Université de Montréal

Déterminants moléculaires de la scoliose idiopathique de l'adolescent

par

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Déterminants moléculaires de la scoliose idiopathique de l'adolescent

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Résumé

La scoliose est la déformation de la colonne vertébrale la plus répendue. Elle atteint 3 à 4% de la population pédiatrique et dans 85% des cas, aucune cause n'a été identifiée. Ces cas sont appelés idiopathiques et les symptômes apparaissent durant la puberté; d'où le terme de 'scoliose idiopathique de l'adolescent (SIA). Cette pathologie atteint le plus souvent les jeunes filles, en nombre et en sévérité. Ces dernières années, plusieurs hypothèses ont été proposées afin d'élucider l'étiologie de cette pathologie. Celles-ci ont mis de l'avant différents facteurs génétiques, biochimiques, méchaniques, neurologiques, musculaires ou hormonaux. Plusieurs études ont rapporté des formes familiales de scoliose, soutenant la thèse d'une prédisposition génétique. Nous avons démontré que les patients souffrant de SIA présentent un défaut de signalisation cellulaire médiée par les protéines Gi et un taux élevé d'ostéopontine (OPN) circulante. En utilisant une approche de type 'gène candidat', nous avons montré que la protéine tyrosine phosphatase μ (PTPμ) régule l'activité du complexe d'intégrines α5/β1 (récepteur de l'OPN) via la protéine kinase PIPKIγ. Dans ce but, nous avons utilisé des cultures primaires d'ostéoblastes issues de biopsies de patients et de cas traumatiques comme sujets contrôles. Les biopsies ossscuses de patients ont été obtenues lors de l'intervention chirurgicale à partir des vertébres T3 à L4, selon les différentes procédures. Les biopsies issues de cas traumatiques proviennent d'autres types d'os (tibia, crête iliaque, fémur). Les profils d'expression du gène PTPRM (codant pour la protéine PTPμ) ont été étudiés par PCR quantitative (qPCR). Les taux de protéines PTPμ ont été analysés par immunoprécipitation suivi d'un western blot. Pour évaluer le rôle de cette protéine, nous avons bénéficié d'un modèle murin. Machida et al. ont démontré qu'il existe un taux plus élevé de scoliose parmi les souris C57Bl/6 bipèdes obtenues suite à l'amputation des membres supérieurs, sous anesthésie, cinq semaines après la naissance. Nous avons utilisé des cultures primaires d'ostéoblastes issues de la colonne

vertébrale de souris C57Bl/6 bipèdes, délétées du gène PTPRM (souris dites 'KO'), afin d'évaluer le niveau de signalisation cellulaire spécifique des protéines Gi par un test fonctionnel: la technique de spectroscopie cellulaire di-électrique (SCD). Selon nos données, 85% des souris bipédales 'KO' pour le géne PTPRM développent une scoliose (modérée à sévère) contre 55% des souris contrôles C57Bl6 bipèdes. De plus, les niveaux de PTPμ exprimée par les ostéoblastes de 34 patients SIA se trouvent diminués par comparaison à 17 sujets contrôles. Nos études de souris bipèdes ont montré que l'inactivation du gène PTPRM augmente l'incidence et la sévérité de la scoliose, sans pour autant affecter les taux circulant d'OPN ou l'expression de ses récepteurs. Par ailleur, dans ce même contexte, nous avons remarqué une augmentation de l'intéraction entre l'OPN et l'intégrine β1 en l'abscence du gène PTPRM. Les cellules issues de ces souris bipèdes KO montrent une réduction dans leurs niveaux de signalisation cellulaire médiée par les protéines Gi après stimulation par l'OPN. Cette diminution est en grande partie récupérée après traitement des cellules par un siRNA spécifique de la protéine PIPK1γ, substrat de PTPμ qui favorise la fixation de ligands aux intégrines. Ces études apportent les premières indications que la perte d'expression de PTPμ est impliquée dans le développement de la SIA, en amplifiant probablement l'effet inhibiteur de l'OPN sur la signalisation cellulaire médiée par les protéines Gi.

Ces études permettent une meilleure compréhension de l'étiologie de la SIA. Elles pourraient avoir une contribution importante dans le développement futur de méthodes diagnostique et thérapeuthique dans le but d'arrete l'apparition et l'évolution de la maladie chez les enfants atteints.

Mots-clés : Scoliose idiopathique adolescente (SIA), protéines G, signalisation, osteopontin, protéine tyrosine phosphatase μ (PTP μ)

Abstract

Adolescent idiopathic scoliosis (AIS) is the most common form of scoliosis that affects 3-4% of the global pediatric population. In more than 85% of cases, no specific cause can be identified. Such cases are called idiopathic and occur mostly during adolescence. AIS affects mainly females in number and severity. Over the past years, many hypotheses were proposed to explain the etiology of AIS, including genetic, biochemical, mechanics, neurological, muscular and hormonal factors. Several studies have reported a high incidence of scoliosis in some families, which argues for a genetic cause of this disease. We demonstrated that AIS patients have a Gi protein signaling defect and exhibit high levels of circulating Osteopontin (OPN). The goal of this thesis is to identify the mechanisms regulating OPN signaling activity in AIS patients. We have used a candidate gene driven approach and discovered that protein tyrosine phosphatase μ (PTP μ) regulates α5β1 integrin (a known OPN receptor) through phosphate kinase type 1 gamma (PIPK1γ). To achieve our goal, we have used primary osteoblast cell cultures derived from AIS patients and biopsies from control subjects. Bone specimens were obtained intraoperatively from vertebras (varying from T3 to L4 according to the surgical procedure performed) while with trauma cases used as non-scoliotic controls, bone specimens were obtained from other anatomical sites (tibia, femur or iliac crest). Expression profiles of the RPTPM gene (encoding for PTPμ) were studied by qPCR. On the other hand, PTPμ protein levels were determined by immunoprecipitation followed by western blot. To evaluate the role of this protein in AIS etiopathogenesis, we took advantage of an animal model exhibiting a higher scoliosis incidence when maintained in a bipedal state. [1], [2] Bipedal surgeries were performed on C57Bl/6 mice after weaning (5-weeks after birth) by amputation of the forelimbs and tail under anesthesia as reported by Oyama et al. (2006). [1] We used the same approach with PTPμ knockout mice and primary osteoblast culture were derived from

the spine of these mice to assess Gi protein signaling through a functional assay termed Cellular Dielectric Spectroscopy (CDS).

Bipedal PTPμ knockout mice develop scoliosis more often (85%) in number and severity, than control C57Bl/6 bipedal mice (55%). Interestingly, functional analysis of osteoblasts derived from PTPμ KO mice by CDS method showed a flaw in the transmission of Gi protein coupled receptor signaling similar to a specific AIS patient subgroup. Furthermore, the clinical relevance of PTPμ was strengthened by the fact that a decrease in the gene expression level of PTPμ was observed in 34 AIS patients when compared to 17 control subjects. Such a decrease was also confirmed at the protein level. We demonstrated that genetic deletion of PTPμ enhances the incidence and severity of scoliosis without affecting plasma levels of OPN or the expression of its receptors. In contrast, increased interaction of OPN with β1 integrin was noticed in cells depleted of PTPμ. Furthermore, reduction of Gi- protein coupled receptor GiPCR signaling by OPN was also enhanced in these cells, while their response to GiPCR stimulation was improved with siRNA of phosphatidylinositol-phosphate kinase type 1 gamma (PIPK1γ), a PTPμ substrate that favours ligand binding to integrins. These studies provide the first indication that the loss of PTPμ influences the nature of idiopathic scoliosis, possibly by amplifying the inhibitory effect of OPN on GiPCR signaling.

This study allows a better understanding of AIS etiology and could have an impact for the future development of innovative diagnostic methods and eventual pharmacological approaches in order to prevent AIS and stop its progression in affected children.

Keywords : Adolescent idiopathic scoliosis (AIS), G proteins, signaling, osteopontin and protein tyrosine phosphatase μ (PTP μ)

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Abbreviation:

ACs:	Adenylate cyclases
AIS:	Adolescent Idiopathic Scoliosis
AJs:	Adherens junctions
ATP:	Adenosine triphosphate
BL:	Black
BMPs:	Bone morphogenetic proteins
BSP-1:	Bone sialoprotein I
cAMP:	Cyclic adenosine monophosphate
CD44:	Cluster of differentiation 44
cdc25:	cell division cycle 25
cDNA:	Complementary deoxyribonucleic acid
CDS:	Cellular dielectric spectroscopy
cGMP:	Cyclic Guanosine triphosphate
DAG:	Diacylglycerol
DNA:	Deoxyribonucleic acid
ECM:	Extracellular matrix
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinases
ETA-1:	Early T-lymphocyte activation
FN:	Fibronectin
GBM:	Glioblastoma multiform
GDP:	Guanosine diphosphate
GPCR:	G protein coupled receptor
GiPCR:	G inhibitory protein coupled receptor
GTP:	Guanosine triphosphate
Gαi:	G alpha inhibitory
Gαs:	G alpha stimulatory
Ig:	Immunoglobulin

IL:	Interleukin
IP3:	Inositol trisphosphate
JM:	Justamembrane
JNK:	c-Jun N-terminal kinases
MAM:	Meprin-A5 antigen-PTP domain
MKP-1:	Mitogen-activated protein kinase phosphatase 1
MT1:	Melatonin receptor 1
MT2:	Melatonin receptor 2
NF-kB:	Nuclear factor kappa B
OMIM:	Online Mendelian Inheritance in Man
OPG:	Osteoprotegerin
OPN:	Osteopontin
PDGF:	Platelet derived growth factor
PI3K:	Phosphatidylinositide 3-kinases
PIP2:	Phosphatidylinositol 4, 5-bisphosphate
PIPK1γ:	Phosphatidylinositol-phosphate kinase type 1 γ
PKCδ:	Protein kinase C delta
PLC β:	Phospholipase C beta
PLCγ1:	Phospholipase C gamma 1
PNX:	Pinealectomized
PTH:	Parathyroid hormone
PTPμ:	Protein tyrosine phosphatase mu
PTPases:	Protein tyrosine phosphatases
PTPRM:	Receptor-type tyrosine-protein phosphatase mu
PTX:	Pertussis toxin
RGD:	Arginine- Glycine- Aspartic acid
SHP-1:	Src-homology 2 domain–containing PTPase 1
SPP1:	Secreted phosphoprotein 1
SRS:	Scoliosis Research Society

TGF:	Transforming growth factor
TNF:	Tumor necrosis factor
TRAIL:	Tumor necrosis factor -related apoptosis-inducing ligand
α:	Alpha
β:	Beta
γ:	Gamma

I dedicate this work to my family:

To my father's soul
To my mother
To my wife, my son and my daughter
To my brothers and my sister

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GENERAL INTRODUCTION

1 RATIONALE OF THE PROJECT

Adolescent idiopathic scoliosis (AIS) is a complex spinal deformity of high prevalence and of extreme variability that occurs most commonly in girls rather than in boys, around the time of puberty. [3], [4], [5] Approximately 4 % of teenagers are affected worldwide and the disease demonstrates a rapid onset with a clinical course which is often unpredictable. In its extreme form, AIS is manifested by a pronounced lateral curvature accompanied by various co-morbidities and dangerous complications, such as cardiopulmonary failure which may cause death. [6], [7] Although this disease has been clinically recognized for centuries, the standard of care for patients has not changed during the last three decades in any significant manner. As a first approach, patients are treated with braces to limit the progression of the spinal deformity, and then as a last resort patients are faced with invasive spinal fusion surgery. However, constraints associated with bracing and complications from surgery, as well as changes in life style caused by these conservative treatments highlight the need for new therapeutic options. Efforts toward developing therapeutic options that could target the cause of AIS and offer a better outcome for patients are largely limited by the disease's multifactorial origin. [8], [9], [10] Indeed, despite decades of intensive research, no unified cause has emerged, to explain the exact pathomechanism of AIS. Nevertheless, there is an emerging concept that Gi protein coupled receptor signaling Dysfunction via Gi, proteins, combined with high circulating osteopontin (OPN) levels, is a decisive event in the development and progression of AIS. Therefore, the identification of molecular determinants regulating such pathological process and a full understanding of the mechanistic aspect of Gi-mediated signaling under high plasma OPN conditions could provide a molecular platform for the development of innovative pharmacological options and personalized strategies for the treatment of AIS.

REVIEW OF LITERATURE

Scoliosis: Definition and types of scoliosis

Scoliosis can be defined as an abnormal lateral curvature of the spine (as defined in Webster's College Dictionary), and is also known as a three-dimensional deformity of the spine with lateral and vertebral rotation, where the Cobb angle is greater than 10 degrees as measured using the Cobb method on a standing radiography. Scoliosis can be classified as non-structural or structural.

The non-structural scoliosis occurs in a structurally normal spine with a lateral curve, developed as a secondary response to a problem occurring elsewhere in the body. This type of scoliosis can arise due to differing leg lengths or a pelvic tilt. These flexion deformities of the hip or knee are often referred to as postural or compensatory scoliosis. The spinal curvature in non-structural scoliosis can be resolved while the patient is seated or lying down. [11]

The structural scoliosis is the type where the spine has a lateral curve with a vertebral rotation. This form of scoliosis does not resolve when the patient lies down or sits upright and is the most known and familiar form. [12] Structural scoliosis is sub-classified to congenital, neurological, myopathic, traumatic and idiopathic. The congenital scoliosis refers to congenital vertebral anomalies occurring at birth and leads to a lateral curvature of the spine. The neurological and myopathic scoliosis are usually classified in a category called neuromuscular scoliosis. In this type of scoliosis, the spinal curvature is secondary to

a neurological or muscular disease, [13] like spina bifida and Duchenne muscular dystrophy. [12], [14] Idiopathic scoliosis is the most common form of spinal deformity for which the cause remains unknown. Idiopathic scoliosis is further classified according to the age of onset. The three subgroups of idiopathic scoliosis are infantile (1 day - 3 years), juvenile (4- 9 years) and adolescent (10-18 years). [15]

Adolescent idiopathic scoliosis (AIS)

AIS occurs during the time of puberty, when a child experiences a rapid growth. [15] AIS affects mainly females, both in number and severity, suggesting a possible contribution of estrogens.

According to the Terminology Committee of the Scoliosis Research Society (SRS) (1976) and Online Mendelian Inheritance in Man (OMIM 181800), AIS represents 80% of all observed spinal deformities, affecting 3-4% of the global pediatric population. [16], [17], [18], [19] The annual estimation report of the National Scoliosis Foundation demonstrated that AIS incurs more than 600,000 visits to physicians in North America; with 30,000 children requiring a brace and 38,000 who undergo a spinal fusion surgery. In the United States, the estimated cost of treating patients hospitalized with a diagnosis of idiopathic scoliosis in 2004 was over $3 billion dollars. [20], [21] According to the National Scoliosis Foundation statistics, there are six millions persons in the United States afflicted with scoliosis, and there is still no cure.

Diagnosis of AIS

In order to diagnose AIS correctly, it is important for doctors to determine the nature and symptoms of the deformity. An initial evaluation is performed, which includes physical examination, risk assessment based on familial history of the disease, as well as imaging techniques if deemed necessary. This evaluation enables the physicians to determine if the spinal deformity is primary or secondary to other diseases or disorders.

Physical examination

For the screening of scoliosis in schools, physicians, still today, use the Adam's forward bend test (figure 1). This physical examination is also used in the offices of pediatricians and primary care physicians. This test, which does not require any equipment whatsoever, has been used regularly since 1865 for the screening of spine curvature. Nevertheless, in 2005 the US Preventive Services Task Force determined that the test was ineffective for the detection of spinal curvatures and recommended against routine IS screening. [22]

Another widely used method for the physical examination of scoliosis is the use of a scoliometer (figure 2). Using a scoliometer, a measurment of seven degrees equates to twenty degrees in Cobb angle [23], [24].

These routine tests are not sufficient for the proper diagnosis of idiopathic scoliosis and often give rise to false positives, which can lead to incorrect treatment, such as

unecessary bracing or referral to specialty care. For accurate diagnosis, an imaging test is required.

Normal Spine Abnormal Spine

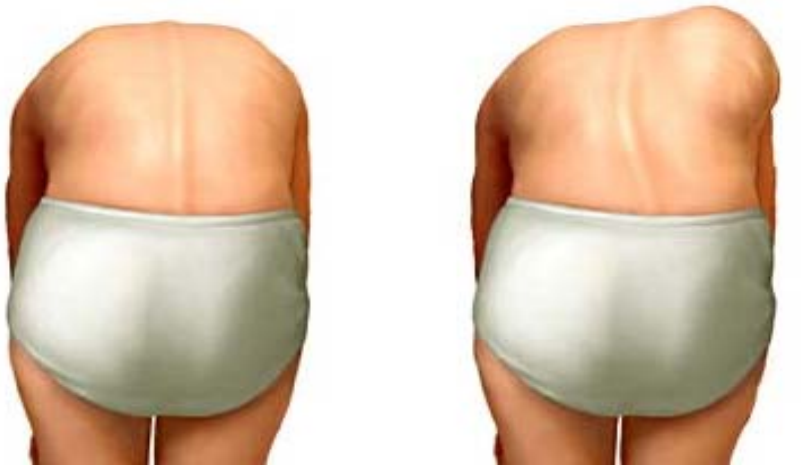

Figure 1: Adam's forward bend test, used for the evaluation of the spinal deformity (adapted from Reamy, et al). [25]

Figure 2: Scoliometer measures the spinal deformity. It is a simple instrument for the detection the spinal curvature (adapted from Franko, et al). [26]

Imaging test

The imaging technique is a more accurate and sensitive method for the detection of the disease, and allows the physicians to follow the progression of the spinal deformity. Additional tests are ordered, in the event of abnormal neurologic examinations, history of severe pain, or presence of rare left thoracic curves, to screen for other factors such as tumors. Additional tests may include X-rays, Computed Tomography (CT) scan or Magnetic Resonance Imaging (MRI). [27] The x- rays are an effective method to establish the severity of the disease and follow its progression by measuring the Cobb angle. However, special precautions need to be taken to limit radiological exposure to avoid the development of tumors.

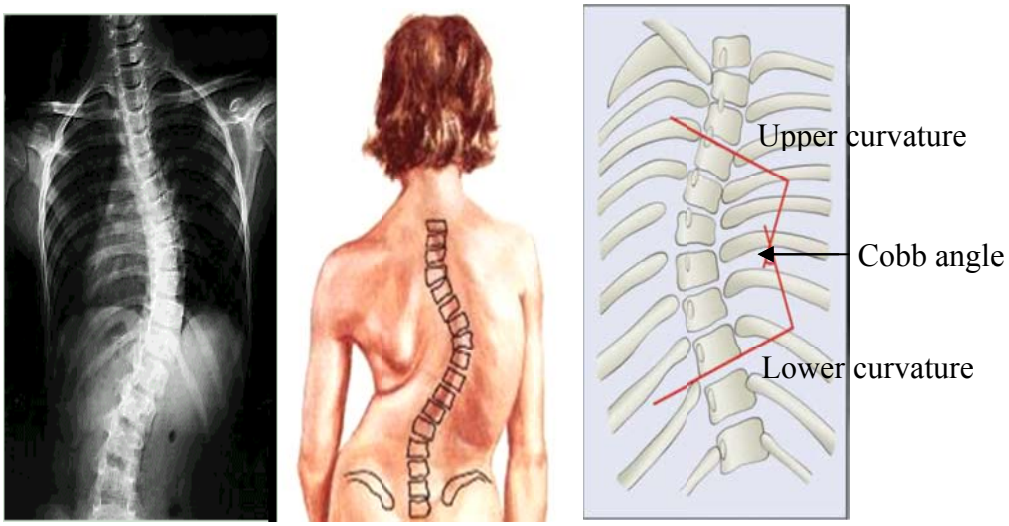

Figure 3: Cobb angle measurement.
The Cobb angle as shown above is the intersection between the right angle of the upper curvature and the lower curvature (adapted from Greiner et al). [28]

Scoliscore

Scoliscore is a recent genetic test developed for the prognosis of AIS using saliva from the patients and then performing genetic analysis. By using 53 genetic markers associated with spinal curve progression in AIS patients, curve progression can be predicted. The positive value of this test remains to be proven, as well as its clinical utility. This test is also limited to Caucasian girls aged between 9 and 13 years old. [29], [30]

Treatment of AIS

Two main treatment options exist for AIS today: bracing and surgery (figure 4). To date, no pharmacological treatment exists. Spine curvatures with Cobb angles between 10° and 25° are simply followed by the orthopaedic surgeons, referring to this period as the

stage of observation. Treatment of IS usually begins when the deformity has developed to 25° to 35°, using different types of braces. In cases where the spine continues to deform (≥ 45°), spinal surgery is the only remaining solution. [31], [11], [32], [33], [34] Alternatively, other treatments such as biofeedback, electric stimulation, physical therapy or chiropractic care have not been shown to alter the natural history of scoliosis. [35], [36] Despite many studies on its etiology, the cause of AIS remains unclear. Therefore, a better understanding of the etiopathogenesis of the disease will enable us to better diagnose, prognose and facilitate treatment.

(A) (B)

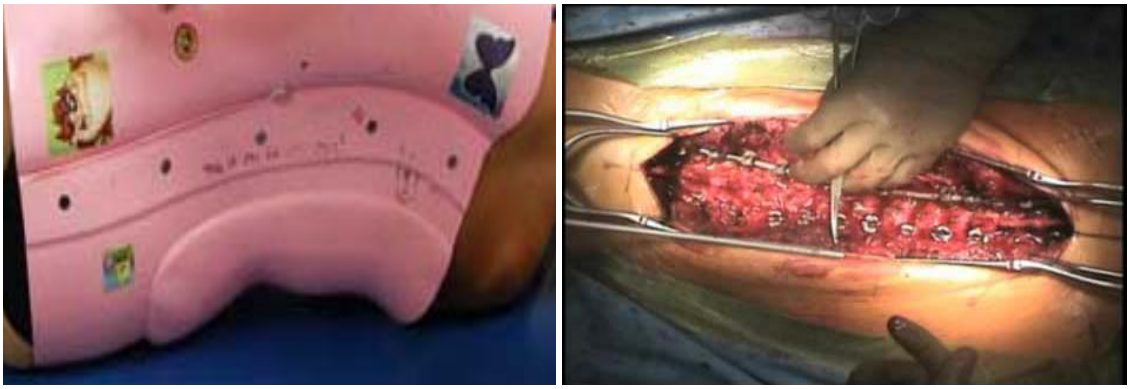

Figure 4: Scoliosis treatment by bracing and surgery.
The right panel (A) shows a brace which treats children with spinal deformities (25° to 35°) while the left panel (B) shows the surgery intervention to treat children with spinal deformities (≥ 45°) (adapted from Moe et al). [37]

Etiology of AIS

Even though widespread research hypotheses have been proposed, and many studies undertaken to examine the roles of the nervous system, environmental factors, spinal growth abnormalities, endocrine system, connective tissue abnormalities, hormonal factors and genetic factors in the etiology of the AIS, none of these studies really explain the cause of IS. [38], [39] [40], [41]

Many studies were undertaken to investigate the effects of the connective tissues on AIS. Researchers studied the collagen and the elastic fiber, the main structural elements of the spinal column. Others showed that proteoglycan and collagen contents are abnormal in the intervertebral disc of AIS patients, [42], [43] which raised controversy. [44] Another study suggested that the abnormalities of these elements are secondary to AIS and not the main cause. [45] Furthermore, elastic fiber abnormalities were observed in the skin and in the spinal ligaments of AIS. [46], [47] As a whole, most researchers postulated that these abnormalities are secondary to the spinal deformity. [48], [49]

Over the past years, the neurological abnormalities associated with AIS were investigated. It was thought that an abnormal sway pattern caused scoliosis but [50] the augmentation of the sway pattern now appears to be secondary to the scoliosis. [51] Machida and coworkers postulated that the development of the scoliosis in pinealectomized (PNX) chicken is due to somatosensory evoked potentials abnormalities. [52] On the other

hand, another group postulated that PNX leads to melatonin reduction, in turn affecting the nervous system maturation. [53]

Many studies were undertaken to assess the role of growth factors in AIS etiopathogenesis. These studies demonstrated that there is no difference between AIS and controls when growth hormone levels were increased. [54] Trontelj et al. demonstrated that the height of the vertebral bodies in girls is greater than in boys. [55] Further investigations are required to study the effects of these growth factors, hormones and their modulators.

Biomechanical factors were also studied in AIS. The mechanical properties of the spinal tissues, the way that the spine is supported and the forces which lead to abnormal loading may also contribute to scoliosis. However, researchers postulated that these factors too, are rather secondary to AIS. [56]

The similarity between the contractile systems of the platelets and the skeletal muscle led some researchers to postulate that the abnormalities of the contractile system could be involved in AIS etiology. The elevation of the platelet intracellular calcium and phosphorous in AIS patients was reported. [57] The elevation of calcium was observed in the dense bodies of platelets and they suggested that the defect of the calcium transport in the cell membrane or contractile complex could lead to spinal deformity. [57] Calmodulin, a well known calcium modulated protein, is a key player, which mediates calcium function and regulation in many processes in the eukaryotic organism. The calcium flux via the sarcoplasmic reticulum is mediated by the interaction of the calmodulin with (actin and

myosin) the contractile system proteins of the platelets and the skeletal muscle. Kindsfater et al. demonstrated that the augmentation of the platelet calmodulin levels in AIS patients is associated with curve severity. The patients with a progressive curve had higher levels of platelet calmodulin than those exhibiting a stable curve. [58] This demonstration was confirmed by Lowe in 2002. [59] Machida et al. also postulated that there is link between the increase of the platelet calmodulin levels in AIS patients and the reduction of the melatonin levels, which lead to the belief that any change of the metabolism or synthesis of melatonin could lead to AIS. [60]

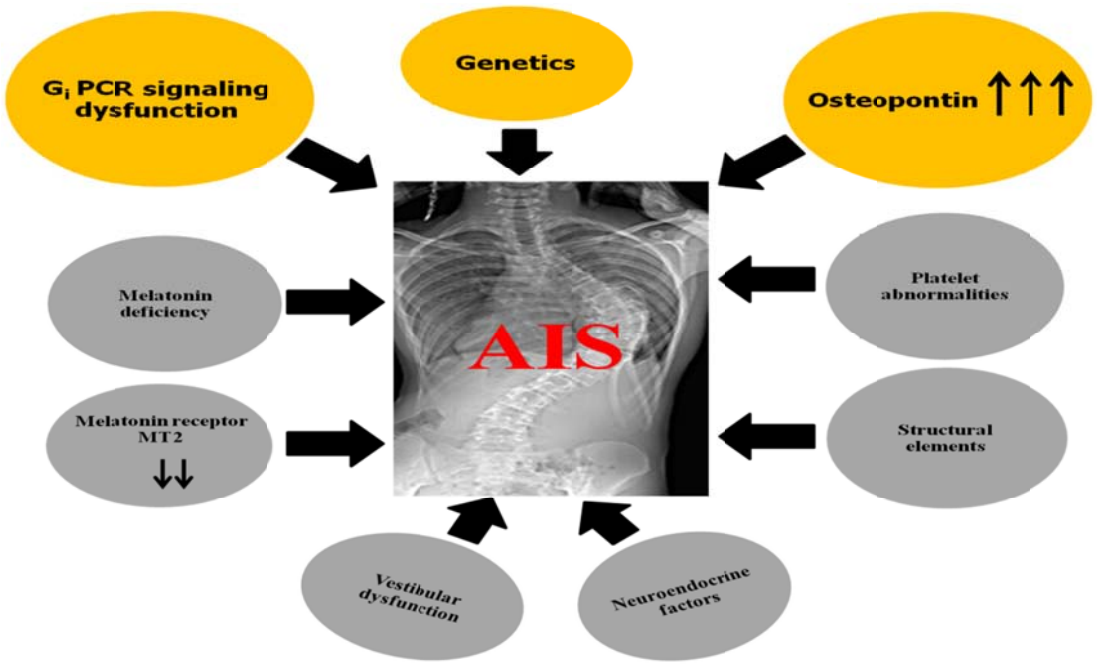

Figure 5: AIS etiology

This figure summarizes the different axes of research focusing on the etiopathogenesis of AIS. The factors in orange represent topics of research investigated in Dr. Moreau's laboratory.

Genetic Factors of AIS

Extensive research is being done on the role of the genetic factors in the development of AIS. Indeed some studies indicate a higher prevalence of AIS among the relatives of AIS patients when compared to the normal unaffected population. [61], [62], [63], [64]

The main conclusion of most genetic studies (table 1) regarding AIS is its multi-factorial nature and possible crosstalk with environmental factors, which remain to be identified. However, for most, if not all of these studies, the number of patients and families needs to be increased and results replicated in distinct cohorts to confirm their validity and clinical utility.

Table 1: Summary of genetic studies for AIS

Year	Authors	Subjects	Key finding
1968	Wynne-Davies	114 IS patients with their family's history	IS has a dominant or a multiple-gene pattern of inheritance. [65]
1975	Robin and Cohen	Five generations of one individual family with adolescent idiopathic	IS has autosomal or multiple-gene inheritance pattern. [66]
2002	Chan et al	Seven Chinese families with 25 affected members	Chromosome 19q13.3 associated with the disease. [67]
2005 & 2008	Miller et al & L Ocaka, et al	202 families with idiopathic scoliosis individuals & 25 multi-generational British families	Chromosomal regions on chromosome 6, 9, 16 and 17 associated with the disease. [68], [69]
2009	Gurnett et al & Raggio et al	13 affected and 10 unaffected family members & 7 multiplex families	Chromosome 18q is associated with IS, [70] another study showed that the disease is associated with Chromosome 12p. [71]
2010	Ward et al and Ogilvie J	277 girls with low risk of AIS 257 girls with higher risk of AIS 163 boys with high risk of AIS All the boys and girls are Caucasian	The genetic markers associated with the severity of the curve. [72], [73]
2012	Gorman et al	50 studies reviewed, both pedigree (linkage) and population based (association)	The association of some genes with the bone formation, metabolism puberty and growth, connective tissue structure and melatonin signaling pathways. [64]

Animal models of AIS

Animal models are important to enable us to understand many human diseases. These models help in the development of suitable treatments. The challenges are to find the suitable animal model representing the disease. In AIS, many animal models were investigated as shown in tables 2 and 3. These animal models were used to investigate many hypotheses like the role of melatonin, genetic, neurological and mechanical factors in AIS etiology. Most of the animal data collected supports the role of a melatonin deficiency as a cause of AIS. However the work of Cheung et al. [74] on monkeys raised controversy, suggesting that melatonin deficiency causes AIS only in lower animals. Gorman et al. demonstrated that guppies could be a relevant animal model for the study of scoliosis. In fact, this model is the only one that naturally develops scoliosis [75] while the curvature in all other scoliosis animal models is induced through surgical interventions. [2], [76]

Table 2: Summary of the surgical animal models for AIS

Melatonin models				
Year	**Authors**	**Animal model**	**Experiment**	**Observation**
1959	Marie-Jeanne Thillard	Chickens	Removal of pineal gland	Pinealectomized chickens developed scoliosis. [77]
1983	Dubousset et al	Chickens	Removal of pineal gland	Pinealectomized chickens developed scoliosis, referring to the deficiency of the melatonin in these chickens. [78]
1993	Machida et al	Chickens	Intramuscular implantation of the pineal body in pinealectomized chickens	No scoliosis was observed in these chickens. [79]
1994	Machida et al	Scoliotic Chickens	1-Treatment of PNX chicken with serotonin 2- Treatment of PNX chicken with melatonin	1- The group treated with serotonin developed scoliosis in 73.3 %. 2- The group treated with melatonin developed scoliosis in 20 %. [53]
1999	Machida et al	Rat	1- Control 2- Removal of pineal gland in quadrupedal rats 3- Removal of pineal gland in bipedal rats implanted with melatonin pellet 4- Removal of pineal gland in bipedal rats	1- 0 % developed scoliosis, 2- 0 % developed scoliosis, 3- 10 % developed scoliosis, 4- 100 % developed scoliosis. They concluded that the bipedal condition in rats is essential for the development of scoliosis like in chickens and humans. [80]

Table 2 cont'd

Year	Authors	Animal model	Experiment	Observation
2001	Bagnall et al	Chickens	Study of the relationship between melatonin levels and scoliosis in the chicken	The decrease in melatonin levels in young chickens led to scoliosis development.[81]
2005	Cheung et al	Monkeys	Removal of pineal gland	None of the pinealectomized (PNX) monkeys developed scoliosis. They concluded that the melatonin deficiency leads to scoliosis in the lower animals only and not in humans. [36]
2006	Machida et al and Oyama et al	C57Bl/6j mice (naturally deficient in melatonin)	Removal of the forelimbs of these mice (bipedal model)	Bipedal C57Bl/6j mice developed scoliosis; is considered a good model for the researchers who are working on scoliosis. [2], [82], [1]
2009	Fagan et al	Chickens	Removal of the pineal gland from one chicken group and used another group as a control without removal of pineal gland	75% of pinealectomized chickens developed scoliosis. Chickens developed scoliosis naturally in 19%. [83]
2011	Kono et al	Chickens	Removal of the pineal gland from two groups of chicken, one treated with melatonin	Deficiency of melatonin is suggested to lead to the development of scoliosis and osteoporosis. [84]

Table 2 cont'd

Neurological models				
Year	**Authors**	**Animal model**	**Experiment**	**Observation**
1989	Suk et al	Rabbits	Removal of the anterior and posterior rhizotomies separately and together in different cohorts	They postulated that anterior root paralysis and posterior root paralysis may induce scoliosis. [85]
2009	Lambert et al	Xenopus laevis (frog)	At the larva stage, removal of the labyrinthine end organ	The young frog after the metamorphosis developed spinal curvature similar to AIS. [86]

Mechanical models				
Year	**Authors**	**Animal model**	**Experiment**	**Observation**
1980	Beguiristain et al	Pigs	Epiphysiodesis in 4 to 5 consecutive vertebrae of neurocentral cartilage using cancellus screws	Pigs developed structural scoliosis. [87]
1991	Poussa et al	Rabbits	Use of an external splint to stimulate lordosis	- Developed lordosis in the thoracolumbar junction - 53 % developed scoliosis - Lordosis is a precondition for development of scoliosis. [88]
1997	Mente et al	Rats	Application of external ring fixators to check the mechanical effect (asymmetrical load) on the spine	-Vertebral wedging is formed - Rats developed scoliosis. [89]
2009	Zhang et al	Goat	Tethering of the goats unilaterally by pedicle screws and contralateral rib resections	The goats developed scoliosis and the curvature increased over time and is similar to the idiopathic deformity. [90]

Table 3: Genetic models for AIS

Genetics models				
Year	**Authors**	**Animal model**	**Experiment**	**Observation**
2001	Blanco et al	BDL mice	Ky knockout mice	Kyphoscoliosis and degenerative myopathy suggesting that this ky protein (muscle/ brain) is important for muscle growth, and myopathy should be checked in IS patients. [91]
2007, 2010 & 2011	Gorman et al	Guppy	Investigation of the spine curvature phenotype through the different generations	- Naturally developed scoliosis - Spinal curvature similar to IS in human - Suitable model to study the genetics associated with spinal curvature in IS patients. [92], [75], [93]

Clinical implication of melatonin deficiency

Despite the fact that most of the animal data pointed to a crucial role of melatonin deficiency in spinal deformity formation, the majority of the clinical results (table 4) clearly demonstrated that melatonin levels were normal in AIS patients.

