must be carefully selected and titrated by the attending physician based upon clinical factors unique to each patient or by a nutritionist. The optimal daily dose

will be determined by methods known in the art and will be influenced by factors such as the age of the patient and other clinically relevant factors. In addition, patients may be taking medications for other diseases or conditions. The other medications may be continued during the time that the agent that decreases OPN and/or  $\alpha_5\beta_1$  is given to the patient, but it is particularly advisable in such cases to begin with low doses to determine if adverse side effects are experienced.

**[00105]** The present invention also relates to the detection of a CD44 risk allele (e.g., comprising one or more SNPs) in a subject in need thereof. In accordance with the present invention, the CD44 risk allele comprises a SNP located in the coding regions of CD44 and can thus be detected at the genomic, mRNA or protein level. SNP genotyping is the measurement of genetic variations of single nucleotide polymorphisms (SNPs) between members of a species (e.g., humans). A SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is >1%). SNP genotyping can be performed using methods well-known in the art such as sequencing (e.g., minisequencing, pyrosequencing), hybridization-based methods (using probes complementary to the SNP site which are hybridized under high stringency conditions), enzyme-based methods, Single strand conformation polymorphism (SSCP), Temperature gradient gel electrophoresis, Denaturing high performance liquid chromatography, High-resolution melting of the entire amplicon, using DNA mismatch-binding proteins, or the SNPlex™ platform. Non limiting examples of hybridization-based methods include Dynamic allele-specific hybridization and use of Molecular beacons. Non-limiting examples of enzyme-based methods include, Restriction fragment length polymorphism (RFLP), PCR-based methods (e.g., ARMS), Flap endonuclease (e.g., Invader), primer extension, 5'-nuclease (TaqMan™ assay), oligonucleotide ligation assays.

**[00106]** In an embodiment, methods of the present invention comprise methods for i) determining the risk of developing a scoliosis; and ii) methods of stratifying subjects having scoliosis comprising determining the presence of a CD44 risk allele comprising SNP rs1467558 or a mutation/SNP/marker in linkage disequilibrium therewith.

**[00107]** In particular embodiments of the invention, linkage disequilibrium (LD) is defined by a specific quantitative cutoff. As described in detail herein, linkage disequilibrium can be quantitatively determined by measures such as  $r^2$  and  $|D'|$ . As a consequence, certain embodiments of the invention relate to substitute markers in linkage disequilibrium by a measure within a certain range specified by particular values of  $r^2$  and/or  $|D'|$ . In an embodiment, LD is characterized by numerical values for  $r^2$  of greater than 0.1. In another embodiment, LD is characterized by numerical values for  $r^2$  of greater than 0.5. In another embodiment, LD is characterized by numerical values for  $r^2$  of greater than 0.8. In another embodiment, LD is characterized by numerical values for  $r^2$  of 0.9 or more.

### **Linkage Disequilibrium**

**[00108]** The natural phenomenon of recombination, which occurs on average once for each chromosomal pair during each meiotic event, represents one way in which nature provides variations in sequence (and biological

function by consequence). It has been discovered that recombination does not occur randomly in the genome; rather, there are large variations in the frequency of recombination rates, resulting in small regions of high recombination frequency (also called recombination hotspots) and larger regions of low recombination frequency, which are commonly referred to as Linkage Disequilibrium (LD) blocks (Myers, S. et al., *Biochem Soc Trans* 34:526-530 (2006); Jeffreys, A.J., et al., *Nature Genet* 29:217-222 (2001); May, C.A., et al., *Nature Genet* 31:272-275(2002)).

**[00109]** Linkage Disequilibrium (LD) refers to a non-random assortment of two genetic elements. Thus, the term "linkage disequilibrium" refers to a non-random genetic association between one or more allele(s) of two different polymorphic DNA sequences, that is due to the physical proximity of the two loci. Linkage disequilibrium is present when two DNA segments that are very close to each other on a given chromosome will tend to remain unseparated for several generations with the consequence that alleles of a DNA polymorphism (or marker) in one segment will show a non-random association with the alleles of a different DNA polymorphism (or marker) located in the other DNA segment nearby. This situation is encountered throughout the human genome when two DNA polymorphisms that are very close to each other are studied. Linkage disequilibrium and the use thereof in inheritance studies is well known in the art to which the present invention pertains as exemplified by publications such as Risch and Merikangas, *Science* 273: 1516-1517 (1996); Maniatis, *Methods Mol Biol.* 376: 109-21 (2007) and Borecki et al., *Adv Genet* 60: 51-74 (2008).

**[00110]** For example, if a particular genetic element (e.g., an allele of a polymorphic marker, or a haplotype) occurs in a population at a frequency of 0.25 (25%) and another element occurs at a frequency of 0.25 (25%), then the predicted occurrence of a person's having both elements is 0.125 (12.5%), assuming a random distribution of the elements. However, if it is discovered that the two elements occur together at a frequency higher than 0.125, then the elements are said to be in linkage disequilibrium, since they tend to be inherited together at a higher rate than what their independent frequencies of occurrence (e.g., allele or haplotype frequencies) would predict. Roughly speaking, LD is generally correlated with the frequency of recombination events between the two elements. Allele or haplotype frequencies can be determined in a population by genotyping individuals in a population and determining the frequency of the occurrence of each allele or haplotype in the population.

**[00111]** Many different measures have been proposed for assessing the strength of linkage disequilibrium (LD). Most capture the strength of association between pairs of biallelic sites. Two important pairwise measures of LD are  $r^2$  (sometimes denoted  $\Delta$ ) and  $|D'|$ . Both measures range from 0 (no disequilibrium) to 1 ('complete' disequilibrium), but their interpretation is slightly different.  $|D'|$  is defined in such a way that it is equal to 1 if just two or three of the possible haplotypes are present, and it is  $<1$  if all four possible haplotypes are present. Therefore, a value of  $|D'|$  that is  $<1$  indicates that historical recombination may have occurred between two sites (recurrent mutation can also cause  $|D'|$  to be  $<1$ , but for single nucleotide polymorphisms (SNPs) this is usually regarded as being less likely than recombination). The measure  $r^2$  represents the statistical correlation between

two sites, and takes the value of 1 if only two haplotypes are present.

**[00112]** The  $r^2$  measure is arguably the most relevant measure for association mapping, because there is a simple inverse relationship between  $r^2$  and the sample size required to detect association between susceptibility loci and SNPs. These measures are defined for pairs of sites, but for some applications a determination of how strong LD is across an entire region that contains many polymorphic sites might be desirable (e.g., testing whether the strength of LD differs significantly among loci or across populations, or whether there is more or less LD in a region than predicted under a particular model).

**[00113]** One approach of measuring LD across a region is to use the measure  $r$ , which was developed in population genetics. Roughly speaking,  $r$  measures how much recombination would be required under a particular population model to generate the LD that is seen in the data. This type of method can potentially also provide a statistically rigorous approach to the problem of determining whether LD data provide evidence for the presence of recombination hotspots. For the methods and procedures described herein, a significant  $r^2$  value can be at least 0.1 such as at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.75, 0.8, 0.85, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99 or 1.0. In one preferred embodiment, the significant  $r^2$  value can be at least 0.8. Alternatively, linkage disequilibrium as described herein, refers to linkage disequilibrium characterized by values of  $|D'|$  of at least 0.2, such as 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.85, 0.9, 0.95, 0.96, 0.97, 0.98 or 0.99. Thus, linkage disequilibrium represents a correlation between alleles of distinct markers. It is measured by correlation coefficient or  $|D'|$  ( $r^2$  up to 1.0 and  $|D'|$  up to 1.0). Linkage disequilibrium can be determined in a single human population, as defined herein, or it can be determined in a collection of samples comprising individuals from more than one human population. In one embodiment of the invention, LD is determined in a sample from one or more of the HapMap™ populations (Caucasian, African, Japanese, Chinese), as defined (<http://www.hapmap.org>). In one such embodiment, LD is determined in the CEU population of the HapMap™ III samples.

**[00114]** If all polymorphisms in the genome were identical at the population level, then every single one of them would need to be investigated in association studies. However, due to linkage disequilibrium between polymorphisms, tightly linked polymorphisms are strongly correlated, which reduces the number of polymorphisms that need to be investigated in an association study to observe a significant association. Genomic LD maps have been generated across the genome, and such LD maps have been proposed to serve as framework for mapping disease-genes (Risch, N. and Merkiangas, K, *Science* 273:1516-1517 (1996); Maniatis, N., et al., *Proc Natl Acad Sci USA* 99:2228-2233 (2002); Reich, DE et al, *Nature* 411:199-204 (2001)).

**[00115]** It is now established that many portions of the human genome can be broken into series of discrete haplotype blocks containing a few common haplotypes; for these blocks, linkage disequilibrium data provides little evidence indicating recombination (see, e.g., Wall, J.D. and Pritchard, J.K., *Nature Reviews Genetics* 4:587-597 (2003); Daly, M. et al., *Nature Genet.* 29:229-232 (2001); Gabriel, S.B. et al., *Science* 296:2225-2229 (2002); Patil, N. et al., *Science* 294: 1719-1723 (2001); Dawson, E. et al., *Nature* 411:544-548 (2002); Phillips, M.S. et

al., Nature Genet 33:382-387 (2003)).

**[00116]** There are two main methods for defining these haplotype blocks: Blocks can be defined as regions of DNA that have limited haplotype diversity (see, e.g., Daly, M. et al., Nature Genet. 29:229-232 (2001); Patil, N. et al., Science 294: 1719-1723 (2001); Dawson, E. et al., Nature 418:544-548 (2002); Zhang, K. et al., Proc. Natl. Acad. Sci. USA 99:7335-7339 (2002)), or as regions between transition zones having extensive historical recombination, identified using linkage disequilibrium (see, e.g., Gabriel, S.B. et al., Science 296:2225-2229 (2002); Phillips, M.S. et al., Nature Genet. 33:382-387 (2003); Wang, N. et al., Am. J. Hum. Genet. 7 :1227-1234 (2002); Stumpf, M.P., and Goldstein, D.B., Curr. Biol. 13: 1-8 (2003)). More recently, a fine-scale map of recombination rates and corresponding hotspots across the human genome has been generated (Myers, S., et al., Science 310:321-32324 (2005); Myers, S. et al., Biochem Soc Trans 34:526530 (2006)). The map reveals the enormous variation in recombination across the genome, with recombination rates as high as 10-60 cM/Mb in hotspots, while closer to 0 in intervening regions, which thus represent regions of limited haplotype diversity and high LD. The map can therefore be used to define haplotype blocks/LD blocks as regions flanked by recombination hotspots. As used herein, the terms "haplotype block" or "LD block" includes blocks defined by any of the above described characteristics, or other alternative methods used by the person skilled in the art to define such regions.

**[00117]** Haplotype blocks can be used to map associations between phenotype and haplotype status, using single markers or haplotypes comprising a plurality of markers.

**[00118]** The main haplotypes can be identified in each haplotype block, and then a set of "tagging" SNPs or markers (the smallest set of SNPs or markers needed to distinguish among the haplotypes) can then be identified. These tagging SNPs or markers can then be used in assessment of samples from groups of individuals, in order to identify association between phenotype and haplotype. If desired, neighboring haplotype blocks can be assessed concurrently, as there may also exist linkage disequilibrium among the haplotype blocks. Thus, the skilled person can readily identify SNPs/variants/markers in linkage disequilibrium with the CD44 risk allele identified herein and can use this marker in linkage disequilibrium to stratify subjects and to determine risk of developing scoliosis.

**[00119]** When the SNP is translated at the protein level, the mutation/variation can be detected using well-known protein detection methods such as ELISA, immunofluorescence, Western blot, sequencing, electrophoresis, HPLC, MS, etc.

**[00120]** The present invention also relates to kits. Without being so limited, it relates to kits for i) increasing GiPCR signaling in cells; ii) stratifying scoliotic subjects, and/or iii) predicting whether a subject is at risk of developing a scoliosis comprising an isolated nucleic acid (e.g., a primer or probe specific for (which is substantially complementary to or hybridizes to  $\alpha_5$  (SEQ ID NO:24),  $\beta_1$  (SEQ ID NO:22), CD44 (wild type (SEQ ID NO:19) or SNP risk allele (e.g., 2301→T (CT) variant) and/or OPN (SEQ ID NO:26)) a protein or a ligand such as

an antibody (for  $\alpha_5$ ,  $\beta_1$ , CD44 (wild type or SNP risk allele (e.g., 230I→T variant) and/or OPN) in accordance with the present invention as described above. 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. Kits of the present invention may also contain instructions to use these probes and or antibodies to stratify scoliotic subjects or predict whether a subject is at risk of developing a scoliosis.

[00121] 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.

[00122] 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".

[00123] The terms "such as" are used herein to mean, and is used interchangeably with, the phrase "such as but not limited to".

[00124] The present invention is illustrated in further details by the following non-limiting examples.

### **EXAMPLE 1**

#### **Materials and Methods**

##### **Experimental animal models**

[00125] The Institutional Review Board for the care and handling of animals used in research (CHU Sainte-Justine) has approved the protocol in accordance with the guidelines of the Canadian Council of Animal Care.

[00126] Breeding pairs of C57Bl/6j mice devoid of either OPN (OPN-null mice) or CD44 (CD44-null mice) were obtained from Dr. Susan Rittling (Rutger University, NJ, USA) and were backcrossed for more than 10 generations in C57Bl/6j background to establish our own colonies, while C57Bl/6j mice were used as wild-type control mice (Charles-River, Wilmington, MA, USA). C57Bl6/6j mouse strain was used because it is naturally deficient in melatonin (Von Gall et al., 2000) and exhibits high circulating OPN levels (Aherrahrou et al., 2004). These mice develop scoliosis when they are maintained in a bipedal state. (Machida et al., 2006). Bipedal surgeries were performed after weaning (5-weeks after birth) by amputation of the forelimbs and tail under anesthesia as reported previously. (Machida et al., 2006). Similarly bipedal C57Bl/6j CD44-null mice were generated.

**Derivation of primary osteoblast cultures**

[00127] Bone specimens from mice were obtained from the spine after euthanasia. Bone fragments were reduced to smaller pieces with a cutter in sterile conditions. The small bone pieces were incubated in  $\alpha$ MEM medium containing 10% fetal bovine serum (FBS; certified FBS, Invitrogen, Burlington, ON, Canada) and 1% penicillin/ streptomycin (Invitrogen) at 37°C in 5% CO<sub>2</sub>, in a 10-cm<sup>2</sup> culture dish. After one month, osteoblasts emerging from the bone pieces were separated from the remaining bone fragments by trypsinization. RNA was extracted from the osteoblasts using the TRIzol™ method, (Invitrogen). Expression profiles were studied by qPCR. Transcript expression was assessed with the Stratagene™ Mx3000P (Agilent Technologies, La Jolla, CA).

**Functional evaluation of G proteins**

[00128] The functional evaluation of Gi, Gs and Gq proteins was assessed by CDS assays using osteoblasts cells derived from C57Bl/6j WT and C57Bl/6j OPN<sup>-/-</sup> mice (bipedal and quadrupedal) using CellKey™ apparatus, as previously described (Akoume et al., 2010, and WO 2010/040234 to Moreau et al.).

**Osteopontin enzyme-linked immunosorbent assays**

[00129] Peripheral blood samples were collected in EDTA-treated tubes and then centrifuged. Plasma samples were derived then aliquoted and reserved frozen at -80°C until thawed and analyzed. The concentrations of plasma OPN were measured by enzyme-linked immunosorbent assays (ELISA) as said by protocols provided by the manufacturer (IBL, Hamburg, Germany). This OPN ELISA kit measures total concentration of both phosphorylated and non-phosphorylated forms of OPN in plasma. All ELISA tests were performed in duplicate and the optical density was measured at 450 nm using an DTX880 microplate reader (Beckman Coulter, USA).

**Quantitative reverse transcription-polymerase chain reaction (qPCR)**

[00130] Thermo-Script™ reverse transcriptase (Invitrogen) was used to reverse transcribe mRNA into cDNA (1mg total concentration). Several dilutions were tested to choose the concentration that yielded the most efficient amplification. The human primers used were the following:

- [00131]  $\beta$ -actin forward 5'-GGAAATCGTGCGTGACAT-3' (SEQ ID NO: 1),
- [00132]  $\beta$ -actin reverse 5'-TCATGATGGAGTTGAAGGTAGTT-3' (SEQ ID NO: 2),
- [00133] CD44 forward 5'-AGCATCGGATTTGAGACCTG-3' (SEQ ID NO: 3),
- [00134] CD44 reverse 5'-TGAGTCCACTTGGCTTTCTG-3' (SEQ ID NO: 4),
- [00135]  $\beta$ 1 integrin forward 5'-ATGTGTCAGACCTGCCTTG-3' (SEQ ID NO: 5),
- [00136]  $\beta$ 1 integrin reverse 5'-TTGTCCCGACTTTCTACCTTG-3' (SEQ ID NO: 6),
- [00137]  $\alpha$ v integrin forward 5'-GTCCCCACAGTAGACACATATG-3' (SEQ ID NO: 7),
- [00138]  $\alpha$ v integrin reverse 5'-TCAACTCCTCGCTTTCCATG-3' (SEQ ID NO: 8).

[00139] Each amplification was performed in duplicate using 5ml of diluted cDNA, 7.5ml of 3mM primer solution and 12.5ml of 2X QuantiTect™ SYBR Green PCR Master Mix (QIAGEN Inc, Ontario, Canada). All reaction mixes were run on Mx3000P™ system from Stratagene (Agilent Technologies Company, La Jolla, CA) and analyzed with MxPro™ QPCR Software also from Stratagene. Relative quantification was calculated with the delta CT method using  $\beta$ -actin as the endogenous control.

#### **Cell lines and siRNA transfection**

[00140] MC3T3-E1 cells were used to check the effect of the knockdown of OPN and its receptors. MC3T3-E1 osteoblasts cells were transiently transfected in serum-free medium, using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions and functional experiments were performed 48h post transfection. The sequence of RNA oligonucleotides used for the knockdown of different genes are, ssp1 (encodes OPN) (CCA CAG CCA CAA GCA GUC CAG AUU A (SEQ ID NO: 9)), integrin  $\beta$ 1 (CCU AAG UCA GCA GUA GGA ACA UUA U (SEQ ID NO: 10)), integrin  $\beta$ 3 (CCU CCA GCU CAU UGU UGA UGC UUA U (SEQ ID NO: 11)), integrin  $\alpha$ 5 (CCG AGU ACC UGA UCA ACC UGG UUC A (SEQ ID NO: 12)), integrin  $\alpha$ 8 (GAG AUG AAA CUG AAU UCC GAG AUA A (SEQ ID NO: 13)), integrin  $\alpha$ v (GAC UGU UGA GUA UGC UCC AUG UAG A (SEQ ID NO: 14) and CD44 (GAA CAA GGA GUC GUC AGA AAC UCC A (SEQ ID NO: 15)). Two other siRNAs against integrin  $\alpha$ 5 were tested (UCC ACC AUG UCU AUG AGC UCA UCA A (SEQ ID NO:16) and UCA GGA GCA GAU UGC AGA AUC UUA U (SEQ ID NO:17)). Comparable results were obtained with all siRNAs.

#### **The evaluation of the effect of OPN on GPCR and Gi proteins expression**

[00141] Gi proteins isoforms expression levels were determined using Western blot technique. MC3T3-E1 cells were treated with PBS or rOPN 0.5 $\mu$ g/ml overnight (R&D systems Inc., Minneapolis, USA). These cells were washed with cold PBS 1x then lysed in RIPA buffer (25 mM Tris.Hcl pH7.4, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) added with 5 mM NaVO<sub>4</sub> and protease inhibitor cocktail (Roche molecular Biochemicals, Mannheim, Germany). Immunoblots were performed with primary antibodies directed specifically against Gi<sub>1</sub>, Gi<sub>2</sub>, Gi<sub>3</sub>, Gs, Phosphoserine, integrin  $\beta$ 1 (Santa Cruz Biotechnology, Santa Cruz, CA), Mu opioid receptor (MOR) (abcam Toronto, ON, Canada), Lysophosphatidic acid receptor 1 (LPAR1) (assaybiotech Inc., USA), melatonin receptor 2 (MT2) and peroxidase-conjugated secondary antibody. Bands were then visualized using SuperSignal™ chemiluminescent substrate (Pierce, Rockford, IL).

#### **Effect of OPN on interaction of Gi proteins with GPCR and $\beta$ 1 integrin**

[00142] MC3T3-E1 cells were treated with PBS or rOPN (0.5 $\mu$ g/ ml) then the whole cell proteins (1mg) were incubated with anti-MOR (Abcam Toronto, ON, Canada), anti-MT2 or anti- $\beta$ <sub>1</sub> integrin (Santa Cruz Biotechnology), plus protein G beads for immunoprecipitation (IP) at 4 °C overnight. Purified proteins were loaded on 10 % gel after IP, then, processed for gel transferring to PVDF membrane and 5 % BSA blocking. Membranes were exposed to antibodies specific for Gi<sub>1</sub>, Gi<sub>2</sub>, Gi<sub>3</sub>, MOR, MT2 and  $\beta$ <sub>1</sub> integrin at 4 °C overnight and, subsequently, treated with secondary antibody at room temperature for 1 h. Bands were visualized using SuperSignal™ chemiluminescent (Pierce, Rockford, IL) and quantified by densitometric scanning.

**[00143] Whole exome sequencing Identification of SNP in AIS subjects associated with increased risk of spinal deformity progression**

**[00144]** A Whole Exome Sequencing (WES) study was performed using Agilent targeted exon capture and Life technologies SOLiD 5500TM for sequencing. GEMINI1TM was used for SNV (Single Nucleotide Variant) annotations. A SNP (rs1467558) was identified in AIS subjects with more severe AIS, which changes the amino acid isoleucine 230 to a threonine in the CD44 coding sequence.

#### **Statistical analysis**

**[00145]** Data are presented as mean  $\pm$  SE, and were analyzed by ANOVA or Student's t test using GraphPad™ Prism 4.0 software. Multiple comparisons of means were performed with one-way ANOVA followed by a post-hoc test of Dunnett. Only P values < 0.05 were considered significant.

### **EXAMPLE 2**

#### **Genetic deletion of OPN protects bipedal C57Bl/6 from scoliosis**

**[00146]** The bipedal C57Bl/6 mice are widely used as animal model to study the pathophysiological events leading to idiopathic scoliosis. These mice rapidly develop spinal deformity following 40 weeks of bipedal ambulation (Oyama et al., 2006). To determine whether OPN is involved in the development of idiopathic scoliosis, female wild-type (WT) and OPN knockout (OPN<sup>-/-</sup>) C57Bl/6 mice were amputated from forelimbs and tails at 1 month of age and subjected to bipedal ambulation for 36 weeks to induce scoliosis. Representative radiographs of the spine as taken at the end of the experiment period are shown in **Fig. 1, A-D**. As expected, scoliosis did not develop in any of the WT or OPN<sup>-/-</sup> quadrupedal mice. However, lateral curvature was apparent in all bipedal WT mice. The convexity of curve was directed to either side, with no consistent preference. In contrast, analysis of radiographs did not yield evidence of scoliosis in bipedal OPN<sup>-/-</sup> mice. All bipedal OPN<sup>-/-</sup> mice had straight spine indistinguishable from the quadrupedal mice. These data strongly emphasize that genetic OPN deletion protects bipedal C57Bl/6 mice from scoliosis.

**[00147]** To provide further evidence for this hypothesis, OPN was measured in plasma from bipedal mice at 12, 24 and 36 weeks after surgery (**Fig. 1 E**), and compared with plasma OPN levels in age-matched quadrupedal mice. Whereas OPN could be detected neither in quadrupedal nor in bipedal OPN<sup>-/-</sup> mice (data not shown), bipedal WT showed significantly higher plasma OPN than quadrupedal WT mice at all indicated time points. The maximum values were observed at the thirty-sixth postoperative week.

### **EXAMPLE 3**

#### **OPN deficiency improves Gi protein-mediated receptor signal transduction**

**[00148]** It was next determined whether lack of OPN can influence Gi protein-mediated signaling. For this purpose, osteoblasts from WT and OPN<sup>-/-</sup> mice were screened for their response to DAMGO, somatostatin, oxymethazolin and apelin to activate opioid, somatostatin, alpha2-adrenergic and APJ receptors, respectively. These receptors are well known to mediate signal transduction through Gi proteins. Results illustrated in **Fig. 1,**

**F-I** show that all four compounds caused a concentration-dependent increase of response in osteoblasts from WT and OPN<sup>-/-</sup> mice. However, in each case, the magnitude of response was greater in OPN<sup>-/-</sup> than in WT osteoblasts, which suggests that the activation of Gi proteins is facilitated in OPN<sup>-/-</sup> osteoblasts. Also, while the magnitude of response was similar in osteoblasts from quadrupedal and bipedal OPN<sup>-/-</sup> mice, a significant difference was observed between the responses from quadrupedal and bipedal WT mice (Data not shown). The extent of response was much lower in osteoblasts from bipedal WT mice.

**[00149]** To corroborate these results with the function of Gi proteins, osteoblasts were treated with pertussis toxin (PTX), which ADP-ribosylates Gi proteins and disrupts their interaction with receptors (Gilman, 1987); (Moss et al., 1984). Results illustrated in **Fig. 1, G-M** show that cellular responses to each of the four tested agonists, were largely reduced by PTX pre-treatment in osteoblasts from either phenotype. However, the extent of reduction was higher in OPN<sup>-/-</sup> osteoblasts. Notably, the differences in the magnitude of response between WT and OPN<sup>-/-</sup> osteoblasts were completely abrogated by PTX treatment. Collectively, these data indicate that OPN deficiency enhances Gi protein-mediated receptor signal transduction in osteoblasts cells.

**[00150]** To determine whether loss of OPN specifically enhances Gi protein signaling, we examined the effect of loss of OPN on signaling initiated by other G proteins such as Gs and Gq. We used isoproterenol and desmopressin to activate Gs through beta-adrenergic and vasopressin receptors, respectively; while bradykinin and endothelin-1 were used to activate Gq through their cognate receptors. Results in **Fig. 1, N and O** show that osteoblasts from OPN<sup>-/-</sup> mice were less responsive to Gs stimulation than those from WT mice, whereas the Gq stimulation elicited in WT and OPN<sup>-/-</sup> osteoblasts with comparable magnitude. These findings are strongly indicative that OPN deficiency exclusively improves Gi protein-mediated signaling.