Table 4: Clinical data representing the role of melatonin in scoliosis

Year	Authors	Methodology	Key finding
1996	Machida et al	Melatonin levels in AIS patients were measured during 24 hours and at night time compared to the controls.	Melatonin levels in the AIS patients were lower than the controls. They concluded that melatonin plays a vital role in the curvature of the spine. [60]
1996	Bagnall et al	Melatonin levels in AIS patients and controls were measured.	No difference among the AIS patients and the controls was observed. [94]
1998	Fagan et al	Melatonin in AIS patients and controls was measured.	No difference among the AIS patients and the controls was observed. [95]
2000	Brodner et al	Melatonin in AIS patients and controls was measured.	The levels of melatonin were similar in AIS patients and controls. [96]
2003	Morcuende et al	Association between the adolescent idiopathic scoliosis and melatonin receptor MT2 studied	No association between the disease and chromosome 4 observed, and no mutation found in the coding region of the gene for the human melatonin receptor. [97]
2004	Moreau et al	cAMP was measured in osteoblast derived from 41 AIS patients and 11 controls after stimulation with forskolin in presence of increasing doses of melatonin	1- AIS patients were classified into three functional groups according to their functional responses to melatonin. 2- Melatonin signaling dysfunction in AIS is caused by an impairment of Gi proteins. [1], [98], [99], [100]

Table 4 cont'd

Year	Authors	Methodology	Key finding
2007	Suh et al	Metabolism of the pineal gland in the AIS patients in comparison with the control subjects studied	The pineal gland metabolism was normal in AIS patients. [101]
2007	Qui et al	Verification of the melatonin receptor in AIS patients	Melatonin receptor MT1 was not associated with AIS occurrence but MT2 was. Expression of the MT2 receptor expressed asymmetrically in the AIS patients. [102], [103], [104]
2007	Azeddine et al	cAMP measured as a secondary messenger in 90 AIS patients and 10 controls	They confirmed what Moreau et al concluded in 2004. [105]
2010	Akoume et al	A new technique called cellular dielectric spectroscopy (CDS) was used to verify the melatonin signaling in the AIS patients	Moreau et al. results confirmed. AIS patients were classified into three different functional groups. Gi signaling defect found. [106]
2013	Yim et al	Expression of melatonin receptors MT1& MT2 verified.	MT2 expression shown to be low in AIS girls. This abnormal expression shown to be associated to an abnormal systemic skeletal growth (abnormal arm span). [107]

Melatonin signaling in AIS

Melatonin is a neuro-hormone synthesized mainly by the pineal gland in the brain. [108], [109] Its secretion is stimulated by the dark and inhibited by the light, and is an important regulator of the circadian rhythm. Melatonin is a highly important antioxidant molecule [110], [111] and its biological effects are through its receptors. In humans and other mammals, melatonin classically binds to two G protein-coupled receptors (GPCR) called melatonin receptor 1 (MT1 or MTNR1A or Mel_{1A}) and melatonin receptor 2 (MT2 or MTNR2B or Mel_{2B}), whereas it binds to Xenopus melatonin receptors in non-mammalian species. Melatonin acts through the activation of G_i protein coupled receptors which inhibits the adenylate cyclase activity and stops the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). [112], [113], [114] Chan et al. showed that in MCF-7 cells melatonin stimulates the Gs and increases the cAMP accumulation, suggesting a coupling between MT1 or MT2 and Gs, activating adenylate cyclase. [115] Melatonin receptor 1 (MT1) also binds to G_q protein, which activates phospholipase C (PLC), which in turn converts the phosphatidylinositol 4, 5-bisphosphate PIP2 into secondary messengers, IP3 and diacylglycerol (DAG) (figure 6). [116], [117]

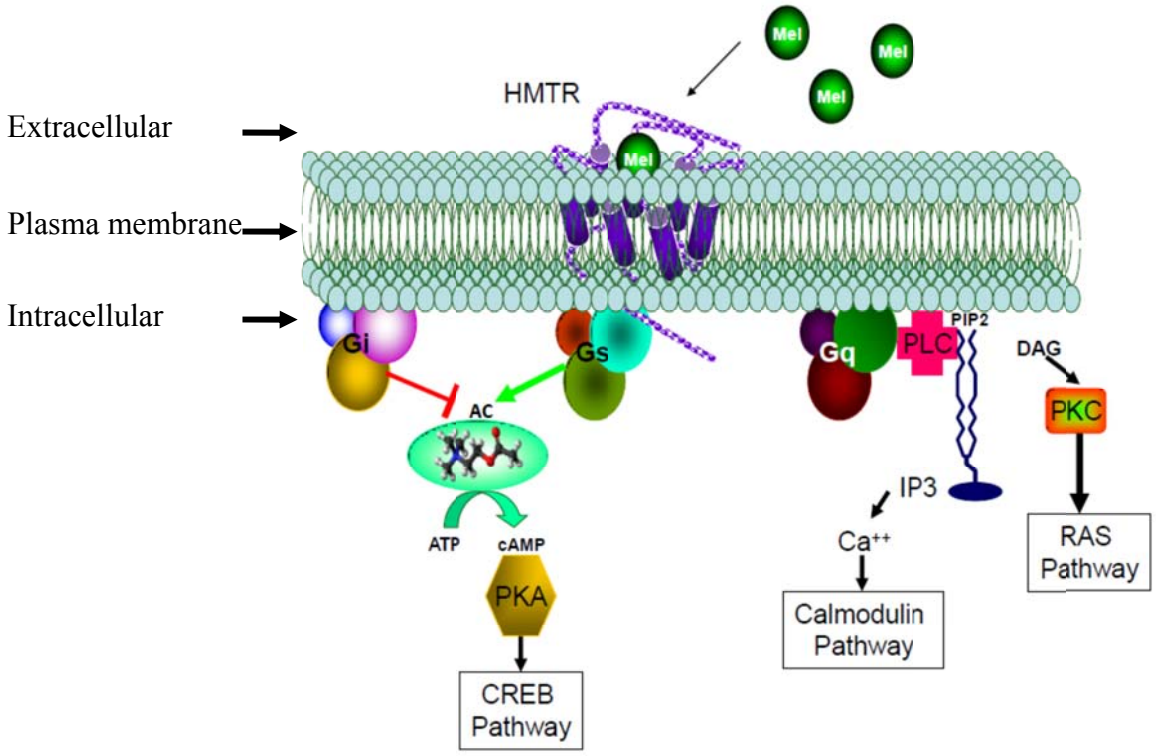

Figure 6: Melatonin signaling through Gi, Gs and Gq proteins (adapted from Slominski et al) [113] HMTR = human melatonin receptor (MT1 & MT2), Mel= melatonin, PLC=phospholipase c, PKC= protein kinase C, PKA= protein kinase A, AC= Adenylate cyclase, DAG= Diacylglycerol, IP3= Inositol trisphosphate & PLC= Phospholipase C

Our team demonstrated that melatonin signaling dysfunction occurs in AIS patients and involves a differential impairment of Gi proteins (figure 7). Additionally, AIS patients responded differently when their osteoblasts were treated with different concentrations of melatonin, the level of cAMP was higher when compared to normal cells. The use of the cellular dielectric spectroscopy (CDS) technique led our team to the same results. [106]

This finding led to the classification of AIS patients into three functional subgroups according to the variability of their cellular response. [16], [106]

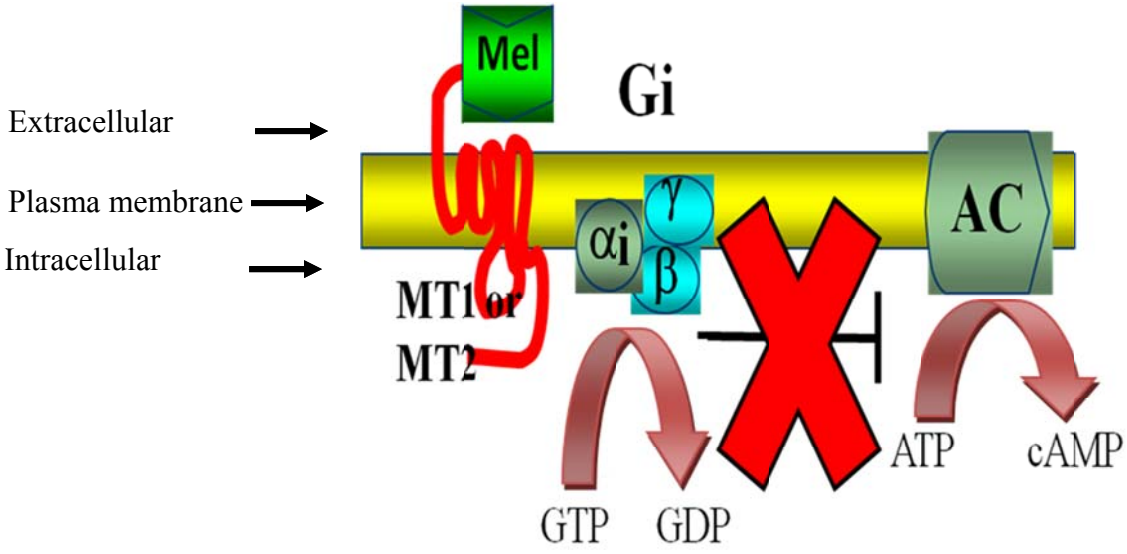

Figure 7: Melatonin signaling dysfunction in AIS
Melatonin binds to either melatonin receptor 1 (MT1) or melatonin receptor 2 (MT2). However in AIS patients $G\alpha_i$ proteins do not inhibit adenylate cyclases or the conversion of ATP to cAMP, culminating in the increase of cAMP in AIS patients.

In osteoblasts derived from AIS patients classified as functional subgroup 1 (FG1), Letellier et al. showed that a preferential coupling of MT2 receptor with Gs alpha protein and in the presence of 17-β-estradiol (10^{-10}M) such coupling was reduced, suggesting an improvement in Gi-signaling. However, osteoblasts from AIS patients classified as FG2 or FG3 subgroups exhibited a more profound Gi signaling defect in the presence of 17-β-estradiol (10^{-10} M). [118]

G proteins

G proteins represent small guanine nucleotide-binding proteins that play an important role in signal transduction of many membrane receptors termed G protein coupled receptor (GPCR). G proteins act as molecular switches, transferring the signal from the outside of the cell to the inside. The signal is transferred to the cell through several effectors such as enzymes, peptides, hormones or channel ions. G proteins are involved in many physiological processes and their malfunction has been found in many pathologies. [119] The G proteins are heterotrimeric proteins which consist of three subunits called alpha (α), beta (β) and gamma (γ). [120] The G proteins are classified according to their α subunit: $G\alpha_{i/o}$, $G\alpha_{q}$, $G\alpha_{12}$ and $G\alpha_{s}$. There are 16 genes which encode α subunits, 5 genes encode β subunits and 12 genes encode γ subunits in humans. Each alpha subunit binds with one beta and one gamma subunit. [121], [122] This classification has gone under review following the discovery in recent years of the roles of the βγ subunits. [123]

The largest subunit is the α subunit (33-55 kDa), which forms a complex with the β and γ subunits when it is linked to a guanosine diphosphate (GDP). This is called the inactive form of G proteins. Once the GDP converts to guanosine triphosphate (GTP), the α subunit dissociates from β and γ subunits and the G protein becomes active. It is in the GDP form that it is recognized by the proper receptor, which is activated by specific effectors and this receptor is called G protein coupled receptor (GPCR). During the interaction of the GPCR and the G protein, GDP is dissociated from the α subunit and is replaced by GTP, resulting in a conformational change and dissociation of the α subunit

from the βγ subunits. It is at this moment that the α and βγ subunits are able to bind to the effector proteins and control their respective functions. The alpha subunits have GTPase activity which regress the G protein to the inactive form. [124], [125], [126], [127], [125]

G proteins play a key role in many biological processes. The impairment of G protein signaling is associated with several diseases. Albright's hereditary osteodystrophy (Pseudohypoparathyroidism type 1) is an example and is associated with low serum calcium, high serum phosphate, high parathyroid hormone (PTH), bone abnormalities and mental retardation, due to the lack of response to the parathyroid hormone. This disease is caused by a mutation in $G\alpha_s$ protein leading to a loss of function. [128], [129] Conversely, the McCune–Albright syndrome, which is characterized by premature puberty as well as bone and skin pigmentation abnormalities, is caused by an activating mutation of the α subunit of Gs proteins. [130] $G\alpha_{i2}$ deficient mice were used as the animal model to study inflammatory bowel disease because they developed similar symptoms, and thus led to the discovery of pharmaceutical treatments. [131] There are two bacterial toxins impeding the G-protein inactivation-activation cycle and these toxins are associated with severe diseases. These toxins play a crucial role in understanding of G-protein signaling. Pertussis toxin (PTX) affects the respiratory system and causes severe coughing spells ending with Whooping cough. PTX catalyzes the ribosylation of adenosine diphosphate (ADP) of Gi proteins not Gs or Gq, and hinders the coupling of the GiPCR to the Gi proteins. Conversely, cholera toxin blocks the GTPase activity of Gs α- subunit through its internal ADP-ribosylation for the arginine receptors and Gs protein becomes constitutively

activated causing severe dehydration and diarrhea. [119], [132], [133] These toxins help researchers to study the specificity of agonists to G protein coupled receptors and their cognate G proteins.

Osteopontin (OPN) and AIS

Osteopontin (OPN) is also known as secreted phosphoprotein 1 (SPP1), bone sialoprotein I (BSP-1 or BNSP) and early T-lymphocyte activation (ETA-1). [134], [135], [136], [137]

The presence of OPN in skeletal muscle, proprioceptive sensory organs, postural control centers such as the cerebellum, and inner ear structures are of particular interest, since AIS patients also exhibit defects in postural control, proprioception and equilibrium. [138], [139], [140]

OPN is an extracellular matrix protein with high negative charges. It is composed of approximately 300 amino acid residues. Among the mammalian species, OPN cDNA exhibits a high degree of homology. OPN contains different binding sites, which includes a polyaspartic acid motif to be able to bind to the hydroxyapatite and the calcium channel. [141], [142] It includes two heparin binding domains and contains an arginine-glycine-aspartic acid (RGD) motif. It also has sites for N – O glycosylation in addition to the serine – threonine phosphorylation sites. These sites generate functional forms of OPN in different tissues. Alternatively, OPN binds to different receptors like integrin beta 1 (β1),

integrin beta 3 (β3), integrin beta 5 (β5) and CD44 isoforms. This diversity of interactions gives OPN different functions in different tissues.

OPN is expressed in different tissues like cartilage, dentin, cementum, skeletal muscle, sensory organ, cerebellum, inner ear, sweat and salivary gland, the pancreatic and bile ducts, the activated lymphocytes and macrophages, the gut and the kidney. [143], [144] OPN is expressed in the bone through osteoblast, osteocyte and osteoclast cells. [145] OPN is expressed by fibroblastic cells in the wound healing sites and the connective tissues. The secreted protein was observed in the biological fluids like blood, breast milk, urine and the seminal fluid. [143], [146], [147], [148], [149] OPN expression is affected by many factors such as cytokines, hormones and growth factors, factors which affect OPN transcription, translation and its posttranslational modifications. The regulation of OPN expression is not completely understood. Vitamin D is known to stimulate OPN expression in bone cells. Conversely, Vitamin D deficient mice have low OPN mRNA expression. Platelet derived growth factor (PDGF), transforming growth factor βs (TGF-βs), inflammatory cytokines, bone morphogenetic proteins (BMPs), angiogensin II and epidermal growth factor (EGF) are factors that up-regulate OPN expression. [144] The tumor necrosis factor α (TNF α) and interleukin-1β (IL-1β) are strong stimulators of OPN expression. On the other hand, OPN is down-regulated by bisphosphonates in the bone and by cGMP dependent protein kinase in the vascular smooth muscle cells. [142], [141]

Many studies have been undertaken to better understand the biological functions of OPN. OPN plays an important role in calcification, development, bone remodeling, immunoprotection and skeletal tissue homeostasis. OPN protects the cells from apoptosis and stimulates the proliferation and the survival of cells, especially smooth muscle and epithelial cells. This was shown by inducing apoptosis, by serum deprivation, in endothelial cells incubated in a plate coated with OPN. [150], [151], [152], [153]

There are studies that show that OPN plays a crucial role in bone remodeling, a process in which osteoclasts remove old bone and osteoblasts form new bone. Both osteoblasts and osteoclasts express OPN and it is found in cement lines (bone formation sites) and at the bone surface. The migration of the osteoclasts is stimulated by OPN through its receptors, CD44 and integrin αvβ3, aside from the stimulation of CD44 expression, which is important for motility. [154], [155]

OPN protects endothelial cells through the activation of nuclear factor kappa B (NF-kB) when it binds with integrin alpha v beta 3 (integrin αvβ3) and this stimulates osteoprotegerin (OPG). OPG blocks apoptosis when it forms a complex with the TNF-related apoptosis-inducing ligand (TRAIL). [156], [157] Non-adherent cells on the other hand, are protected against apoptosis by OPN through the CD44 receptor and the activation of the PI3K-Akt signaling pathway. [158] Taken together, we deduce that OPN can protect different types of the cells from apoptosis through different receptors and different pathways.

OPN is a multifunctional cytokine and is involved in many adult pathological conditions. [144], [141] Levels of OPN in the blood, OPN biosynthesis and OPN signaling are associated with different diseases. (Table 5)

Table 5: OPN is associated with many diseases

OPN abnormalities	**Pathogenesis**
Serum OPN elevation	- Many types of cancer, such as breast cancer [159] - Associated to cancer severity [159]
Plasma OPN elevation	- Coronary artery disease [160] - Systemic lupus erythematosus [161] - Osteoarthritis [162] - Prostate cancer, breast cancer and myeloma [163], [164], [165], [166]
OPN biosynthesis stimulation	- Cardiovascular disease - Renal disease (kidney stone - mineralization disorder) - Oncogenes association like Src and Ras [141]
OPN signaling	In autoimmune diseases:- A- Through integrin beta 3, IL-12 expression is stimulated B- Through CD44, IL-10 expression is attenuated [167], [168]

OPN receptors

OPN exhibits different functions through its interactions with distinct receptors. This can be explained by the fact that OPN binds to its receptors through different binding sites. These receptors are mainly CD44 and integrin receptors.

CD44 is the receptor for hyaluronic acid and also binds to OPN. CD44 is expressed in different types of the cells like osteocytes, fibroblasts, osteoblasts, epithelial and endothelial cells and smooth muscle cells. [142] There are many types of cancer like glioma, prostate, leukemia initiating cells and breast cancer that express the CD44 receptor. This receptor was shown to play a role in the stimulation of the invasion and metastasis in different types of cancer. [169], [170]

Integrins are other known receptors for OPN. They are transmembrane heterodimeric receptors which contain alpha (α) and beta (β) subunits. Integrins play a role in cell adhesion, allowing the cell to attach to the extracellular matrix (ECM), as well as a role in cell signaling. [171], [172]

The integrins have 18 α subunits and 8 β subunits, which associate together, to give no less than 24 heterodimers. [173] These heterodimers play a role in biological processes. One of the most widely expressed integrins is integrin β1 which has 12 complexes and invokes the binding of the cell with the matrix protein. [173] OPN and integrins can protect the cells from programmed cell death (apoptosis) and this was observed in solid cancers, for instance breast cancer. It was observed that integrin β1 inhibits apoptosis of cancer cells

normally induced by vincristine and paclitaxel, used as chemotherapy by stimulating apoptosis, leading to a drug resistance. [174] Other studies found that high expression of integrin β1 is associated with poor prognosis due to drug resistance in small cell lung cancer. [175], [176]

The integrin receptors play important roles in OPN-mediated cell adhesion through receptors like integrin beta 1, integrin beta 3 and integrin beta 5. [177], [178], [179] Some of these integrin receptors bind to OPN through the RGD motif like αvβ1, αvβ3,αvβ5 α5β1, and α8β1 but there are other integrin receptors interacting with OPN through a different amino acid sequence, like α4β1 and α9β1. CD44 binds to OPN in a RGD independent manner. [180], [181], [182], [183], [184], [185], [186], [187]

The activation of the integrins requires an interaction with talin. Talin has the ability to bind to the cytoplasmic tail of the integrins leading to a conformational change of the latter by the separation of the heterodimer subunits of the cytoplasmic tails. This conformational change of the integrins allows their binding to cognate agonists. [188] Talin needs to be phosphorylated to bind and activate the integrins. The phosphorylation of the talin needs serine/ threonine kinases to be phosphorylated, and this process is regulated by protein tyrosine phosphatases (PTPases). [189], [190]

Protein tyrosine phosphatases (PTPases)

Protein tyrosine phosphatases (PTPases) are enzymes removing the phosphate group from phosphorylated tyrosine residues. PTPases play a vital role in the regulation of the signal transduction, which controls many biological processes like differentiation, proliferation and apoptosis. [191] The human genome encodes approximately 112 PTPases. The specific function of these phosphatases is dependent on their substrate and their catalytic domain. [192]

PTPases are classified according to their function, which mainly removes the phosphate from the tyrosine residues. However, other PTPases target the serine and threonine residues, and thus these PTPases have a dual function. PTPases work on the phospholipids as well as phosphoproteins. [193]

The PTPases are further classified according to their localization: Non receptor PTPases, which are intracellular, and Receptor-like PTPases, which are transmembranous and contain PTPase domains, have an extracellular domain, a transmembrane region and catalytic cytoplasmic domain. In some cases, the extracellular domains of the receptor PTPases contain MAM domains, immunoglobulin-like domains and fibronectin type III (FN-III) repeats. While the cytoplasmic region contains two copies of the PTPase domain, only one has enzymatic activity while the other remains inactive. [194], [195]

PTPases and their roles in the pathogenesis

Once the identification and classification based on function and location is defined, it is necessary to discuss the association between PTPases and the pathogenesis (table 6).

Table 6: PTPases and their roles in diseases

PTPase	**Pathogenesis**	**The mechanism**
Cell division cycle 25 (cdc25) family members	Overexpressed in malignant tumor	- This family plays a role in DNA repair and apoptosis. - They allow the replication of DNA of the damaged cells - cdc25 A or B associated with the poor prognosis and not cdc25 C. [196], [197]
Mitogen-activated protein kinase phosphatase 1 (MKP-1)	Overexpressed in solid tumors like prostate cancer, pancreatic cancer, breast cancer and ovarian cancer	- MKP-1 is PTPase with a dual specific activity. - MKP-1 plays a role in the protection of these solid tumors from apoptosis [198], [199], [200], [201], [202] - MKP-1 is associated with the early transformation and poor prognosis. [200], [202]

The knockdown of the MKP-1 expression led to the reduction of the tumorigenicity in mice, the increase of programmed cell death and the decrease of cell growth in the cancer cells. [203], [204]

Src-homology 2 domain–containing PTPase 1 (SHP-1) is an example of a PTPase acting as a negative regulator. SHP-1 is a negative regulator for immune cell activation and cytokine signaling. [205], [206]

Hence, Protein tyrosine phosphatase mu (PTPμ) plays a crucial role in AIS; in the following section I will describe its structure and functions.

Protein tyrosine phosphatase mu (PTPμ): structure, function and role in diseases.

In 1991 Gebbink et al, isolated 5.4 kb of mouse cDNA, which encodes for a protein called receptor protein tyrosine phosphatase mu (RPTPμ), which belong to the receptor-like protein tyrosine phosphatases family. RPTPμ has an extracellular region [contains a meprin-A5 antigen-PTP mu (MAM) domain, an Ig-like domain and four fibronectin type III-like repeats], a single transmembrane region, a long juxtamembrane domain and two tyrosine phosphatase domains, D1 and D2 (D1 is catalytically active, while D2 is not) (figure 8). Then in 1993, Suijkerbuijk RF et al. mapped the human receptor-like protein tyrosine phosphatase gene (PTPRM) to 18p11.2 using fluorescence *in situ* hybridization. [207], [208]

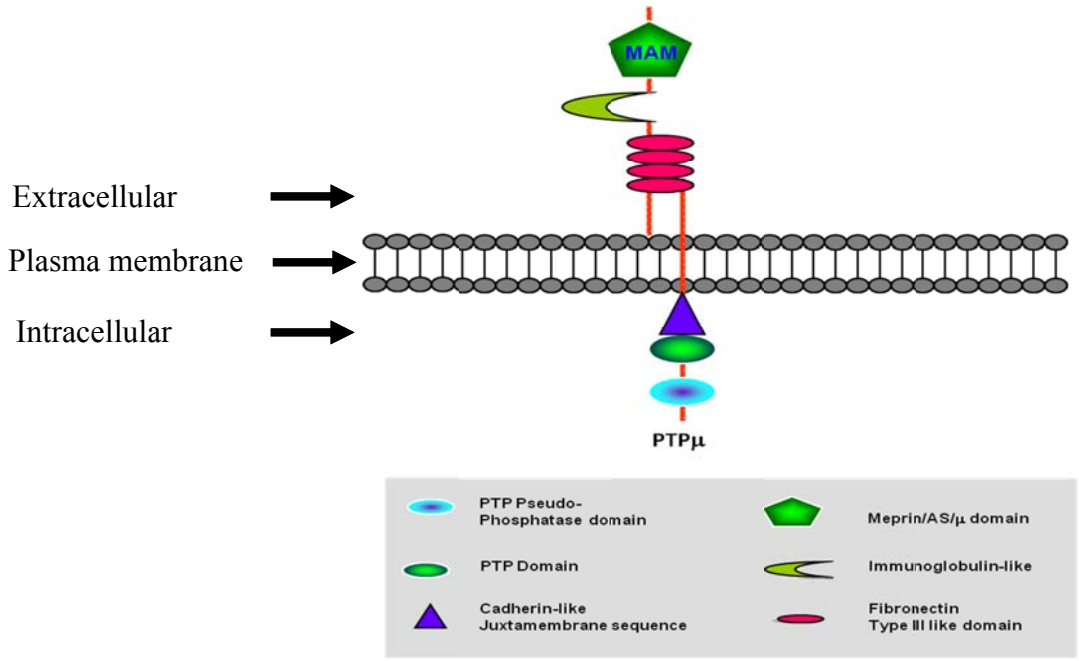

Figure 8: PTPμ structure.
RPTPμ has an extracellular region [contains a meprin-A5 antigen-PTPμ (MAM) domain, an Ig-like domain and four fibronectin type III-like repeats], a single transmembrane region, a long juxtamembrane domain and two tyrosine phosphatase domains (D1 and D2).

PTPμ-cadherins-catenins are proteins associated with cell adhesion. The cadherin proteins are important in the stabilization of cell-cell junctions and called adherens junctions. The stabilization of the cell-cell junctions occurs through the interaction with catenins (alpha, beta and p120) which then binds to actin cytoskeleton. This process inhibits the growth of the actin, thus keeping the cells fixed. PTPμ is a regulator for the cadherins-catenins complex which interacts and/or dephosphorylates E-cadherin, N-cadherin and p120 catenin. PTPμ is thus important in the cell-cell adhesion and cell-cell junctions. [209], [210], [211], [212]

It was demonstrated that PTPμ regulates integrins through the dephosphorylation of the phosphatidylinositol-phosphate kinase type 1 γ (PIPK1γ). The integrin needs a phosphorylated talin to be activated, and PIPK1γ plays this role. PIPK1γ needs to be phosphorylated to be activated and this occurs in order to stimulate the formation of adherens junctions (AJs). Once integrin completes its role, PTPμ dephosphorylates PIPK1γ, inactivating it and indirectly inactivating β integrins. [189] [213], [190] Another group demonstrated that β integrins regulate the expression of PTPμ and cell-cell adhesion. [214]

PTPμ also plays a role in the regulation of the neurite outgrowth. It was shown that the presence of PTPμ as a substrate in the dish led to neurite outgrowth stimulation. Also, the blocking of PTPμ in the developing retina affects the development of the neural retina. The dephosphrylation of the tyrosine residues, which is required for both temporal neurite repulsion and mediation of nasal neurite outgrowth, needs PTPμ. This tyrosine residue dephosphrylation is achieved through the main elements of the cadherin-catenin complex and this regulates the axonal migration. [215], [216], [217], [218], [219] Several related proteins were discovered such as protein kinase C delta (PKCδ), phospholipase C gamma 1(PLCγ1) and others. The first two proteins were found to be important substrates for PTPμ and which participate in the regulation of neurite outgrowth and cell migration. [220], [221] PTPμ downregualtion was observed in the Glioblastoma multiform (GBM) and interestingly, the expression level of PTPμ diminished in breast cancer. It is also noteworthy, that PTPμ is important for cell invasion and migration through the control of ERK and JNK tyrosine phosphorylation. [222], [223]

GENERAL OBJECTIVE

A series of studies from our laboratory initially demonstrated the occurrence of a melatonin signaling dysfunction in osteoblasts from progressive AIS patients, due to a hyperphosphorylation of Gi proteins. The differential degree of this defect among AIS patients allowed their classification into three functional subgroups. In addition, our studies have demonstrated that this defect occurs before the development of the spinal deformity. In parallel, analysis of plasma of AIS patients has revealed high levels of circulating OPN in both non-progressive and progressive AIS patients, as well as in asymptomatic children with a family history of AIS. Our general objective is to examine whether there is a cause-and-effect relationship between these findings in order to gain more insights into the potential pathomechanisms leading to the development and progression of AIS.

Key issues

The pharmacological aspect of melatonin is not simple. Indeed, melatonin has been demonstrated to regulate and influence a variety of biological functions by initiating signal transduction through two high affinity receptors coupled to G proteins from the Gi and Gq families. [224], [225] Our demonstration that Gi protein-mediated melatonin signaling is reduced at different degrees among AIS patients is suggestive of a disparity in melatonin signaling pathways. This signaling disparity does not seem to be related to melatonin receptors, [97], [16] but rather lies at the Gi protein level. Indeed, we have attributed the reduced melatonin signaling to the phosphorylation of Gi proteins by demonstrating an increased phosphorylation of serine residues through the three Gi_1, Gi_2 and Gi_3 isoforms in

osteoblasts from AIS patients. [16] However, a rational link between the hyperphosphorylation of Gi proteins and the different degrees of responses among AIS patient groups has not been established and many questions remain to be answered.

Hypothesis 1

Since Gi proteins interact with a variety of receptors expressed in many cell types throughout the body, we hypothesized that the reduced ability of Gi proteins to transduce extracellular signals from receptors is neither confined to osteoblasts nor specific to melatonin receptors, but constitutes a systemic and generalized defect in AIS.

In the first manuscript the following working hypotheses were investigated:

(1) The activity of melatonin receptor varies among functional groups of AIS patients,

(2) The affinity of melatonin receptor for its ligands varies among functional groups of AIS patients,

(3) The pattern of interaction between melatonin receptor and Gi proteins differs among functional groups of AIS patients,

(4) The defect occurs in cells other than osteoblasts,

(5) The defect is extended to other receptors coupled to Gi proteins,

(6) The phosphorylation pattern of Gi protein isoforms varies among functional groups of AIS patients,

(7) The hyperphosphorylation is due to increased kinase or decreased phosphatase activities,

(8) The involved kinases or phosphatases differ among functional groups of AIS patients,

(9) The defect impacts the signaling of other G proteins such as Gs and Gq proteins,

(10) The defect is a hereditary condition.

Hypothesis 2

Since deficient melatonin signaling through Gi proteins occurs before the spine deformity appears and given that OPN is elevated in plasma from asymptomatic children and patients affected with AIS, we hypothesized that OPN contributes to the onset and development of this disease by interfering with Gi protein-mediated signaling.

The second manuscript focused mainly on the possible link between OPN and Gi-coupled receptor signaling dysfunction occurring in AIS patients. In the second manuscript the following working hypotheses were investigated:

(1) A link exists between this defect and the high circulating OPN levels associated with AIS,

(2) OPN contributes to the development of AIS,

(3) The receptors may or may not be required for the contribution of OPN,

(4) The incidence and severity of AIS are influenced by changes in the interaction of OPN with its receptors.

Hypothesis 3

Since the elevation of OPN was observed in AIS patients and correlates with disease severity and the occurrence of a Gi-coupled receptor signaling impairment. We hypothesized that, other molecules, like PTPμ, contribute to the AIS pathomechanism by modulation of OPN signaling activity.

The third manuscript focused mainly on the identification and characterization of the molecular mechanisms governing the cellular response to OPN, and which cause the Gi signaling defect. In the third manuscript the following working hypotheses were investigated:-

(1) A link exists between high circulating OPN levels associated with AIS and PTPμ,

(2) PTPμ affects the Gi protein signaling defect caused by OPN,

(3) PTPμ regulates the interaction of OPN with its receptors,

(4) PTPμ leads OPN to favour α5β1 integrin and not the others integrins.

The first manuscript: Disrupted Gi-coupled receptor signaling occurs in adolescent idiopathic scoliosis

Previously by using two different experimental methods we demonstrated that a melatonin signaling dysfunction occurs in AIS patients and involves an impairment of Gi proteins, leading to their stratification into three functional groups according to the variability of their cellular response. In this manuscript, we demonstrated the presence of systemic dysfunction of Gi-protein coupled receptor signaling in AIS patients by using different type of cells (osteoblasts, PBMCs and myoblasts) and varying concentrations of different agonists operating through distinct receptors coupled to Gi proteins.

In this first manuscript, I contributed in the experimental design and in the generation of the results. More specifically I did the immunoprecipitation and western blots experiments and I have done siRNA transfections for some functional experiments. I reviewed and revised the manuscript, and approved the final manuscript as submitted.

Dr. Marie-Yvonne Akoume conceptualized and designed the study, performed the CellKey^{TM} manipulations using CDS technique, some of the siRNA transfections carried out the initial analyses, drafted the initial manuscript, and approved the final manuscript as submitted.

Mr. Maxime Veillette performed the CellKey^{TM} manipulation using CDS technique and approved the final manuscript as submitted.

Dr. Cedric Julien and Ms. Anita Franco performed the CellKey^{TM} manipulation using CDS technique reviewed and revised the manuscript, and approved the final manuscript as submitted.

Dr. Hubert Labelle, Dr. Benoit Poitras, Dr. Guy Grimard, Dr. Jean Ouellet, Dr. Stephan Parent, Dr. Giovanni Lombardi, Dr. Alessandra Colombini, Dr. Giuseppe Banfi and Dr. Marco Brayda-Bruno contributed in the diagnosis of the patients, donated us the bone specimens from the AIS patients and the controls subjects and approved the final manuscript as submitted.

Dr. Alain Moreau contributed to the study conception, the analysis and interpretation of the data. He critically revised the manuscript for important intellectual content and approved the final manuscript as submitted.

DISRUPTED Gi-COUPLED RECEPTOR SIGNALING OCCURS IN ADOLESCENT IDIOPATHIC SCOLIOSIS

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Abstract

We have previously stratified adolescent idiopathic scoliosis (AIS) patients into three functional groups (FG1, FG2 and FG3) representing distinct biological endophenotypes and have developed a cell-based assay evaluating melatonin signaling through Gi proteins. Here we report that the differential reduction of Gi protein activity among AIS groups contributes to a systemic and generalized signaling defect perturbating all Gi-coupled receptors due to a selective phosphorylation of Gi alpha subunit isoforms involving distinct kinases. AIS patients can be stratified into these three biological endophenotypes by any agonist that initiate Gi-mediated signaling, and represent an inheritable trait conserved among affected family members. Evaluation of the clinical outcomes of AIS patients according to their biological endophenotypes in Canadian and Italian cohorts revealed that those classified in FG2 endophenotype are more susceptible to developing severe scoliosis, while those in FG1 endophenotype present a much lower risk of disease progression. Early detection 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.

INTRODUCTION

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. Most patients requiring treatment are females (90%) and AIS is extremely heterogeneous in its clinical course **(Kane, 1977); (Weinstein, 1989); (Lonstein, 1994).** Unfortunately, patients today are treated in a substantially manner to those of twenty or thirty years ago – observation (deformities between 10° to 25°), bracing (25° to 35°) and spinal surgery (≥ 45°) as last resort **(Negrini et al., 2010); (Fazal and Edgar, 2006).** Indeed, one of the challenges for orthopaedic surgeons is to identify this last category of patients so that appropriate treatment can be provided early to prevent surgical complications and cardiac and/or respiratory problems. Nowadays, 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 sequellaes.