#### **EXAMPLE 4**

##### **Extracellular OPN disrupts Gi protein-mediated receptor signal transduction**

**[00151]** To further investigate the role of OPN in Gi protein-mediated receptor signaling, several lines of experiments were performed using MC3T3-E1 osteoblastic cell line. Since these cells express OPN, was first examined whether depletion of OPN by siRNA influences the capacity of GiPCR ligands to induce cell signaling as measured by CDS in MC3T3-E1 cells. Results illustrated in **Fig. 2 A** show that the integrated response to DAMGO was significantly greater in cells depleted of OPN. Similar results were obtained when cells were stimulated with oxymethazolin. Interestingly, blockade of secreted OPN, by exposing cells to neutralizing OPN antibody, also increased response to both compounds (**Fig. 2 B**). These results suggest that the extracellular OPN disrupts GiPCR signaling. To confirm this hypothesis, cells were treated with exogenous recombinant OPN (rOPN) prior to DAMGO and oxymethazolin stimulation. In each case, rOPN caused decrease in the integrated response in a concentration-dependent manner, which was prevented by OPN antibody (**Fig. 2, C-D**).

**EXAMPLE 5****CD44 is not involved in the inhibition of GiPCR signaling by extracellular OPN**

[00152] OPN is known to function by interacting with a variety of cell surface receptors, including CD44 and many integrins (Weber et al., 1996); (Rangaswami et al., 2006). Since CD44 is considered as a key receptor for OPN, was first explored whether CD44 is responsible for the inhibitory effect of OPN on GiPCR signaling. For this purpose, the interaction between OPN and CD44 was interfered, using CD44 antibody. As shown in **Fig. 3 A**, OPN reduced the integrated response to DAMGO in cell pre-treated with IgG control or CD44 antibody. Similar results were obtained when cells were stimulated with oxymethazolin (**Fig. 3 A**), suggesting that the blockade of CD44 did not prevent the GiPCR signaling reduction induced by OPN and even potentiated its effect. To exclude the possibility that the lack of prevention of OPN inhibition by CD44 antibody was due to its inefficacy, the cells pre-treated with IgG control or CD44 antibody were stimulated with increasing concentrations of hyaluronic acid (HA), a high affinity ligand of CD44. As shown in **Fig. 3 B**, the integrated response induced by HA was completely abrogated by pre-treatment with CD44 antibody. Moreover, the effect of CD44 antibody on response to HA stimulation was concentration-dependent (**Fig. 3 C**). These data clearly indicate that CD44 antibody was active.

[00153] The small interfering RNA (siRNA) approach was further used to knockdown the expression of CD44, and the efficiency of siRNA transfection was demonstrated by quantitative real-time PCR and Western blot analysis (**Fig. 3, D and E**). The deletion of CD44 by siRNA did not prevent the inhibitory effect of OPN on response to DAMGO or oxymethazolin (**Fig. 3, F**). Consistent with these findings, rOPN treatment caused a concentration-dependent decrease in response to both DAMGO and oxymethazolin in osteoblasts from bipedal CD44 knockout (CD44<sup>-/-</sup>) mice (**Fig. 3, G and H**). Moreover, these osteoblasts were less responsive to DAMGO or oxymethazolin when compared with osteoblasts from quadrupedal (CD44<sup>-/-</sup>) mice (**Fig. 3, I and J**).

[00154] Further experiments in wild type and CD44<sup>-/-</sup> cells in the presence or absence of HA confirmed that the inhibitory effect of OPN is exacerbated in the absence of CD44 and intensified in the presence of HA, a known CD44 ligand (**Figure 3 K to P**). CD44 is not the cause of GiPCR signaling defect, rather this effect is due to OPN. However since CD44 binds to OPN, inhibition of CD44 or its effects (by HA, CD44 antibody, anti-CD44 siRNA or using CD44<sup>-/-</sup> cells) exacerbates the damaging effects of OPN.

[00155] Collectively, these data indicate without ambiguity that CD44 is not involved in the inhibition of GiPCR signaling by OPN and its presence reduces the effect of OPN on GiPCR signaling.

**EXAMPLE 6****RGD-dependent integrins mediate the inhibitory effect of OPN on GiPCR signaling**

[00156] Was next examined the possible requirement of integrins. Given that OPN contains an arginine-glycine-aspartate (RGD)-motif that engages a subset of cell surface integrins and a serine-valine-valine-tyrosine-glutamate-leucine-arginine (SVVYGLR)-containing domain that interacts with other integrins, it was of interest to examine whether OPN action required a specific domain-dependent integrin. For this purpose, small synthetic

peptides were used to compete with integrin binding of the RGD or SVVYLRG sequences in OPN. BIO1211 was used to selectively inhibit  $\alpha_4\beta_1$  integrin, the only SVVYLRG-containing integrin present in osteoblasts. As shown in **Fig. 4 A**, OPN action on GiPCR signaling was not significantly influenced by BIO1211 in MC3T3-E1 cells. Response to DAMGO or oxymethazolin was reduced by rOPN in the absence or presence of BIO1211, suggesting that integrins with SVVYLRG recognition specificity are not entailed in this process. In contrast, coincubation of cells with rOPN and RDG peptide completely prevented the inhibitory effect of rOPN on response to DAMGO and oxymethazolin. **Fig. 4, B and C** illustrate that this preventive effect of RDG was concentration-dependent. Increasing concentrations of RGD resulted in a progressive attenuation of the inhibitory effect of OPN, reversing the effect of OPN when concentrations went beyond 10 $\mu$ g/ml. Similarly, incubation of osteoblasts from WT mice with high concentrations of RGD demonstrated significant increase of response to DAMGO or oxymethazolin (**Fig. 4, D**), suggesting efficient inhibition of the effect of secreted OPN by RGD. Consistent with this view, RGD was without effect when osteoblasts from OPN<sup>-/-</sup> mice were used (**Fig. 4, E**), indicating that the effect of RGD is strictly dependent on the presence of OPN.

**[00157]** Collectively, these data suggest that the inhibitory effect of OPN on GiPCR signaling is mediated by one or more RGD-dependent integrin expressed in osteoblasts.

#### **EXAMPLE 7**

##### **Identification of integrins involved in the inhibition of GiPCR signaling by OPN**

**[00158]** Having established a probable role for RGD-dependent integrins in the inhibitory effect of OPN on GiPCR signaling in osteoblasts, specific integrins were examined to determine whether they could mediate this effect. For this purpose, antibodies were used to selectively neutralize the subunits of  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_5\beta_1$ , and  $\alpha_8\beta_1$ , the RGD-dependent integrins that have been shown to be expressed in osteoblasts (Hughes et al., 1997); (Gronthos et al., 1997); (Grzesik and Robey, 1994); (Clover et al., 1992); (Moursi et al., 1997); (Pistone et al., 1996). **Fig. 5, A and B** show that the inhibitory effect of rOPN on response to DAMGO and oxymethazolin was attenuated at different degrees, but not abolished, by  $\beta_3$ ,  $\beta_5$ ,  $\alpha_v$  and  $\alpha_8$  antibodies. Conversely, antibodies against  $\alpha_5$  or  $\beta_1$  reversed the inhibitory effect of rOPN on response to both DAMGO and oxymethazolin. Cells pre-treated with these antibodies showed an increase in the magnitude of the integrated response in the presence of rOPN compared to untreated cells, in sharp contrast to the decrease induced by rOPN in cell pre-treated with IgG. These results suggest that  $\alpha_5$  and  $\beta_1$  integrins are the primary mediators of the inhibitory effect of OPN on GiPCR signaling.

**[00159]** To further support this hypothesis, the siRNA approach was used. When compared with untreated cells, MC3T3-E1 transfected with scrambled siRNA demonstrated less response, while cells transfected with  $\alpha_5$  or  $\beta_1$  integrins siRNA demonstrated high response in the presence of rOPN. Cells transfected with both  $\alpha_5$  and  $\beta_1$  integrin siRNA demonstrated a further increase (**Fig. 5 C**). Also, silencing of both integrin subunits simultaneously resulted in increased response to DAMGO and oxymethazolin in osteoblasts from bipedal WT and CD44<sup>-/-</sup> mice (**Fig. 5, D and E**). Effective silencing of integrin expression was supported by q-RT-

PCR (Fig. 5, F). In all cases, transfection of cells with other integrins resulted in a partial attenuation of the inhibitory effect of OPN on GiPCR signaling (**data not shown**). Taken together, these results are the convincing evidence that  $\alpha_5\beta_1$  is the main integrin dimer involved in mediating the inhibitory effect of OPN on GiPCR signaling in osteoblasts.

### **EXAMPLE 8**

#### **OPN is without effect on expression of Gi proteins and their cognate receptors**

[00160] Given that the level of GPCRs is critical for their signaling, it was of interest to examine whether the defective GiPCR signaling induced by OPN is the consequence of a quantitative reduction of these receptors. For this purpose, MC3T3-E1 cells were treated with PBS or rOPN and the cell lysates were analyzed by Western blot for the expression of three different GPCRs; including mu-opioid (MOR), lysophosphatidic acid type 1 (LPA1R) and melatonin type 2 (MT2R) receptors. As showed in **Fig. 6 A**, all three receptors were immunodetected in MC3T3-E1 cells and the level of each receptor was not modified by rOPN treatment. Consequently, the effect of rOPN on the expression of Gi proteins was examined. Relative to PBS-treated cells, there was no significant effect of rOPN treatment on the quantity of Gi proteins as determined by immunoblotting using antibodies against Gi<sub>1</sub>, Gi<sub>2</sub> and Gi<sub>3</sub> isoforms (**Fig. 6 B**). The qPCR analysis also revealed no significant difference in the mRNA expression of any of these isoforms between PBS- and rOPN-treated cells (**Fig. 6 C**). These results exclude the possibility that rOPN reduces the expression of receptors or Gi proteins.

### **Example 9**

#### **OPN reduces the availability of Gi proteins for their cognate receptors**

[00161] Gi proteins have been shown to be recruited by  $\beta_1$  integrins via an adaptor molecule and to mediate integrin signaling (Green et al., 1999). It is notable that when different receptors signal through the same subfamily of G proteins, they share the same limited pool of G proteins. Therefore, if OPN enhances the recruitment of Gi proteins in the  $\beta_1$  integrin complex, reduction in the amount of Gi proteins in GiPCR complex would be expected. To evaluate this possibility, cells lysates were immunoprecipitated with antibodies against MO, MT2 or  $\beta_1$  integrin receptors, and the presence of Gi proteins in each precipitate was examined by Western blot using antibodies specific for Gi<sub>1</sub>, Gi<sub>2</sub> or Gi<sub>3</sub> isoform. **Fig. 6 D, E and F** shows that the relative amount of Gi<sub>1</sub> isoform immunoprecipitated with MO or MT2 receptor was reduced in rOPN-treated cells compared to PBS-treated cells. Similar observations were noted for Gi<sub>2</sub> and Gi<sub>3</sub> isoforms. In contrast, the amount of each of these Gi protein isoforms was elevated in the  $\beta_1$  integrin precipitates following rOPN treatment. These results suggest that OPN causes a kidnapping of Gi proteins and reduces their availability for GiPCRs.

### **Example 10**

#### **OPN enhances the phosphorylation of Gi proteins**

[00162] Given that the defective GiPCR signaling associated with idiopathic scoliosis has causatively been related to increased phosphorylation of Gi proteins (Moreau et al., 2004), it was of interest to examined whether OPN influences the phosphorylation status of Gi proteins. Accordingly, MC3T3-E1 cells were treated with

rOPN, then cell lysates were immunoprecipitated with antibodies against Gi<sub>1</sub>, Gi<sub>2</sub> or Gi<sub>3</sub> protein isoforms, and the phosphorylation level was examined by Western blot using anti-phospho serine/threonine or anti-tyrosine antibodies. Results revealed the presence of phosphorylated serine and tyrosine residues in Gi<sub>1</sub> precipitates obtained from cells treated with PBS or rOPN. However, band density was higher in OPN-treated cells. Similar observations were noted in Gi<sub>2</sub> and Gi<sub>3</sub> precipitates (**Fig. 7 A**). These results show that OPN enhances the phosphorylation of Gi proteins at serine and tyrosine residues.

**[00163]** To support the involvement of  $\alpha_5\beta_1$  integrin in this process, cells were pre-treated with antibody against  $\alpha_5\beta_1$  integrin prior to rOPN treatment. Western blot analysis revealed that the Gi phosphorylation induced by rOPN treatment was attenuated by anti- $\alpha_5\beta_1$  integrin blocking antibody (**Fig. 7 A**).

**[00164]** To further associate these findings with the decreased GiPCR signaling in scoliosis, Gi proteins were examined directly to determine whether they undergo increased phosphorylation in osteoblasts from scoliotic mice. The immunoprecipitated of Gi<sub>1</sub>, Gi<sub>2</sub> and Gi<sub>3</sub> proteins were probed with an anti-phospho-serine/threonine or an anti-phospho-tyrosine specific antibody and it was observed that the level of phosphorylation of each Gi isoform was significantly greater in osteoblasts from scoliotic bipedal WT or CD44<sup>-/-</sup> mice compared with those from quadrupedal control mice (**Fig. 7B**). Importantly, phosphorylation levels were attenuated by anti- $\alpha_5\beta_1$  integrin blocking antibody (data not shown). These results provide convincing evidence that GiPCR signaling in scoliotic mice is associated with Gi protein phosphorylation induced by OPN via  $\alpha_5\beta_1$  integrin engagement.

### **Example 11**

#### **Phosphorylation of Gi proteins induced by OPN involves various kinases**

**[00165]** To identify which kinases are involved in the phosphorylation of Gi proteins induced by OPN, FAK, MEK, ERK1/2, P38, JNK, PI3K, Src, PKC and CaMKII were pharmacologically inhibited. Cell extracts prepared from MC3T3-E1 that had been treated with rOPN in the presence or absence of an inhibitor specific for each kinase were subjected to immunoprecipitation and Western blot analysis. Results in **Fig. 7 C-E** show that an inhibitor of PKC (Gö6983) attenuated level of serine phosphorylation in the Gi protein precipitates, but was without effect on tyrosine phosphorylation levels. However, both serine and tyrosine phosphorylation were attenuated by inhibitors of FAK (FAK inhibitor-14), Scr (PP2), CaMKII (KN93), PI3K (wortmannin-data not shown), MEK (PD98059), ERK1/2 (FR180204), JNK (SP60125) and P38 (SB203580). These results indicate that various kinases are directly or indirectly involved in the phosphorylation of Gi proteins induced by OPN.

### **Example 12**

#### **The effects of OPN on Gi and Gs proteins favour GsPCR signaling**

**[00166]** Several reports indicate that inhibition of Gi proteins disrupts signal from GiPCR and enhances GsPCR signaling (Itoh et al., 1984); (Katada et al., 1985); (Wesslau and Smith, 1992). On the other hand, it has been demonstrated that the tyrosine phosphorylation of Gs protein by Src also enhances GsPCR signaling,

presumably by increasing activity of Gs protein and its association with receptor (Hausdorff et al., 1992); (Chakrabarti and Gintzler, 2007).

[00167] Taken into account these considerations and having observed that genetic deletion of OPN inversely influenced response to Gi and Gs stimulation (**Fig. 1**), it was of interest to examine the effect of OPN on the functional and phosphorylation status of Gs protein and on its interaction with receptors. For this purpose, cells were subjected to isoproterenol and desmopressin in the presence of rOPN or PBS. As expected, response to isoproterenol was higher in rOPN than in PBS-treated cells. Similar results were obtained when cells were stimulated with desmopressin, indicating that OPN increases response to GsPCR stimulation (**Fig. 8 A**). Interestingly, immunoprecipitates of Gs proteins probed with anti-tyrosine antibody exhibited higher intensity in rOPN-treated cells compared to PBS-treated cells (**Fig. 8 B**). More interestingly, rOPN treatment enhances the presence of Gs proteins in the immunoprecipitates of MT2 and MO receptors (**Fig. 8 C-D**).

[00168] Collectively, these results demonstrate that OPN enhances not only the GsPCR signaling, but also the tyrosine phosphorylation of Gs protein and its association with receptors.

[00169] Assuming that inhibition of Gi protein and phosphorylation of Gs protein are responsible for the increased GsPCR signaling associated with rOPN, the next experiments aimed to determine the contribution of each parameter. For this purpose, cells were treated with PTX to mimic disruption action of rOPN and compared to cells treated with rOPN alone or in combination with Src inhibitor (PP2) to prevent tyrosine phosphorylation. Results in **Fig. 8 E** show that rOPN-treated cells were more responsive to isoproterenol stimulation than PTX-treated cells by 30 %. This modest difference was abolished by Src inhibitor (PP2). Similar observations were noted when cells were stimulated with desmopressin. These results indicate that Gs phosphorylation contributes only part of the stimulatory effect of rOPN on GsPCR signaling and that disruption of the inhibitory signal from GiPCR represents the main cause of this phenomenon.

#### **Example 13:**

#### **Identification of a CT SNP in AIS subjects associated with increased risk of spinal deformity progression and effect on GiPCR signaling**

[00170] A Whole Exome Sequencing (WES) study was performed on cell samples from AIS subjects and a SNP (rs1467558) was identified in AIS subjects with severe AIS, which changes the amino acid isoleucine 230 to threonine in the CD44 coding sequence.

[00171] Next, the effect of the CT SNP on GiPCR signaling was assessed. Osteoblasts from severe AIS patients (who underwent surgery) carrying the CT SNP and osteoblasts from healthy controls with wild type CD44 (CC) were stimulated with LPA in the presence of recombinant OPN (rOPN). The GiPCR defective signaling effect in response to the same rOPN treatment was reduced 50% more in mutated CD44 (CT) osteoblasts as compared to in wild type CD44 (CC) osteoblasts (**Fig. 9 A**). Comparable results have been obtained using

different concentrations of OPN. The CT mutation in CD44 thus increases the sensitivity of scoliotic patients to the damaging effects of OPN on GiPCR signaling and thereby contributes the more severe phenotype observed in these subjects.

**[00172]** On the basis of findings presented herein and the available evidences, and without being so limited, a hypothetical scenario schematized in **Fig. 9**, explains the mechanism by which OPN reduces GiPCR signaling and contributes to the development of spinal deformity. According to the proposed mechanism, upon OPN binding,  $\alpha_5\beta_1$  integrin recruits a part of Gi proteins from the common pool, then promotes different signaling pathways leading to the activation of various kinases, which directly or indirectly phosphorylate a part of the remaining Gi proteins in the common pool. These events simultaneously cause depletion of pool and inactivation of different isoforms, leading to the reduction in the amount of the functional Gi proteins necessary for the efficient GiPCR signaling. This would reduce the inhibitory control on Gi protein and favour GsPCR signaling, which is exacerbated by phosphorylation of Gs protein by Src. The exaggerated GsPCR signaling increases bone formation (Hsiao et al., 2008) and reduce myoblast proliferation (Marchal et al., 1995), leading to an imbalance between bone mass and muscular strength around the spine. Thus, spine will be throwing out of the balance and the lateral curvature will appear. Since signal transduction through Gs proteins enhances OPN mRNA and protein levels (Nagao et al., 2011), the exaggerated GsPCR signaling would sustain the high production of OPN and amplify events driving the development of spinal deformity. Although additional mechanisms could be operable, the contribution of GsPCR signaling is further supported by the addition of spinal deformity to the constellation of orthopaedic problems commonly associated with Fibrous Dysphasia of bone, an uncommon disease caused by congenital mutation in Gs protein (Leet et al., 2004).

**[00173]** Results presented herein demonstrate that absence of CD44 expression potentiates the effect of OPN and further reduces GiPCR signalization in cells (**Figure 3**). Furthermore, HA was shown to potentiate this effect. CD44 is known to bind to OPN and HA. Finally, in AIS subjects with severe scoliosis (without being bound to any particular theory), CD44 could act by sequestering a portion of the OPN pool available to interact with the integrin receptor. Under circumstances where less CD44 is available to bind to OPN (e.g., under circumstances where expression levels of CD44 are reduced, where OPN's affinity for CD44 is reduced or where binding to OPN is blocked (e.g., by an antibody specific for CD44 or by another ligand such as HA)), bioavailability of OPN to  $\alpha_5\beta_1$  is increased and GiPCR signalization is further reduced. Conversely, increasing CD44/sCD44 expression (availability to bind to OPN) could contribute to reduce OPN's binding to integrins and increase GiPCR signalization in cells.

**[00174]** The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

## REFERENCES :

1. Kane, W. J. (1977) Scoliosis prevalence: a call for a statement of terms. *Clinical orthopaedics and related research*, 43-46.
2. Dickson, R. A. (1992) The etiology and pathogenesis of idiopathic scoliosis. *Acta orthopaedica Belgica* 58 Suppl 1, 21-25.
3. Machida, M. (1999) Cause of idiopathic scoliosis. *Spine* 24, 2576-2583.
4. Burwell, R. G. (2003) Aetiology of idiopathic scoliosis: current concepts. *Pediatric rehabilitation* 6, 137-170.
5. Aherrahrou, Z., S.B. Axtner, P.M. Kaczmarek, A. Jurat, S. Korff, L.C. Doehring, D. Weichenhan, H.A. Katus, and B.T. Ivandic. 2004. A locus on chromosome 7 determines dramatic up-regulation of osteopontin in dystrophic cardiac calcification in mice. *The American journal of pathology* 164:1379-1387.
6. Akoume, M.Y., B. Azeddine, I. Turgeon, A. Franco, H. Labelle, B. Poitras, C.H. Rivard, G. Grimard, J. Ouellet, S. Parent, and A. Moreau. 2010. Cell-based screening test for idiopathic scoliosis using cellular dielectric spectroscopy. *Spine (Phila Pa 1976)* 35:E601-608.
7. Azeddine, B., K. Letellier, S. Wang da, F. Moldovan, and A. Moreau. 2007. Molecular determinants of melatonin signaling dysfunction in adolescent idiopathic scoliosis. *Clinical orthopaedics and related research* 462:45-52.
8. Berg, K.A., G. Zardeneta, K.M. Hargreaves, W.P. Clarke, and S.B. Milam. 2007. Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. *Neuroscience* 144:889-897.
9. Brown, E.J., and W.A. Frazier. 2001. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* 11:130-135.
10. Bushfield, M., B.E. Lavan, and M.D. Houslay. 1991. Okadaic acid identifies a phosphorylation/dephosphorylation cycle controlling the inhibitory guanine-nucleotide-binding regulatory protein Gi2. *The Biochemical journal* 274 ( Pt 2):317-321.
11. Chakrabarti, S., and A.R. Gintzler. 2007. Phosphorylation of Galphas influences its association with the micro-opioid receptor and is modulated by long-term morphine exposure. *Molecular pharmacology* 72:753-760.
12. Chiba, S., M.M. Rashid, H. Okamoto, H. Shiraiwa, S. Kon, M. Maeda, M. Murakami, M. Inobe, A. Kitabatake, and A.F. Chambers. 1999. The role of osteopontin in the development of granulomatous lesions in lung. *Microbiology and immunology* 44:319-332.
13. Clover, J., R.A. Dodds, and M. Gowen. 1992. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. *Journal of cell science* 103 ( Pt 1):267-271.
14. Denhardt, D., E. Burger, C. Kazaneki, S. Krishna, C. Semeins, and J. Klein-Nulend. 2001a. Osteopontin-deficient bone cells are defective in their ability to produce NO in response to pulsatile fluid flow. *Biochemical and biophysical research communications* 288:448-453.
15. Denhardt, D.T., C.M. Giachelli, and S.R. Rittling. 2001b. Role of osteopontin in cellular signaling and toxicant injury. *Annual review of pharmacology and toxicology* 41:723-749.
16. Denhardt, D.T., and X. Guo. 1993. Osteopontin: a protein with diverse functions. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 7:1475-1482.
17. Denhardt, D.T., M. Noda, A.W. O'Regan, D. Pavlin, and J.S. Berman. 2001c. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *The Journal of clinical investigation* 107:1055-1061.
18. Fitzpatrick, L., A. Severson, W. Edwards, and R. Ingram. 1994. Diffuse calcification in human coronary arteries. Association of osteopontin with atherosclerosis. *Journal of Clinical Investigation* 94:1597.

19. Giachelli, C.M., N. Bae, M. Almeida, D.T. Denhardt, C.E. Alpers, and S.M. Schwartz. 1993. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *The Journal of clinical investigation* 92:1686-1696.
20. Giachelli, C.M., L. Liaw, C.E. Murry, S.M. Schwartz, and M. Almeida. 1995. Osteopontin expression in cardiovascular diseases. *Annals of the New York Academy of Sciences* 760:109-126.
21. Giachelli, C.M., R. Pichler, D. Lombardi, D.T. Denhardt, C.E. Alpers, S.M. Schwartz, and R.J. Johnson. 1994. Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. *Kidney international* 45:515-524.
22. Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annual review of biochemistry* 56:615-649.
23. Green, J.M., A. Zhelesnyak, J. Chung, F.P. Lindberg, M. Sarfati, W.A. Frazier, and E.J. Brown. 1999. Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. *The Journal of cell biology* 146:673-682.
24. Gronthos, S., K. Stewart, S.E. Graves, S. Hay, and P.J. Simmons. 1997. Integrin Expression and Function on Human Osteoblast-like Cells. *Journal of Bone and Mineral Research* 12:1189-1197.
25. Grzesik, W.J., and P.G. Robey. 1994. Bone matrix RGD glycoproteins: immunolocalization and interaction with human primary osteoblastic bone cells in vitro. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 9:487-496.
26. Hausdorff, W.P., J.A. Pitcher, D.K. Luttrell, M.E. Linder, H. Kurose, S.J. Parsons, M.G. Caron, and R.J. Lefkowitz. 1992. Tyrosine phosphorylation of G protein alpha subunits by pp60c-src. *Proceedings of the National Academy of Sciences of the United States of America* 89:5720-5724.
27. Hsiao, E.C., B.M. Boudignon, W.C. Chang, M. Bencsik, J. Peng, T.D. Nguyen, C. Manalac, B.P. Halloran, B.R. Conklin, and R.A. Nissenson. 2008. Osteoblast expression of an engineered Gs-coupled receptor dramatically increases bone mass. *Proceedings of the National Academy of Sciences* 105:1209-1214.
28. Hughes, P.E., M.W. Renshaw, M. Pfaff, J. Forsyth, V.M. Keivens, M.A. Schwartz, and M.H. Ginsberg. 1997. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 88:521-530.
29. Illario, M., V. Amideo, A. Casamassima, M. Andreucci, T. di Matola, C. Miele, G. Rossi, G. Fenzi, and M. Vitale. 2003. Integrin-dependent cell growth and survival are mediated by different signals in thyroid cells. *The Journal of clinical endocrinology and metabolism* 88:260-269.
30. Itoh, H., F. Okajima, and M. Ui. 1984. Conversion of adrenergic mechanism from an alpha- to a beta-type during primary culture of rat hepatocytes. Accompanying decreases in the function of the inhibitory guanine nucleotide regulatory component of adenylate cyclase identified as the substrate of islet-activating protein. *The Journal of biological chemistry* 259:15464-15473.
31. Katada, T., A.G. Gilman, Y. Watanabe, S. Bauer, and K.H. Jakobs. 1985. Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *European journal of biochemistry / FEBS* 151:431-437.
32. Katagiri, Y.U., J. Sleeman, H. Fujii, P. Herrlich, H. Hotta, K. Tanaka, S. Chikuma, H. Yagita, K. Okumura, M. Murakami, I. Saiki, A.F. Chambers, and T. Ueda. 1999. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. *Cancer research* 59:219-226.
33. Leet, A.I., E. Magur, J.S. Lee, S. Wientroub, P.G. Robey, and M.T. Collins. 2004. Fibrous dysplasia in the spine: prevalence of lesions and association with scoliosis. *The Journal of Bone & Joint Surgery* 86:531-537.
34. Liaw, L., D.E. Birk, C.B. Ballas, J.S. Whitsitt, J.M. Davidson, and B.L. Hogan. 1998. Altered wound healing in mice lacking a functional osteopontin gene (spp1). *The Journal of clinical investigation* 101:1468-1478.