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. In absence of

reliable AIS phenotypes, we have undertaken a research program to understand 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. It is clear that the same changes are coming for many common chronic diseases and scoliosis is no exception. Previously, we have reported the existence of a differential melatonin signaling dysfunction 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).** These results suggested a differential reduction of melatonin signaling through a hypofunctionality of Gi proteins among AIS patients. More recently, we provided further support to this hypothesis using 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).** We found that the cellular response following melatonin receptor stimulation by melatonin was mainly Gi-dependent in normal osteoblasts and was reduced at different extents in osteoblasts derived from AIS patients **(Akoume et al., 2010).** We have attributed this reduced melatonin signaling to posttranslational modifications of Gi alpha subunits by demonstrating an increased phosphorylation of serine residues of Gi_1, Gi_2 and Gi_3 isoforms in osteoblasts from AIS patients **(Moreau et al., 2004).**

In this study, we provide the first clinical evidence to our knowledge that a differential disruption of Gi alpha subunits occurs in AIS and demonstrate that such

impairment is caused by a serine phosphorylation of distinct Gi isoforms leading to the classification of AIS patients into three biological endophenotypes representing inheritable traits. Heritability was clearly demonstrated with the detection of the same endophenotype in all family members affected by scoliosis. Evaluation of the clinical outcomes of AIS patients according to their biological endophenotypes reveals in two distinct cohorts (Canadians and Italians) that AIS patients classified in FG2 endophenotype are more susceptible to developing severe scoliosis, while those in FG1 endophenotype present a much lower risk of disease progression. In mechanistic *in vitro* studies, we further characterize that hypofunctionality of Gi proteins occurring in AIS led to (a) a systemic differential signaling defect affecting Gi-coupled receptors in different cell types, (b) an enhancement of Gs- coupled receptor signaling while Gq-coupled receptor signal transduction was not compromised, and (c) pharmacological inhibition of selected kinases rescued or improved Gi-signaling impairment at the cellular level and such response varied in function of each biological endophenotype. Our findings provided the first evidence to our knowledge that a differential disruption of Gi-signaling underscores AIS pathogenesis and represent a rational basis for the development of innovative prognostic tools and pharmacological therapies.

Results

Demographic and clinical characteristics of French-Canadian and Italian cohorts. The French-Canadian cohorts consisted of 956 consecutive adolescents with AIS and 240 aged-matched controls without a family history of scoliosis. The absence of spine abnormalities

was confirmed in all control subjects, while a total of 162 AIS patients exhibited curvatures greater than 45° and 794 AIS patients had curvature between 10° and 44°. In the Italian cohort, the moderate curves (Cobb angle 10° - 44°) were apparent in 61 AIS patients and the severe curves (Cobb angle > 45°) in 78 AIS patients. All AIS patients were age-matched with control subjects in both Canadian and Italian cohorts (Table 1).

Clinical outcomes of AIS patients according to their functional classification. Patients were classified according to the response degree of their PBMCs to iodomelatonin stimulation as indicated in material and method section. Of 956 AIS patients from the Canadian cohort, 243 were classified in functional group 1 (FG1), 353 in functional group 2 (FG2) and 310 in functional group 3 (FG3). The prevalence of all three functional groups was comparable among low to moderate cases (Cobb angle 10° - 44°). However, the FG2 was predominant among severe cases (Cobb angle > 45°) with a proportion of 56 % compared to 31 % and 13 % for FG3 and FG1, respectively. Similar profile of distribution was observed in the Italian cohort in whom surgery was required for 61 % of FG2, 36 % of FG3 and 3% of FG1 AIS patients. Collectively, these results strengthen the view that clinical outcomes vary among AIS patients and suggest that the risk of severe progression is higher for FG2, moderate for FG3 and low for FG1 (table 2 and table 3).

Each functional group represents a potential hereditary trait. Since the hereditary or genetic basis of AIS has consistently been claimed **(Riseborough and Wynne-Davies, 1973); (Blank et al., 1999); (Roach, 1999)**, we tested the possibility that the biological

defect characterizing each functional group may be a hereditary condition. For this purpose, 25 individuals from 6 unrelated families were examined. Pedigrees are shown in **supplementary figure S1.** At least two individuals were affected in each family. The classification has revealed that all affected family members belonged to the same functional group and so displayed similar biological defect (Table 4). However, neither pattern nor severity of curve was group specific (Table 4). This suggests that each functional group represents a biological endophenotype that co-segregates within families independently of curve type and magnitude of spinal deformity.

Deficit in cellular response to melatonin in AIS is associated with a generalized impairment of Gi protein-mediated receptor signaling. Melatonin is well known to regulate a variety of biological functions by initiating signal transduction through high affinity receptors coupled to Gi proteins **(Hardeland, 2009)**. We hypothesized that the melatonin signaling dysfunction in AIS was possibly due to changes affecting the melatonin receptor/Gi protein coupling. Since the agonist binding to the receptor increases the efficiency of receptor/G protein coupling **(Mukai et al., 1992)**, we first examined whether the affinity of melatonin for its receptors varies among AIS patients groups. For this purpose, concentration-response curves were generated for melatonin using osteoblasts from control subjects and compared with those of AIS patients of each biological endophenotype. As illustrated in Figure 1A, melatonin caused a concentration-dependent increase of response in all control and AIS groups reaching a plateau at 1μM, with the higher magnitude in control group and lower but at varying degree within the three AIS

groups. The order of magnitude of the maximum response from highest to lowest responses was control group, FG3, FG2 and FG1. Despite this apparent discrepancy in the maximum response between groups, the half-maximum response was observed at similar concentrations in control and AIS groups. These observations suggest that the affinity of melatonin receptor for its agonist is preserved in AIS.

Then, we examined the possibility of changes in the activity of melatonin receptor by testing three agonists that have different efficacy to activate melatonin receptor. We compared the response produced with their maximum concentration (10 uM) in osteoblasts from control subjects and AIS patients of each functional group. Results illustrated in Figure 1B show that the three agonists, melatonin, idomelatonin and phenylmelatonin evoked various degree of response in osteoblats from control and AIS subjects. The response to each agonist was lower in all AIS groups compared to the control group. However, the magnitude and the reduction due to AIS in each group reflected the efficacy of agonists, the order of agonist potency being phenylmelatonin > iodomelatonin > melatonin, as previously reported by other investigators using different methods **(Nonno et al., 1999)**. This suggests that the activity of melatonin receptor is comparable between control subjects and AIS patients and underscore that deficit is beyond the melatonin receptor.

To examine the possibility that the deficit in melatonin signaling could be related to a decreased ability of agonist/receptor complex to activate Gi proteins, we used the G protein antagonist GPAnt-2. This hydrophobic peptide has been shown to inhibit receptor/Gi protein coupling by competing with the activated receptor for interaction with

G proteins **(Mukai et al., 1992)**. Results illustrated in Fig 1C show that GPAnt-2 inhibited the response to melatonin stimulation in a concentration-dependent manner in control and AIS functional groups. At maximal concentration, the extent of reduction is similar in all groups, but at submaximal concentrations, the pattern is clearly different. Concentration-response curves of all three AIS functional groups exhibited a left-shift compared to that of the control group, and the IC50 values were significantly decreased among AIS groups when compared to controls. Taking into a count that GPAnt-2 competes with receptors on various G proteins, we further selectively decreased the amount of Gi proteins by incubating osteoblasts with increasing concentration of pertussis toxin (PTX). We found that PTX treatment reduced the cellular response to melatonin in FG2 and FG3 osteoblasts exhibiting a pattern similar to the one obtained with GPAnt-2. In contrast, PTX treatment enhanced the response to melatonin in FG1 osteoblasts at maximal concentration (Fig. 1D). These data show that the reduced melatonin signaling in AIS is most likely due to a decrease in the sensibility of Gi proteins for melatonin receptors, and indicate that the melatonin receptors may also interact with Gs in AIS patients classified in FG1. This later point is in agreement with a previous report showing that melatonin receptor MT2 physically interacts with Gs alpha subunit in AIS patients classified in FG1 endophenotype **(Letellier et al., 2008)**.

To determine if the signaling dysfunction through Gi proteins as measured in AIS osteoblasts was restricted to melatonin receptors, we performed a comparative study with various synthetic compounds activating selectively other receptors coupled to Gi proteins. Five compounds were used including, apelin-17, BP554 maleate, lysophosphatidic acid

(LPA), UK14304 and somatostatin to activate endogenous APJ, serotonin 5-HT_{1A}, LPA_1, alpha2-adrenergic and somatostatin (sst) receptors, respectively. As illustrated in Fig. 2, all tested agonists caused a concentration-dependent increase of the cellular response, reaching a plateau at the same concentration in all control and AIS functional groups. In each case, the magnitude of the signaling response was lower in all AIS groups when compared with the control group, but the EC50 values were almost identical in all groups indicating that the affinity of all tested agonists for their respective receptors is not affected in AIS (Fig. 2G). Interestingly, inhibition curves of GPAnt-2 (Figure 3) or PTX (Figure 4) generated with any of the five synthetic agonists tested revealed curve patterns similar to those obtained with melatonin. In each case, GPAnt-2 reduced the IC50 values in AIS groups when compared to control group (Fig. 3G). Similar responses were also observed when GPAnt-2 was competed with mastoparan-7 (Fig. 3F), which directly activates Gi proteins by mimicking agonist-activated receptor **(Higashijima et al., 1990)** Moreover, response to mastoparan was almost abolished in all AIS and control groups following treatment with high concentrations of PTX, further pointing the abnormality at the level of Gi proteins. Collectively, these data strengthen the concept that agonist/receptor interaction is not affected in AIS, and reveal that AIS patients can be functionally stratified with any compounds that activate Gi protein-mediated signaling pathways. On the other hand, by extending our analysis to other cell types such as skeletal myoblasts and peripheral blood mononuclear cells (PBMCs) from the same set of controls and AIS patients, we found a pattern of response similar to that obtained in osteoblats for each agonist tested (**Online**

Supplementary Figures S2 and S3). Overall, these findings are strongly indicative of a systemic and generalized impairment of Gi protein-mediated receptor signaling.

Reduction in Gi protein function selectively influences Gs protein function in AIS. Several studies using PTX treatment have revealed that abolition of Gi protein function enhances the function of Gs protein, presumably by removing the inhibitory input from Gi proteins (**Itoh et al., 1984**), **(Anand-Srivastava et al., 1987), (Anand-Srivastava, 1989), (Wesslau and Smith, 1992)**. Accordingly, if Gi protein function is reduced in AIS, enhanced Gs protein function would be expected. This possibility was evaluated by screening osteoblasts from control and AIS patients for their response to isoproterenol and desmopressin, which activate beta-adrenergic and vasopressin (V2) receptors, respectively. Both receptors mediate signal transduction through Gs proteins. Results illustrated in (Fig. 5A-B) show that cellular responses initiated by both agonists were significantly enhanced in osteoblasts from AIS patients when compared with control osteoblasts. For each functional group, increased response to Gs stimulation inversely mirrored the reduced response induced after Gi protein stimulation, suggesting a functional imbalance between Gi and Gs proteins. To further illustrate this divergence, values of the responses to melatonin and isoproterenol were reported as differences (Δ) between response to Gi and Gs protein stimulation. As illustrated in Figure 5C, response to Gi stimulation predominated in a concentration-dependent manner in control group. Similar pattern was observed in FG3 group, while no apparent imbalance was observed in FG2 group. In contrast, FG1 group exhibited predominance for response to Gs stimulation. These data indicate that Gs protein is

functionally affected in AIS according to the aberration degree of Gi protein function, revealing a profile of imbalance between Gi and Gs protein function specific to each AIS group.

To extend our studies on the functional status of Gq protein, osteoblasts were stimulated with bradykinin and endothelin-1. Both agonists elicited similar responses at various concentrations in osteoblasts derived from control subjects and AIS patients, demonstrating that receptor signaling through Gq protein remains largely intact in AIS (Fig. 5D-E). It appears that reduction in Gi protein function in AIS exclusively influences Gs protein function.

Divergences in the reduction degree of Gi protein function among AIS groups are not due to changes in expression of Gi protein isoforms. The three isoforms of Gi proteins, termed Gi_1, Gi_2 and Gi_3 share the same proprieties and most membranous receptors that interact with Gi proteins are able to initiate signals via each of these isoforms. However, the amplitude of the response depends on the capacity of Gi isoforms to mediate the signal transduction while some Gi isoforms seem more efficient than other. We hypothesized that the differences in the degree of response characterizing the three AIS biological endophenotypes, is due to the differential alteration of Gi proteins isoforms. To address this hypothesis, we first sought to examine whether the functional changes observed are due to changes in the expression of G proteins. The qPCR analysis revealed no significant change in expression of any isoform of Gi proteins (Gi_1, Gi_2 and Gi_3) and Gs protein between control and AIS osteoblasts (Fig. 6A). Assuming that PCR-amplification of the different

isoforms was equally efficient, it appears that Gi_1 and Gi_2 were the most abundant isoforms of Gi proteins in control and AIS osteoblasts, while the expression level of Gi_3 isoform was less and quite similar to that of Gs protein. At the protein level, these isoforms have revealed no difference between control and any AIS group (Fig. 6B). These results indicate that the functional changes observed in osteoblasts from AIS patients were unlikely due to aberration in the expression levels of Gi protein isoforms.

Differential phosphorylation patterns affect Gi protein isoforms in AIS. Activity of Gi proteins is acutely regulated by phosphorylation, a process that limits their ability to transduce signals **(Casey et al., 1995); (Katada et al., 1985); (Kozasa and Gilman, 1996); (Lounsbury et al., 1991); (Lounsbury et al., 1993); (Morishita et al. 1,995); (Morris et al., 1995); (Yatomi et al., 1992)**. We previously reported an increased phosphorylation of serine residues of the three Gi_1, Gi_2 and Gi_3 isoforms of Gi proteins in osteoblasts from AIS patients **(Moreau et al., 2004)**. To examine whether the functional disruption in Gi signaling occurring in AIS can be related to the differential pattern of Gi phosphorylated isoforms, we immunoprecipitated Gi isoforms from control subjects and AIS patients from each biological endophenotype and probed them with anti-phospho-serine antibody (Fig. 7). Compared with control group, only Gi_1 and Gi_3 isoforms were phosphorylated in FG3 group, while Gi_1 and Gi_2 only were phosphorylated in FG2 group. However, the three Gi_1, Gi_2 and Gi_3 isoforms were phosphorylated in FG1 group. These data provide evidence for a relationship between the phosphoprylation pattern of Gi

isofoms and the heterogeneous defect of Gi proteins in AIS and are undoubtedly indicative of a difference in the functional status of Gi protein isoforms among AIS groups

We confirmed the identity of Gi isoforms responsible for the residual response in each AIS group using small interference RNA (siRNA) approach to knockdown individually or in combination the expression of Gi_1, Gi_2 Gi_3 and Gs prior to stimulate osteoblasts with apelin, LPA or somatostatin. Silencing of each gene reduced by 75-85 % the expression of the corresponding mRNA in osteoblasts from control and the three AIS groups (Fig. 8E-H). In control group, response to any tested agonist was not significantly affected by Gi_1, Gi_2 Gi_3 siRNA when transfected alone, but was almost abolished when transfected together (Fig. 8A). Response to each tested agonist was reduced by at least 75% by silencing Gi_3 alone in FG2 osteoblasts, and by 90% by silencing Gi_1 alone in FG3 osteoblasts confirming that the residual response to Gi stimulation is mediated by Gi_3 and Gi_1 isoforms in FG2 and FG3 groups, respectively. In FG1 osteoblasts, response to each agonist was reduced by 50% following the depletion of all Gi isoform by siRNA, and was not affected by the knockdown of individual Gi isoform. In contrast, the depletion of Gs alone reduced the cellular response to any tested agonist by 50% in this AIS group, and was devoid of any effect in control, FG2 and FG3 AIS group. This agrees with the idea that Gi-coupled receptors switch from Gi to Gs in FG1 group (**Letellier et al., 2008**), and indicates that the residual response to Gi stimulation in this functional group is an additive effect of Gi-receptors coupling simultaneously to Gs and Gi proteins.

Identification of serine/threonine k*inases contributing to Gi proteins hypofunctionality in AIS.* In order to identify putative serine/threonine kinases phosphorylating Gi isoforms in AIS, we treated control and AIS osteoblasts with a panel of serine/threonine kinase inhibitors prior to their stimulation with two distinct agonists (LPA and somatostatin). Of note, Gi stimulation induced via LPA or somatostatin receptor activation by their agonists in control osteoblasts was not affected by any kinase inhibitors (Fig. 9). In contrast, signaling response was significantly increased in FG1 osteoblasts by Gö8963, which inhibits several isoforms of PKC and also by STO-609 acetate, which inhibits three isoforms of calmodulin kinase (CaMK1, CaMK2, CaMK4), but was unaffected by KN93 that inhibits selectively CaMK2. However, both CaMK inhibitors STO-609 acetate and KN93 increased the signalling response in osteoblasts from FG2 group, while Gö8963 was devoid of significant effect in this AIS group. Cellular response in FG3 osteoblasts was not improved by inhibition of neither of PKC nor CaMK. In contrast, the inhibition of Casein Kinase 2 (CK2) with D4476 increased response in osteoblasts from FG1 and FG3 groups, but not in FG2 osteoblasts, while the inhibition of PKA with H89 increased the signalling response in all AIS groups. Expression analysis had revealed a selective increase in the expression levels of PKCε, PKCη and CaMK1-δ in FG1 osteoblasts when compared to control group (Fig. 10). Neither FG2 nor FG3 osteoblasts have exhibited high expression levels of any examined kinase. In contrast, the expression level of CaMK2N1, a natural CaMK2 inhibitor, was significantly decreased in FG2 osteoblasts, suggesting that the regulatory system of CaMK2 activity is affected in this AIS group. Collectively, these

results show that selective functional alteration of Gi protein isoforms in AIS involves distinct kinases.

DISCUSSION

Gi protein-mediated receptor signaling is impaired in AIS. Heterotrimeric G proteins play a pivotal role in signal transduction in a variety of cell systems by coupling many GPCRs types and subtypes to intracellular effectors. The increasing interest in clinical perspectives of altered G protein function has yielded important findings about the involvement of G proteins in processes leading to certain human diseases. In each case, abnormalities in G protein function have been associated with a signaling dysfunction. We have initially demonstrated, using two different experimental methods, the occurrence of a differential melatonin signaling dysfunction in AIS patients, leading to their stratification into three functional groups **(Moreau et al., 2004); (Azeddine et al., 2007); (Moreau et al., 2009); (Akoume et al., 2010)**. In this study, we observed that osteoblasts, myoblasts and PBMCs from the same AIS patients displayed similar cellular responses in presence of varying concentrations of different agonists operating through distinct receptors coupled to Gi proteins. This demonstrates that the abnormality affecting Gi protein function in AIS is not specifically or exclusively related to melatonin receptors, and strongly argues for a generalized and systemic disturbance of Gi-mediated receptor signaling. This is further supported by the fact that EC50 values measured for each agonist remained comparable between control and AIS functional groups in different cell types, which strongly imply that agonist-receptor interactions are not impaired in AIS. Conversely, treatment with

GPAnt-2, an antagonist that competes with GPCRs for interaction with G proteins, showed also a differential response toward each agonist tested as evidenced by decreased IC50 values in osteoblasts derived from AIS patients when compared to control ones. This reflects indirectly the level of residual Gi activity in each AIS functional group, FG1 being the most affected followed by FG2 and to a lesser extent FG3 group. Consistently, the result with mastoparan-7, which directly stimulates Gi proteins, further substantiates the hypothesis that the defect lies at the level of the Gi proteins because control and AIS functional groups were clearly distinguishable by the extent of their maximal response to mastoparan-7 stimulation with patterns similar to those observed with melatonin.

Functional status of Gi and Gs proteins are imbalanced in AIS. Classical paradigm has emerged from studies with PTX indicating that reduction in Gi protein function leads to increased Gs protein activity. Results presented here are in agreement with such paradigm and reveals a parallel relationship between reduced Gi and increased Gs protein functions in AIS it was interesting to note that profile of the functional imbalance between Gi and Gs protein was specific to each AIS groups, indicating that AIS patients can be clearly classified with respect to the profile of imbalance between response to Gi and Gs protein stimulation. Such approach advantageously eliminates the necessity to use control subjects and allows to monitoring patient responses over time.

Differential alteration of Gi protein isoforms among AIS groups. Several lines of evidence support that events leading to the impaired function of G proteins are dependent on disease

and can involve altered G protein expression **(Gawler et al., 1987); (Bushfield et al., 1990)**. However, no significant changes were detected in Gi and Gs isoforms expression at the mRNA and protein levels in AIS. Subsequently, we focused our attention on serine phosphorylation of Gαi proteins since it inhibits Gαi activity **(Houslay and Milligan, 1997); (Moreau et al., 2004)**. It was interesting to observe distinct patterns of phosphorylation of Gi isoforms among AIS groups. Indeed, all Gi isoforms were phosphorylated on serine residues in FG1 osteoblasts, whereas only Gi_1 and Gi_2 in FG2 group, and Gi_1 and Gi_3 in FG3 group were phosphorylated. The detection of unphosphorylated Gi_3 and Gi_2 isoforms respectively in FG2 and FG3 groups could explain their higher Gi signaling activity when compared to FG1 group. Nevertheless, FG2 osteoblasts exhibit a much weaker residual Gi signaling activity when compared to FG3 osteoblasts, which could be explained by the fact that Gi_3 isoform is less abundant in human osteoblasts as demonstrated in the present study. The selective depletion of Gi_3 and Gi_1 isoforms in FG2 and FG3 osteoblasts respectively almost abolished their cellular responses to three distinct Gi-coupled receptors in a similar manner, further confirming the functional status of Gi_3 and Gi_1 isoforms in these two AIS groups. It is tempting to speculate that the loss of function of other Gi isoforms may be compensated by Gi_3 isoform in FG2 and by Gi_1 in FG3 group, which is consistent with the concept that each Gi isoform can partially rescue Gi signaling (**Hurst et al., 2008**). However, our previous data obtained with cAMP assays showed that FG2 osteoblasts were unable to inhibit cAMP production induced by forskolin stimulation **(Moreau et al., 2004); (Azeddine et al., 2007)**. Accordingly, the identification of Gi_3 as the remaining functional Gi isoform in this AIS

group is agreement with several reports indicating that Gi_3 does not contribute to the inhibition of cAMP production **(Chen and Manning, 2001); (McClue et al., 1992).**

It is of interest that depletion of all Gi isoforms by siRNA in control, FG2 and FG3 osteoblasts translated in a 90% reduction in GPCR signaling activity elicited by three distinct agonists, while knockdown of Gs protein had no effect. More interestingly, depletion of the three Gi isoforms in FG1 osteoblasts reduced only by 50% the signaling activity of these distinct GPCRs. These findings, together with results from our experiments with high PTX concentrations, suggest the presence of a compensatory mechanism involving other G proteins in FG1 osteoblasts. Conceptually, it is possible that phosphorylation of all Gi isoforms allows or facilitates the coupling of Gs proteins to Gi-coupled receptors in FG1 osteoblasts. Such possibility was clearly demonstrated by the simultaneous deletion of Gs and all Gi isoforms, which almost abrogated completely the signaling activity in FG1 osteoblasts. On the other hand, the three GPCRs tested did not activated Gs mediated signaling in FG2 and FG3 osteoblasts, which strongly suggest that Gs protein access to these receptors is limited by the presence of Gi_3 and Gi_2 isoforms, which are not phosphorylated in these groups. Of note, depletion of Gs proteins alone did not abolish the signaling activity in FG1 osteoblasts despite the fact that all three Gi isoforms were phosphorylated suggesting a possible heterogeneity in the number and position of serine residues phosphorylated among AIS functional groups. It is conceivable that phosphorylated Gi proteins in FG1 osteoblasts still exhibit some residual activity as opposed to phosphorylated Gi isoforms in FG2 and FG3 groups.

*Contributions of PKC and CaMK in Gi-coupled GPCR signaling disruption in AIS. B*y the application of specific kinase inhibitors, PKC, PKA and CaMK were shown to be involved in the differential Gi agonists induced responses in AIS functional groups. It has been reported that PKC can phosphorylate Gi_1 and Gi_2, but not Gi_3 in vitro leading to their inactivation activity **(Murthy et al., 2000)**. Pharmacological inhibition of PKC with Gö6983 caused an increased signaling response to LPA and somatostatin agonists in FG1 osteoblasts, but not in FG2 and FG3 osteoblasts. It is possible that phosphorylation of Gi_1 and Gi_2 isoforms in FG2 and FG3 might be due to other serine/threonine kinases, such as PKA. Indeed, it has been shown that PKA phosphorylates Gi_2 **(Bushfield et al., 1991)**. Our observation of significant increase in response to Gi stimulation in all AIS groups following inhibition of PKA with H89 indicates that the Gi defect in AIS involves, at least in part, the action of PKA. A possible role for Calmodulin kinase (CaMK) as a contributor to Gi defect cannot be ruled out since the pre-treatment of AIS osteoblasts with STO-609 acetate to inhibit three isoforms of Calmodulin kinase (CaMK1, CaMK2 and CaMK4), increases the response in FG1 and FG2 osteoblasts significantly. Interestingly, the fact that the selective inhibition of CaMK2 enhanced response in FG2, but not in FG1, suggests that this CaMK isoform as a specific player for FG2. Of particular interest was the different level of expression of PKC and CaMK isoforms among AIS groups. We noted that PKC-ε, PKC-η and CaMK1-δ isoforms were significantly up-regulated in FG1, but unchanged in FG2 and FG3. All other PKC and CaMK isoforms were not significantly altered in any of the AIS groups (Online Supplementary Figures S5 and S6). The increase in PKC-ε, PKC-η and CaMK1-δ expression in FG1 would be expected to enhance their activity, and

consequently their inhibitory effect, thus justifying the increased response observed in this AIS group following their inhibition. In contrast, the normal levels of CaMK2 was not consistent with the hyperresponsiveness obtained in FG2 following the inhibition of this kinase with KN93. However, its inhibitor, CaMK2N1, was significantly downregulated in this AIS functional group, suggesting that a possible dysregulation of CaMK2 activity in FG2. Collectively, these observations suggest a selective involvement of PKC-ε, PKC-η and CaMK1-δ for FG1 and CaMK2 for FG2, in the phosphorylation of serine residue of the concerned Gi protein isoforms. No evidence was obtained to suggest the involvement of these kinases in the phosphorylation of Gi proteins in FG3. In contrast, despite the presence of normal levels of casein kinase (CK) isoforms, we postulate that this serine/threonine kinase may be responsible for the phosphorylation of serine residue of Gi_3 isoform. The increased response obtained in FG1 and FG3 following the inhibition of CK1 with D4476, but not in FG2 in which Gi_3 is not phosphorylated, might offer support to this assumption. Nevertheless, further work is needed before this aspect can be clarified.

Potential usefulness of Gi protein defect in predicting prognosis and treatment of AIS. The progression of AIS is often unpredictable and manifestations of this disease become life threatening when curve exceed 70 degrees, making surgery inevitable. Indeed, there is consensus about surgery treatment in a minority of patients with spinal curvature greater than 45 degrees. Gratifying results associated with this treatment when performed at early ages continually stimulate interests in the development of prognostic approaches to predict progressive disorders requiring surgery. Previously, we have validated classification of

individuals on the basis of Gi protein defect as prognostic test to identify sooner the asymptomatic children at risk of developing AIS. In the current study, we report that patients classified in FG2 are more susceptible of significant AIS progression as demonstrated by the higher prevalence of this endophenotype group among severe AIS cases. The similarity of this distribution pattern between Canadian and Italian cohorts strongly suggests that FG2 can serve as reference endophenotype to predict the type of patients at risk to require surgery in both Canadian and Italian populations. However, the application of this reference in other population needs to be validated.

The current concept considers AIS as a multigene dominant condition with a strong pattern of inheritance **(Riseborough and Wynne-Davies, 1973); (Blank et al., 1999); (Roach, 1999).** Results presented here support such concept as evidenced by the classification of all affected family members in the same endophenotype group. Interestingly, the genealogical information for one of six families enrolled in the study allowed us to detect the feature as far as the proband's grandmother. Based on these finding, it can be envisaged that, besides the affected individuals, other at risk family members could also be classified before the development of spinal deformity and then, if required, surgery treatment could be provided in timely manner. Indeed, physicians commonly prescribe bracing to all patients at risk of progression. However, some patients still receive failure outcome and have often to be confronted with surgery when spine becomes more rigid or more deformed and consequently results are less fruitful. It could be very beneficial if evaluation of Gi protein defect could also predict the outcome of brace treatment. In this point, due to the small number of patients wearing brace among severe cases enrolled in the present study, we do

not have solid data to demonstrate that FG2 can also represent patients likely to be resistant to brace treatment. To address this issue, we have undertaken a study of large cohort of patients, followed in longitudinal, with the intention of determining if certain endophenotype groups will better respond to brace treatment and also if certain types of braces will provide more fruitful results to specific endophenotype groups. Nevertheless, the present study provides convincing evidences that evaluation of Gi protein defect can help clinicians in the decision regarding the surgery treatment. Overall, results confirm our functional classification test and demonstrate the potential usefulness of this classification in predicting clinical outcome of AIS patients. Furthermore, these data offer a molecular platform for developing the pharmacological options for the personalized treatments.

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METHODS

French-Canadian patients (Montreal's cohort)

This study was approved by the institutional review boards of The Sainte-Justine University Hospital, The Montreal Children's Hospital, and The Shriners Hospital for Children. Three populations including children with AIS, families of children with AIS and control subjects were enrolled in the study. Healthy children recruited in Montreal's elementary schools and Trauma cases were used as controls. The recruitment was approved by the Montreal English school Board, The Affluent School Board and all institutional review Board mentioned above. Parents or legal guardians of all participants with or without AIS gave their informed written consent, and minors gave their assent. All participants were examined by one of the seven orthopedic surgeons (H.L., B.P., C-H.R., G.G., J.O., M.B-B., S.P.) participating in this study.

Italian patients (Milano's cohort)

A total of 139 consecutive AIS patients and 103 controls subjects were enrolled at the IRCCS Istituto Ortopedico Galeazzi (Milano, Italy) and represent a replication cohort. This replication study was approved by the institutional review board of IRSCC and The Sainte-Justine University Hospital. All patients were examined by one orthopedic surgeon (M.B-B).

Cell preparation and culture

Osteoblasts were isolated from bone specimens obtained intraoperatively from vertebras (varying from T3 to L4 according to the surgical procedure performed) and from other anatomical sites (tibia or femur) in AIS patients and trauma control cases, respectively as previously described **(Moreau et al., 2004)**.

Myoblasts were isolated from biopsy specimen of skeletal muscle obtained from AIS and trauma control patients. Each biopsy specimen was cleared of remains fatty and connective tissue prior to be cut into smaller pieces. The tissue pieces were then transferred into PBS solution containing 0.01% collagenase, and digested for 45 min at 37 ºC. After dilution with culture media (1:1), the solution was filtered in sterile conditions through a nylon filter of 45 µm prior to the centrifugation at 280 x g for 5 min at room temperature. The pallet containing myoblasts was suspended in culture media (alpha-MEM) supplemented with 20% foetal bovine serum (certified FBS; Invitrogen, Burlington, ON, Canada), and 1% penicillin/ streptomycin (Invitrogen). After two weeks, culture media was replaced by fresh culture media supplemented with 10% FBS and 1% penicillin/ streptomycin and myoblasts were allowed to grow until confluence.

Peripheral blood mononuclear cells (PBMC) were extracted from blood obtained from patients and control groups, as previously described **(Akoume et al., 2010)**.

Functional classification

Patients were classified by evaluating the functional status of melatonin signaling in PBMC with cellular dielectric spectroscopy (CDS), using CellKey^{TM} apparatus, as previously described (**Akoume et al., 2010**). The impedance that reflects the cellular changes resulting from ligand/receptor interaction was measured for 15 minutes to monitor the cellular response to 14^{-4}M iodomelatonin stimulation. The classification was achieved according to the following value ranges of impedance: between 0 and 40 ohms for FG1, between 40 and 80 ohms for FG2 and between 80 and 120 ohms for FG3. All control cases exhibiting the response extent less than 120 ohms were excluded from the study.

Functional evaluation of G proteins

The functionality of Gi, Gs and Gq proteins was evaluated by CDS assay in osteoblasts, myoblasts and PBMCs from the same individuals, using CellKey™ apparatus, as previously described **(Akoume et al., 2010)**.

RNA interference

All Gi_1, Gi_2, Gi_3, Gs and scrambled siRNA were obtained from Ambion (Ambion USA). The sequences used for gene silencing are shown in supplemental Table 3. Osteoblasts from control subjects and AIS patients 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 gene knockdown was evaluated by quantitative real-time PCR (qPCR).

Quantitative real-time PCR

RNA was isolated from osteoblasts using TRIzol reagent (Invitrogen) according to the manufactur's protocol. Total RNA (1µg) was reverse-transcribed into cDNA using Tetro cDNA synthesis Kit (Bioline). Following cDNA synthesis, qPCR was performed using a PCR master mix containing QuantiTect SYBR Green PCR Master Mix (QIAGEN, Ontario, Canada). Transcript expression was assessed with the Stratagene Mx3000P (Agilent Technologies, La Jolla, CA) and calculations were performed according to the ΔΔCT method using β-actin as internal control. The sequences of the forward and reverse

primers used for identification of human mRNA of our interest genes are shown in supplemental Table 4.

G protein expression in osteoblasts

Expression levels of Gi proteins isoforms and Gs protein were determined using standard western blotting technique. In brief, osteoblasts from AIS patients and trauma control cases were lysed in RIPA buffer (25 mM Tris.Hcl pH7.4, 150 mMNaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) added with 5 mM$NaVO_4$ and protease inhibitor cocktail (Roche molecular Biochemicals, Mannheim, Germany). Immunoblots were performed with primary antibodies directed specifically against Gi_1, Gi_2, Gi_3 or Gs (Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated secondary antibody. Bands were then visualized using SuperSignal chemilunescent substrate (Pierce, Rockford, IL)

Assessment of phosphorylation of Gi protein isoforms in osteoblasts

Comparative studies were performed to examine the level of phosphorylation and to identify the phosphorylated Gi isoform with immunoprecipitated proteins using isoform specific antibodies and, subsequently, probing the level of phosphorylation with a phospho-serine/threonine specific antibody (Santa Cruz Biotechnology). Whole cell proteins (1mg) were incubated with anti-Gi_1, anti-Gi_2 or anti-Gi_3 (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 nitrocellulose membrane and 5 % BSA blocking. Membranes were exposed to phospho-serine/threonine specific antibody at 4 °C overnight and, subsequently, treated with secondary antibody at room temperature for 1 h. Bands were visualized using SuperSignal cheminescence, and quantified by densitometric scanning.

Statistical analysis

Data are presented as mean ± 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.

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Figure Legends

Figure 1 The functionality of melatonin receptor is not impaired in AIS. (A) Comparison of concentration-response curves for melatonin between control and AIS functional groups. Data were normalised to maximal response in cells from control subjects. (B) Comparison of response to melatonin and its analogues. Cells were stimulated with the same concentration (1 uM) of melatonin, iodomelatonin or phenylmelatonin. The impedance represented in y-axis as dziec, indicates the resistance (ohms) of the cells toward the electric current applied by the Cellkey apparatus and represents the integrated cellular response. (C, D) Inhibition curves for response to melatonin following a treatment of (C) 4h with GPAnt-2 and (D) 16 hours with pertussis toxin. (E) EC_{50} and IC_{50} values of melatonin and GPAnt-2 in each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs control group.

Figure 2 The AIS functional groups are distinguished by the degree of response to various specific agonists of Gi-coupled receptors in osteoblasts. (A, B, C, D, E, and F) Agonists and targeted receptors are indicated in left corner of each panel. Data were normalised to maximal response in cells from control subjects. (G) EC_{50} values of tested compounds in each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs control group.