35. Machida, M., J. Dubousset, T. Yamada, J. Kimura, M. Saito, T. Shiraishi, and M. Yamagishi. 2006. Experimental scoliosis in melatonin-deficient C57BL/6J mice without pinealectomy. *Journal of pineal research* 41:1-7.
36. Machida, M., I. Murai, Y. Miyashita, J. Dubousset, T. Yamada, and J. Kimura. 1999. Pathogenesis of idiopathic scoliosis. Experimental study in rats. *Spine (Phila Pa 1976)* 24:1985-1989.
37. Marchal, S., I. Cassar-Malek, J.P. Magaud, J.P. Rouault, C. Wrutniak, and G. Cabello. 1995. Stimulation of avian myoblast differentiation by triiodothyronine: possible involvement of the cAMP pathway. *Experimental cell research* 220:1-10.
38. Moreau, A., S. Forget, B. Azeddine, D. Angeloni, F. Fraschini, H. Labelle, B. Poitras, C.-H. Rivard, and G. Grimard. 2004. Melatonin signaling dysfunction in adolescent idiopathic scoliosis. *Spine* 29:1772-1781.
39. Moss, J., P. Bruni, J.A. Hsia, S.C. Tsai, P.A. Watkins, J.L. Halpern, D.L. Burns, Y. Kanaho, P.P. Chang, E.L. Hewlett, and et al. 1984. Pertussis toxin-catalyzed ADP-ribosylation: effects on the coupling of inhibitory receptors to the adenylate cyclase system. *Journal of receptor research* 4:459-474.
40. Moursi, A.M., R.K. Globus, and C.H. Damsky. 1997. Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro. *Journal of cell science* 110 ( Pt 18):2187-2196.
41. Murry, C.E., C.M. Giachelli, S.M. Schwartz, and R. Vracko. 1994. Macrophages express osteopontin during repair of myocardial necrosis. *The American journal of pathology* 145:1450-1462.
42. Murthy, K.S., J.R. Grider, and G.M. Makhlof. 2000. Heterologous desensitization of response mediated by selective PKC-dependent phosphorylation of G(i-1) and G(i-2). *American journal of physiology. Cell physiology* 279:C925-934.
43. Nagao, M., T.N. Feinstein, Y. Ezura, T. Hayata, T. Notomi, Y. Saita, R. Hanyu, H. Hemmi, Y. Izu, S. Takeda, K. Wang, S. Rittling, T. Nakamoto, K. Kaneko, H. Kurosawa, G. Karsenty, D.T. Denhardt, J.P. Vilaradaga, and M. Noda. 2011. Sympathetic control of bone mass regulated by osteopontin. *Proceedings of the National Academy of Sciences of the United States of America* 108:17767-17772.
44. O'Brien, K.D., J. Kuusisto, D.D. Reichenbach, M. Ferguson, C. Giachelli, C.E. Alpers, and C.M. Otto. 1995. Osteopontin is expressed in human aortic valvular lesions. *Circulation* 92:2163-2168.
45. Oates, A., R. Barraclough, and P. Rudland. 1997. The role of osteopontin in tumorigenesis and metastasis. *Invasion & metastasis* 17:1.
46. Ophascharoensuk, V., C.M. Giachelli, K. Gordon, J. Hughes, R. Pichler, P. Brown, L. Liaw, R. Schmidt, S.J. Shankland, C.E. Alpers, W.G. Couser, and R.J. Johnson. 1999. Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apoptosis. *Kidney international* 56:571-580.
47. Oyama, J., I. Murai, K. Kanazawa, and M. Machida. 2006. Bipedal ambulation induces experimental scoliosis in C57BL/6J mice with reduced plasma and pineal melatonin levels. *Journal of pineal research* 40:219-224.
48. Pistone, M., C. Sanguineti, A. Federici, F. Sanguineti, P. Defilippi, F. Santolini, G. Querze, P.C. Marchisio, and P. Manduca. 1996. Integrin synthesis and utilization in cultured human osteoblasts. *Cell biology international* 20:471-479.
49. Rangaswami, H., A. Bulbule, and G.C. Kundu. 2006. Osteopontin: role in cell signaling and cancer progression. *Trends in cell biology* 16:79-87.
50. Reinholt, F.P., K. Hultenby, A. Oldberg, and D. Heinegard. 1990. Osteopontin—a possible anchor of osteoclasts to bone. *Proceedings of the National Academy of Sciences of the United States of America* 87:4473-4475.
51. Shangguan, Y., K.E. Hall, R.R. Neubig, and J.W. Wiley. 2003. Diabetic neuropathy: inhibitory G protein dysfunction involves PKC-dependent phosphorylation of Galpha. *Journal of neurochemistry* 86:1006-1014.

52. Sodek, J., B. Ganss, and M. McKee. 2000. Osteopontin. *Critical Reviews in Oral Biology & Medicine* 11:279-303.
53. Strassheim, D., and C.C. Malbon. 1994. Phosphorylation of Gi alpha 2 attenuates inhibitory adenylyl cyclase in neuroblastoma/glioma hybrid (NG-108-15) cells. *The Journal of biological chemistry* 269:14307-14313.
54. Von Gall, C., A. Lewy, C. Schomerus, B. Vivien-Roels, P. Pevét, H.W. Korf, and J.H. Stehle. 2000. Transcription factor dynamics and neuroendocrine signalling in the mouse pineal gland: a comparative analysis of melatonin-deficient C57BL mice and melatonin-proficient C3H mice. *European Journal of Neuroscience* 12:964-972.
55. Weber, G.F., S. Ashkar, M.J. Glimcher, and H. Cantor. 1996. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271:509-512.
56. Wesslau, C., and U. Smith. 1992. The inhibitory GTP-binding protein (Gi) regulates the agonistic property of beta-adrenergic ligands in isolated rat adipocytes. Evidence for a priming effect of cyclic AMP. *The Biochemical journal* 288 (Pt 1):41-46.
57. Letellier K, Azeddine B, Parent S, Labelle H, Rompré PH, Moreau A, Moldovan F. Estrogen cross-talk with the melatonin signaling pathway in human osteoblasts derived from adolescent idiopathic scoliosis patients. *J Pineal Res.* 2008 Nov;45(4):383-93.
58. Verdonk E, Johnson K, McGuinness R, Leung G, Chen YW, Tang HR, Michelotti JM, Liu VF. Cellular dielectric spectroscopy: a label-free comprehensive platform for functional evaluation of endogenous receptors. *Assay Drug Dev Technol.* 2006 Oct;4(5):609-19.

**CLAIMS:**

1. A method of increasing GiPCR signalization in the cells of a subject in need thereof comprising administering to the subject an effective amount of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity, whereby GiPCR signalization is increased in the cells of the subject.
2. The method of claim 1, further comprising administering to the subject an effective amount of an inhibitor of OPN expression and/ or activity.
3. The method of claim 2, wherein the inhibitor of OPN activity is an OPN antibody.
4. The method of claim 2, wherein the inhibitor of OPN expression is a siRNA specific to OPN.
5. The method of any one of claims 1-4, wherein the inhibitor of  $\alpha_5\beta_1$  activity is (a) an antibody that binds specifically to integrin subunit  $\alpha_5$ ; (b) an antibody that binds specifically to integrin subunit  $\beta_1$ ; (c) an antibody that binds specifically to integrin subunits  $\alpha_5\beta_1$ ; (d) a molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin; or (e) any combination of at least two of (a) to (d).
6. The method of claim 5, wherein said molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a RGD peptide or derivative thereof.
7. The method of claim 5, wherein said molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a peptide fragment of OPN comprising a RGD motif.
8. The method of claim 7, wherein the peptide fragment of OPN comprises the amino acid sequence GRGDSVYGLRS (SEQ ID NO: 18).
9. The method of any one of claims 1-4, wherein the inhibitor of  $\alpha_5\beta_1$  expression is an siRNA specific to  $\alpha_5$  or  $\beta_1$ .
10. The method of any one of claims 1-9, further comprising administering to the subject i) an effective amount of sCD44 or a fragment thereof which specifically binds to OPN; or ii) a stimulator of sCD44 and/or CD44 expression.
11. Use of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity in the preparation of a medicament for inhibiting GiPCR signalization in cells of a subject in need thereof.
12. Use of an inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity for inhibiting GiPCR signalization in cells of a subject in need thereof.
13. An inhibitor of integrin  $\alpha_5\beta_1$  expression and/or activity for use in inhibiting GiPCR signalization in cells of a

subject in need thereof.

14. The use of claim 11 or 12 or the inhibitor of claim 13, wherein said inhibitor of  $\alpha_5\beta_1$  activity is (a) an antibody that binds specifically to integrin subunit  $\alpha_5$ ; (b) an antibody that binds specifically to integrin subunit  $\beta_1$ ; (c) an antibody that binds specifically to integrin subunits  $\alpha_5\beta_1$ ; (d) a molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin; or (e) any combination of at least two of (a) to (d).

15. The use or inhibitor of claim 14, wherein the molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a RGD peptide or derivative thereof.

16. The use or inhibitor of claim 14, wherein the molecule that specifically blocks the binding of OPN to  $\alpha_5\beta_1$  integrin is a peptide fragment of OPN comprising a RGD motif.

17. The use or inhibitor of claim 16, wherein the peptide fragment of OPN comprises the amino acid sequence GRGDSVYGLRS (SEQ ID NO: 18).

18. The use of claim 11 or 12 or the inhibitor of claim 13, wherein the inhibitor of integrin  $\alpha_5\beta_1$  expression is an siRNA specific to  $\alpha_5$  or  $\beta_1$ .

19. A composition comprising the inhibitor as defined in any one of claims 13 to 18, and a pharmaceutical carrier.

20. The composition of claim 19, for increasing GiPCR signaling in cells of a subject in need thereof.

21. The method of any one of claims 1-10, the use of any one of claims 11, 12, and 14 to 18, the inhibitor of any one of claims 13-18, or the composition of claim 20, wherein the subject is a subject diagnosed with a scoliosis or at risk of developing a scoliosis.

22. The method, use, inhibitor, or composition of claim 21, wherein the scoliosis is an idiopathic scoliosis.

23. The method, use, inhibitor, or composition of claim 21, wherein the scoliosis is adolescent idiopathic scoliosis (AIS).

24. A method of determining the risk of developing a scoliosis in a subject comprising:

- (i) providing a cell sample isolated from the subject;
- (ii) detecting, in the cell sample from the subject, the presence of at least one copy of a CD44 risk allele which introduces a mutation at amino acid position 230 of CD44 or a marker in linkage disequilibrium therewith; and
- (iii) determining that the subject is at risk of developing a scoliosis when at least one copy of the risk allele or marker in linkage disequilibrium therewith is detected in the cell sample from the subject.

25. The method of claim 24, wherein the risk allele comprises SNP rs1467558 and the mutation changes an isoleucine at position 230 of CD44 to a threonine.
26. A method of stratifying a subject having scoliosis comprising:
- (i) providing a cell sample isolated from the subject;
  - (ii) detecting, in the cell sample from the subject, the presence of at least one copy of a CD44 risk allele which introduces a mutation at amino acid position 230 of CD44; and
  - (iii) (a) stratifying the subject into a first AIS subclass when at least one copy of the CD44 risk allele or a marker in linkage disequilibrium therewith is detected in the cell sample from the subject; or  
(b) stratifying the subject into a second AIS subclass when the CD44 risk allele or marker in linkage disequilibrium therewith is not detected in the cell sample from the subject.
27. The method of claim 26, wherein the mutation changes an isoleucine at position 230 of CD44 to a threonine.
28. The method of any one of claims 24 to 27, wherein the at least one copy of the CD44 risk allele consists of two copies of the CD44 risk allele and the subject is homozygote for the mutation.
29. The method of any one of claims 24 to 28, wherein the sample is a nucleic acid sample.
30. The method of any one of claims 24 to 29, wherein said sample is a protein sample.
31. A kit for performing the method as defined in any one of claims 24 to 30 comprising:
- (i) a nucleic acid probe or primer for detecting a CD44 risk allele comprising a mutation in a codon encoding amino acid 230 of CD44; and/or
  - (ii) a protein ligand which specifically detects a mutation at amino acid 230 of CD44.
32. The kit of claim 31, wherein the nucleic acid probe or primer comprises a nucleic acid sequence which specifically hybridizes to a nucleic acid encoding a threonine at amino acid position 230 of CD44.
33. The kit of claim 31, wherein the protein ligand is an antibody specific for a CD44 protein comprising a threonine at amino acid position 230.

FIG 1

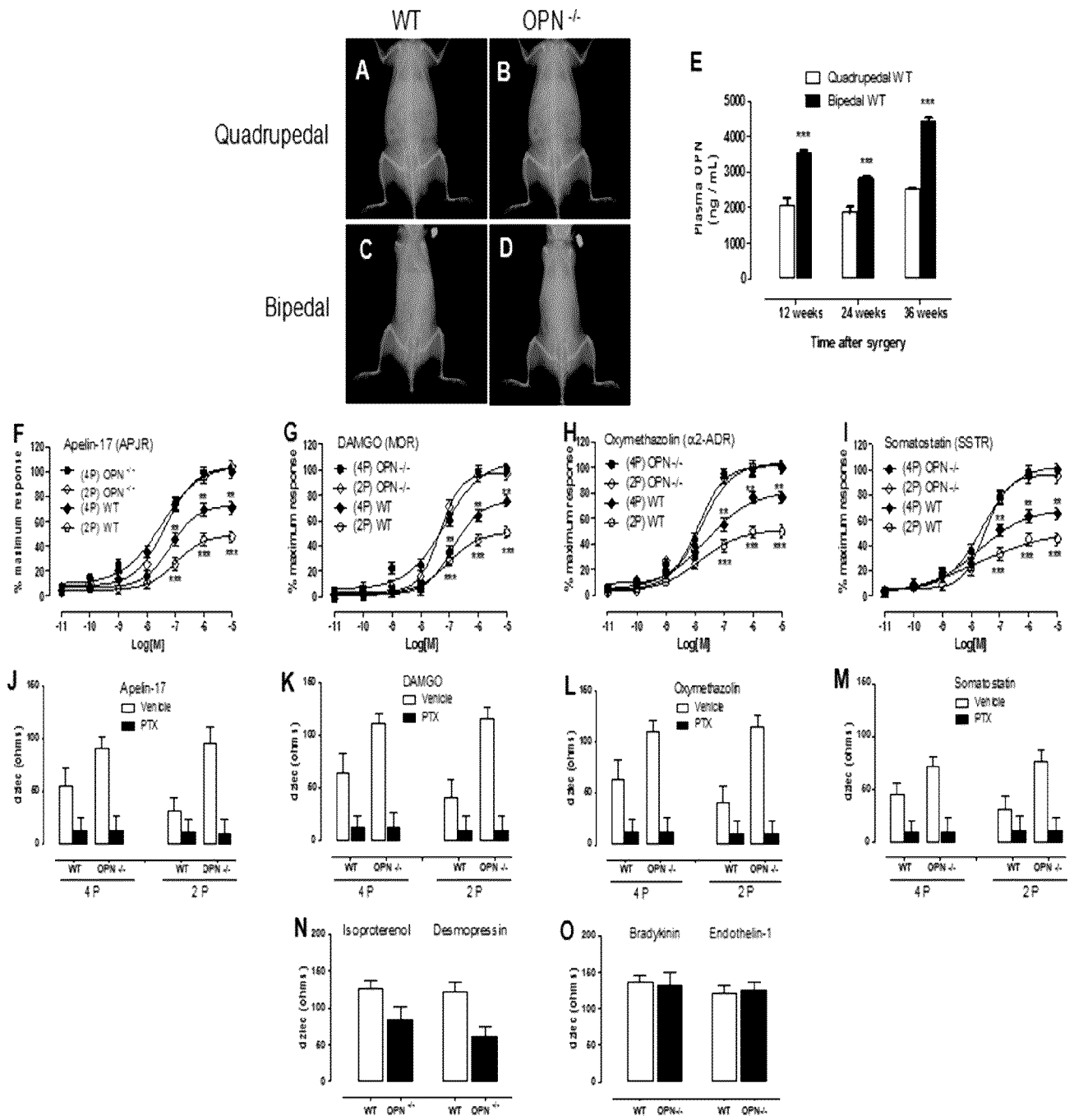


FIG 2

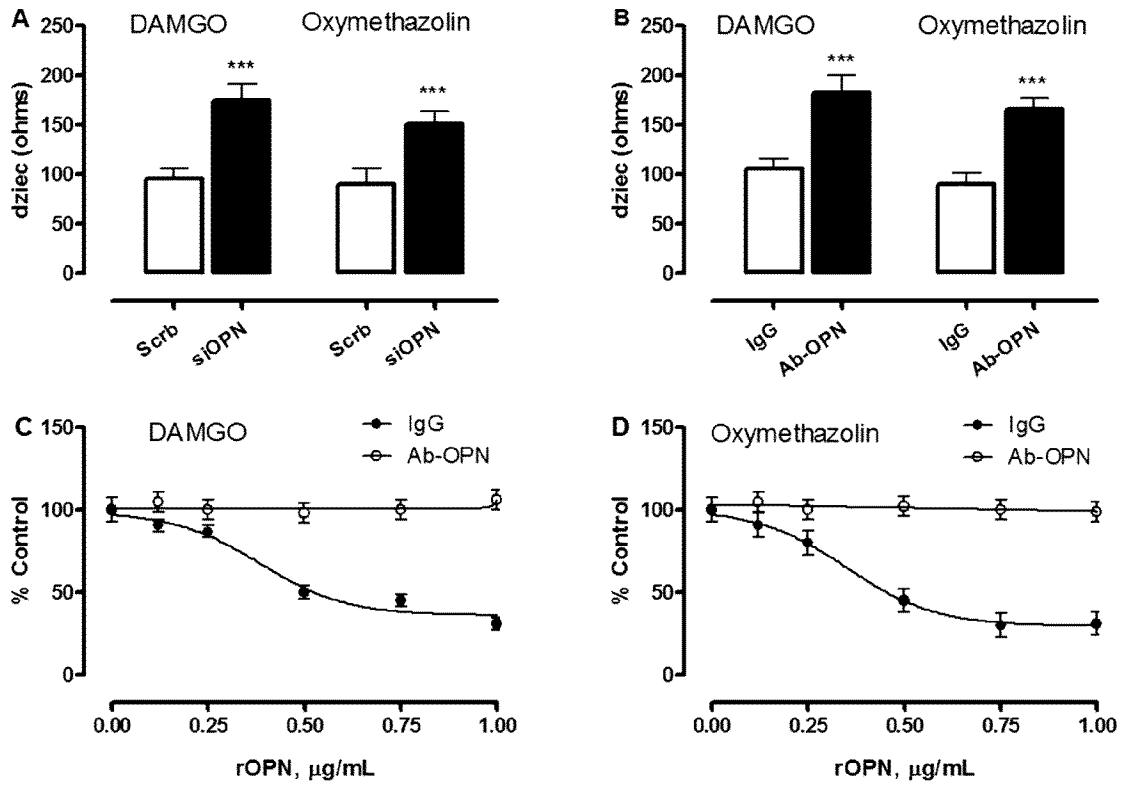


FIG 3

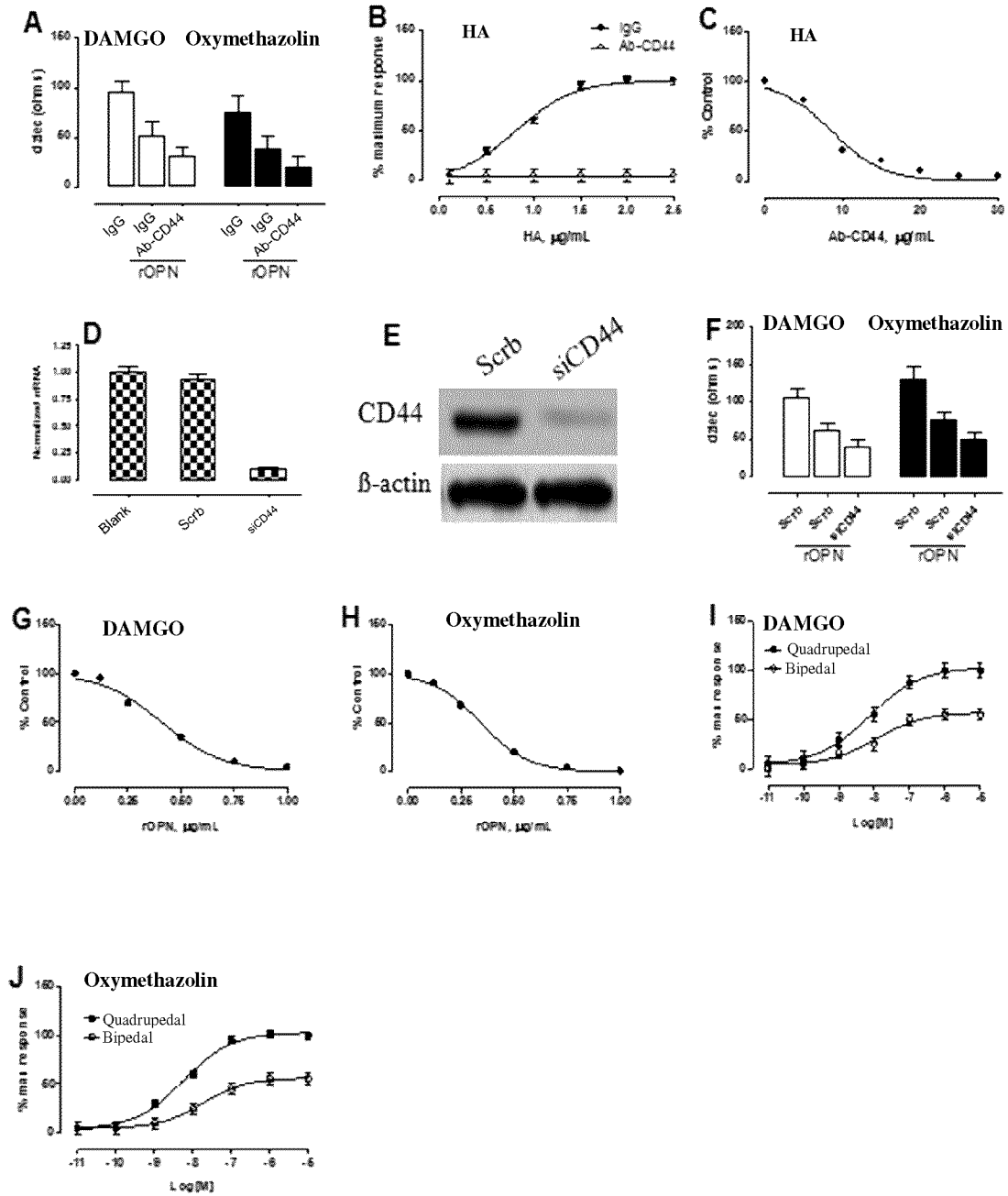
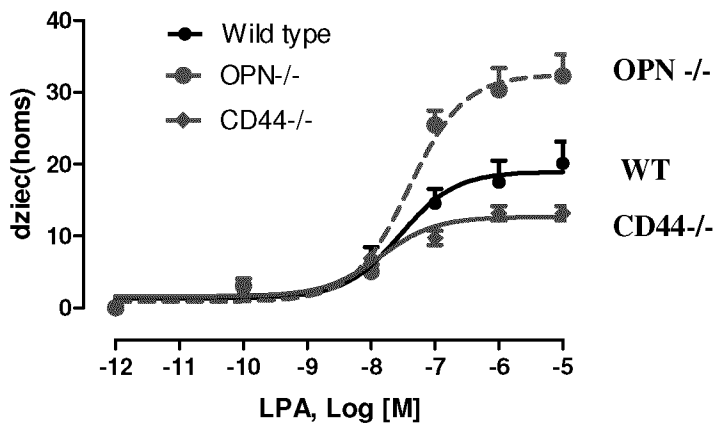
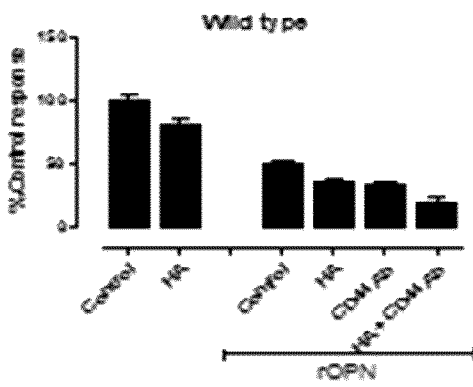


FIG 3 (continued)

K



L



M

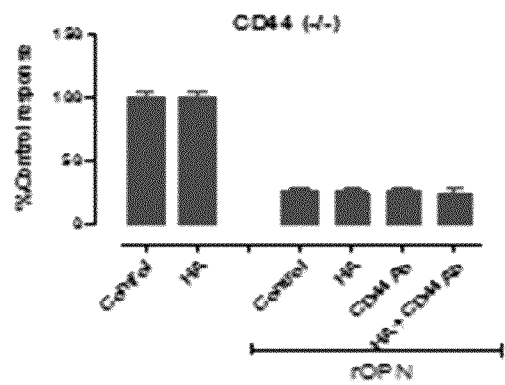
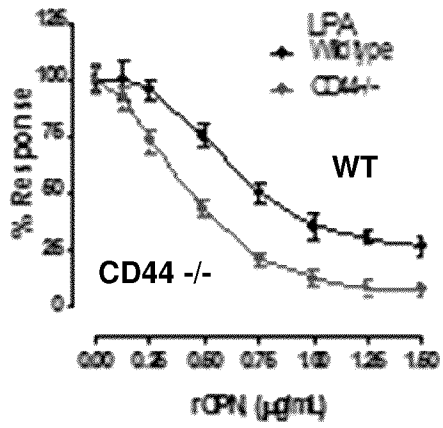
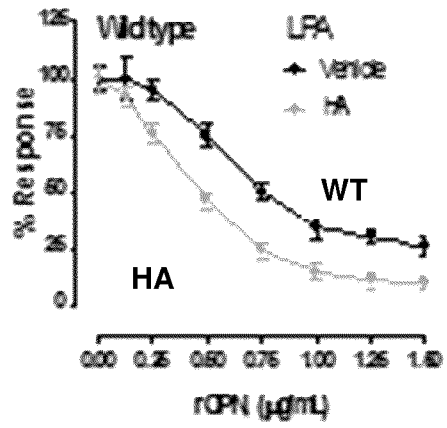