Figure 3 Inhibition curves of GPAnt-2 on response to various selective agonists of Gi-coupled receptors. (A, B, C, D, E, and F) Osteoblasts from control subjects or AIS patients of different groups were preincubated with varying concentrations of GPAnt-2 for

4h prior stimulation with 1 uM of specific synthetic agonist. The tested agonists and targeted receptors are indicated in left corner of each panel. Data were normalised to maximal response in cells from control subjects. (G) Table of IC_{50} values GPAnt-2 for each tested compound in each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

Figure 4 Inhibition curves of PTX on response to various selective agonists of Gi-coupled receptors. (A, B, C, D, E, and F) Osteoblasts from control subjects or AIS patients of different groups were preincubated with varying concentrations of PTX for 16h prior stimulation with 1 uM of specific synthetic agonist. The tested agonists and targeted receptors are indicated in left corner of each panel. Data were normalised to maximal response in cells from control subjects. Data are expressed as mean ± SE of n = 12 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

Figure 5 Functional status of Gs and Gq proteins in osteoblasts from control and AIS functional groups. The functionality of Gs protein was evaluated by challenging cells with (A) Isoproterenol or (B) Desmopressin. (C) The difference between response to Gi and Gs stimulation was calculated at various concentrations, and the functionality of Gq was assessed by challenging cells with Bradykinin (D) or Endothelin-1 (E). Receptor subtype targeted by the indicated agonist is put between parentheses. Data were normalised to maximal response in cells from control subjects. (F) EC_{50} values of tested compounds in

each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

Figure 6 Expression of Gi and Gs proteins is similar between AIS functional groups. (A) Total RNA were extracted from osteoblasts and qPCR was used to compare mRNA expression levels of Gi_1, Gi_2, Gi_3 and Gs genes in control relative to the AIS functional groups. β-actin was used as internal control. (B) Lysates were obtained from osteoblasts of each functional group. Equal amonut of proteins (40 ug) of each lysate was resolved by 10 % SDS-PAGE and imminoblated for Gi1, Gi2, Gi3 or Gs proteins.

Figure 7 Differential phosphorylation pattern of Gi protein isoforms in AIS functional groups. Whole osteoblasts cells from control or AIS patients were subjected to immunoprecipitation with antibody of (A) Gi_1, (B) Gi_2 or (C) Gi_3 and these precipitates were resolved by 10 % SDS-PAGE and immunoblotted for phospho-serine/threonine specific antibody. The bands were quantified by densitometric scanning. Values are expressed as mean ± SE, of n = 6 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

Figure 8 Differential effects of Gi and Gs siRNA on response to Gi stimulation in AIS functional groups. Osteoblasts from (A) control subjects, (B) FG3, (C) FG2 and (D) FG1 were transfectected with Gi_1, Gi_2, Gi_3, Gs siRNA alone or in combination, or with scrambled siRNA, as indicated in Material and Method section. Efficiency of siRNA was verified with qPCR in each functional group (B, D, and F) 48 hours after transfection, and

response to Gi stimulation was evaluated by challenging cells with Apelin-17, LPA or Somatostatin. Data were normalised to response in cells transfected with scrambled siRNA, and are expressed as mean ± SE, of n = 6 patients in each functional group. * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs control group.

Figure 9 Effect of various serine/threonine kinase inhibitors on response to Gi stimulation in osteoblasts from control and AIS functional groups. Cells were treated with PKA inhibitor H89 (5 uM),PKC inhibitor Gö6983 (5 uM), CaMK (1,2,4) inhibitor STO-609 acetate (5 uM), CaMK-2 inhibitor KN93 (5 uM), CK1 inhibitor D4476 (5 uM) or vehicle for 1 hour prior to the stimulation with (A) LPA (10^{-6} M) or (B) Somatostatin (10-6 M). Data were normalised to response in osteoblasts treated vehicle, and are expressed as mean ± SE, of n = 12 patients in each functional group. * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs control group.

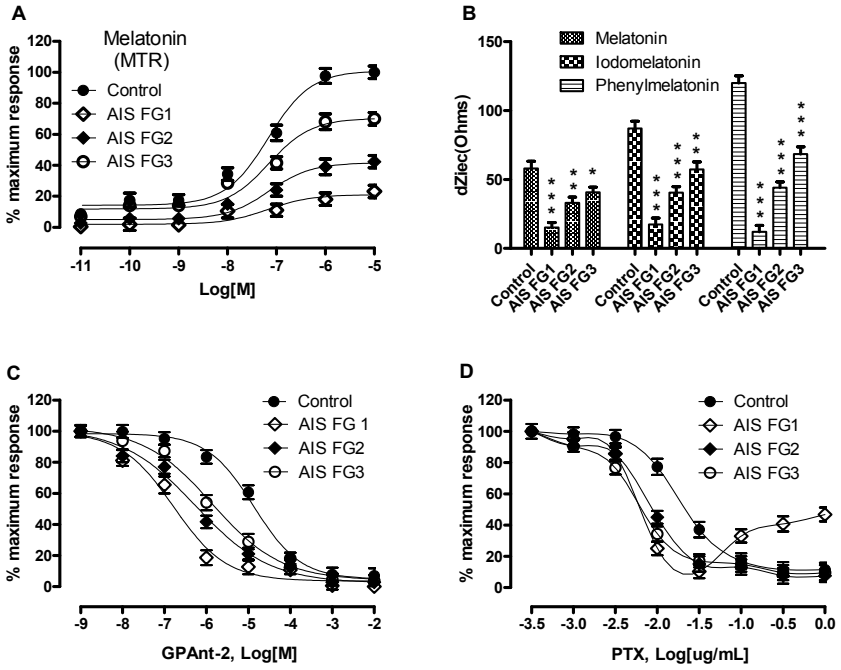
Figure 10 Expression of various serine/threonine kinases in AIS. (A) Total RNA were extracted from osteoblasts and qPCR was used to compare mRNA expression levels of PKC, PKA, CaMK and CK isoforms in control relative to the AIS functional groups. β-actin was used as internal control. (B) Lysates were obtained from osteoblasts of each functional group. Equal amount of proteins (40 ug) of each lysate was resolved by 10 % SDS-PAGE and imminobloted for proteins of indicated kinase isoforms.

Supplemental figure S1 Representative pedigrees of families with a positive history of AIS. The circles represent females and the squares represent males. The symbol for affected individuals is filled in and the indicated numbers correspond to individuals listed in Table 4 that illustrates their functional groups and their clinical data

Supplemental figure S2 The AIS functional groups are distinguished by the degree of response to various specific agonists of Gi-coupled receptors in myoblasts. (A, B, C, D, E, F) (A, B, C, D, E, and F) Agonists and targeted receptors are indicated in left corner of each panel. Data were normalised to maximal response in cells from control subjects. (G) EC_{50} values of tested compounds in each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

Supplemental figure S3 The AIS functional groups are distinguished by the degree of response to various specific agonists of Gi-coupled receptors in PBMCs. (A, B, C, D, E, F) (A, B, C, D, E, and F) Agonists and targeted receptors are indicated in left corner of each panel. Data were normalised to maximal response in cells from control subjects. (G) EC_{50} values of tested compounds in each functional group. Data are expressed as mean (± SE) of n = 12 patients per group. * P < 0.05, **P < 0.01, ***P < 0.001, vs control group.

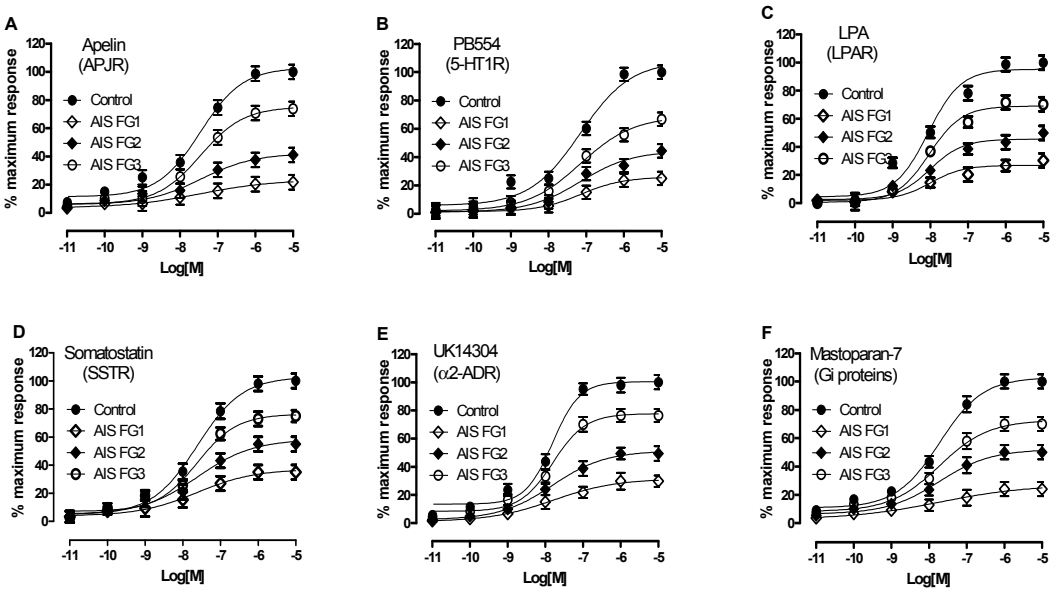
Figure 1



E

	Control	FG1	FG2	FG3
Melatonin				
EC_{50} (nM)	68.46 ± 0.36	66.68 ± 0.91	70.6 ± 0.43	69.89 ± 0.51
GPAnt-2				
IC_{50} (uM)	13 ± 0.19	0.16 ± 0.03***	0.54 ± 0.03***	1.37 ± 0.46***

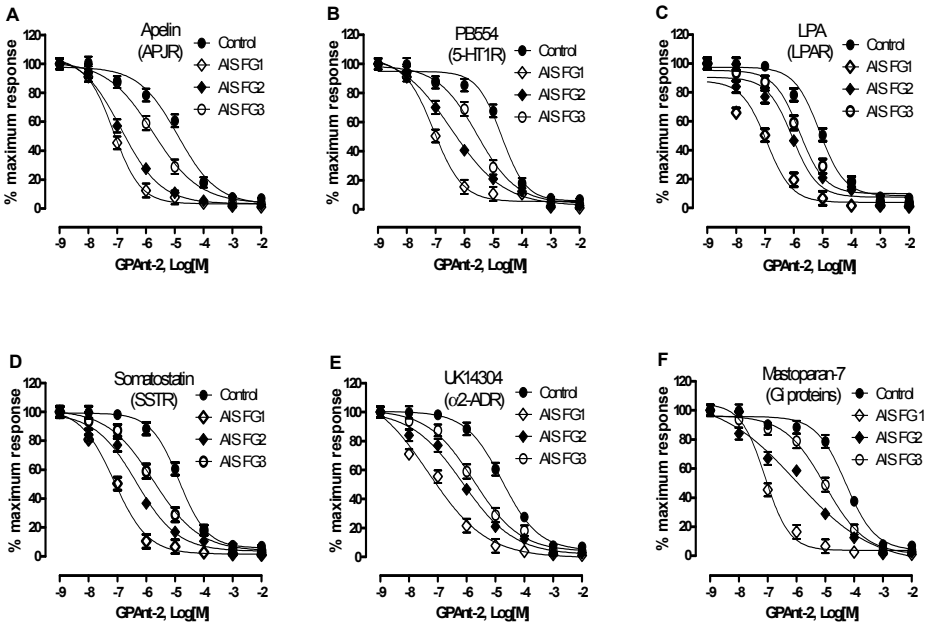
Figure 2



G

	Control	FG1	FG2	FG3
	EC50 (nM)	EC50 (nM)	EC50 (nM)	EC50 (nM)
Apelin-17	34.37 ± 0.30	34.07 ± 2.64	36.07 ± 0.93	35 ± 0.39
BP554	69.57 ± 0.65	71.78 ± 0.28	70.45 ± 0.64	70.12 ± 0.49
LPA	8.762 ± 0.49	10.13 ± 0.63	9.85 ± 0.44	9.35 ± 0.23
Somatostatin	24.32 ± 0.25	23.93 ± 1.01	24.75 ± 0.62	24.35 ± 0.27
UK14304	15.46 ± 0.22	16.19 ± 1.18	15.34 ± 0.55	15.88 ± 0.28
Mastoparan	18.98 ± 0.29	19.14 ± 1.90	18.35 ± 0.52	18.84 ± 0.33

Figure 3



G

	Control	FG1	FG2	FG3
	IC_{50} (uM)	IC_{50} (uM)	IC_{50} (uM)	IC_{50} (uM)
Apelin-17	12.8 ± 0.27	0.07 ± 0.01***	0.13 ± 0.23***	1.76 ± 0.30***
BP554	19.6 ± 0.19	0.09 ± 0.02***	0.35 ± 0.3***	2.76 ± 0.27***
LPA	7.86 ± 0.17	0.11 ± 0.03***	0.89 ± 0.22***	1.58 ± 0.23***
Somatostatin	13.7 ± 0.15	0.08 ± 0.02***	0.45 ± 0.27***	1.76 ± 0.30***
UK14304	17.1 ± 0.19	0.05 ± 0.02***	0.71 ± 0.31***	1.76 ± 0.30***
Mastoparan	51.3 ± 0.20	0.08 ± 0.02***	0.99 ± 0.15***	9.63 ± 0.27***

Figure 4

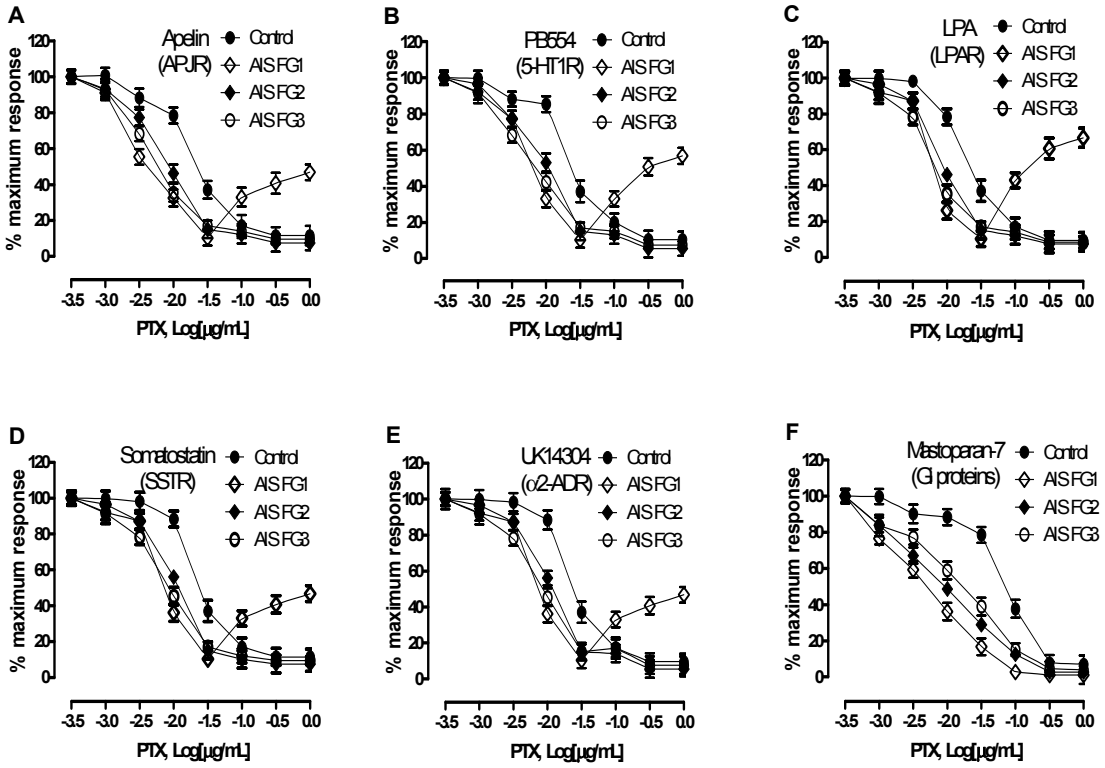
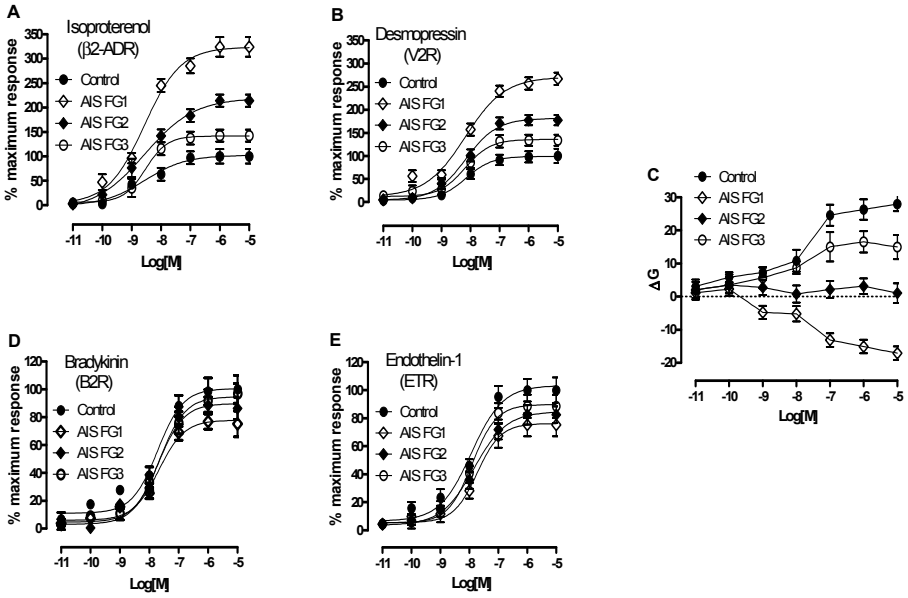

A
Apelin (APJR)
Control
AISFG1
AISFG2
AISFG3
% maximum response
PTX, Log[µg/mL]
B
PB554 (5-HT1R)
C
LPA (LPAR)
D
Somatostatin (SSTR)
E
UK14304 (α2-ADR)
F
Mastoparan-7 (Gi proteins)

Figure 5



F

	Control	FG1	FG2	FG3
	EC50 (nM)	EC50 (nM)	EC50 (nM)	EC50 (nM)
Isoproterenol	2.79 ± 0.59	2.7 ± 0.23	2.77 ± 0.46	2.94 ± 0.32
desmopressin	6.98 ± 0.37	6.6 ± 0.25	6.82 ± 0.25	6.5 ± 0.33
Bradykinin	18.92 ± 0.29	20.91 ± 0.27	19.87 ± 0.23	22.42 ± 0.27
Endothelin-1	11.67 ± 0.49	18.58 ± 0.30	14.41 ± 0.26	12.56 ± 0.26

Figure 6

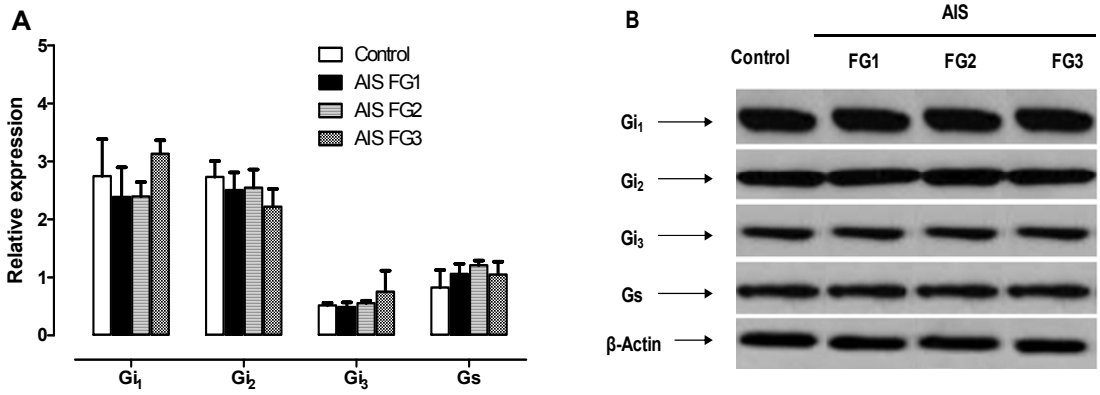

A
Relative expression
0
1
2
3
4
5
Control
AIS FG1
AIS FG2
AIS FG3
Gi1
Gi2
Gi3
Gs
B
AIS
Control
FG1
FG2
FG3
Gi1
Gi2
Gi3
Gs
β-Actin

Figure 7

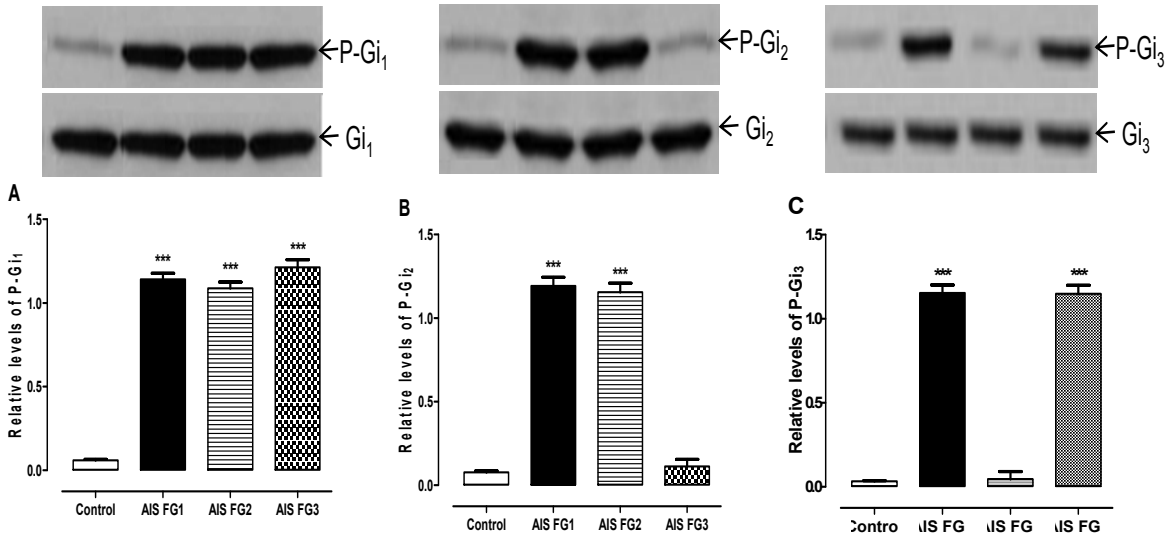

P-Gi1
Gi1
P-Gi2
Gi2
P-Gi3
Gi3
A
B
C
Relative levels of P-Gi1
Relative levels of P-Gi2
Relative levels of P-Gi3
***
Control
AIS FG1
AIS FG2
AIS FG3

Figure 8

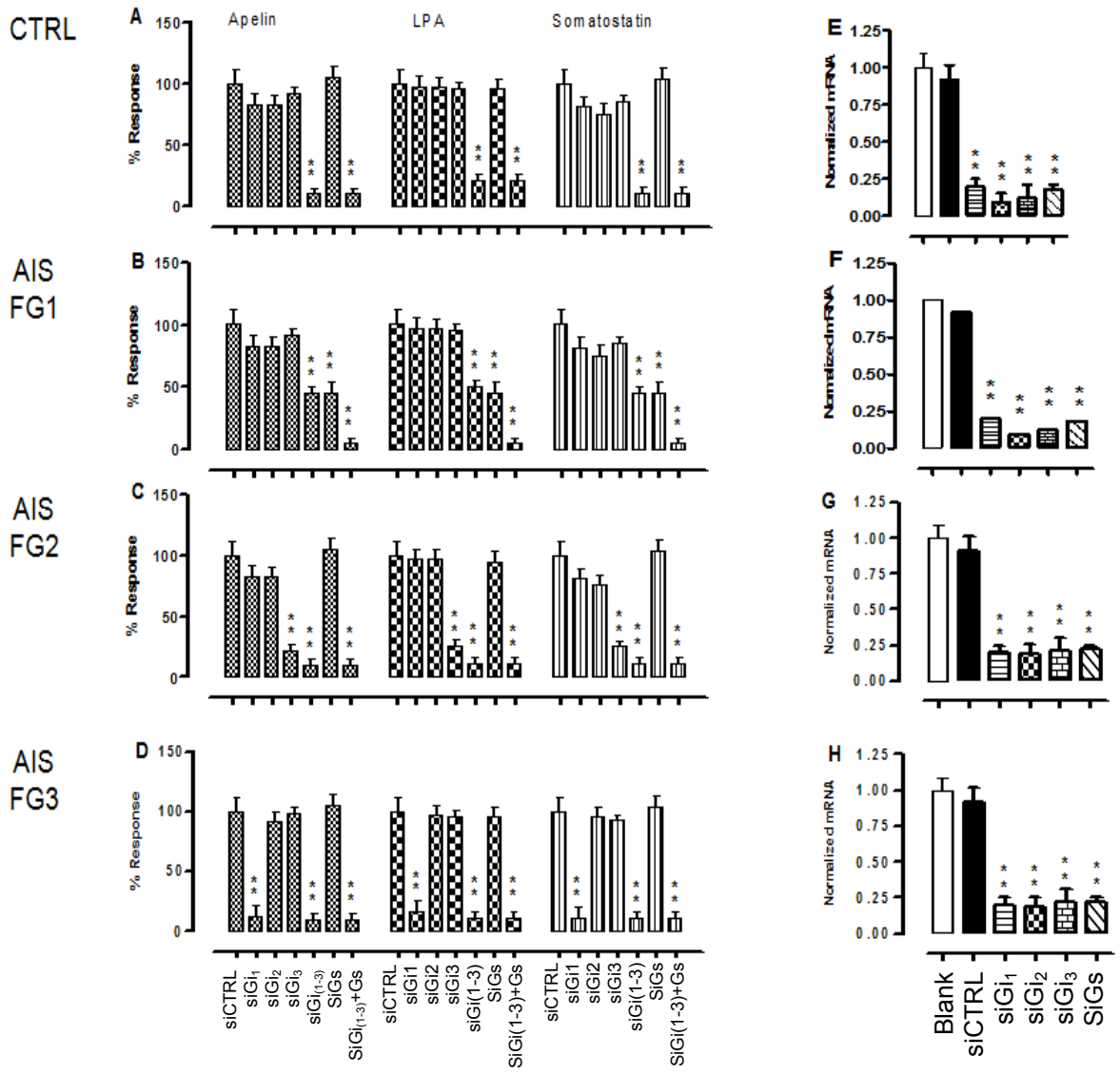

CTRL
AIS FG1
AIS FG2
AIS FG3
A
B
C
D
Apelin
LPA
Somatostatin
% Response
E
F
G
H
Normalized mRNA
siCTRL
siGi1
siGi2
siGi3
siGi(1-3)
SiGs
SiGi(1-3)+Gs
Blank
siCTRL
siGi1
siGi2
siGi3
SiGs

Figure 9

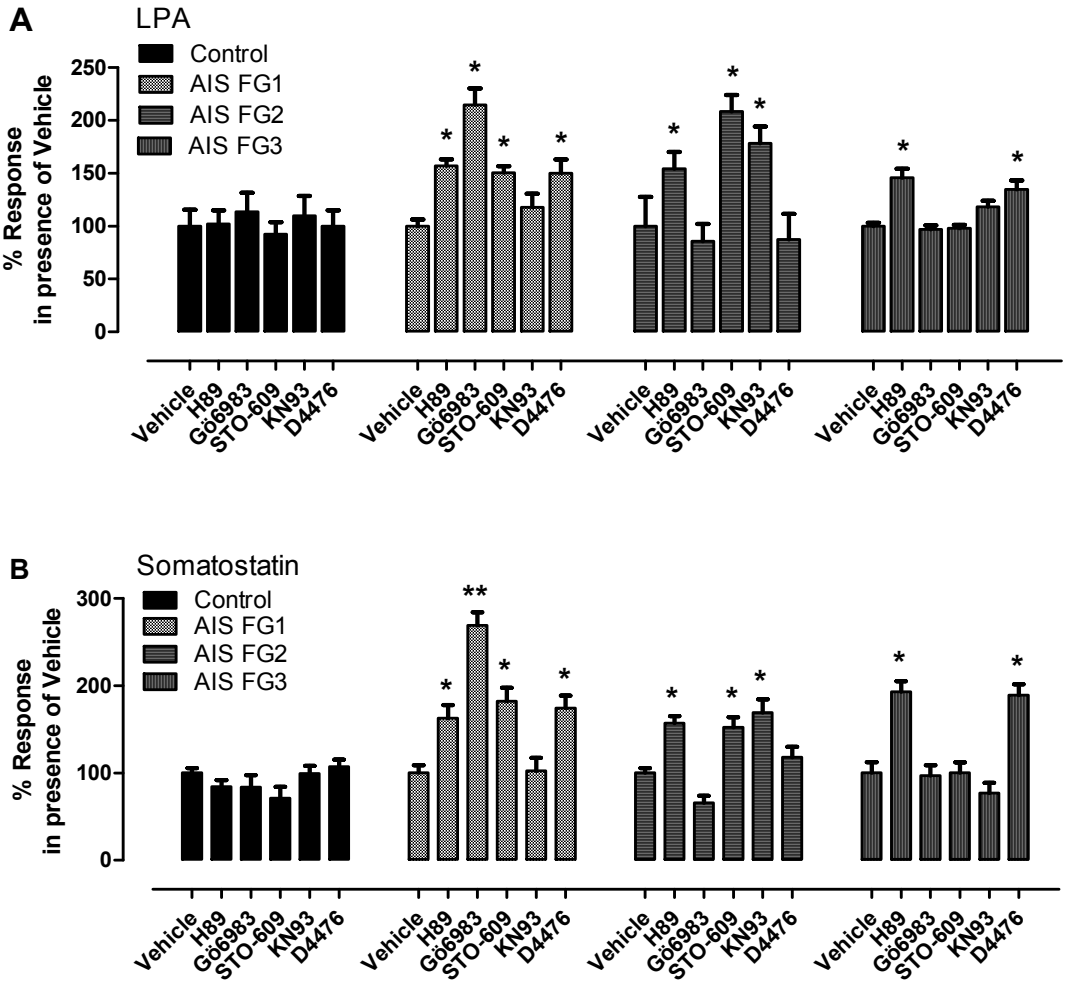

A
LPA
Control
AIS FG1
AIS FG2
AIS FG3
% Response in presence of Vehicle
250
200
150
100
50
0
Vehicle
H89
Gö6983
STO-609
KN93
D4476
B
Somatostatin
300
200
100
0

Figure 10

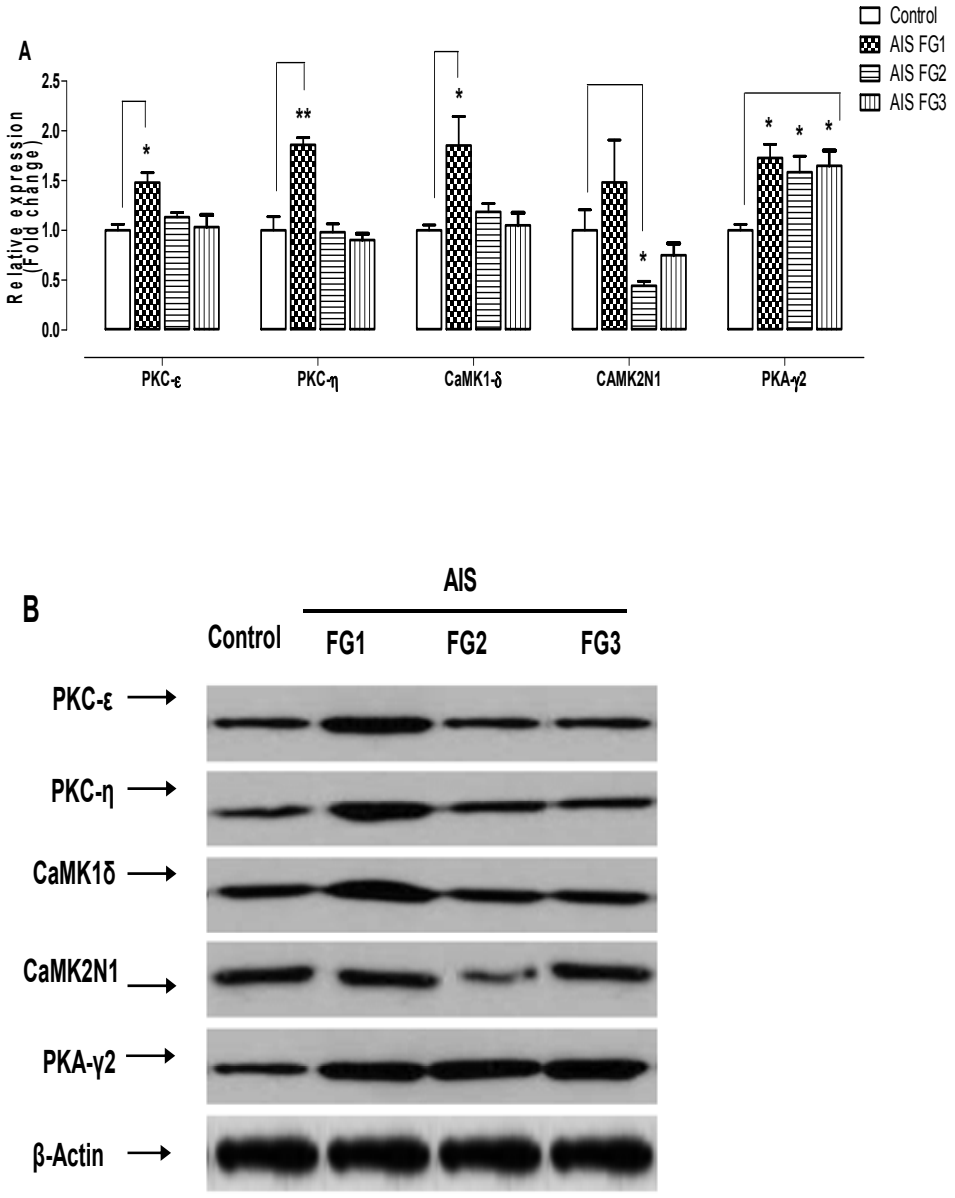

A
Relative expression (Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Control
AIS FG1
AIS FG2
AIS FG3
PKC-ε
PKC-η
CaMK1-δ
CAMK2N1
PKA-γ2
B
AIS
Control
FG1
FG2
FG3
PKC-ε
PKC-η
CaMK1δ
CaMK2N1
PKA-γ2
β-Actin

Table 1
Demographic data of AIS patients and healthy control subjects

	Canadian Cohort		Italian Cohort	
	N	**Mean Age (years)**	**N**	**Mean Age (years)**
Healthy control subjects	240	12.4 ± 3.2	103	11.0 ± 1.6
AIS patients				
Cobb Angle < 44 %	794	13.0 ± 2.6	78	13.6 ± 2.8
Cobb Angle > 45 %	162	15.1 ± 2.1	61	13.8 ± 2.2

Table 2
Clinical data of AIS patients classified into functional groups

	Canadian Cohort						
	Cobb Angle < 44 °		Cobb Angle > 45 °		Curve type		
	N (%)	Cobb Angle (°)	N (%)	Cobb Angle (°)	Single N (%)	Double N (%)	Triple N (%)
FG1	222 (28)	20.0 ± 10.1	24 (13)	60.8 ± 12.4	97 (51)	85 (45)	7 (4)
FG2	262 (33)	21.5 ± 9.5	45 (56)	60.0 ± 10.8	144 (48)	140 (46)	19 (6)
FG3	310 (39)	18.9 ± 9.4	26 (31)	60.7 ± 12.6	206 (54)	157 (41)	19 (5)