FIG 3 (continued)

N



O



P

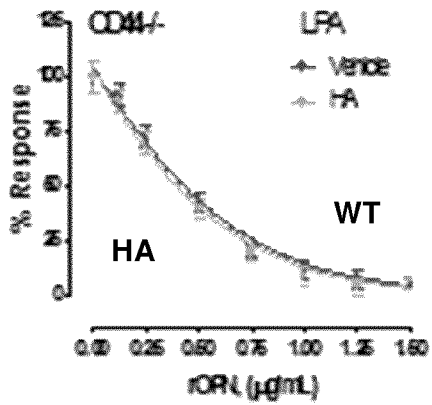


FIG 4

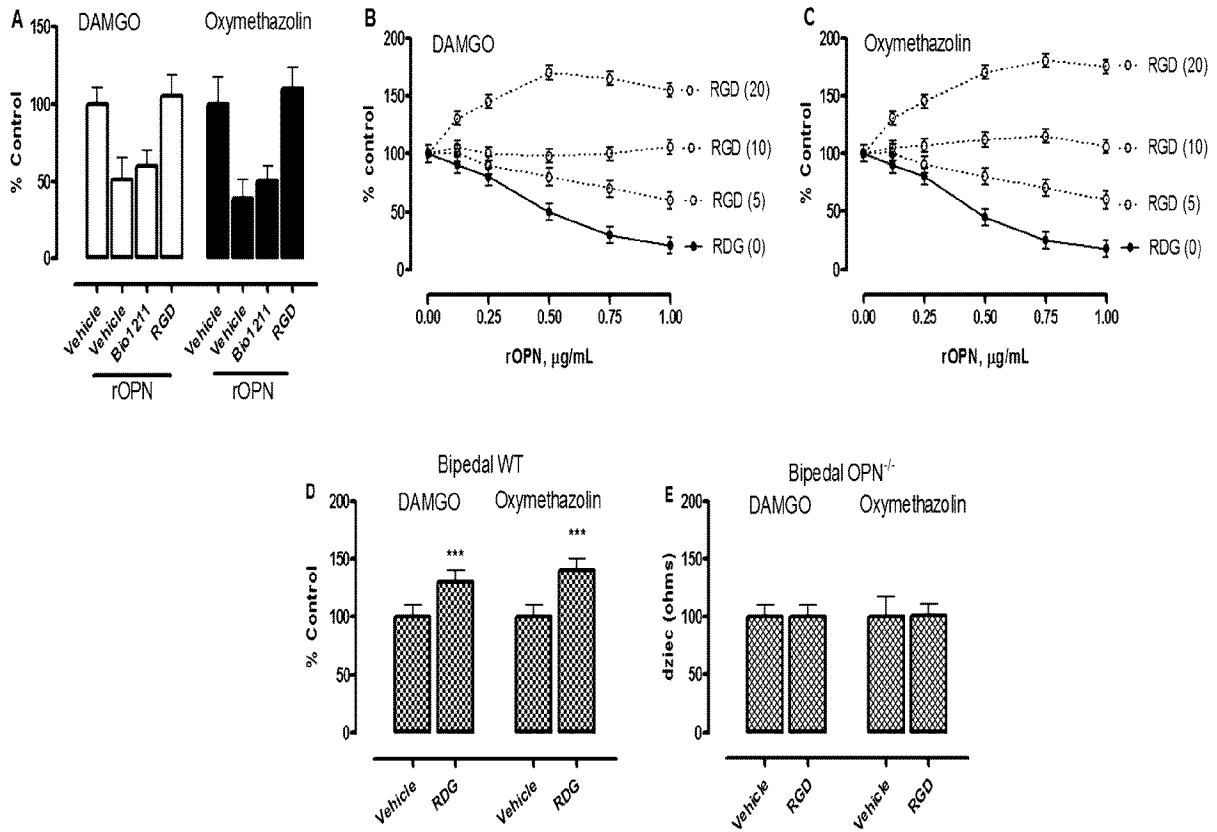
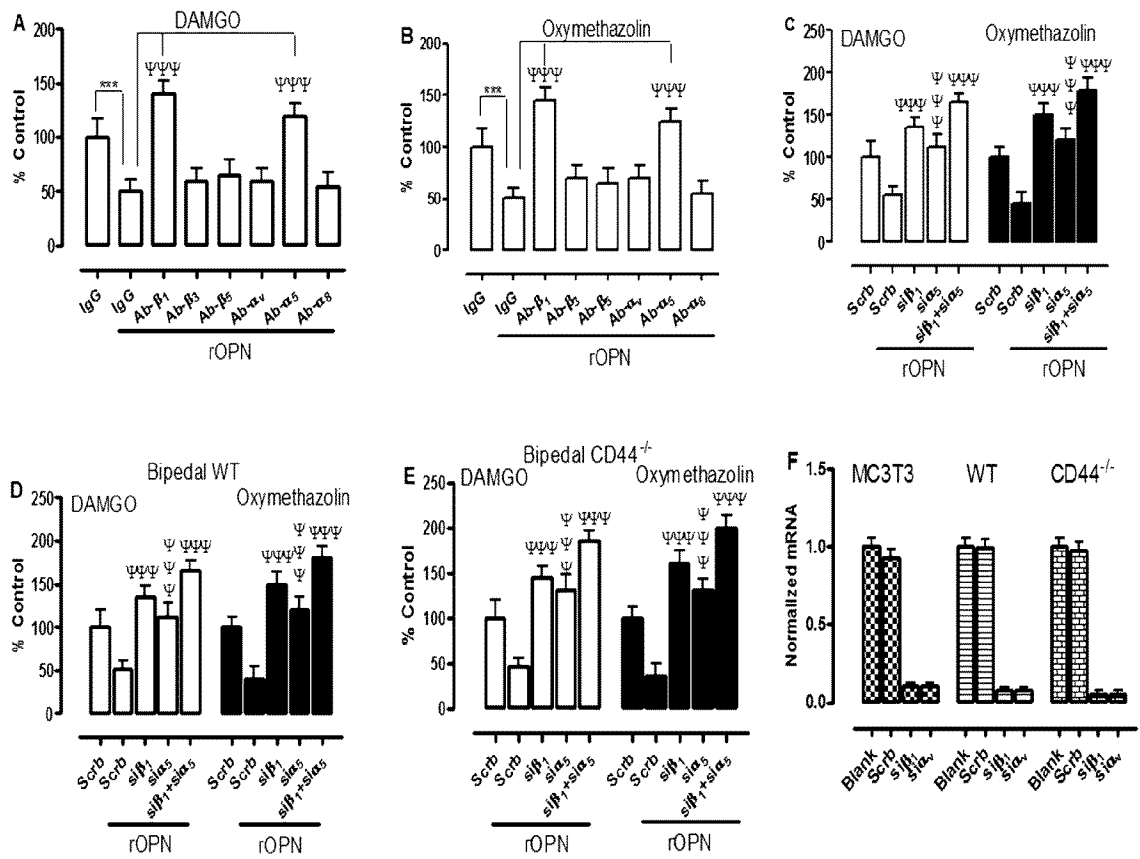
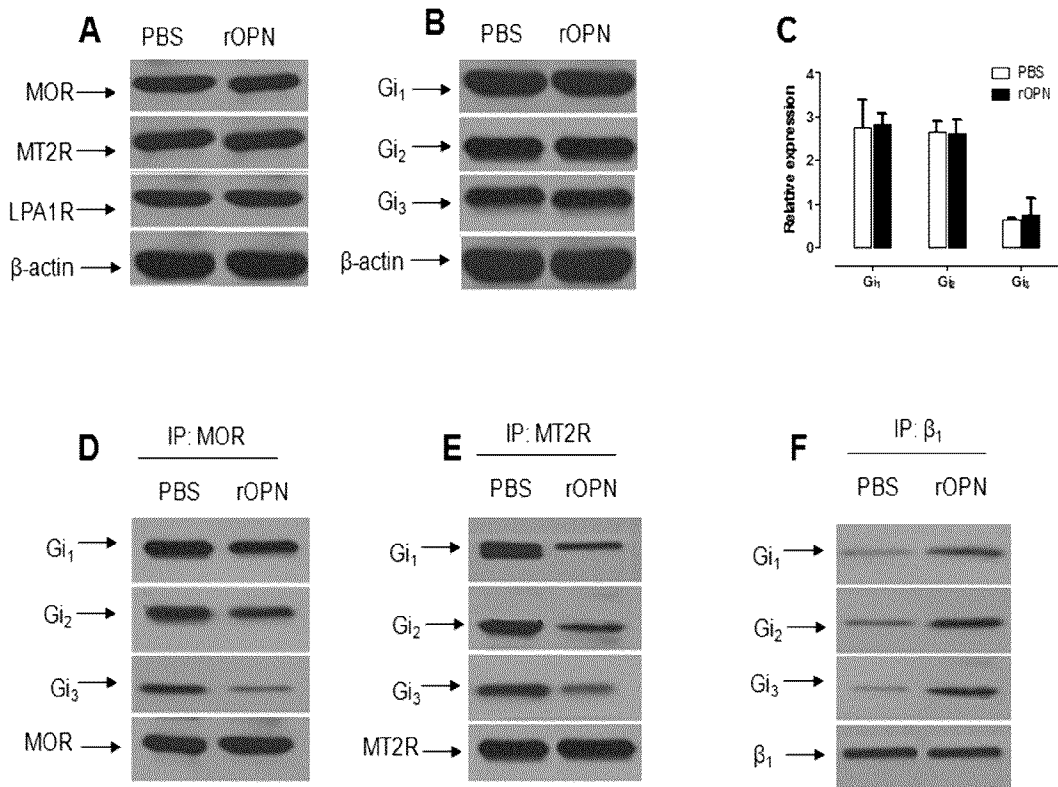


FIG 5



**FIG 6**





**FIG 8**

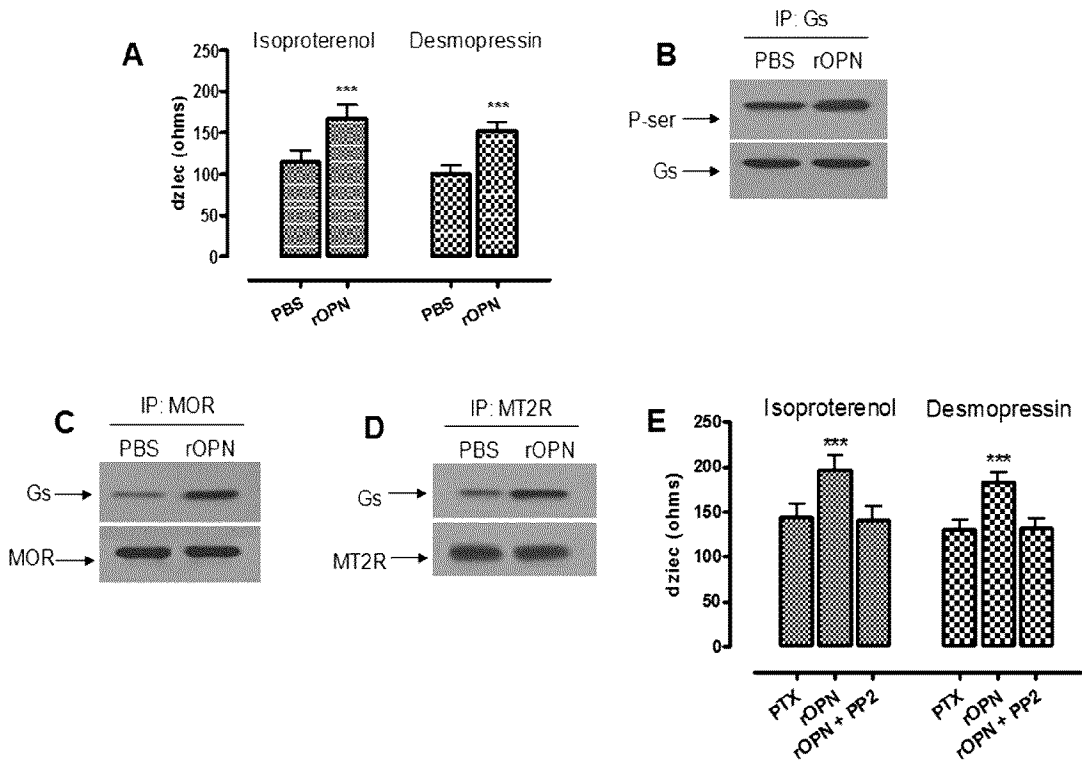
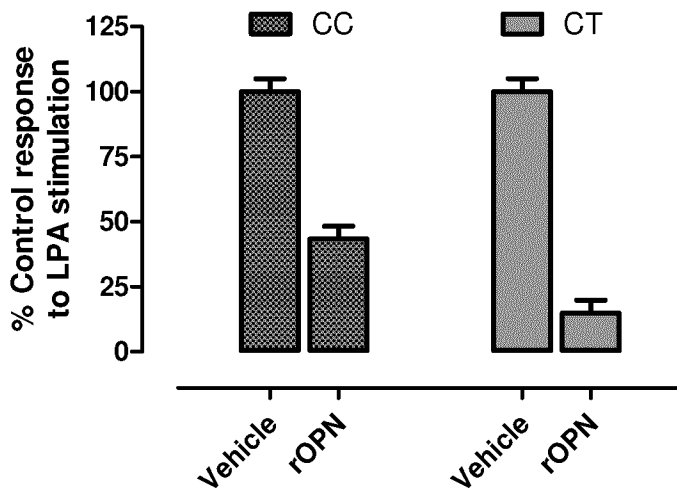