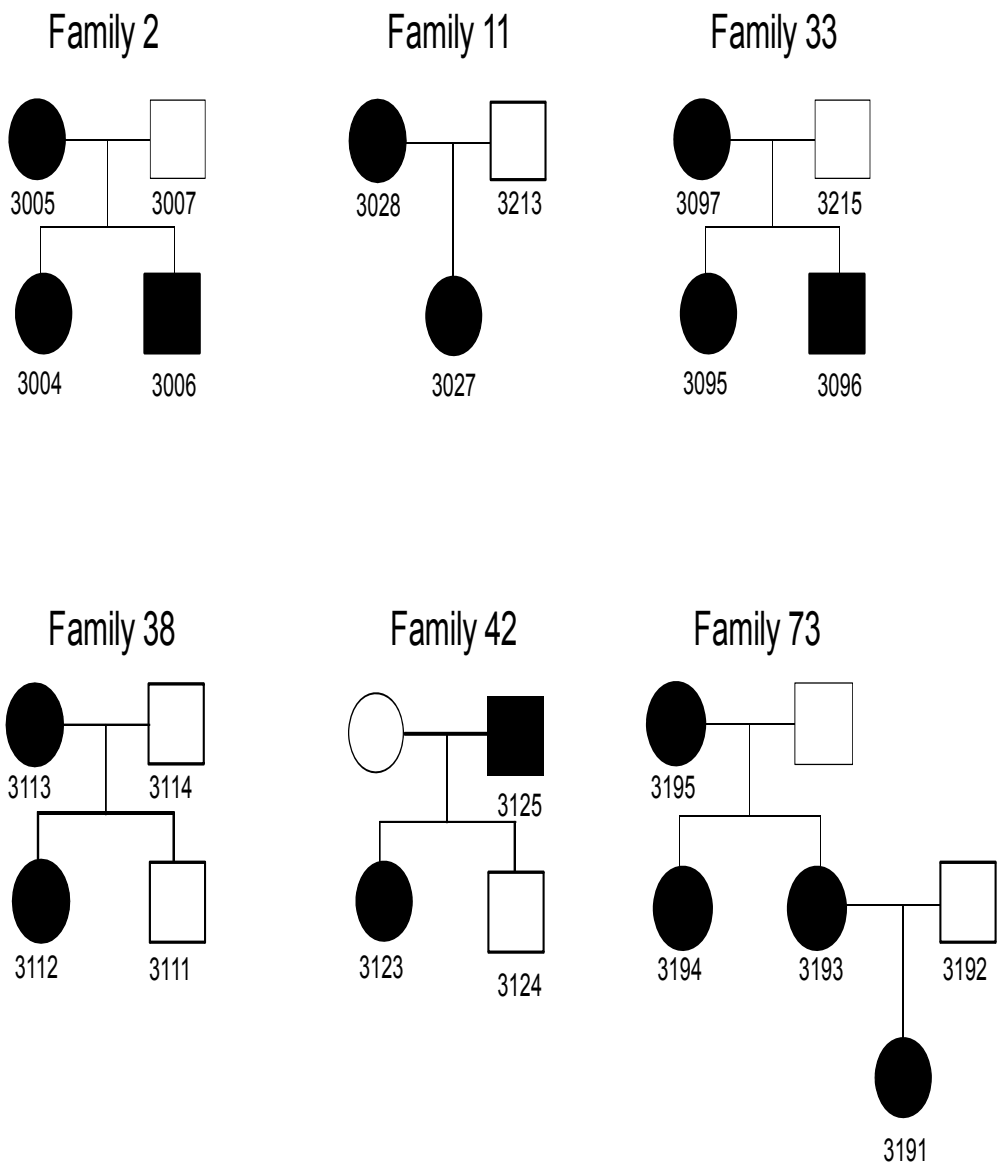
Table 3
Clinical data of AIS patients classified into functional groups

	Italian Cohort						
	Cobb Angle < 44 °		Cobb Angle > 45 °		Curve type		
	N (%)	Cobb Angle (°)	N (%)	Cobb Angle (°)	Single N (%)	Double N (%)	Triple N (%)
FG1	222 (28)	20.0 ± 10.1	24 (13)	60.8 ± 12.4	97 (51)	85 (45)	7 (4)
FG2	262 (33)	21.5 ± 9.5	45 (56)	60.0 ± 10.8	144 (48)	140 (46)	19 (6)
FG3	310 (39)	18.9 ± 9.4	26 (31)	60.7 ± 12.6	206 (54)	157 (41)	19 (5)

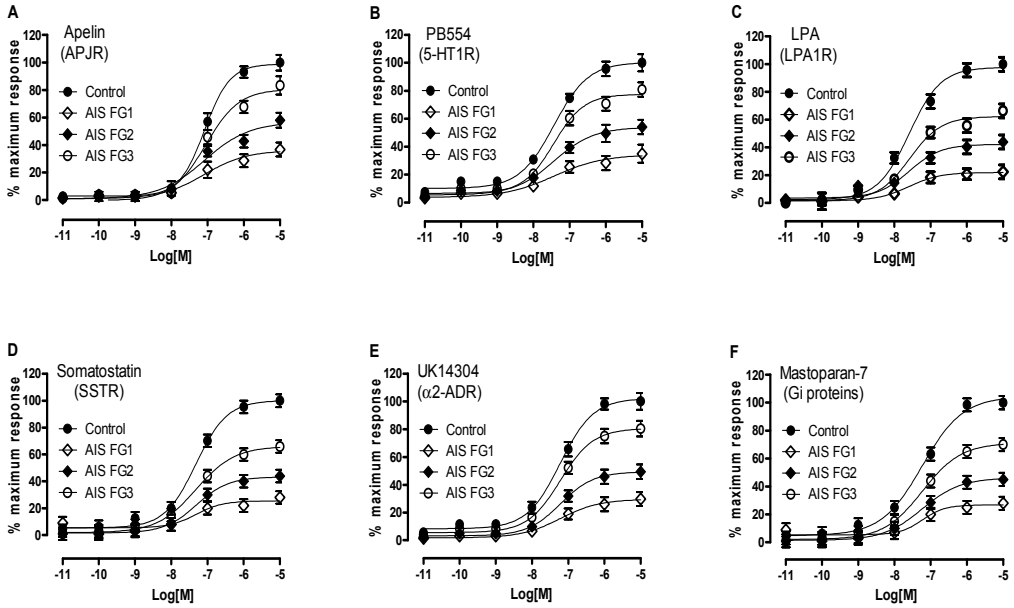
Table 4: Functional groups and clinical data of affected members from studied families

Functional subgroup	Family ID	Case Number	Gender	Age	Cobb Angle	Curve Pattern
FG1	2	3004	Female	12,8	8 - 8	-
FG1	2	3005	Female	mother	NA	Scoliosis
FG1	2	3006	Male	9,4	12	Left thoracolumbar
-	2	3007	Male	father	0	-
FG2	11	3027	Female	16,5	16	Left Lumbar
FG2	11	3028	Female	NA	NA	Scoliosis
-	11	3213	Male	NA	0	-
-	33	3095	Male	14,4	0	-
FG2	33	3096	Female	12,3	15	Right thoracolumbar
FG2	33	3097	Female	mother	NA	Scoliosis
-	33	3215	Male	father	0	-
FG3	38	3111	Male	14,0	0	-
FG3	38	3112	Female	16,5	14	Right Thoracic
FG3	38	3113	Female	mother	NA	Scoliosis
-	38	3114	Male	father	0	-
FG2	42	3123	Female	13,3	8 – 14	Double major
-	42	3124	Male	17,5	4	-
FG2	42	3125	Male	father	NA	Scoliosis
FG2	73	3191	Female	9,5	10	Left thoracolumbar
-	73	3192	Male	father	0	-
FG2	73	3193	Female	mother	NA	Scoliosis
FG2	73	3194	Female	aunt	NA	Scoliosis
FG2	73	3195	Female	grand-mother	NA	Scoliosis

Supplementary Figure S1

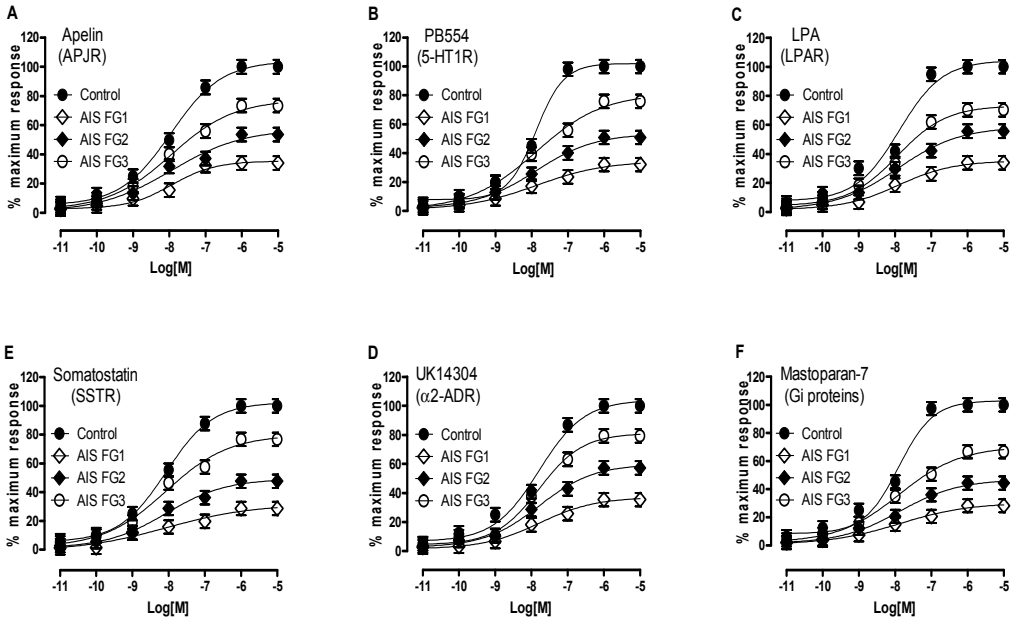


Supplementary Figure S2



G	Control	FG1	FG2	FG3
	EC50 (nM)	EC50 (nM)	EC50 (nM)	EC50 (nM)
Apelin-17	82.62 ± 0.17	80.16 ± 0.73	81.5 ± 0.54	82.83 ± 0.29
BP554	37.01 ± 0.22	37.63 ± 0.98	36.87 ± 0.48	36.26 ± 0.28
LPA	25.91 ± 0.19	25.38 ± 0.32	25.9 ± 0.55	26.61 ± 0.36
Somatostatin	48.06 ± 0.19	49.19 ± 1.06	49.56 ± 0.18	48.35 ± 0.35
UK14304	59.85 ± 0.22	60.65 ± 0.73	61.27 ± 0.39	60.76 ± 0.25
Mastoparan	58.08 ± 0.26	59.66 ± 0.82	57.65 ± 0.13	58.85 ± 0.33

Supplementary Figure S3



G	Control	FG1	FG2	FG3
	EC50 (nM)	EC50 (nM)	EC50 (nM)	EC50 (nM)
Apelin-17	11.12 ± 0.26	11.61 ± 0.54	11.46 ± 0.79	11.3 ± 0.47
BP554	13 ± 0.28	13.58 ± 0.45	13.13 ± 0.26	13.43 ± 0.45
LPA	13.04 ± 0.64	12.17 ± 0.39	12.17 ± 0.34	11.75 ± 0.39
Somatostatin	8.45 ± 0.11	8.34 ± 1.34	8.34 ± 0.47	8.5 ± 0.46
UK14304	16.28 ± 0.45	16.5 ± 0.42	16.5 ± 0.36	16.26 ± 0.31
Mastoparan	12.48 ± 0.43	13.11 ± 0.47	13.11 ± 0.37	12.97 ± 0.50

Supplementary Table S1: siRNA sequences used in this study

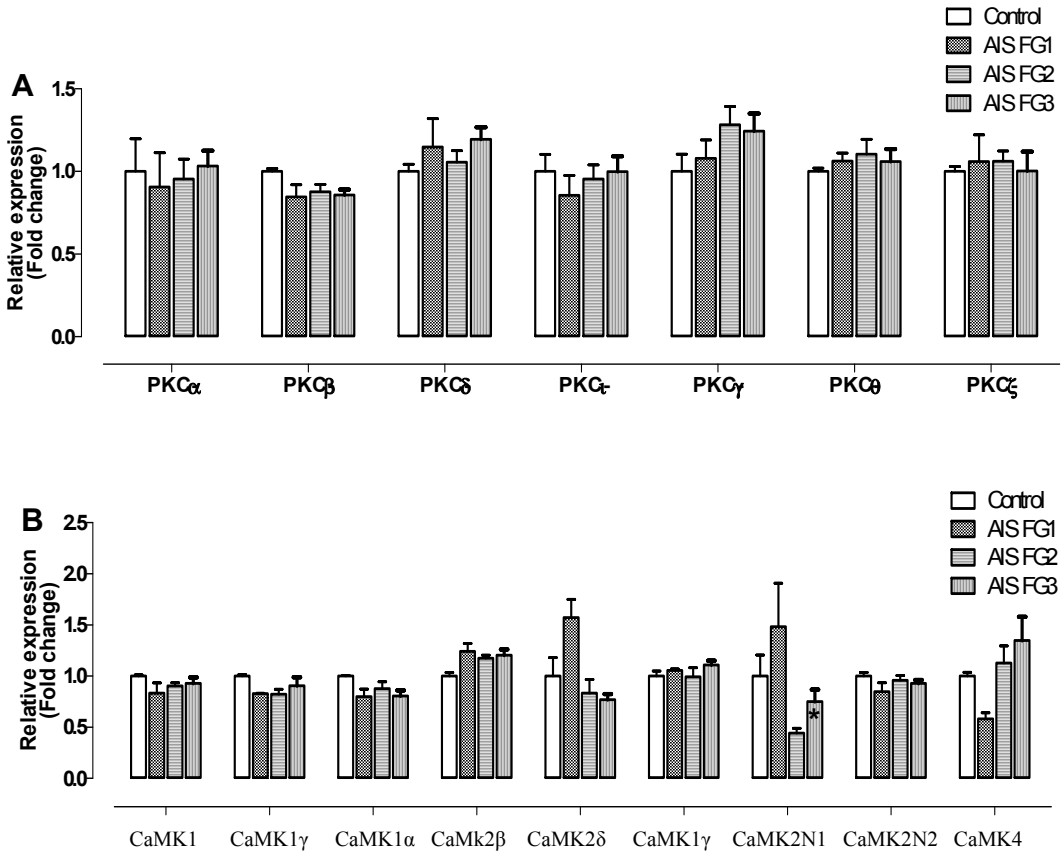
Gene symbol	siRNA sequence	Cat. Number
GNAi1	GAGAUUGUGGAAAGAUAGUGGUGUA	1299003
GNAi2	GAGGACCUGAAUAAGCGCAAAGACA	1299003
GNAi3	UCAGCUCAAUGAUUCUGCUUCAUAU	1299003
GNAS	ACAACAUGGUCAUCCGGGAGGACAA	1299003

Supplementary Table S2: List of the forward and reverse sequences used in this study

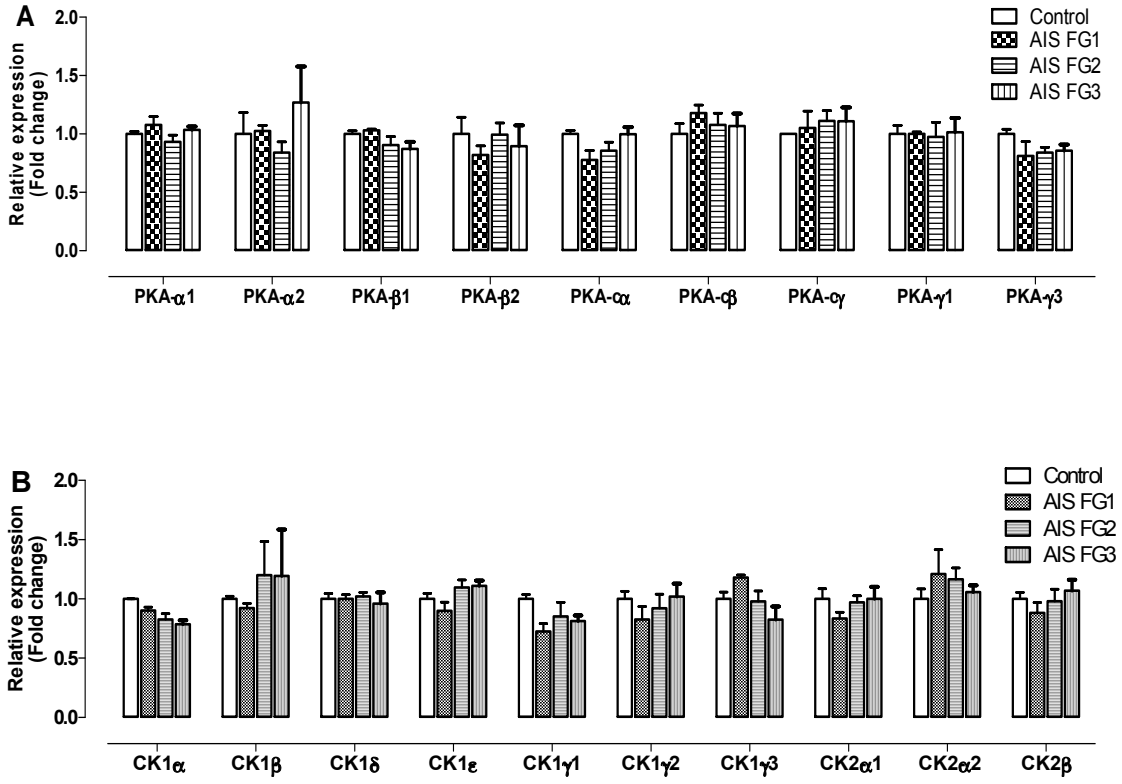
	Forwards	Reverses
Gi1	AGGGCTATGGGGAGGTTGAAGAT	ACTCCAGCAAGTTCTGCAGTCA
Gi2	AGGGAATACCAGCTCAACGACTCA	TGTGTGGGGATGTAGTCACTCTGT
Gi3	GAGAGTGAAGACCACAGGCATT	CGTTCTGATCTTTGGCCACCTA
Gs	GAGACCAAGTTCCAGGTGGACA	GATCCACTTGCGGCGTTCAT
Gq	ATCAGAACATCTTCACGGCC	AAAGCAGACACCTTCTCCAC
PKC-α (PKCA)	CGGAATGGATCACACTGAGAAG	ACATAAGGATCTGAAAGCCCG
PKC-β (PKCB)	TTCCCGATCCCAAAAGTGAG	GTCAAATCCCAATCCCAAATCTC
PKC-δ (PKCD)	GCTTCAAGGTTCACAACTACATG	ACCTTCTCCCGGCATTTATG
PKC-ε (PKCE)	CCTACCTTCTGCGATCACTG	TACTTTGGCGATTCCTCTGG
PKC-η (PKCHt)	GTAAATGCGGTGGAACTTGC	ACCCCAATCCCATTTCCTTC
PKC-I (PKCI)	GAGAAGCATGTGTTTGAGCAG	GGAAGTTTTCTTTGTCGCTGC
PKC-γ (Gamma)	ACGAAGTCAAGAGCCACAAG	GTCGATGAACCACAAAGCTG
PKC-θ (PKCQ)	CGGATTTTGGAATGTGCAAGG	CATAAAGGAGAACCCCGAAGG
PKC-ζ (PKCZ)	TGCTTACATTTCCTCATCCCG	CGCCCGATGACTCTGATTAG
CaMK1-δ (CaMK1D)	AATGGAGGGCAAAGGAGATG	AAGATGTAGGCAATCACTCCG
CaMK1-γ (CaMK1G)	CTTGAGAAGGATCCGAACGAG	TTGCCTCCACTTGCTCTTAG
CaMK2-α (CaMK2A)	CAGTTCCAGCGTTCAGTTAATG	TTCGTGTAGGACTCAAAATCTCC
CaMK2-β (CaMK2B)	CTCTGACATCCTGAACTCTGTG	CCGTGGTCTTAATGATCTCCTG
CaMK2-δ (CaMK2D	GGCACACCTGGATATCTTTCTC	AGTCTGTGTTGGTCTTCATCC
CaMK2-γ (CaMK2G)	AAACAGTCTCGTAAGCCCAG	ATCCCATCTGTAGCGTTGTG
CaMK4	TCGCCTCTCACATCCAAAC	CATCTCGCTCACTGTAATATCCC
CaMK2n1	AGGACACCAACAACTTCTTCG	GGTGCCTTGTCGGTCATATT

	Forwards	Reverses
PKA-α1	CTCAGTTCCTGGAGAAAGATGG	CCCAGTCAATTCATGTTTGCC
PKA-α2	ATGGAATATGTGTCTGGAGGTG	TGGTTTCAGGTCTCGATGAAC
PKA-β1	GAGTAAACTTCCCCTCACCAG	TCCACTGACCATCCACAAAG
PKA-β2	CACTGTTATCCGCTGGTCTG	CTTGTATTGGTGCTCTCCCTC
PKA-cα	CAAGGACAACTCAAACTTATACATGG	CAGATACTCAAAGGTCAGGACG
PKA-cβ	CCTTTCCTTGTTCGACTGGAG	TGAGCTGCATAGAACCGTG
PKA-cγ	CCGGATCTCCATCAATGAGAAG	TTCAATCCAACCCTCCCATC
PKA-γ1	GCGCATTCTGAAGTTCCTCA	AAAATCCCCAGAGCCACATAG
PKA-γ2	TTGCCCGTTATTGACCCTATC	CGTTCCTATTCCAAGCTCATCC
PKA-γ13	TGACTGCACTGGACATCTTTG	TGGTTGTAGGTTTGCTGGG
CK-1α	TGTCGGAGGGAAATATAAACTGG	GGCCTTCTGAGATTCTAGCTTC
CK-1γ1	AGGTGGAGGTAGTGGAGG	GTACAATTGAGTCAGAGTCCCC
CK-1γ2	GTGATGTTCTAGCCACAGAGG	CCCTTTCCCTCCTTTCTTGTC
CK-1γ3	GTTCAAATGCACCCATCACAG	AGTAACTCCCCAGGATCTGTC
CK-2α1	GTATGAGATTCTGAAGGCCCTG	CCAAACCCCAGTCTATTAGTCG
CK-2α2	CGATACGACCATCAACAGAGAC	TCGCTTTCCAGTCTTCATCG

Supplementary Figure S4



Supplementary Figure S5



The second manuscript: Osteopontin contributes to the development of idiopathic scoliosis

We previously observed the elevation of circulating OPN levels in patients affected with adolescent idiopathic scoliosis (AIS) or asymptomatic children at risk of developing AIS. This observation supports our hypothesis that OPN plays a crucial role in the pathogenesis of AIS. In this manuscript we demonstrated how OPN plays an important role in the development of spinal deformity through its interaction with $\alpha_5\beta_1$ integrin. We took the advantage of C57Bl/6 bipedal mouse model which developed scoliosis after amputation of forelimbs and tails and we found that these bipedal scoliotic mice exhibited higher plasma osteopontin and lower response to GiPCR stimulation when compared to non-scoliotic quadrupedal mice. The genetic depletion of OPN prevented spinal deformities in bipedal mice and improved the response to GiPCR.

In this second manuscript, I contributed in the experimental design and in the generation of the results. More specifically I did the immunoprecipitation and western blots experiments and I have done siRNA transfections for some functional experiments. I reviewed and revised the manuscript, and approved the final manuscript as submitted.

Dr. Marie-Yvonne Akoume conceptualized and designed the study, performed the CellKey^{TM} manipulation using CDS technique, some of the siRNA transfections carried out the initial analyses, drafted the initial manuscript, and approved the final manuscript as submitted

Mr. Saadallah Bouhanik contributed in the generation of the bipedal mice and their phenotypic characterization and approved the final manuscript as submitted.

Ms. Anita Franco contributed to the ELISA experiments and approved the final manuscript as submitted.

Dr. Alain Moreau contributed to the study conception, the analysis and interpretation of the data. He critically revised the manuscript for important intellectual content and approved the final manuscript as submitted.

OSTEOPONTIN CONTRIBUTES TO THE DEVELOPMENT OF IDIOPATHIC SCOLIOSIS

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ABSTRACT

Idiopathic scoliosis is a three-dimensional spinal deformity of unknown origin. We have previously associated this condition with a defective Gi protein-coupled receptor (GiPCR) signaling and reported increased circulating osteopontin (OPN) levels in affected individuals. Here we provide the first evidence that lack of OPN leads to improvement in GiPCR signaling and confers a protection against the development of spinal deformity. We further showed that OPN reduces GiPCR signaling *in vitro* via $\alpha_5\beta_1$ integrin engagement. Our extensive investigation revealed that the mechanism by which OPN interferes with signaling of GiPCR is likely a depletion of functional Gi proteins necessary for these receptors. We propose a scenario that includes the sequestration of a part of Gi proteins by integrin β_1 subunit and the inactivation of the remaining Gi proteins following their phosphoryaltion by various kinases engaged in the signaling cascade of $\alpha_5\beta_1$ integrin. For the first time, a critical role for OPN is demonstrated in the development of idiopathic scoliosis.

INTODUCTION

Osteopontin (OPN) is a multifunctional glycoprotein greatly synthesized in variety of cell types and secreted into body fluids in response to injury **(Sodek et al., 2000); (Denhardt et al., 2001a)**. OPN is also abundantly produced in pathological conditions **(Chiba et al., 1999); (Fitzpatrick et al., 1994); (Katagiri et al., 1999); (Liaw et al., 1998); (Ophascharoensuk et al., 1999); (Reinholt et al., 1990)** and has been implicated in the pathogenesis of numerous diseases **(Denhardt and Guo, 1993); (Giachelli et al., 1993); (Giachelli et al., 1994); (Giachelli et al., 1995); (Murry et al., 1994); (O'Brien et al., 1995); (Oates et al., 1997)**. We have previously reported high circulating levels of OPN in patients with idiopathic scoliosis. However, whether OPN is involved in the development of idiopathic scoliosis has not been explored in depth.

Idiopathic scoliosis is generally defined as a three-dimensional deformity of the spine characterized by lateral curvature combined with vertebral rotation. Despite extensive research, the aetiology and pathophysiology of this disease remain largely unknown. Current view maintains that many factors are involved in the genesis of idiopathic scoliosis and practically every structure of the body has been incriminated, while no single biological process driving the pathogenesis has been identified. Nevertheless, recent works from our laboratory suggest that this unsolved mystery may implicate the deficiency in Gi protein-mediated signal transduction. Indeed, we have demonstrated that various compounds activating Gi proteins, either directly or via receptors failed to efficiently induce cell signaling in cells from scoliotic patients and asymptomatic children as

measured by cellular dielectric spectroscopy (CDS) and cAMP assay **(Moreau et al., 2004); (Azeddine et al., 2007); (Akoume et al., 2010).** Moreover, approximately 33 % of asymptomatic children diagnosed with a defective Gi protein function have developed scoliosis many years later **(Akoume et al., 2010).**

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., 2006a); (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. Our findings reveal that OPN is of key importance in the development of idiopathic scoliosis likely by disrupting Gi protein-mediated signaling via a dual mechanism requiring β1 integrin engagement.

MATERIALS AND METHODS

Derivation of primary osteoblast cultures

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 αMEM medium containing 10% fetal bovine serum (FBS; certified FBS, Invitrogen, Burlington, ON, Canada) and 1% penicillin/ streptomycin (Invitrogen) at 37°C in 5% CO_2, in a 10-cm^2 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

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 $OPN^{-/-}$ mice (bipedal and quadrupedal) using CellKey™ apparatus, as previously described (**Akoume et al., 2010**).

Osteopontin enzyme-linked immunosorbent assays

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).

Generation of bipedal C57Bl/6j OPN-null mice and bipedal C57Bl/6j CD44-null mice

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., 2006b).** 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., 2006b).** Similarly bipedal C57Bl/6j-CD44-null mice were generated.

Cell line and transfections

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 oligo used for the knockdown of different genes are, ssp1 (encode OPN) (CCA CAG CCA CAA GCA GUC CAG AUU A), integrin β1 (CCU AAG UCA GCA GUA GGA ACA UUA U), integrin β3 (CCU CCA GCU CAU UGU UGA UGC UUA U), integrin α5 (CCG AGU ACC UGA UCA ACC UGG UUC A), integrin α8 (GAG AUG AAA CUG AAU UCC GAG AUA A), integrin αv (GAC UGU UGA GUA UGC UCC AUG UAG A) and CD44 (GAA CAA GGA GUC GUC AGA AAC UCC A).

Quantitative reverse transcription-polymerase chain reaction (qPCR)

Thermo-Script reverse transcriptase (Invitrogen) was used to reverse 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:

β-actin forward 5'-GGAAATCGTGCGTGACAT-3',

β-actin reverse 5'-TCATGATGGAGTTGAAGGTAGTT-3',

CD44 forward 5'(AGCATCGGATTTGAGACCTG)3',

CD44 reverse 5'(TGAGTCCACTTGGCTTTCTG)3',

β1 integrin forward 5'(ATGTGTCAGACCTGCCTTG)3',

β1 integrin reverse 5'(TTGTCCCGACTTTCTACCTTG)3',

αv integrin forward 5'(GTCCCCACAGTAGACACATATG)3',

αv integrin reverse 5'(TCAACTCCTCGCTTTCCATG)3'.

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 Strata-gene. Relative quantification was calculated with the delta CT method using β-actin as the endogenous control.

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

Gi proteins isoforms expression levels were determined using western blot technique. MC3T3-E1 cells were treated with PBS or rOPN 0.5µg/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 mMNaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) added with 5 mM$NaVO_4$ and protease inhibitor cocktail (Roche molecular Biochemicals, Mannheim, Germany). Immunoblots were performed with primary antibodies directed specifically against Gi_1, Gi_2, Gi_3, Gs, Phosphoserine, integrin β1 (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 β1 integrin

MC3T3-E1 cells were treated with PBS or rOPN (0.5µg/ ml) then the whole cell proteins (1mg) were incubated with anti- MOR (Abcam Toronto, ON, Canada), anti-MT2 or anti- β_1 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_1, Gi_2, Gi_3, MOR, MT2 and β_1 integrinat 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.

Statistical analysis

Data are presented as mean ± SE. Multiple comparisons of means were performed with one-way analysis of variance (ANOVA) followed by a post-hoc test of Dunnett, using GraphPad Prism 4.0 software. Only P values < 0.05 were considered significant.

RESULTS

Genetic deletion of OPN protects bipedal C57Bl/6 from scoliosis

The bipedal C57Bl/6 mice are widely used as animal model to study the pathophysiological events leading to idiopathic scoliosis. To determine whether OPN is involved in the development of idiopathic scoliosis, female wild-type (WT) and OPN

knockout ($OPN^{-/-}$) 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 $OPN^{-/-}$ quadrupedal mice. However, lateral curvature was apparent in 55 % of bipedal WT mice. The convexity of curve was directed to either side, with no consistent preference. In contrast, analysis of radiographs yielded no evidence of scoliosis in bipedal $OPN^{-/-}$ mice. All bipedal $OPN^{-/-}$ mice had straight spine indistinguishable from the quadrupedal mice. These data strongly emphasize that genetic OPN deletion protects bipedal C57Bl/6 mice from scoliosis and suggest a role for OPN in the development of scoliosis.

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 $OPN^{-/-}$ 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.

OPN deficiency improves Gi protein-mediated receptor signal transduction

Since we have previously associated idiopathic scoliosis with defective Gi protein function, it was of interest to examine whether lack of OPN can influence Gi protein-mediated signaling. For this purpose, osteoblasts from WT and $OPN^{-/-}$ 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 $OPN^{-/-}$ mice. However, in each case, the magnitude of response was greater in $OPN^{-/-}$ than WT osteoblasts, which may suggest that the activation of Gi proteins is facilitated in $OPN^{-/-}$ osteoblasts. On the other hand, while the magnitude of response was similar in osteoblasts from quadrupedal and bipedal $OPN^{-/-}$ mice, a significant difference was observed in response between those from quadrupedal and bipedal WT mice. The extent of response was much lower in osteoblasts from bipedal WT mice.

To corroborate these results with the function of Gi proteins, we treated osteoblasts with pertussis toxin (PTX), which ADP-rybosylates Gi proteins and disrupts their interaction with receptors **(Gilman, 1987); (Moss et al., 1984)**. Results illustrated in (**Fig. 1, J-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 high in $OPN^{-/-}$ osteoblasts. Notably, the differences in the magnitude of response between WT and $OPN^{-/-}$ osteoblasts were completely abrogated by PTX treatment. Collectively, these data indicate that OPN deficiency enhances Gi protein-mediated receptor signal transduction in osteoblasts cells.

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 $OPN^{-/-}$ mice were less responsive to Gs stimulation than those from WT mice, whereas the Gq stimulation elicited in WT and $OPN^{-/-}$ osteoblasts with comparable magnitude. These findings are strongly indicative that OPN deficiency exclusively improves Gi protein-mediated signaling.

Extracellular OPN disrupts Gi protein-mediated receptor signal transduction

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, we 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).**

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

OPN is well 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, we first explored whether CD44 is responsible for the inhibitory effect of OPN on GiPCR signaling. For this purpose, we first interfered the interaction between OPN and CD44, 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. To exclude the possibility that the lack of prevention 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.

We further used the small interfering RNA (siRNA) approach 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**). We found that 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 ($CD44^{-/-}$) mice (**Fig. 3, G and H)**. Moreover, these osteoblasts were less responsive to DAMGO or oxymethazolin when compared with osteoblasts from quadrupedal ($CD44^{-/-}$) mice (**Fig. 3, I and J**).

Collectively, these data indicate without ambiguity that CD44 is not involved in the inhibition of GiPCR signaling by OPN and support the view that there is a relationship between deficient GiPCR signaling and the development of scoliosis

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

We 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 integrins. For this purpose, small synthetic peptides were used to compete with integrin binding of the RGD or SVVYGLR sequences in OPN. We used BIO1211 to selectively inhibit $\alpha_4\beta_1$ integrin, the only SVVYGLR-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 SVVYGLR recognition specificity is 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 10μg/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 $OPN^{-/-}$ mice were used **(Fig. 4, E)**, indicating that the effect of RGD is strictly dependent on the presence of OPN.

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

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

Having established a probable role for RGD-dependent integrins in the inhibitory effect of OPN on GiPCR signaling in osteoblasts, we attempted to examine specific integrins that could mediate this effect. For this purpose, we used antibodies 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 β_3, β_5, α_v and α_8 antibodies. Conversely, antibodies against α_5 or β_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 α_5 and β_1 integrins are the primary mediators of the inhibitory effect of OPN on GiPCR signaling.

To further support this hypothesis, we used siRNA approach. When compared with untreated cells, MC3T3-E1 transfected with scrambled siRNA demonstrated less response, while cells transfected with α_5 or β_1 integrins siRNA demonstrated high response in the presence of rOPN. Cells transfected with both α_5 and β_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 $CD44^{-/-}$ 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 dimmer involved in mediating the inhibitory effect of OPN on GiPCR signaling in osteoblasts.

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

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 (MO), lysophosphatidic acid type 1 (LPA1) and melatonin type 2 (MT2) 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, we examined the effect of rOPN on the expression of Gi proteins. 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 Gi1, Gi2 and Gi3 isoforms (**Fig. 6 B**). The qPCR analysis also revealed no significant difference in 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.

OPN reduces the availability of Gi proteins for their cognate receptors

Gi proteins have been shown to be recruited by β_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 β_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 β_1 integrin receptors, and the presence of Gi proteins in each precipitate was examined by western blot using antibodies specific for Gi_1, Gi_2 or Gi_3 isoform. (**Fig. 6 D, E and F)** shows that the relative amount of Gi_1 isoform immunoprecipitated with MO or MT2 receptor was reduced in rOPN-treated cells compared to PBS-treated cells. Similar observations were noted for Gi_2 and Gi_3 isoforms. In contrast, the amount of each of these Gi protein isoforms was elevated in the β_1 integrin precipitates following rOPN treatment. These results suggest that OPN causes a kidnapping of Gi proteins and reduces their availability for GiPCRs.

OPN enhances the phosphorylation of Gi proteins

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_1, Gi_2 or Gi_3 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_1 precipitates obtained from cells treated with PBS or rOPN. However, band density was higher in OPN-treated cells. Similar observations were noted in

Gi_2 and Gi_3 precipitates (**Fig. 7 A)**. These results show that OPN enhances the phosphorylation of Gi proteins at serine and tyrosine residues.

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**).

To further associate these findings with the decreased GiPCR signaling in scoliosis, we examined directly whether Gi proteins undergone increased phosphorylation in osteoblasts from scoliotic mice. We probed the immunoprecipitated of Gi_1, Gi_2 and Gi_3 proteins with an anti-phospho-serine/threonine or an anti-phospho-tyrosine specific antibody and found that the level of phosphorylation of each Gi isoform was significantly greater in osteoblasts from scoliotic bipedal WT or $CD44^{-/-}$ 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.

Phosphorylation of Gi proteins induced by OPN involves various kinases

To identify which kinases are involved in the phosphorylation of Gi proteins induced by OPN, we pharmacologically inhibited FAK, MEK, ERK1/2, P38, JNK, PI3K, Src, PKC and CaMKII, which were described to be activated by OPN **(Denhardt et al.,**

2001a; Denhardt et al., 2001c) (Denhardt et al., 2001b) Cell extracts prepared from MC3T3-E1 that had been treated with rOPN in the presence or absence of inhibitor specific for each kinase were subjected to immunoprecipitation and western blot analysis. Results in (**Fig. 7 C-E)** show that 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), 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.

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

Several reports indicate that inhibition of Gi proteins disrupts signal from GiPCR and enhances GsPCR signaling **(Itoh et al., 1984); (Katada et al., 1985); (Katada et al., 1985); (Wesslau and Smith, 1992); (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)**.

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 protein probed with anti-tyosine 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 protein in the immunoprecipitates of MT2 and MO receptors (**Fig. 8 C-D**).

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.

Assuming that inhibition of Gi protein and phosphorylation of Gs protein are responsible for the increased GsPCR signaling associated with rOPN, our 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 notified 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.

Discussion

The consideration of OPN as a potential contributor in the pathophysiologic process underlying idiopathic scoliosis is a concept relatively recent that has emerged from the observation of high circulating OPN levels in patients affected with idiopathic scoliosis or asymptomatic children at risk of developing this disease. Results presented in the present study support such concept and demonstrate that OPN plays an important role in the development of spinal deformation by interfering with GiPCR signaling via a dual mechanism involving $\alpha_5\beta_1$ integrin.

Research involving idiopathic scoliosis has focused on many potential mechanisms, but in most cases, the demonstration of a direct link with development of the disease has been hampered by lack of appropriate animal models. A well-established model and largely utilized to explore the pathogenesis of idiopathic scoliosis is bipedal C57Bl/6 mice. These mice rapidly develop spinal deformity following 40 weeks of bipedal ambulation **(Oyama et al., 2006).** A major observation of the present study is that the genetic depletion of OPN prevented spinal deformation in these mice and enhanced the response to GiPCR stimulation in their osteoblasts, suggesting a role for OPN. Consistent with this, we found that scoliotic bipedal mice exhibited higher plasma OPN than non-scoliotic quadrupedal mice and responded less efficiently to the activation of GiPCR. Given these results and the demonstration by *in vitro* assay that OPN reduces response to GiPCR activation; our findings establish a causative relationship between, spinal deformation, GiPCR dysfunction and OPN.

It is well known that OPN functions by binding to CD44 or integrin receptors **(Weber et al., 1996); (Rangaswami et al., 2006).** We found that the inhibitory effect of OPN on GiPCR signaling is mediated by RGD-dependent integrins. There was no evidence for the involvement of CD44 receptor in the effects related to circulating OPN. Indeed, bipedal mice depleted from CD44 exhibited high circulating OPN levels and have developed spinal deformity. Interestingly, the inhibition of the expression of CD44 or of its interaction with OPN resulted in an exacerbated reduction in GiPCR signaling by OPN. These findings suggest that the ability of OPN to bind CD44 may interfere with the activation of integrins. It is possible that the blockade or the absence of CD44 causes a greater access to integrins.

Among the five RDG-dependent integrins characterized in osteoblasts, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_8\beta_1$ **(Hughes et al., 1997); (Gronthos et al., 1997); (Grzesik and Robey, 1994); (Clover et al., 1992); (Moursi et al., 1997); (Pistone et al., 1996),** we identified $\alpha_5\beta_1$ integrin to mediate the inhibitory effect of OPN on GiPCR signaling. This integrin appears to be of particular importance in GiPCR signaling, since it coprecipitates with Gi proteins **(Brown and Frazier, 2001); (Berg et al., 2007)**. In the present study, we found that the amount of Gi proteins increases in the immunoprecipitates of β_1 integrin while it decreases in those of GiPCRs following rOPN treatment. This suggests that rOPN reduces response to GiPCR activation by favouring the kidnapping of Gi proteins by β_1 integrin. However, the modest amount of Gi proteins in β_1 integrin precipitates contrasts with the strong reduction in response to GiPCR activation, suggesting an additional

parameter. In this regard, phosphorylation of Gi proteins has been demonstrated to contribute to diminishing GiPCR signaling in various cell types **(Katada et al., 1985); (Bushfield et al., 1991); (Strassheim and Malbon, 1994); (Murthy et al., 2000); (Shangguan et al., 2003)**. Interestingly, we found that OPN caused an increase in the phosphorylation of Gi proteins. The fact that this increase was prevented by the inhibition of β_1 integrin is strongly suggestive of involvement of the β_1 integrin downstream events being given that integrins do not have intrinsic activity. Consistent with this notion, we found that the rOPN effect on Gi protein phosphorylation was prevented by the inhibition of various molecular intermediates engaged in integrin signaling pathways.

Several reports indicate that integrin activation promotes various signaling pathways, many of which are mediated by tyrosine kinases, which in turn phosphorylate other kinases **(Illario et al., 2003).** Using several inhibitors, our results suggest that FAK, Src, MAPK, PI3K, PKC and CamK participate in the signaling cascade linking β_1 integrin activation by rOPN to Gi protein phosphorylation. Whether each of these kinases directly or indirectly phosphorylates was not examined in the present study. To date, Kinases that have been associated with direct phosphorylation of Gi proteins include the serine/threonine kinase PKC and the tyrosine kinase Scr. It has been shown that Src can phosphorylate most of members of the Gi protein family **(Hausdorff et al., 1992),** whereas PKC phosphorylates only Gi1 and Gi2 proteins **(Murthy et al., 2000).** Our observation that OPN causes increased phosphorylation of the three tested (Gi1, Gi2 and Gi3) Gi proteins isoforms indicates that, beside PKC, other serine/threonine kinases may be involved.

Although our findings suggest that Akt in PI3K downstream as well as MAPK and CaMKII could be good candidates, more definitive studies are required to identify the exact kinases responsible for the phosphorylation of each Gi protein isoform following OPN binding to β_1 integrin.

Nevertheless, on the basis of our collective findings and the available evidences, we present the hypothetical scenario schematized in (**Fig. 9**), to explain the mechanism by which OPN reduces GiPCR signaling and contributes to the development of spinal deformity. We propose that 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 kinase, which directly or indirectly phosphorylate a part of the remaining Gi protein 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).**

In conclusion, we show here for the first time that OPN plays a crucial role in the pathogenesis of idiopathic scoliosis, at least by reducing Gi protein-mediated receptor signaling via a mechanism involving $\alpha_5\beta_1$ integrin.

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Figures Legends

Figure 1 Genetic deletion of OPN protects bipedal C57BL6 from scoliosis by improving Gi protein-mediated receptor signal transduction. (A-D) Female C57Bl/6 wild-type (WT) and OPN knockout ($OPN^{-/-}$) 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. $OPN^{-/-}$ mice did not develop scoliosis in the quadrupedal or even bipedal while WT developed in the bipedal mice. (E) Plasma OPN was detected in C57Bl/6 (WT) and the bipedal showed higher plasma OPN than quadrupedal WT mice. (F-I) GiPCR signaling was checked in osteoblasts from WT and OPN-/- mice using DAMGO, somatostatin, oxymethazolin and apelin. The response was greater in $OPN^{-/-}$ than WT osteoblasts. On the other hand, bipedal WT mice showed lower response than the quadrupedal WT. (G-J) The pre-treatment of osteoblasts from WT and $OPN^{-/-}$ 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 $OPN^{-/-}$ 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 $OPN^{-/-}$ mice were less responsive to Gs stimulation than those from WT mice, whereas no difference in the Gq stimulation elicited in WT and $OPN^{-/-}$ osteoblasts.

Figure 2 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 was blocked by OPN specific antibody in MC3T3-E1 osteoblastic cell line gave the same results as (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 decrease in the integrated response in a concentration-dependent manner, which was prevented by OPN antibody.

Figure 3 CD44 is not involved in the inhibition of GiPCR signaling caused by extracellular OPN. (A) The blockage of CD44 by CD44 specific antibody in MC3T3-E1 osteoblastic cell line and cells treated with rOPN 0.5μg/ml did not affect the Gi signaling defect cause by OPN. DAMGO and oxymethazolin were used as agonist for GiPCR. (B-C) To validate the activity of 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 CD44 antibody. Moreover, the effect of 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 was demonstrated by qPCR and western blot analysis. (F) The knockdown of CD44 led to similar results as (A) and CD44 knockdown did not affect the Gi signaling defect cause by OPN. (G-H) rOPN treatment caused a concentration-dependent decrease in response to both DAMGO and oxymethazolin in osteoblasts from bipedal CD44 knockout (CD44-/-) mice. (I-J) Osteoblasts cells form bipedal ($CD44^{-/-}$) mice were less responsive to DAMG or oxymethazolin when compared with osteoblasts from quadrupedal ($CD44^{-/-}$) mice.

Figure 4 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) by bio1211 which selectively inhibits α4β1 integrin, the only SVVYGLR-containing integrin present in osteoblasts and did not affect on the OPN signaling while coincubation of cells with rOPN and RDG peptide completely prevented the inhibitory effect of rOPN in 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 $OPN^{-/-}$ mice was observed.

Figure 5 Identification of integrins involved in the inhibition of GiPCR signaling by OPN. The blockage of the different integrins in MC3T3-E1 osteoblastic cells (A-B-C) using specific antibodies and (D-E-F) the knockdown of these integrins in C57Bl/6 bipedal WT and $CD44^{-/-}$ was reversed the inhibitory effect of rOPN in response to both DAMGO and oxymethazolin through α5β1 integrin.

Figure 6 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; including mu-opioid (MO), lysophosphatidic acid type 1 (LPA1) and melatonin type 2 (MT2) receptors while Gi_1, Gi_2 and Gi_3 proteins isoforms expression was detected by western blot and qPCR. There was no significant effect of rOPN treatment on the quantity of Gi proteins or GiPCR. (D-E-F) Cells lysates were immunoprecipitated with antibodies against MO, MT2 or β1 integrin receptors, and the presence of Gi proteins in each precipitate was examined by western blot using antibodies specific for Gi_1, Gi_2 or Gi_3 isoform. The amount of each of these Gi protein isoforms was elevated in the β1 integrin precipitates following rOPN treatment.

Figure 7 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 cell lysates were IP by antibodies against Gi_1, Gi_2 or Gi_3 protein isoforms, and WB by anti-phospho serine/threonine or anti-tyrosine antibodies. (B) Osteoblasts from scoliotic bipedal WT or $CD44^{-/-}$ mice and quadrupedal control mice IP by antibodies against Gi1, Gi2 and Gi3 proteins then WB by antibodies against anti-phospho-serine/threonine or an anti-phospho-tyrosine. (C-E) Cell extracts prepared from MC3T3-E1 that had been treated with rOPN in the presence or absence of Kinases inhibitors then IP by antibodies against Gi_1, Gi_2 or Gi_3 protein isoforms, and WB by anti-phospho serine/threonine or anti-tyrosine antibodies.

Figure 8 The effect of OPN on GsPCR proteins. (A) MC3T3-E1 cells were treated with rOPN or PBS (A) stimulated by isoproterenol and desmopressin or (B) IP by antibodies against Gs then WB by antibodies against anti-phospho-serine/threonine or (C-D) IP by antibodies against MOR or MT2R then WB by 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.

Figure 9 OPN reduces GiPCR signaling and contributes to the development of spinal deformity. OPN binding, α5β1 integrin recruits a part of Gi proteins from the common pool, then promotes different signaling pathways leading to the activation of various kinase, which directly or indirectly phosphorylate a part of the remaining Gi protein in the common pool

Figure 1

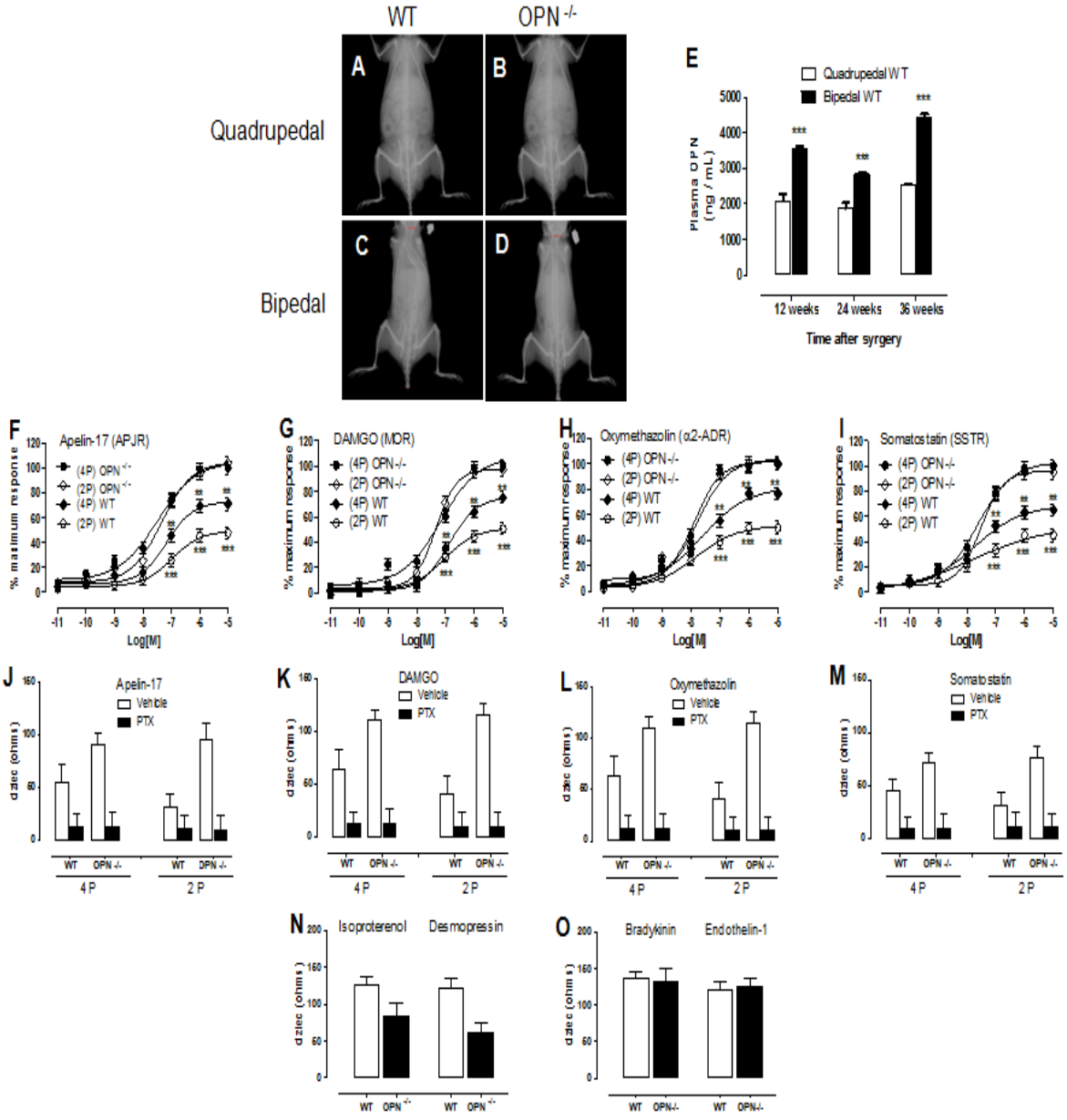

WT
OPN -/-
Quadrupedal
Bipedal
A
B
C
D
E
Quadrupedal WT
Bipedal WT
Plasma OPN (ng / mL)
12 weeks
24 weeks
36 weeks
Time after syrgery
F
Apelin-17 (APJR)
G
DAMGO (MOR)
H
Oxymethazolin (α2-ADR)
I
Somatostatin (SSTR)
% maximum response
Log[M]
(4P) OPN -/-
(2P) OPN -/-
(4P) WT
(2P) WT
J
Apelin-17
K
DAMGO
L
Oxymethazolin
M
Somatostatin
Vehicle
PTX
dZtec (ohms)
WT
OPN -/-
4 P
2 P
N
Isoproterenol
Desmopressin
O
Bradykinin
Endothelin-1

Figure 2

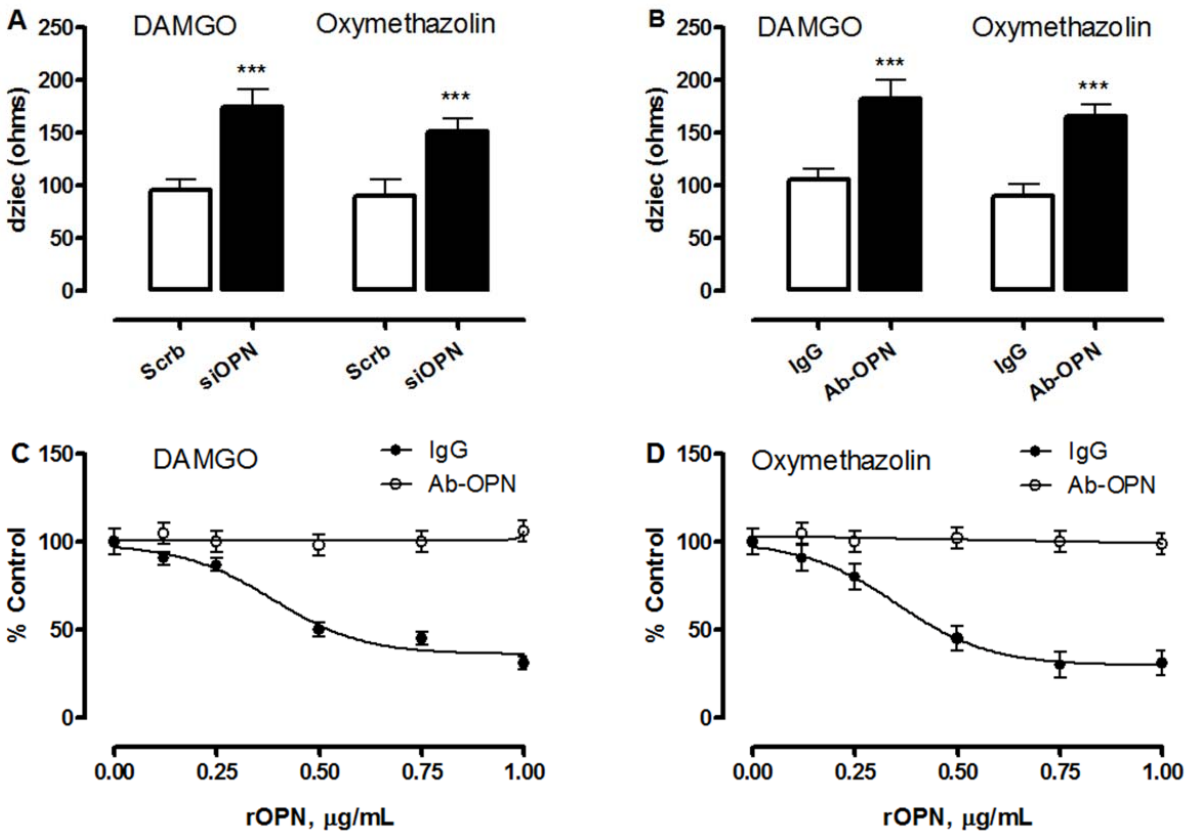

A
DAMGO
Oxymethazolin
dziec (ohms)
250
200
150
100
50
0
***
***
Scrb
siOPN
Scrb
siOPN
B
DAMGO
Oxymethazolin
dziec (ohms)
250
200
150
100
50
0
***
***
IgG
Ab-OPN
IgG
Ab-OPN
C
DAMGO
IgG
Ab-OPN
% Control
150
100
50
0
0.00
0.25
0.50
0.75
1.00
rOPN, µg/mL
D
Oxymethazolin
IgG
Ab-OPN
% Control
150
100
50
0
0.00
0.25
0.50
0.75
1.00
rOPN, µg/mL

Figure 3

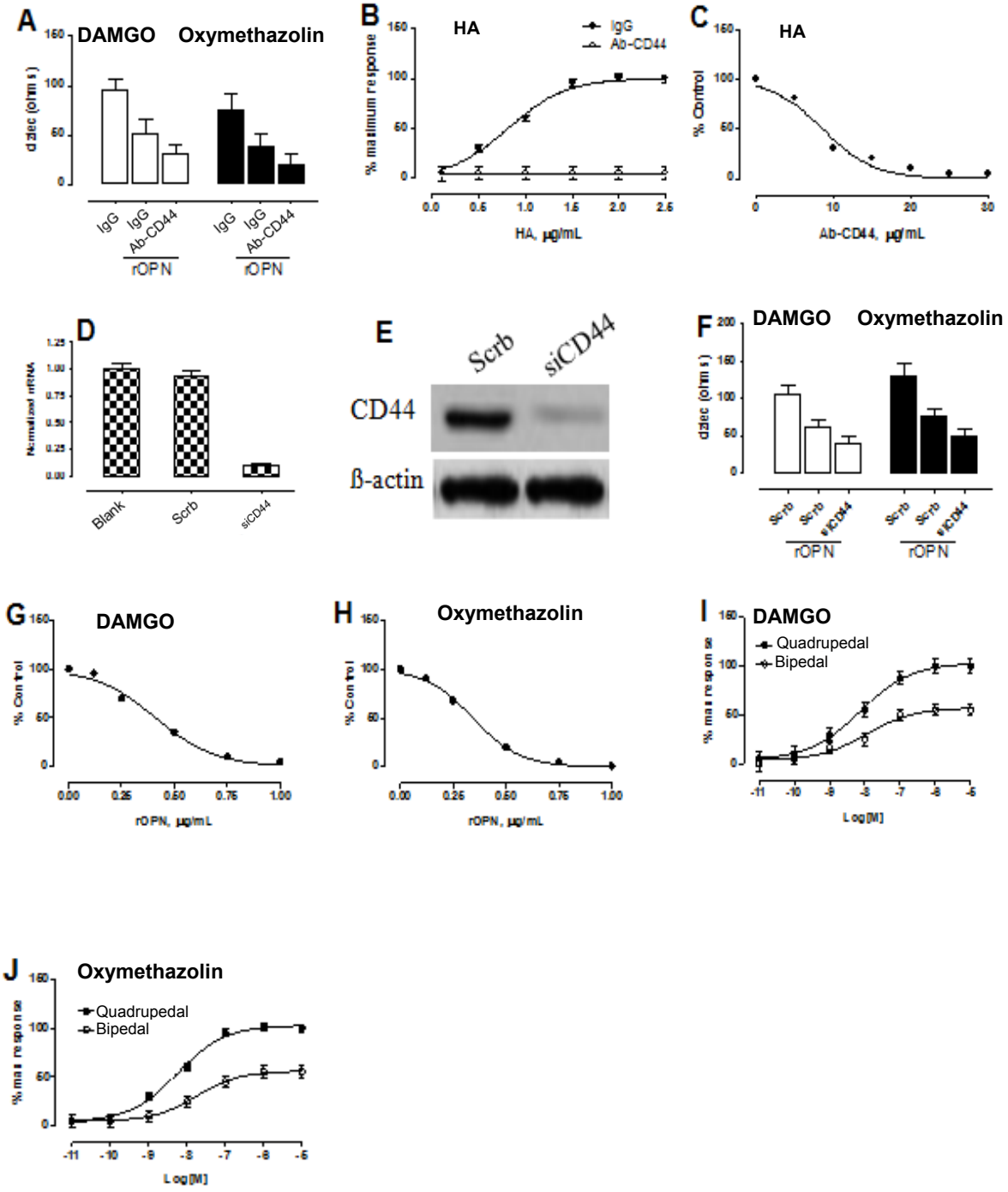

A
DAMGO
Oxymethazolin
IgG
IgG
Ab-CD44
rOPN
B
HA
IgG
Ab-CD44
HA, μg/mL
C
HA
Ab-CD44, μg/mL
D
Blank
Scrb
siCD44
E
Scrb
siCD44
CD44
ß-actin
F
DAMGO
Oxymethazolin
rOPN
G
DAMGO
rOPN, μg/mL
H
Oxymethazolin
rOPN, μg/mL
I
DAMGO
Quadrupedal
Bipedal
Log[M]
J
Oxymethazolin
Quadrupedal
Bipedal
Log[M]

Figure 4

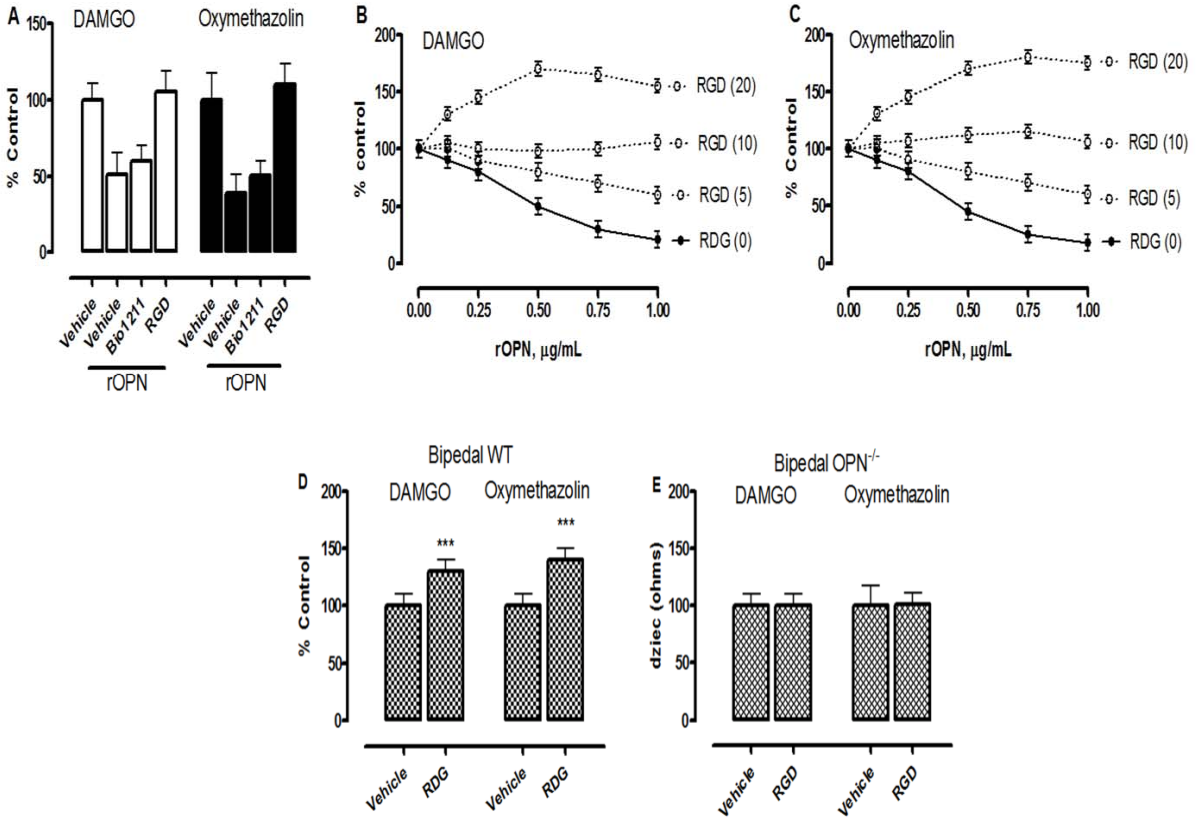

A
DAMGO
Oxymethazolin
% Control
Vehicle
Vehicle
Bio1211
RGD
rOPN
B
DAMGO
% control
RGD (20)
RGD (10)
RGD (5)
RDG (0)
rOPN, µg/mL
C
Oxymethazolin
% Control
RGD (20)
RGD (10)
RGD (5)
RDG (0)
rOPN, µg/mL
D
Bipedal WT
DAMGO
Oxymethazolin
% Control
***
RDG
E
Bipedal OPN-/-
DAMGO
Oxymethazolin
dziec (ohms)
RGD

Figure 5

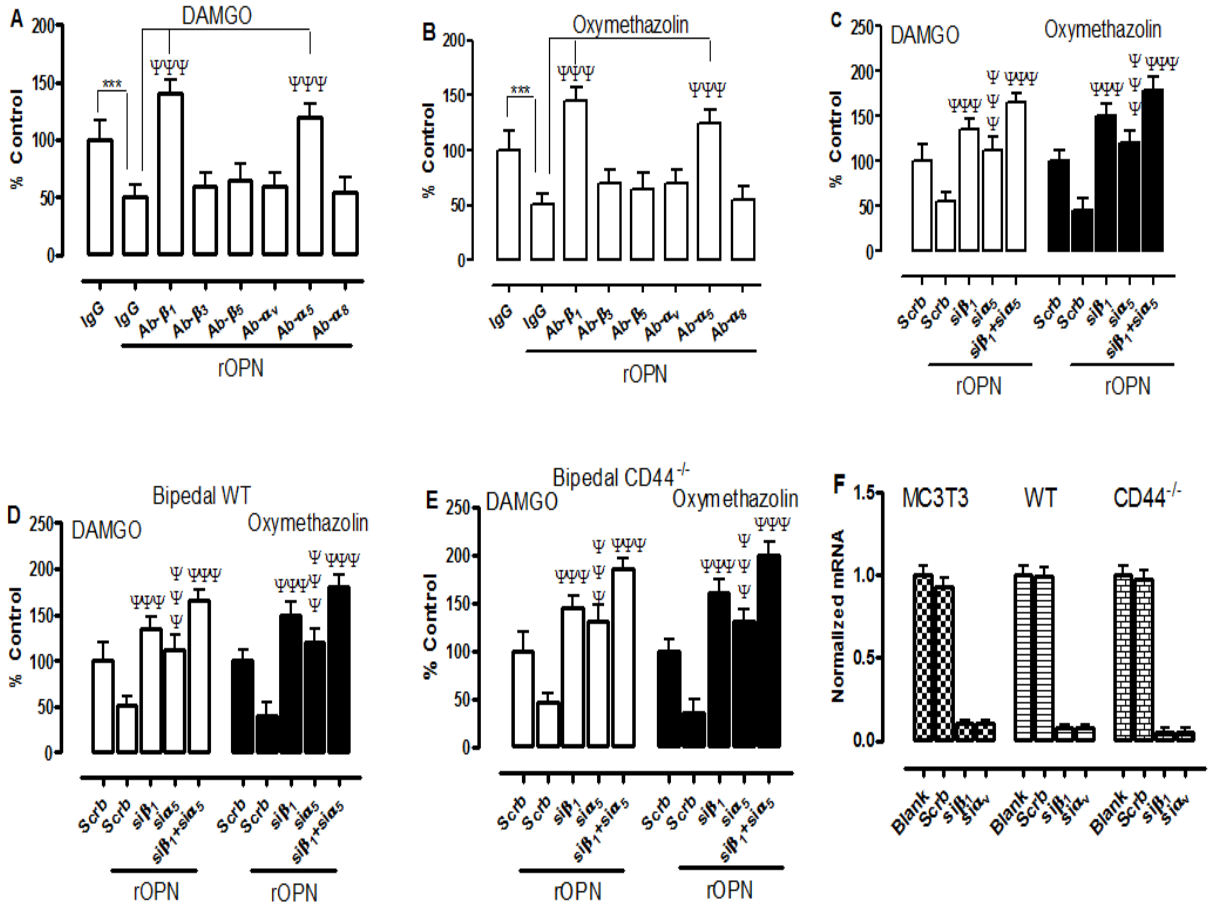

A
DAMGO
% Control
***
ΨΨΨ
IgG
IgG
Ab-β1
Ab-β3
Ab-β5
Ab-αv
Ab-α5
Ab-α8
rOPN
B
Oxymethazolin
% Control
***
ΨΨΨ
IgG
IgG
Ab-β1
Ab-β3
Ab-β5
Ab-αv
Ab-α5
Ab-α8
rOPN
C
DAMGO
Oxymethazolin
% Control
Scrb
Scrb
siβ1
siα5
siβ1+siα5
rOPN
rOPN
D
Bipedal WT
DAMGO
Oxymethazolin
% Control
Scrb
Scrb
siβ1
siα5
siβ1+siα5
rOPN
rOPN
E
Bipedal CD44-/-
DAMGO
Oxymethazolin
% Control
Scrb
Scrb
siβ1
siα5
siβ1+siα5
rOPN
rOPN
F
MC3T3
WT
CD44-/-
Normalized mRNA
Blank
Scrb
siβ1
siαv

Figure 6

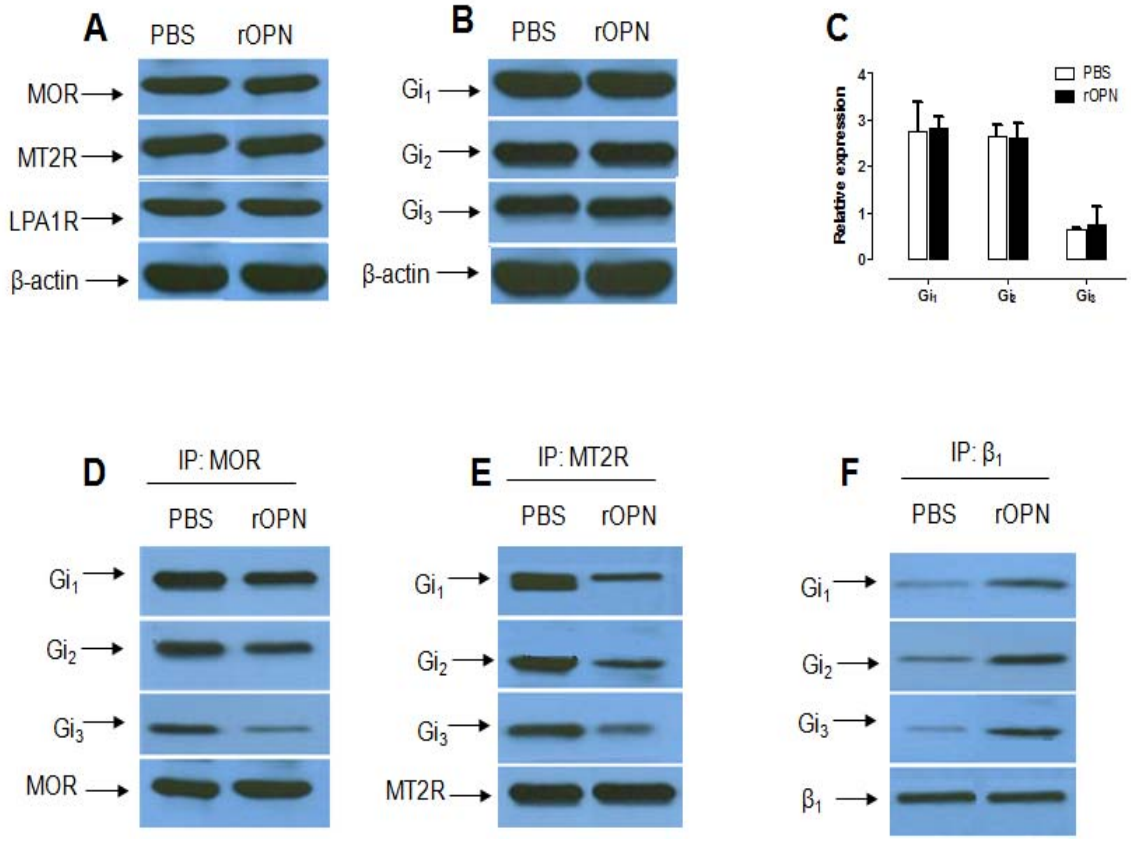

A
PBS
rOPN
MOR
MT2R
LPA1R
β-actin
B
PBS
rOPN
Gi1
Gi2
Gi3
β-actin
C
PBS
rOPN
Relative expression
0
1
2
3
4
D
IP: MOR
PBS
rOPN
Gi1
Gi2
Gi3
MOR
E
IP: MT2R
PBS
rOPN
Gi1
Gi2
Gi3
MT2R
F
IP: β1
PBS
rOPN
Gi1
Gi2
Gi3
β1