FIG 9

A



B

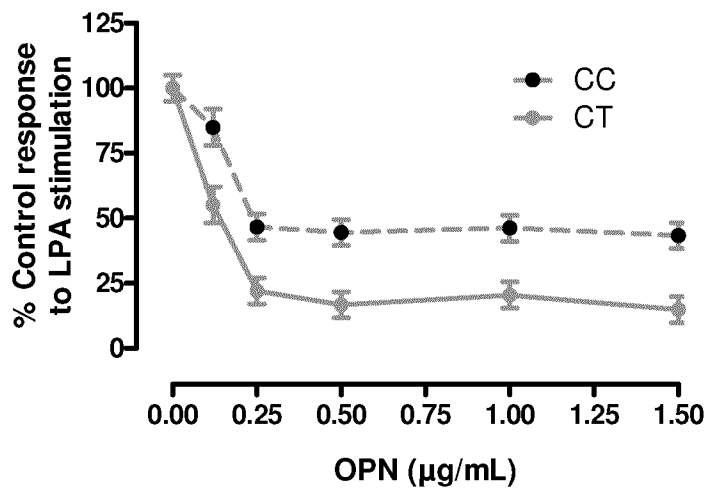
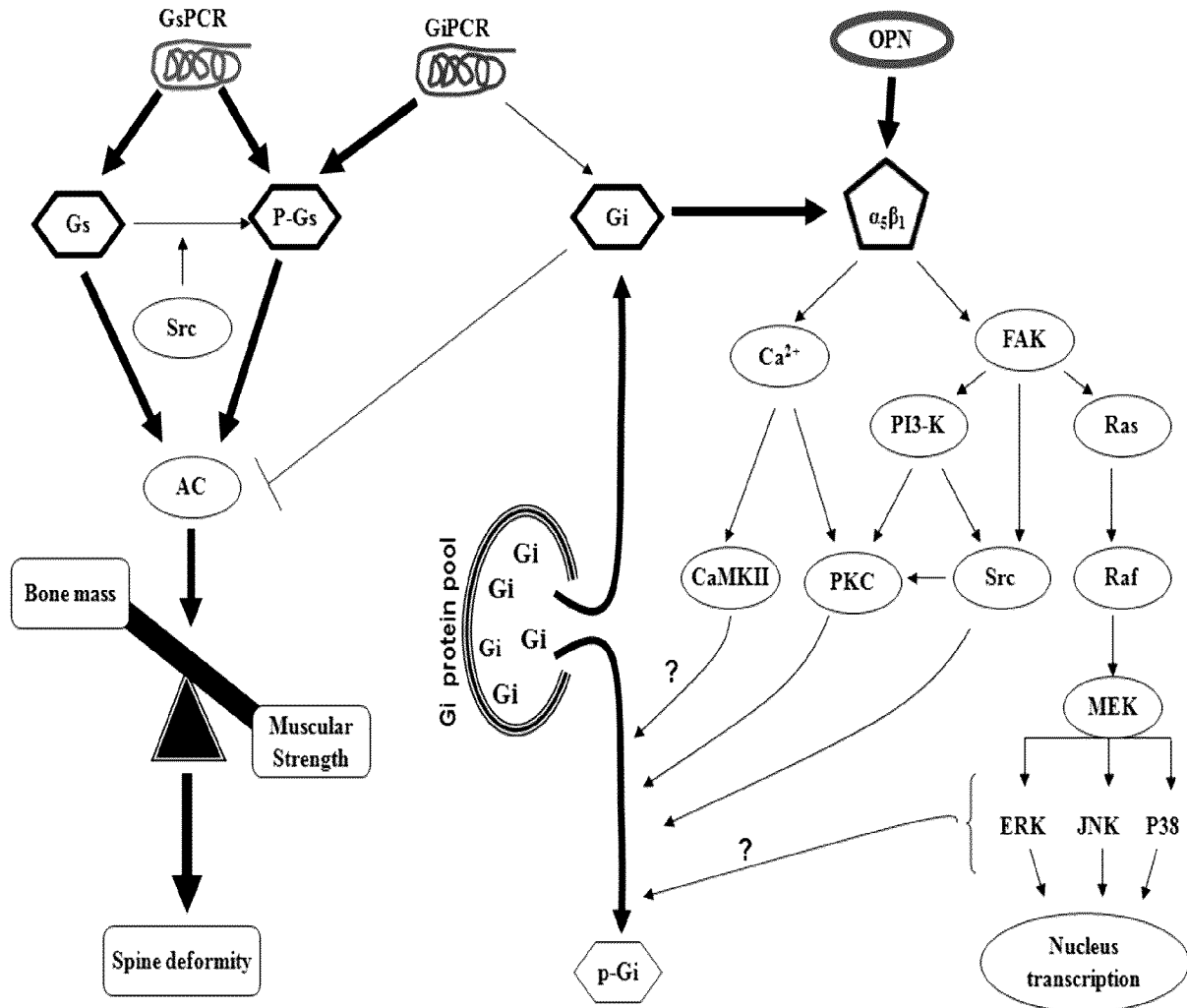


FIG 10



## INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CA2014/050569**

<p>A. CLASSIFICATION OF SUBJECT MATTER          IPC: <b>A61K 39/395</b> (2006.01), <b>A61K 31/713</b> (2006.01), <b>A61P 19/08</b> (2006.01), <b>C07H 21/00</b> (2006.01),  <b>C12Q 1/68</b> (2006.01), <b>G01N 33/48</b> (2006.01), <b>G01N 33/53</b> (2006.01)</p>																							
<p>B. FIELDS SEARCHED</p> <p>Minimum documentation searched (classification system followed by classification symbols)          IPC: <b>A61K 39/395</b> (2006.01), <b>A61K 31/713</b> (2006.01), <b>A61P 19/08</b> (2006.01), <b>C07H 21/00</b> (2006.01),  <b>C12Q 1/68</b> (2006.01), <b>G01N 33/48</b> (2006.01), <b>G01N 33/53</b> (2006.01)</p> <p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p> <p>Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)          Genomequest (GQ-Pat Gold Nuc, Genbank, Geneseq, RefSeq, ENSEMBL mRNA, GQ-Pat Gold Protein, Geneseq - Protein, Genpept, ENSEMBL Protein, Swiss-Prot, RefSeq - Pro, Translated EMBL), Scopus, TotalPatent. Keywords: cd 44; scoliosis; hcam; pgp-1; pgp1; ecm-iii; hutch-1; hutch1; opn; osteopontin; bone sialoprotein 1; bsp-1; bsp1; bnspp; early t-lymphocyte activation; eta-1; eta1; secreted phosphoprotein 1; SPP-1; SPP1; 2ar; rickettsia resistance; ric; integrin alpha5beta1; CD29; CD49e; ; grgdsvvyglrs; rs1467558; spina bifida; cerebral palsy; spinal muscular atrophy, chiari malform*; osteoblast*</p>																							
<p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p> <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>WO2008119170A1 (MOREAU) 9 October 2008 (09-10-2008) Paragraphs [0028]-[0042] and [140]; Table 4; Examples 1 and 4</td> <td>11-17 and 19-23</td> </tr> <tr> <td>Y</td> <td></td> <td>18</td> </tr> <tr> <td>X</td> <td>FARBER, K. et al. Molecular and cellular neurosciences. An alpha5beta1 integrin inhibitor attenuates glioma growth. December 2008 Vol. 39(4). Pages 579-585. ISSN:1044-7431</td> <td>11-14 and 19</td> </tr> <tr> <td>Y</td> <td></td> <td>18</td> </tr> <tr> <td>X</td> <td>ZAHN, G. et al. Assessment of the integrin alpha5beta1 antagonist JSM6427 in proliferative vitreoretinopathy using in vitro assays and a rabbit model of retinal detachment.</td> <td>11-14 and 19</td> </tr> <tr> <td>Y</td> <td>INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE. February 2010. Vol.51(2), pages 1028-1035. ISSN 0146-0404</td> <td>18</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	WO2008119170A1 (MOREAU) 9 October 2008 (09-10-2008) Paragraphs [0028]-[0042] and [140]; Table 4; Examples 1 and 4	11-17 and 19-23	Y		18	X	FARBER, K. et al. Molecular and cellular neurosciences. An alpha5beta1 integrin inhibitor attenuates glioma growth. December 2008 Vol. 39(4). Pages 579-585. ISSN:1044-7431	11-14 and 19	Y		18	X	ZAHN, G. et al. Assessment of the integrin alpha5beta1 antagonist JSM6427 in proliferative vitreoretinopathy using in vitro assays and a rabbit model of retinal detachment.	11-14 and 19	Y	INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE. February 2010. Vol.51(2), pages 1028-1035. ISSN 0146-0404	18
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Y	INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE. February 2010. Vol.51(2), pages 1028-1035. ISSN 0146-0404	18																					
<p><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.</p>																							
<p>* Special categories of cited documents:          "A" document defining the general state of the art which is not considered to be of particular relevance          "E" earlier application or patent but published on or after the international filing date          "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)          "O" document referring to an oral disclosure, use, exhibition or other means          "P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention          "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone          "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art          "&amp;" document member of the same patent family</p>																						
<p>Date of the actual completion of the international search          2 September 2014 (02-09-2014)</p>		<p>Date of mailing of the international search report          18 September 2014 (18-09-2014)</p>																					
<p>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</p>		<p>Authorized officer   <b>Claire Plumb (819) 994-9468</b></p>																					

INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CA2014/050569**

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	COURT, M.H. et al. Candidate gene polymorphisms in patients with acetaminophen-induced acute liver failure. DRUG METABOLISM AND DISPOSITION: THE BIOLOGICAL FATE OF CHEMICALS. January 2014 Vol. 42(1), pages 28-32. Published online 8 October 2013. ISSN: 1873-3492	31-33

## INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CA2014/050569****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.: 1-10 and 24-30  
because they relate to subject matter not required to be searched by this Authority, namely:

Please see page 8: *Continuation of Box II Observations where certain claims were found unsearchable*

2.  Claim Nos.: Please see page 8: *Continuation of Box II Observations where certain claims were found unsearchable*  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

Please see page 8: *Continuation of Box III Observations where unity of invention is lacking*

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.

**Continuation of: Box II Observations where certain claims were found unsearchable***Search restriction due to subject matter:*

Claims 1-10 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. This Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 1-10. Please note that claims 20-23 also encompass embodiments directed at a method for treatment of the human or animal body by surgery or therapy and the search has likewise been restricted for the portions of these claims that encompass method of treatment.

Claims 24-30 are directed to a diagnostic method, which the International Searching Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product as defined in claims 24-30.

*Search restriction due to lack of compliance with disclosure requirement:*

The International Searching Authority has not carried out a search over the full scope of claims 11-20. Claims 11-20 so lack clarity and/or support that a meaningful search over the whole of the claimed scope is impossible. Claims 11-20 encompass a use, an inhibitor, and a composition which target any disease associated with integrin alpha5beta1 and/or osteopontin (OPN). In the present case, the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been established for the parts of the application which appear to be clear and supported, namely a use, an inhibitor, and a composition which inhibits the interaction between integrin alpha5beta1 and OPN, for inhibiting idiopathic scoliosis.

**Continuation of: Box III Observations where unity of invention is lacking**

The requirements of unity of invention are not fulfilled in that there is no technical relationship among the inventions as they do not involve one or more of the same or corresponding technical features. The expression "special technical features" means those features which define a contribution which each of the claimed inventions considered as a whole makes over the prior art.

Claims 11-23 encompass the use, an inhibitor and a composition for inhibiting integrin alpha5beta1 and/or OPN. Claims 31-33 encompass a kit comprising reagents to test for a CD44 mutation, said kit for use in the assessment of the risk of developing scoliosis. The subject matter of claims 11-23 and claims 31-33 does not share any common technical features.

The use of either an OPN or a CD44 inhibitor for the treatment of idiopathic scoliosis is known in the art (see D1 WO2008119170A1 MOREAU et al.). Therefore, the concept of identifying compounds that inhibits the interaction of OPN with its target is known in the art (claims 11-20); D1 further identifies CD44 as a participant in idiopathic scoliosis (claims 31-33).

The claims encompass two separate inventions divided into the following groups:

**Invention 1** - claims 11-23 (all fully) are directed to a use, inhibitor or composition featuring inhibition integrin alpha5beta1 and/or OPN; and

**Invention 2** - claims 31-33 (all fully) a kit for use in determining the risk of developing a scoliosis in a subject featuring the detection of a risk allele at position 230 of CD44.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.

**PCT/CA2014/050569**

<b>Patent Document Cited in Search Report</b>	<b>Publication Date</b>	<b>Patent Family Member (s)</b>	<b>Publication Date</b>
WO2008119170A1	2008-10-09	JP2010522699A	2010-07-08
		ZA200906738A	2010-05-26
		IL201186D0	2010-05-17
		CN101680887A	2010-03-24
		MX2009010400A	2010-02-18
		KR1020100016075A	2010-02-12
		EP2132568A1	2009-12-16
		CA2682114A1	2008-10-09
		AU2008234374A1	2008-10-09