Figure 7

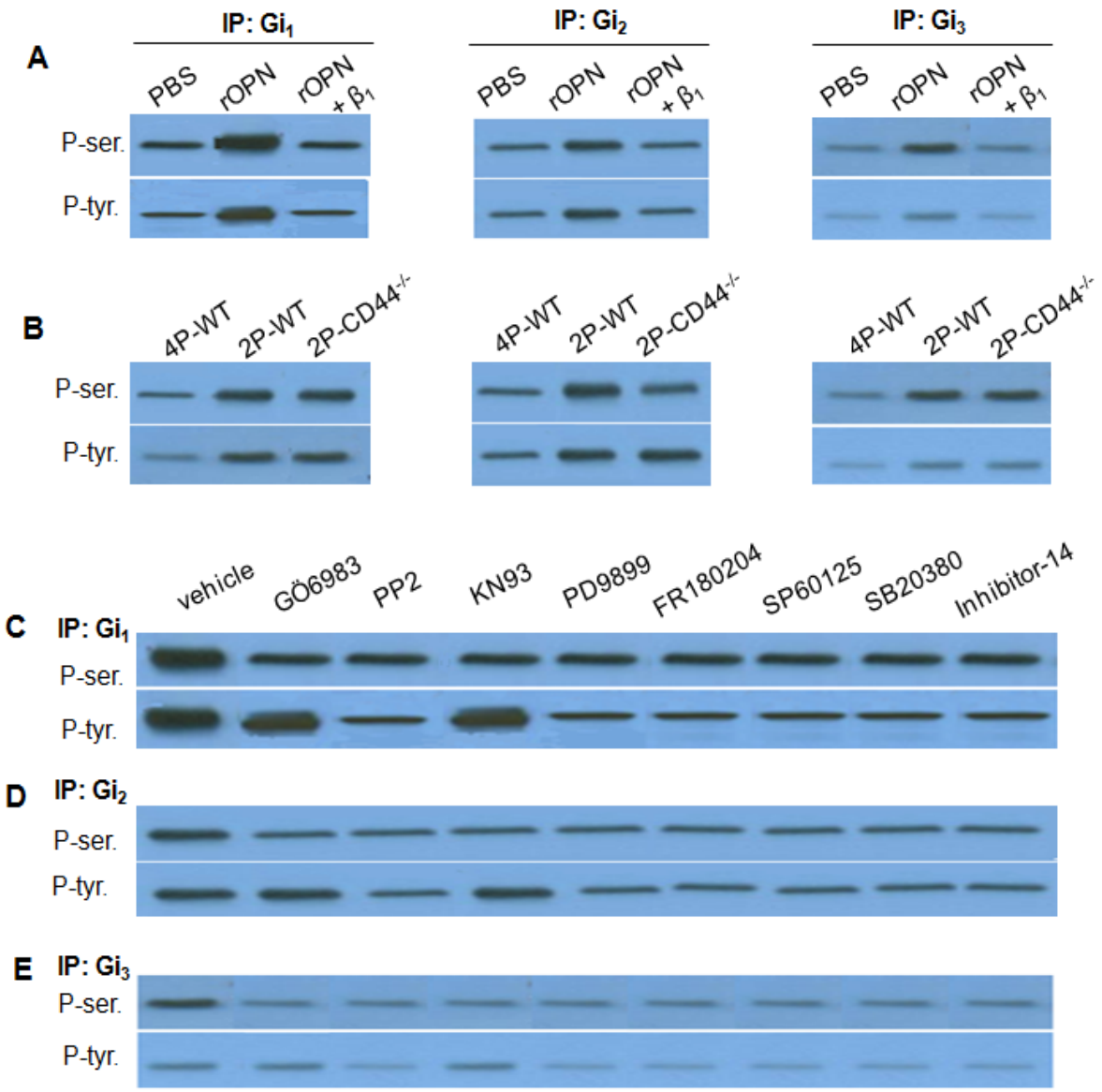

A
IP: Gi1
IP: Gi2
IP: Gi3
PBS
rOPN
rOPN + β1
P-ser.
P-tyr.
B
4P-WT
2P-WT
2P-CD44-/-
C
IP: Gi1
vehicle
GÖ6983
PP2
KN93
PD9899
FR180204
SP60125
SB20380
Inhibitor-14
D
IP: Gi2
E
IP: Gi3

Figure 8

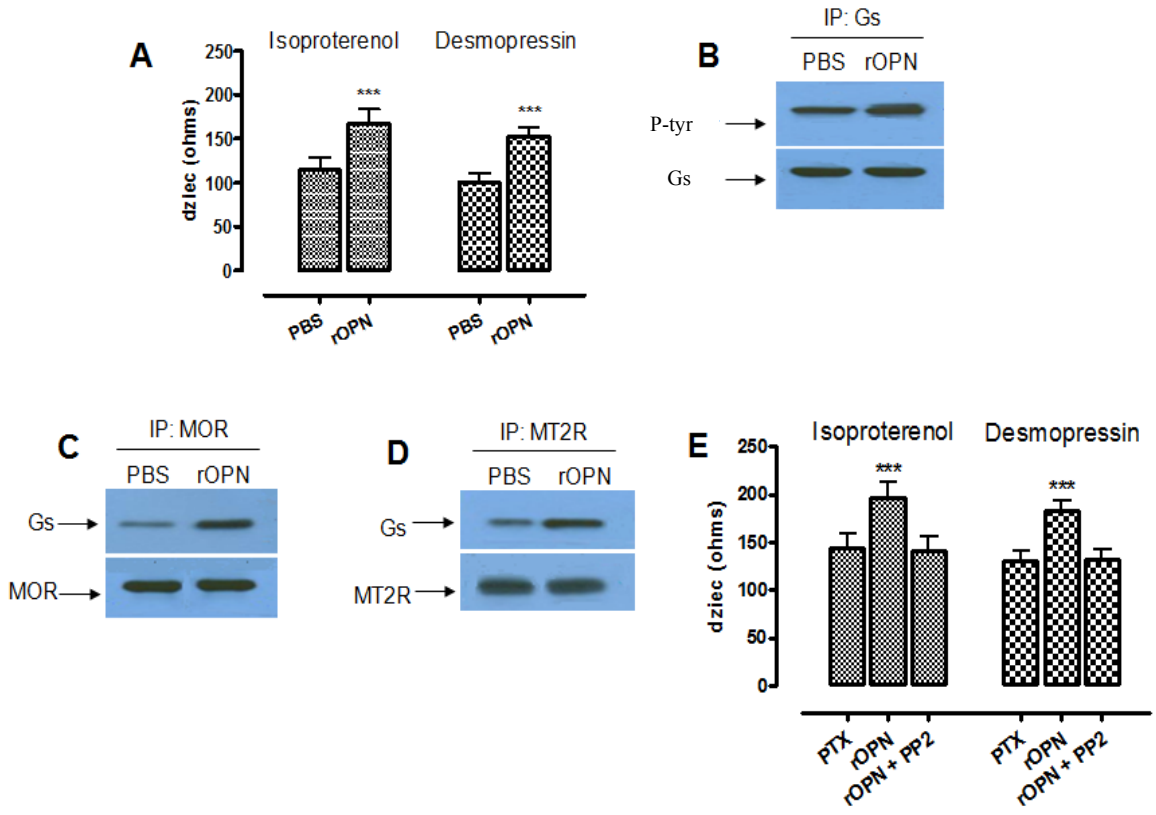
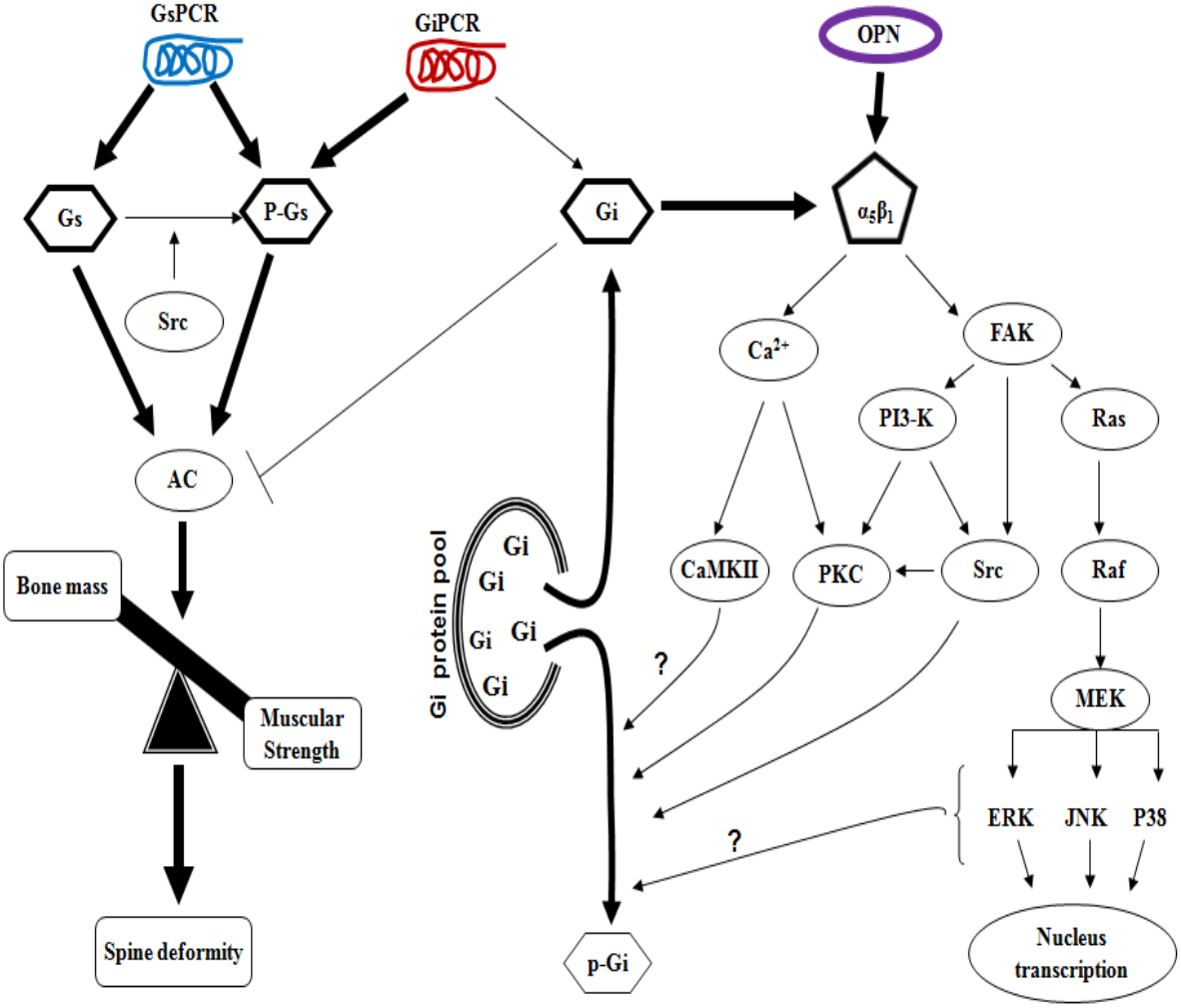

A
Isoproterenol
Desmopressin
dziec (ohms)
250
200
150
100
50
0
***
***
PBS
rOPN
PBS
rOPN
B
IP: Gs
PBS rOPN
P-tyr
Gs
C
IP: MOR
PBS rOPN
Gs
MOR
D
IP: MT2R
PBS rOPN
Gs
MT2R
E
Isoproterenol
Desmopressin
dziec (ohms)
250
200
150
100
50
0
***
***
PTX
rOPN
rOPN + PP2
PTX
rOPN
rOPN + PP2

Figure 9


GsPCR
GiPCR
OPN
Gs
P-Gs
Gi
$\alpha_5\beta_1$
Src
Ca^{2+}
FAK
PI3-K
Ras
AC
Bone mass
Gi protein pool
Gi
Gi
Gi
Gi
Gi
CaMKII
PKC
Src
Raf
?
MEK
Muscular Strength
ERK
JNK
P38
?
Spine deformity
p-Gi
Nucleus transcription

The third manuscript: PTPμ deficiency aggravates the harmful role of osteopontin in Idiopathic Scoliosis

We recently demonstrated how OPN played a vital role in the development of the spinal deformity through its interaction with α5β1 integrin. In this manuscript we demonstrated that the Gi protein coupled receptor (GiPCR) signaling dysfunction mediated by the elevation of plasma OPN in AIS patients is exacerbated by the depletion of PTPμ. The knockout of PTPμ in C57Bl/6 bipedal mice stimulates the incidence and severity of the spinal deformity without affecting plasma levels of OPN or the expression of its receptors. PTPμ is also downregulated in AIS patients. Interestingly, this downregualtion of PTPμ is more prevalent in the severe AIS cases classified in FG2 group when compared to the two other groups and healthy subjects. Although, OPN causes GiPCR signaling dysfunction through binding to α5β1 integrin in scoliotic bipedal mice, PTPμ potentiates the binding of OPN to α5β1 integrin when compared to others OPN receptors. This mechanism mediated by PTPμ is regulated through phosphatidylinositol-phosphate kinase type 1 γ (PIPK1γ).

In this third manuscript, I conceptualized and designed the study, performed the immunoprecipitation and western blots experiments and I have done siRNA transfections, CDS analysis and carried out the initial analyses, drafted the initial manuscript and approved the final manuscript as submitted.

Dr. Marie-Yvonne Akoume contributed in the experimental design and in the generation of the results. More specifically she did CellKey^{TM} experiments for some functional experiments, reviewed and revised the manuscript, and approved the final manuscript as submitted.
Mr. Saadallah Bouhanik contributed in the generation of the bipedal mice and their phenotypic characterization and approved the final manuscript as submitted.
Ms. Anita Franco contributed to the ELISA experiments and approved the final manuscript as submitted.
Dr. Alain Moreau contributed to the study conception, the analysis and interpretation of the data. He critically revised the manuscript for important intellectual content and approved the final manuscript as submitted.

PTPμ DEFICIENCY AGGRAVATES THE HARMFUL ROLE OF OSTEOPONTIN IN IDIOPATHIC SCOLIOSIS

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Phone: 514-345-4931ext 3476; Fax: 514-345-4801

This manuscript will be submitted to the journal of Federation of American Societies for Experimental Biology (FASEB)

ABSTRACT

Recent studies suggest that osteopontin (OPN) plays a critical role in the development of idiopathic scoliosis by reducing Gi protein-coupled receptor (GiPCR) signaling via β1 integrin engagement. Here, we establish a link between severity of scoliosis associated with OPN action and lack of protein tyrosine phosphatase μ (PTPμ), a negative regulator of integrin activation. We demonstrate that genetic deletion of PTPμ enhances the incidence and severity of scoliosis without affecting plasma levels of OPN or the expression of its receptors. In contrast, increased interaction of OPN with β1 integrin was notified in cells depleted of PTPμ. Furthermore, reduction of GiPCR signaling by OPN was also to the higher extent in these cells, while their response to GiPCR stimulation was improved with siRNA of phosphatidylinositol-phosphate kinase type I gamma (PIPK1γ), a PTPμ substrate that favours ligand binding to integrin. These studies provide the first indication that the loss of PTPμ exacerbates spinal deformity progression, possibly by amplifying the inhibitory effect of OPN on GiPCR signaling.

INTRODUCTION

Idiopathic scoliosis is a three-dimensional deformity of the spine with lateral curvature accompanied by a vertebral rotation (1). Despite considerable advances in research involving this disease, the aetiology and pathogenesis remain unclear (2), (3), (4). From the current understanding, it is generally accepted that the cause of idiopathic scoliosis is multifactorial (2), (3), (4) and an emerging concept indicate that biological process driving the pathogenesis may implicate defective GiPCR signaling (5), (6), (7).

Recently, we have identified OPN, a multifunctional cytokine, as a potentially key pathophysiologic contributor in the development of idiopathic scoliosis (Akoume et al., 2013a, 1st manuscript of the thesis). Particularly, we have documented increased plasma OPN levels in patients with idiopathic scoliosis and in bipedal mice, a well-established animal model of this disease. Furthermore, using OPN knockout mice, we demonstrated that a lack of OPN, protects bipedal mice against scoliosis. We further showed that OPN reduces GiPCR signaling in *vitro* via a dual mechanism involving β1 integrin engagement.

It is known that the engagement of integrins by ligands is facilitated by the binding of talin that allows integrins to switch from the low affinity to the high affinity form for ligands (8), (9), (10). Previously, talin binding to integrin was shown to be modulated by PIPK1γ. This enzyme generates the lipid second messenger phosphatidylinositol-4,5 biphosphate (PI4,5P2), which promotes the interaction of talin with integrin (11). Tyrosine phosphorylation of PIPK1γ upon FAK-dependent Src activation is essential for this interaction since it stimulates the catalytic activity of this enzyme, resulting in enhanced

PI4,5P2 production in the vicinity of integrin and further promotion of talin binding to integrin (12), (13). Termination of this process occurs following dephosphorylation by various tyrosine phosphatases among which PTPμ has been identified as the most promising for maintaining PIPK1γ in a hypophosphorylated state (14).

Based on these considerations, we hypothesized that lack of PTPμ may exacerbate the inhibitory effect of OPN on GiPCR signaling and stimulate spinal deformity progression. Our results show that the genetic deletion of PTPμ enhances the incidence and severity of scoliosis, possibly by enhancing the interaction of OPN with β1 integrin. This study highlights the importance of the inhibitory effect of OPN on GiPCR signaling in the pathogenesis of idiopathic scoliosis.

Materials and Methods

Patient recruitment

The Institutional Review Board of The Sainte-Justine Hospital, Montreal, Quebec approved this study. Parents or legal guardians of all participants gave their written informed consent, and minors gave their assent. One of the orthopedic surgeons at the Saint-Justine Children's hospital clinically assessed each patient, and all bone biopsies were collected during corrective surgeries. For AIS patients, bone specimens were obtained intra-operatively from vertebrae (varying from T3 to L4 according to the surgical procedure performed), while with trauma cases (used as non-scoliotic controls), bone specimens were obtained from other anatomical sites (tibia, femur or iliac crest).

Experimental animal models

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.

The bipedal mouse model has been generated as described by Oyama et al (2006) (15). Amputation of the forelimbs and tail was performed under anesthesia after weaning (5-weeks after birth), as reported by Oyama et al. (2006) (15). A Faxitron X-ray instrument (Faxitron MX20- Faxitron Co., Arizona, USA) was used to image and examine the spine of these mice monthly post-weaning, up until their sacrifice at nine months of age. Bipedal surgeries were performed on 120 mice from each wild type, PTPμ knockout (a gift from Dr. Gebbink MF, Laboratory of Thrombosis and Haemostasis, Department of Clinical

Chemistry and Haematology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands), and OPN knockout mice (a gift from Dr. Susan R. Rittling and Dr. David T. Denhard, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854, USA).

Derivation of primary osteoblast cultures

In human subjects, we derived primary osteoblast cell cultures from AIS and control patient biopsies that were obtained intra-operatively. 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 αMEM medium containing 10% fetal bovine serum (FBS; certified FBS, Invitrogen, Burlington, ON, Canada) and 1% penicillin/ streptomycin (Invitrogen) at 37°C in 5% CO_2, in a 10-cm^2 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 of the PTPμ gene was studied by qPCR. Transcript expression was assessed with the Stratagene Mx3000P (Agilent Technologies, La Jolla, CA).

Quantitative reverse transcription-polymerase chain reaction (qPCR)

Thermo-Script reverse transcriptase (Invitrogen) was used to reverse 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:

β-actin forward 5'-GGAAATCGTGCGTGACAT-3',

β-actin reverse 5'-TCATGATGGAGTTGAAGGTAGTT-3',

PTPμ forward 5'-GGCCGGACTTTTGCTAACT-3',

PTPμ reverse 5'-TGTGCTATACGGCTCATCAAA-3',

CD44 forward 5'-AGCATCGGATTTGAGACCTG-3',

CD44 reverse 5'-TGAGTCCACTTGGCTTTCTG-3'

β1 integrin forward 5'-ATGTGTCAGACCTGCCTTG-3',

β1 integrin reverse 5'-TTGTCCCGACTTTCTACCTTG-3',

β3 integrin forward 5'-GGAAAGTCCATCCTGTATGTGG-3'

β3 integrin reverse 5'-GAGTTTCCAGATGAGCAGGG-3'

αv integrin forward 5'-GTCCCCACAGTAGACACATATG-3',

αv integrin reverse 5'-TCAACTCCTCGCTTTCCATG-3',

α1 integrin forward 5'-GACATTTGGATGAACTTTAGTCACC-3',

α1 integrin reverse 5'-GGCAATGGAATTCACGACTTG-3',

α4 integrin forward 5'-GGATGAGACTTCAGCACTCAAG-3',

α4 integrin reverse 5'-GGTGAAATAACGTTTGGGTCTTTG-3',

β3 integrin forward 5'-GGAAAGTCCATCCTGTATGTGG-3',

β3 integrin reverse 5'-GAGTTTCCAGATGAGCAGGG-3',

β5 integrin forward 5'-CTTGCACTCCTGGCTATCTG-3',

β5 integrin reverse 5'-TGCGTGGAGATAGGCTTTC-3',

β8 integrin forward 5'-GATTGGGTTGCTTAAAGTCCTG-3',

β8 integrin reverse 5'-GGTAGGTGACTGCT CTTGTG-3'.

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 Strata-gene. Relative quantification was calculated with the delta CT method using β-actin as the endogenous control.

Isolation of plasma membrane (PM) protein from cell culture

Osteoblasts from human subjects were washed the cell monolayer 3 times with cold PM (plasma membrane) buffer [0.25 M Sucrose, 1mM EDTA and 20 mM Tricine] and 2 ml of cold PMC buffer (PM buffer plus 1X protease inhibitors, 1mM PMSF, 0.4 mM Sodium Orthovanadate) was added. The cells were scraped from the petri dishes and centrifuged at 1000xg for 5 min. The pellet (5 cm) was dissolved in 600 µl of cold PMC buffer. The pellet was then homogenized using ceramic beads (Precellys) 3x 5500xg for 20″ with 2 min between each cycle, and then centrifuged at 1000xg for 10 min at 4°C. The Post-Nuclear Supernatant (PNS) was kept on ice. The pellet was resuspended in 300 µl PMC buffer, the homogenization step was repeated once more and then centrifuged at

1000xg for 10 min at 4°C. The protein concentration was measured; PNS was layered on the top of 15 ml of 30% percoll with PMC buffer (in 25x 89 mm tubes). The samples were centrifuged in a fixed angle rotor at 84,000xg (50.2Ti rotor) for 30 min at 4°C. PM fraction was visible as a band at a distance of 5.7 cm from the bottom of tube. To remove any trace of percoll, the samples were centrifuged in a S45-A rotor in a sorval M150 micro-ultracentrifuge at 105,000xg (TLA100.4 rotor) for 90 min. A tightly packed pellet was formed by the percoll and PM fraction was carefully removed and stored at -80°C for immunoprecipitation and western blot methods. The concentration of protein was measured using, Protein Bio Rad, (Bio-Rad laboratories, California, U.S.A).

Immunoprecipitation and western blot

A pre-clearing step was done to reduce the non-specific binding of proteins to agarose or sepharose beads. Briefly, 25 µl of protein sepharose (A) beads (GE Healthcare Bioscience AB, Canada) were added to the PM protein solution (1.0mg). The mix was incubated for 30 minutes at 4°C with gentle agitation. The solution was then centrifuged at 16,200 xg at 4°C for 1 minute. The pellet was discarded and the supernatant was kept for immunoprecipitation. To immunoprecipitate the PTPµ we added 1µg of anti-PTPµ antibody (SC-25433), (Santa Cruz Biotechnology Inc., Santa Cruz, CA) /1 mg protein. The sample was incubated with the antibody overnight at 4°C with agitation. To each sample, 50µl of protein sepharose (A) beads were added and then mixed gently using wide-mouthed pipette tips. The lysate-beads mixture was incubated at 4°C with agitation for 2

hours. After incubation the tubes were centrifuged, the supernatant was removed and the beads were washed in PM buffer three times (each time centrifuging at 4°C and removing the supernatant). Finally, the supernatant was removed and 50 µl of 3x loading (Laemmli) buffer was added. Samples were boiled at 100°C for 5 minutes to denature the protein and separate it from the protein a beads, then samples were centrifuged and the supernatant was kept for the protein marker. Samples were subjected to 5%-12% gradient SDS–PAGE, transferred to PVDF (polyvinylidene fluoride) membrane and immunoblotted using anti-PTPµ anti-mouse (1:500 dilution of primary antibodies; Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibodies, BioSource Inc. Camarillo, CA). Reactive bands were visualized using an enhanced chemiluminescent kit (BM Chemiluminescent blotting substrate POD) according to the manufacturer's specifications (Roche Diagnostic Corp., Indianapolis, IN). Similarly, in order to validate the interaction between OPN and its cognate receptors, the different receptors for OPN were immunoprecipitated using different antibodies. For each receptor 1µg was added per 1 mg protein these antibodies, integrin β1 (SC-6622), integrin β3 (SC-6627), integrin β5 (SC-5401), integrin α4 (sc- 6589), integrin α5 (sc-166681), integrin α8 (sc-30983) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and integrin αv (4711) (Cell signaling technology, Ontario, Ca). This Immunoprecipitation was followed by western blot using anti-OPN anti mouse (1/2000) (courtesy of Dr. Marc D. McKee, McGill University). The previous antibodies were used for western blot detection (1/ 1000).

Analysis of G protein signaling

The signaling capacity of G proteins was assessed from osteoblast cultures using cellular dielectric spectroscopy (CDS) performed on a CellKey™ apparatus (MDS Sciex, San Francisco, CA), as described in Akoume, et al., 2010. To determine the precise class of G protein activated, we used specific agonists that bind to GPCR and regulate either Gi, Gs or Gq proteins: Lysophosphatidic acid (LPA) (Sigma Aldrich, Canada), Melatonin (Mel) (Sigma Aldrich, Canada) and Somatostatin (Som) (Tocris Bioscience, Canada) were used to test Gi protein coupled receptors; Isoproterenol (Iso) and Vasopressin (Vaso) (both from Tocris Bioscience, Canada) to test Gs protein coupled receptors, and finally Bradykinin (BK) and Endothelin-1 (both from Tocris Bioscience, Canada) to test Gq protein coupled receptors.

Furthermore, Pertussis toxin (PTX) (Sigma Aldrich, Canada) was used to determine if the effect of the agonists was related to G αi proteins. This toxin is produced by *Bordetella pertussis* and it catalyzes the adenosine diphosphate (ADP)-ribosylation of some G-proteins at a cysteine residue near the C-terminus resulting in uncoupling of receptor and G-protein.

siRNA transfection

Primary osteoblast cell cultures from C57Bl/6 WT and PTPμ$^{-/-}$ were cultured (as described above). Lipofectamine RNAimax (Invitrogen) was applied for DNA transfection according to the manufacturer's instructions. The sequence of RNA oligo used for the knockdown of OPN is (CCA CAG CCA CAA GCA GUC CAG AUU A). The cells were

harvested for RNA extraction after 48hrs. The same procedure was followed for phosphatidylinositol-phosphate kinase type I gamma (PIPK1γ). The sequence of RNA oligo used for the knockdown of PIPK1γ is (CCU CCA CAU CGG GAU UGA UAU U).

Osteopontin immunosorbent assays

Peripheral blood samples from mice were collected in EDTA-treated tubes and then centrifuged. Derived plasma samples were aliquoted and kept frozen at -80°C until thawed and analyzed. Plasma concentrations of OPN were measured by capture enzyme-linked immunosorbent assays (ELISA) according to protocols provided by the manufacturer (IBL, Hamburg, Germany). The 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 reading were performed at 450 nm

Statistical analysis

Data are presented as mean ± 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.

RESULTS

Lack of PTPμ influences the nature of scoliosis associated with high plasma OPN in bipedal mice

Amputation of forelimbs and tails induces scoliosis in mice after 40 weeks of bipedal ambulation (16), (15) and increases their plasma OPN levels. Consistent with this approach, scoliosis was induced in female wild type (WT) and PTPμ knockout ($PTP\mu^{-/-}$) mice to examine the impact of PTPμ deficiency on the development of scoliosis under high plasma OPN conditions. Measurements of OPN in plasma from $PTP\mu^{-/-}$ mice were performed each 12 weeks during the experimental period. Results presented in (**Figure 1 A)**, have revealed no significant difference in plasma OPN level between normal scoliotic C57Bl/6 bipedal mice and PTPμ ko ones. At all time points, levels of plasma OPN in WT and $PTP\mu^{-/-}$ mice were similar. Of all mice examined by radiography at the final time point 36^{th} postoperative week, lateral curvature was apparent in 55 % of WT and 85 % of $PTP\mu^{-/-}$ mice, indicating a higher incidence of scoliosis in $PTP\mu^{-/-}$ than in WT mice (**Figure 1 B**). The lateral curvature was also more pronounced in $PTP\mu^{-/-}$ mice, as illustrated by representative radiographs in (**Figure 1 C and D)**, suggesting that scoliosis is more severe in $PTP\mu^{-/-}$ than in WT mice. These data emphasize that lack of PTPμ exacerbates spinal deformity progression and support a link between development of scoliosis and high plasma OPN in bipedal mice.

Lack of PTPμ amplifies the defective GiPCR signaling in bipedal mice

We have recently provided evidences for the occurrence of defective GiPCR signaling in bipedal mice by demonstrating a reduced ability of various GiPCR selective agonists to promote cell signaling as measured by CDS using mouse (Akoume et al., 2013b, 2nd manuscript of the thesis). To examine the impact of PTPμ$^{-/-}$ deficiency on this defect, osteoblasts from WT and PTPμ$^{-/-}$ mice were screened for their response to three GiPCR selective agonists identified in (**Figure 2)**. In agreement with our previous results, all three compounds evoked typical CDS response profiles of GiPCR in WT osteoblasts. Consistent with Gi coupling for their cognate receptors in these cells, response to each of the three tested compounds was blocked by pre-treatment with PTX (**Figure 2 A-F**). Similar results were obtained with PTPμ$^{-/-}$ osteoblasts (**Figure 2 G-L**). Results illustrated in Figure 3 show that all three compounds increased response in a concentration-dependent manner in osteoblasts from WT and PTPμ$^{-/-}$ mice. However, in each case, osteoblasts from PTPμ$^{-/-}$ mice were less responsive than those from WT mice, but EC50 values were similar in both groups (**Figure 3 A-C**). These results suggest that lack of PTPμ affects Gi protein activity independently of the receptor.

To relate these findings to the OPN action, we used the small interference RNA (siRNA) approach to knockdown the expression of OPN in WT and PTPμ$^{-/-}$ osteoblasts. The efficiency of siRNA in these osteoblasts was demonstrated by qPCR and western blot analysis (**Figure 3 D and E**). We found that the deletion of OPN enhanced response to GiPCR stimulation in WT and PTPμ$^{-/-}$ osteoblasts and abrogated the difference in the degree of their responses **(Figure 3 F-H**). These results support a role for OPN in the

defective GiPCR signaling in bipedal mice and suggest that lack of PTPμ exacerbates the inhibitory effect of OPN on GiPCR signaling.

To test this hypothesis, WT and PTPμ$^{-/-}$ osteoblasts were treated with varying concentrations of exogenous recombinant OPN (rOPN) prior to GiPCR stimulation with agonists identified in **(Figure 4 A-C)** in each case, rOPN caused decrease in the integrated response in a concentration-dependent manner, as well in WT as in PTPμ$^{-/-}$ osteoblasts. However, IC50 values were lower in PTPμ$^{-/-}$ compared to WT osteoblasts, suggesting that osteoblasts from PTPμ$^{-/-}$ mice are more sensitive to the inhibitory effect of OPN on GiPCR signaling

Lack of PTPμ influences the interaction of OPN with integrin in osteoblasts

We have previously demonstrated that OPN inhibited GiPCR signaling in osteoblasts cells via a dual mechanism involving $\alpha_5\beta_1$ integrin (Akoume et al., 2013b, 2nd manuscript of the thesis). Since OPN interacts with various receptors among which osteoblasts have been shown to express $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$ integrins and CD44 (17), (18), (19), (20), (21), (22), it was of interest to examine whether $\alpha_5\beta_1$ integrin is also the unique receptor responsible for the effects associated with PTPμ deficiency. For this purpose, we first examined the expression levels of all these mentioned receptors, using a qPCR analysis. As illustrated in (**Figure 5A)**, we found no significant difference in expression of integrin at the mRNA levels between WT and PTPμ$^{-/-}$ osteoblasts. Similar profiles were obtained when protein levels of these receptors were determined by western

blot (**Figure 5 B**). These results exclude the possibility that the effects associated with PTPμ deficiency implicate changes in receptor expression.

Then, we sought to examine if the interaction of OPN with receptor is influenced by PTPμ deficiency. For this purpose, cell lysates from WT and PTPμ$^{-/-}$ osteoblasts were immunoprecipitated with antibodies against OPN receptors and the interaction with OPN was revealed by western blot using antibody specific for OPN indicated in (**Figure 5 C)**. Results showed that OPN was co- immunoprecipitated with all receptors in WT and PTPμ$^{-/-}$ osteoblasts. However, levels of OPN in the β_1 and α_5 integrins immunoprecipitates were increased by more than 30 fold in PTPμ$^{-/-}$ osteoblasts compared to WT osteoblasts, while there was only a moderate difference (0,8 to 2,3 fold) in the levels of other integrins between both cell groups. In contrast, levels of OPN in CD44 immunoprecipitates were similar in WT and PTPμ$^{-/-}$ osteoblasts. These results suggest that loss of PTPμ favours the interaction of OPN with integrins in osteoblasts and indicate that $\alpha_5\beta_1$ integrin is possibly the most promising receptor responsible for the effects associated with PTPμ deficiency.

Silencing of PIPK1γ selectively enhances GiPCR signaling in PTPμ$^{-/-}$ osteoblasts

To understand the molecular basis of the interaction of OPN with integrin in the absence of PTPμ, we gave special attention to PIPK1 because the catalytic activity of PIPK1γ by tyrosine phosphorylation is essential for enhancing the affinity of integrin for ligands, and this enzyme has been shown to be dephosphorylated by PTPμ$^{-/-}$. Therefore, we hypothesized that loss of PTPμ amplifies the reduction in GiPCR signaling through the sustained activation of PIPK1γ. To test this hypothesis, we first examined the phosphorylation status of PIPK1γ in WT and PTPμ$^{-/-}$ osteoblasts. Accordingly, cell lysates were immunoprecipitated with PIPK1γ antibody and probed with phospho-tyrosine antibody. As expected, phosphorylation levels of PIPK1γ were higher in osteoblasts from PTPμ$^{-/-}$ than those from WT mice, while levels of PIPK1γ total form were comparable between both phenotypes (**Figure 6 A**). To examine whether FAK and Src are responsible for the increased phosphorylation of PIPK1γ observed in osteoblasts from PTPμ$^{-/-}$ mice, cells were treated with inhibitor of Src (PP2) and FAK (inhibitor-14) prior to immunoprecipitation assay. As expected, levels of PIPK1γ phosphorylation were attenuated by both treatments. This suggests that the activity of PIPK1γ is abnormally elevated in osteoblasts from PTPμ$^{-/-}$ mice upon FAK and Src action.

To further provide the implication of PIPK1γ in the mechanism of action behind PTPμ deficiency, we used SiRNA approach to knockdown the expression of PIPK1γ in WT and PTPμ$^{-/-}$ osteoblasts prior to initiate GiPCR signaling with somatostatin stimulation. Efficiency of siRNA in WT and PTPμ$^{-/-}$ osteoblasts was confirmed by qPCR and western

blot analysis (**Figure 6 B and C**). Representative index curves illustrated in **(Figure 6 D-H)** show that impedance signatures were not affected in WT and PTPμ$^{-/-}$ osteoblasts depleted of PIPK1γ. Both cells exhibited typical CDS response of GiPCR following somatostatin stimulation. In contrast, the screening at varying concentrations of somatostatin revealed that depletion of PIPK1γ enhanced response to somatostatin stimulation in osteoblasts from PTPμ deficiency mice, but not in those from WT mice. In addition, PTPμ$^{-/-}$ osteoblasts depleted of PIPK1γ exhibited similar degree of response to somatostatin stimulation than WT osteoblasts, indicating that depletion of PIPK1γ abrogates the difference in their ability to respond to GiPCR stimulation.

Collectively, these results suggest that the amplified reduction in GiPCR signaling observed in osteoblasts from PTPμ$^{-/-}$ deficient mice is due to the dysregulation of PIPK1γ activity resulting from loss of PTPμ.

PTPμ is downregulated in osteoblasts from patients with idiopathic scoliosis

To explore the clinical relevance of the loss of PTPμ in the development of idiopathic scoliosis, we first investigate PTPμ expression level in patients with idiopathic scoliosis and healthy control subjects. We found that PTPμ expression was clearly detected in osteoblasts from control subjects but was decreased in those from patients with idiopathic scoliosis, whereas expression levels of β-actin were similar between both groups (**Figure 7 A and B**). We found at least a 50 % decrease in PTPμ mRNA in osteoblasts from scoliotic patients relative to control subjects. Similar results were obtained when cell

lysates were analysed by western blot to examine protein levels of PTPμ (**Figure 7C**). These data clearly indicate that PTPμ is downregulated in patients with idiopathic scoliosis.

DISCUSSION

We have recently established a causative role between circulating OPN and the development of scoliosis using bipedal mice, a well-established model of idiopathic scoliosis. These mice exhibit high plasma OPN levels and are protected from scoliosis by the genetic deletion of OPN (Akoume et al., 2013b, 2nd manuscript of the thesis). Our study has also revealed a defective GiPCR signaling in their osteoblasts and suggested that OPN contributes to pathogenesis of scoliosis in this model by interfering with GiPCR signaling. Results of the present study support such notion and provide the first evidence that severe manifestations of scoliosis under high plasma OPN conditions may implicate decrease in PTPμ protein. Indeed, the present study reveals that bipedal PTPμ$^{-/-}$ mice displayed greater incidence of scoliosis and exhibited more pronounced lateral curvature than bipedal WT mice. There was, however, no association between OPN concentrations and disease severity, since WT and PTPμ$^{-/-}$ mice exhibited similar plasma OPN levels. This observation was strongly suggestive that the relationship between OPN and PTPμ deficiency in bipedal mice extends beyond the OPN production and lies probably at the level of OPN action. In this regard, an earlier study from our laboratory has demonstrated that OPN has an inhibitory effect on GiPCR signaling via a mechanism that requires β_1 integrin engagement. Here, we add to these findings that the PTPμ deficiency leads to the amplification of the inhibitory effect of OPN on GiPCR signaling. We further provided the mechanistic insights

by demonstrating that lack of PTPμ favours the interaction of OPN with β_1 integrin via PIPK1γ action.

It should be noted that the association of integrins with theirs ligands can be regulated by the tyrosine phosphorylation of PIPK1γ upon FAK-dependent Src activation (12), (13) and a recent study has shown that PIPK1γ is dephosphorylated by PTPμ (14). Consistent with these observations, we found increased tyrosine phosphorylated PIPK1γ in osteoblasts from PTPμ$^{-/-}$ mice, and this increased phosphorylation was attenuated by the inhibition of FAK and Src with pharmacological inhibitors. Thus, our data demonstrate that PIPK1γ can be viewed as a key player in the increased reduction of GiPCR signaling observed in the absence of PTPμ. This is further supported by the observation that silencing of PIPK1γ by siRNA abrogated the difference in the degree of response between WT and PTPμ$^{-/-}$ osteoblasts. Collectively, these results suggest that loss of PTPμ causes a dysregulation of PIPK1γ activity, which in turn leads to the amplification of the inhibitory effect of OPN on GiPCR signaling and the subsequent severe lateral curvature.

It was particularly interesting to observe that osteoblasts from PTPμ$^{-/-}$ mice were more sensitive to the inhibitory effect of OPN on GiPCR signaling. This finding together with the fact that PTPμ$^{-/-}$ mice exhibited more severe lateral curvature, support the notion that repression of GiPCR signaling is an important event in biological process driving the development of scoliosis and indicate that decrease of PTPμ protein could underlie the major changes in the pathobiology of scoliosis and play an important role in severe progression of scoliosis in bipedal mice.

Our findings may have important clinical implications since by extending our study to humans, we found that all scoliotic patients examined exhibited lower levels PTPμ protein than control individuals. However, a disparity in PTPμ protein levels was observed among scoliotic patients. Therefore, it is tempting to speculate that patients exhibiting very low PTPμ protein levels will be more sensitive to OPN effect and more likely at risk for severe progression of idiopathic scoliosis.

Early prediction of clinical outcome in individuals affected with idiopathic scoliosis could be crucial to improve the treatment of severe scoliosis. Thus, the expression levels of PTPμ could potentially be used as a marker for predicting the clinical outcome of affected individuals. However, future studies will be necessary to correlate PTPμ expression levels with the risk of scoliosis progression and to better define the thresholds below which PTPμ expression could become rate limiting for severe progression of scoliosis. In this context, we have previously categorized three groups of scoliotic patients according to their degree of alteration in GiPCR signaling (5), (7), (Akoume et al., 2013a. 1st manuscript of the thesis)

On the other hand, we have suggested a genetic factor in the defective GiPCR signaling associated with idiopathic scoliosis, by demonstrating that affected individuals from the same family exhibited the same degree of GiPCR signaling defect and belonged to the same functional group (Akoume et al., 2013a, 2nd manuscript of the thesis). Therefore, it should be expected that loss of PTPμ protein could also contribute to genetic predisposition for sensitivity to the OPN action on GiPCR signaling and to the development of severe idiopathic scoliosis in children of affected parents. Thus, identifying and targeting the

genetic and epigenetic mechanisms underlying PTPμ downregulation in individuals with idiopathic scoliosis will be a first step toward identifying strategy for preventing PTPμ repression.

CONCLUSION

To our knowledge, the present study provides the first evidence that lack of PTPμ can exacerbate spinal deformity progression, possibly by amplifying the inhibitory effect of OPN on GiPCR signaling. In addition, this study identifies dysregulation of PIPK1γ due to the loss of PTPμ as a unique mechanism underlying development of severe lateral curvature under high plasma OPN conditions.

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Figures Legends

Figure 1 Lack of PTPμ influences the nature of scoliosis associated with high plasma OPN in bipedal mice. (A) Plasma OPN was detected in C57Bl/6j Wild type (WT) and PTPμ-/- each 12 weeks during the experimental period. No difference was detected at all time points in the plasma OPN levels of WT and PTPμ$^{-/-}$ mice. The higher values were notified at the 36th postoperative week in both phenotypes. (B, C & D) All the mice were examined by radiography the PTPμ$^{-/-}$ showed increase in the incidence and severity comparing to wild type mice.

Figure 2 Specificity of the agonists to GiPCR signaling. The pre-treatment of osteoblasts from WT and PTPμ$^{-/-}$ mice with PTX blocked the Gi coupling for their cognate receptors in these cells, response to each of the three tested compounds (Apelin- 17 (APJR), Oxymethazolin (α2-ADR) & Somatostatin (SSTR)). (A-B-C) C57Bl/6j Wild type (WT) cells treated with vehicle (D-E-F) C57Bl/6j Wild type (WT) cells treated with PTX (G-H-I) C57Bl/6j (PTPμ$^{-/-}$) cells treated with vehicle (J-K-L) C57Bl/6j (PTPμ$^{-/-}$) Cells treated with PTX. This is indicating that these compounds evoked typical CDS response profiles of GiPCR in WT and PTPμ$^{-/-}$ osteoblasts.

Figure 3 Lack of PTPμ exacerbates the defective GiPCR signaling in bipedal mice.
(A-C) Osteoblasts from PTPμ$^{-/-}$ mice were less responsive than those from WT mice to Apelin-17 (APJR), Oxymethazolin (α2-ADR) & Somatostatin (SSTR). Different

concentrations for each agonist were used leading to increased response in a concentration-dependent manner in osteoblasts from WT and PTPμ$^{-/-}$ mice. (D-E) OPN was knockdown in WT and PTPμ$^{-/-}$ osteoblasts cells and knockdown efficiency was determined by qPCR and western blot. (F-G-H) The lack of PTPμ exacerbates the inhibitory effect of OPN on GiPCR signaling using the same three agonists which mentioned above.

Figure 4 Lack of PTPμ exacerbates the defective GiPCR signaling caused by OPN. (A-B-C) Osteoblasts from WT and PTPμ$^{-/-}$ osteoblasts were treated with varying concentrations of exogenous recombinant OPN (rOPN) prior to GiPCR stimulation with different concentrations of the agonists identified.

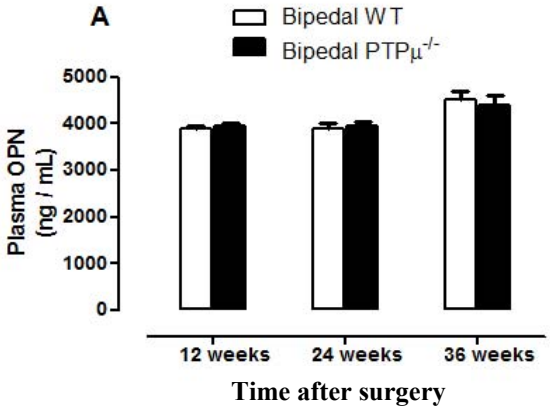
Figure 5 Lack of PTPμ influences the interaction of OPN with integrin in osteoblasts. (A-B) The expression of OPN receptors was determined in WT and PTPμ$^{-/-}$ osteoblasts using qPCR and western blot. (C) Osteoblasts cell lysates of WT and PTPμ$^{-/-}$were immunoprecipitated with antibodies against OPN receptors followed by western blot specific for OPN.

Figure 6 Silencing of PIPK1γ selectively enhances GiPCR signaling in PTPμ$^{-/-}$ osteoblasts. (A) Osteoblasts from PTPμ$^{-/-}$ mice, cells were treated with inhibitor of Src (PP2) and FAK (inhibitor-14) prior to immunoprecipitation assay. Phosphorylation levels of PIPK1γ were attenuated by both treatments while Phosphorylation levels of PIPK1γ were higher in osteoblasts from PTPμ$^{-/-}$ than those from WT mice. (B-C) PIPK1γ was

knockdown using siRNA approach in WT and PTPμ$^{-/-}$ osteoblasts cells and it is approved by the qPCR and western blot. (D-H) PIPK1γ was knockdown in WT and PTPμ-/- osteoblasts cells Osteoblasts depleted of PIPK1γ exhibited similar degree of response to somatostatin stimulation than WT osteoblasts.

Figure 7 PTPμ is downregulated in osteoblasts from patients with idiopathic scoliosis. (A-B-C) The expression of PTPμ was determined in osteoblasts from scoliotic patients relative control subjects using RT-PCT, qPCR and western blot. PTPμ is downregulated in idiopathic scoliosis patients comparing to control subjects.

Figure 1



B

	WT	PTPμ-/-
Total number	60	60
Scoliotic	33 (55 %)	51 (85 %)
Non scoliotic	27 (45 %)	9 (15 %)

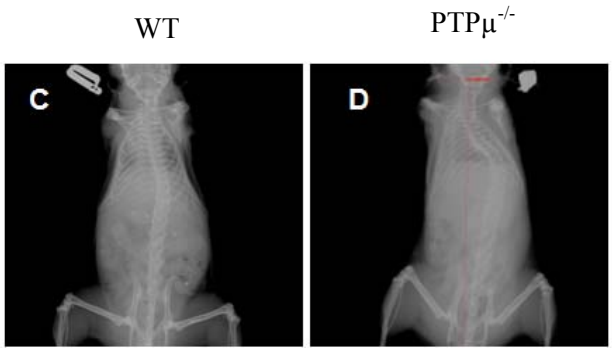


Figure 2

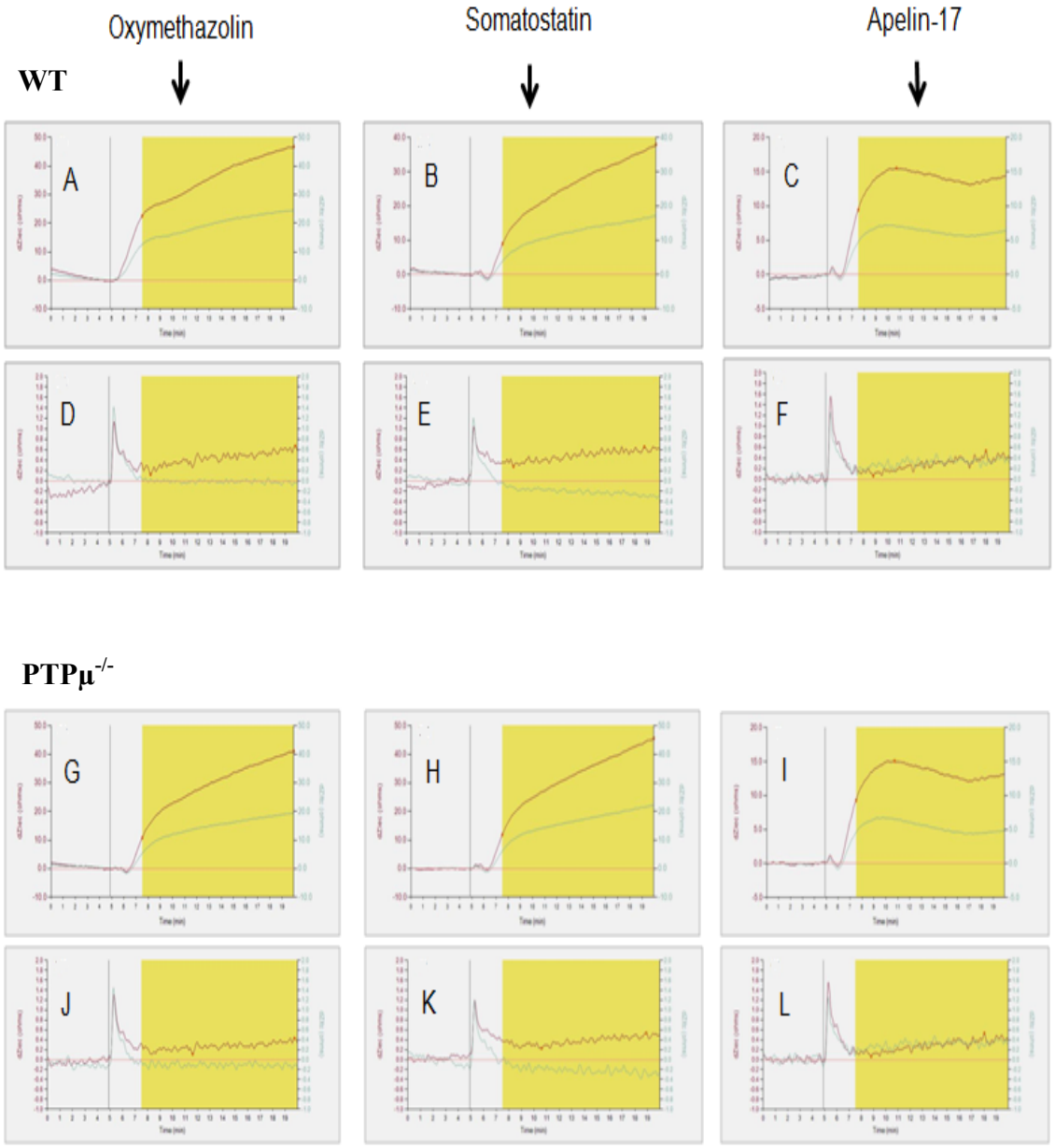

Oxymethazolin
Somatostatin
Apelin-17
WT
A
B
C
D
E
F
PTPμ-/-
G
H
I
J
K
L

Figure 3

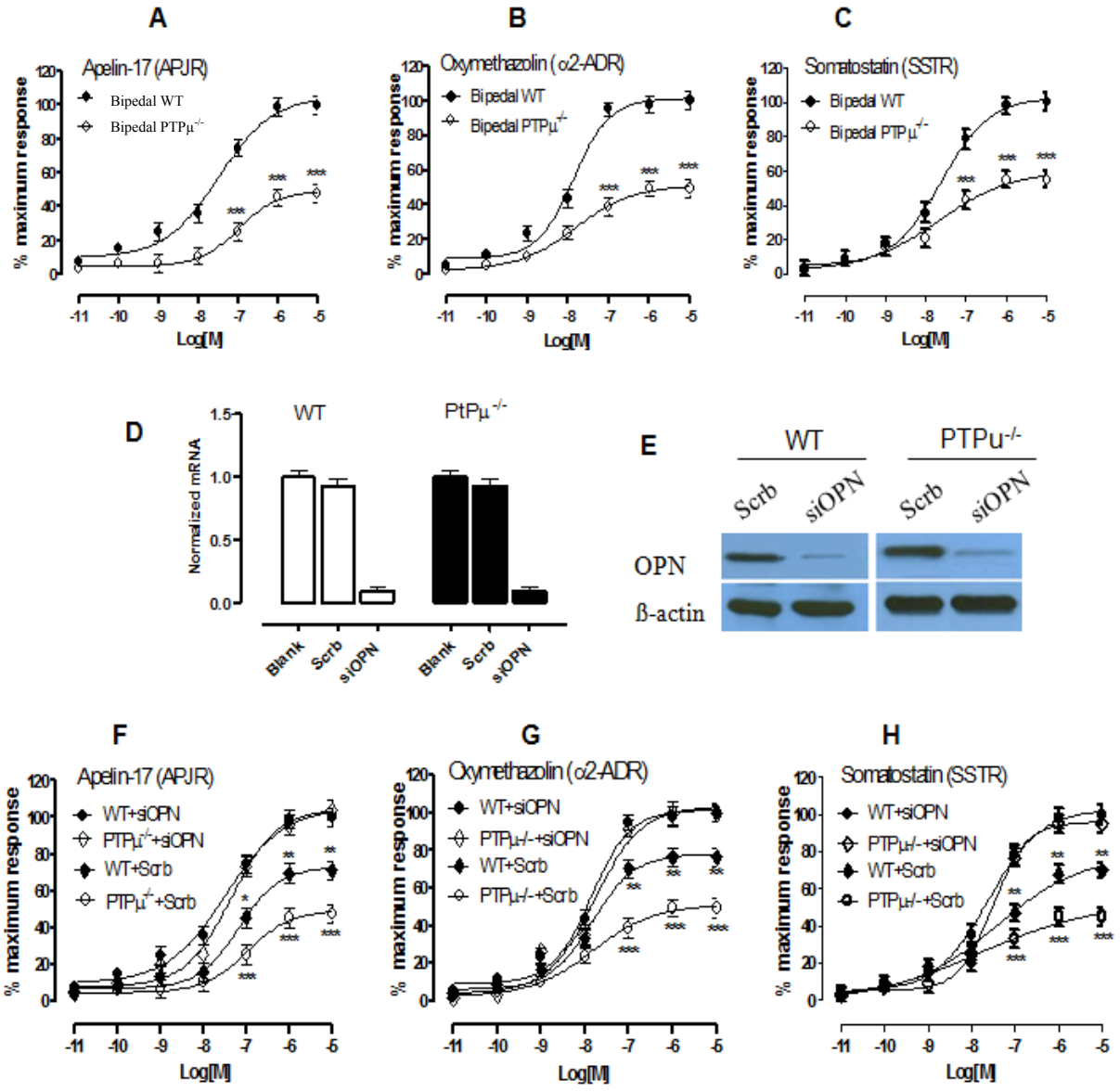

A
Apelin-17 (APJR)
Bipedal WT
Bipedal PTPμ-/-
% maximum response
Log[M]
B
Oxymethazolin (α2-ADR)
Bipedal WT
Bipedal PTPμ-/-
% maximum response
Log[M]
C
Somatostatin (SSTR)
Bipedal WT
Bipedal PTPμ-/-
% maximum response
Log[M]
D
WT
PtPμ-/-
Normalized mRNA
Blank
Scrb
siOPN
E
WT
PTPu-/-
Scrb
siOPN
OPN
ß-actin
F
Apelin-17 (APJR)
WT+siOPN
PTPμ-/-+siOPN
WT+Scrb
PTPμ-/-+Scrb
% maximum response
Log[M]
G
Oxymethazolin (α2-ADR)
WT+siOPN
PTPμ-/-+siOPN
WT+Scrb
PTPμ-/-+Scrb
% maximum response
Log[M]
H
Somatostatin (SSTR)
WT+siOPN
PTPμ-/-+siOPN
WT+Scrb
PTPμ-/-+Scrb
% maximum response
Log[M]

Figure 4

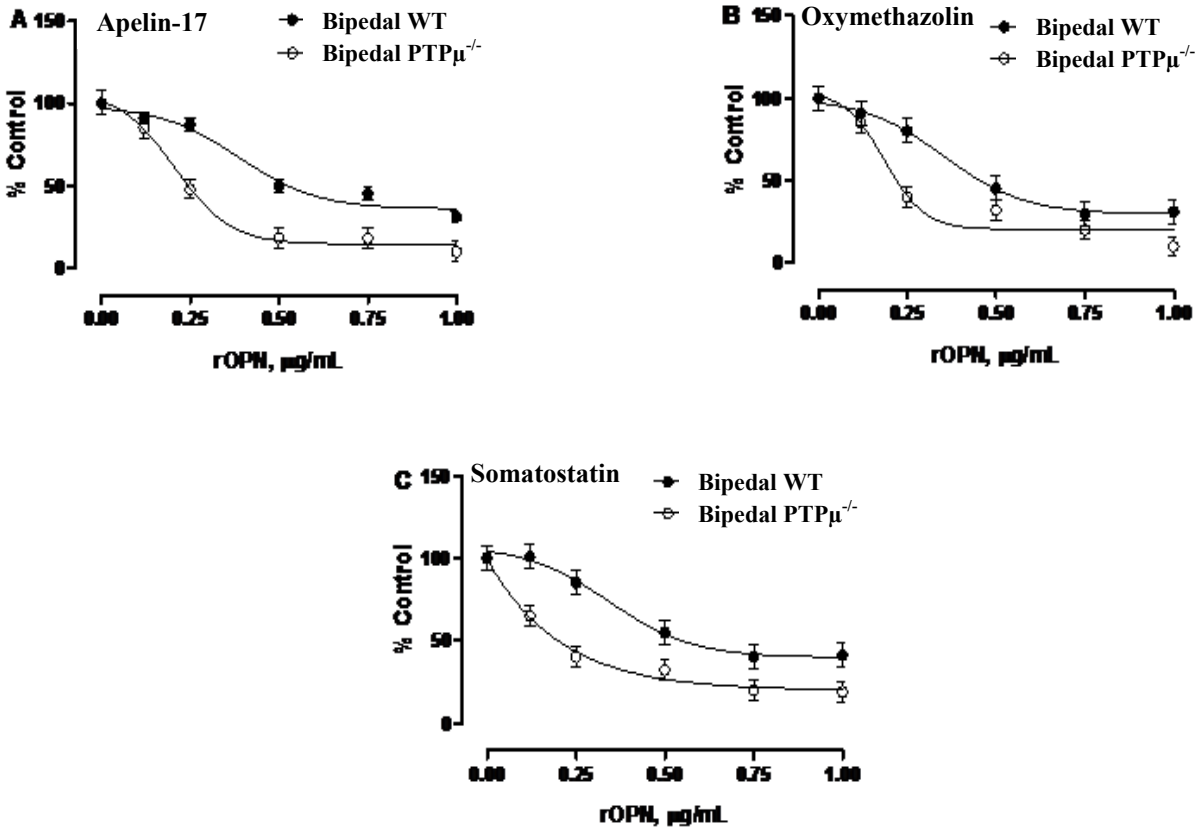

A
Apelin-17
Bipedal WT
Bipedal PTPμ-/-
% Control
150
100
50
0
0.00
0.25
0.50
0.75
1.00
rOPN, μg/mL
B
Oxymethazolin
Bipedal WT
Bipedal PTPμ-/-
% Control
rOPN, μg/mL
C
Somatostatin
Bipedal WT
Bipedal PTPμ-/-
% Control
rOPN, μg/mL

Figure 5

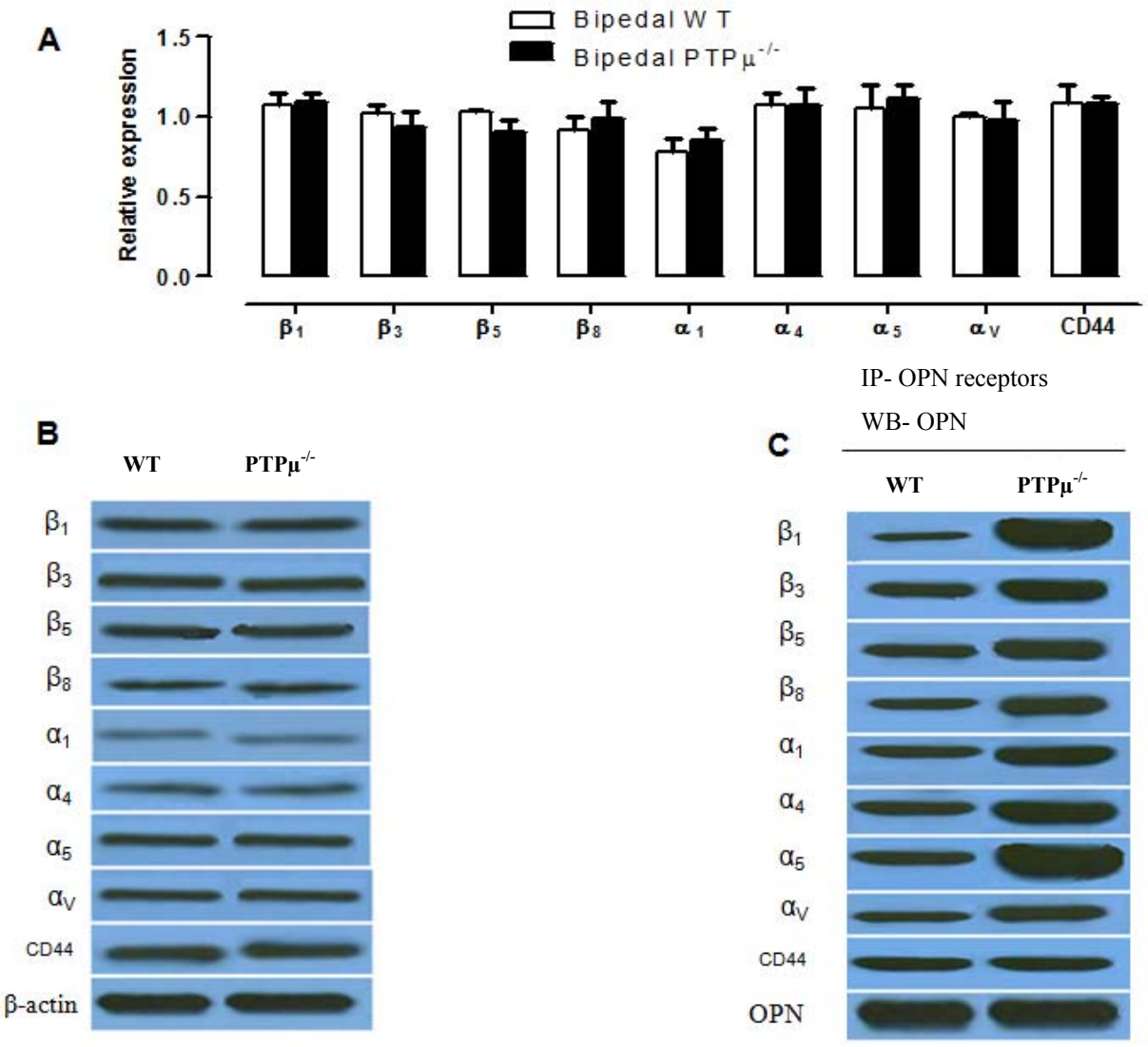

A
Bipedal WT
Bipedal PTPμ-/-
Relative expression
1.5
1.0
0.5
0.0
β1
β3
β5
β8
α1
α4
α5
αV
CD44
B
WT
PTPμ-/-
β1
β3
β5
β8
α1
α4
α5
αV
CD44
β-actin
C
IP- OPN receptors
WB- OPN
WT
PTPμ-/-
β1
β3
β5
β8
α1
α4
α5
αV
CD44
OPN

Figure 6

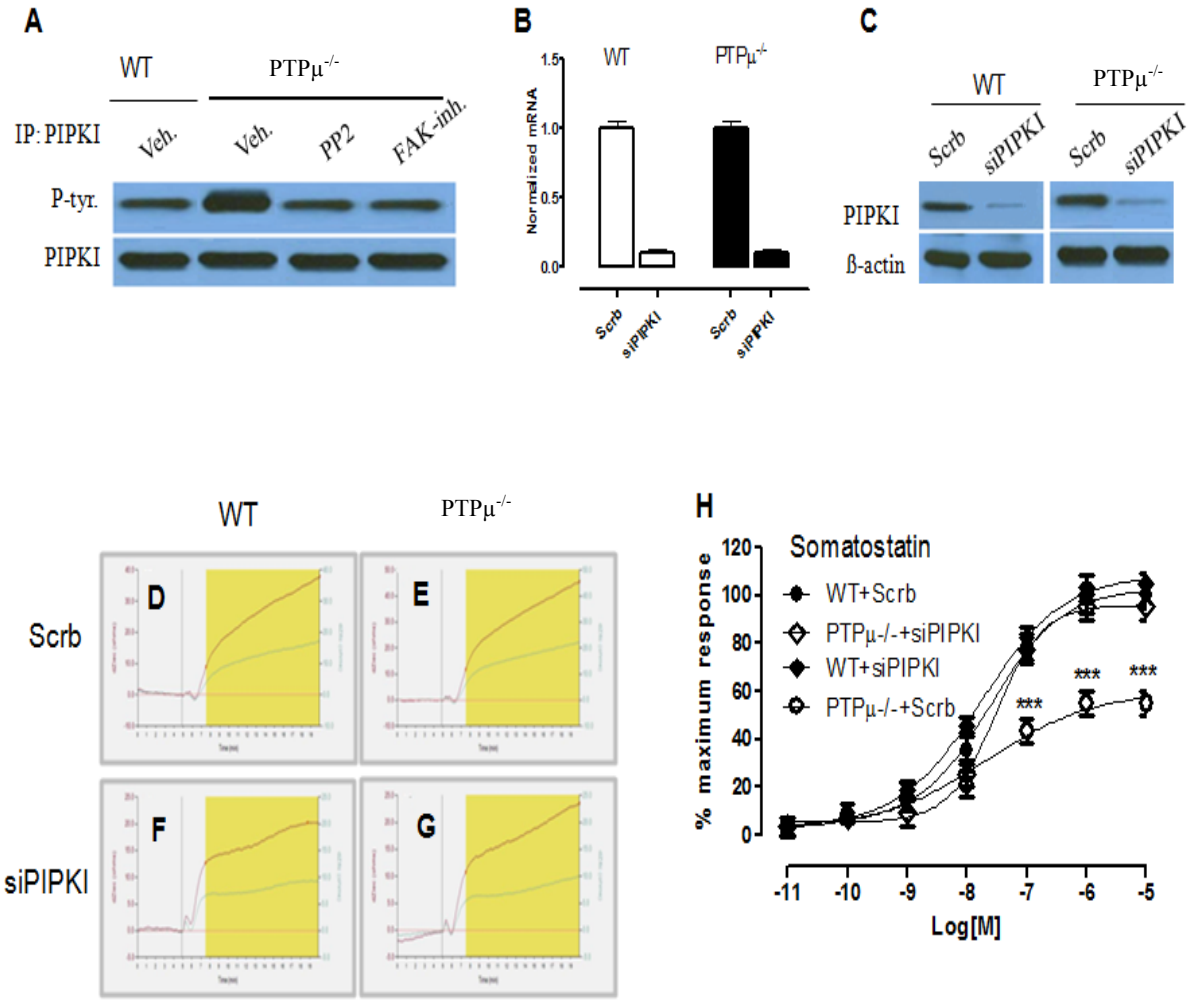
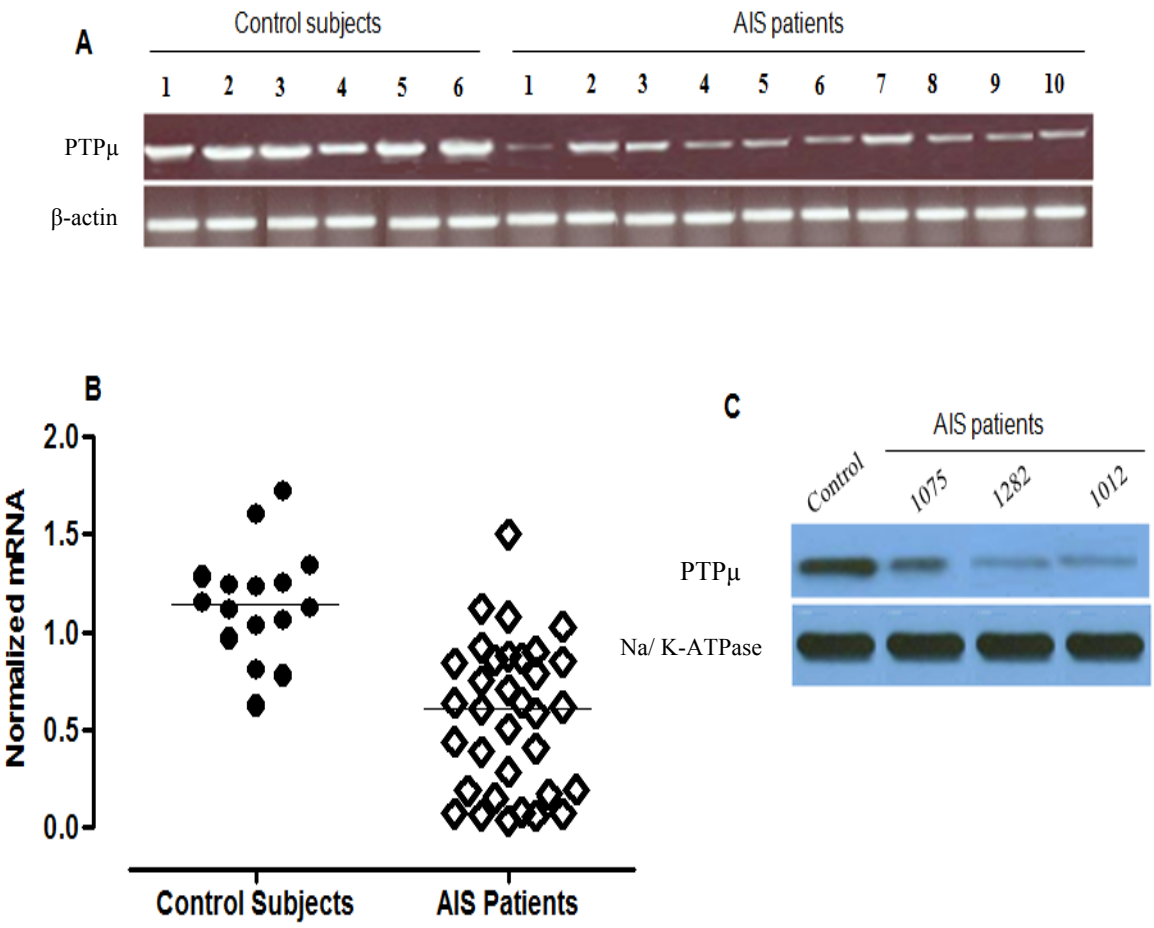

A
WT
PTPμ-/-
IP: PIPKI
Veh.
Veh.
PP2
FAK-inh.
P-tyr.
PIPKI
B
WT
PTPμ-/-
Normalized mRNA
1.5
1.0
0.5
0.0
Scrb
siPIPKI
Scrb
siPIPKI
C
WT
PTPμ-/-
Scrb
siPIPKI
Scrb
siPIPKI
PIPKI
ß-actin
WT
PTPμ-/-
Scrb
siPIPKI
D
E
F
G
H
Somatostatin
WT+Scrb
PTPμ-/-+siPIPKI
WT+siPIPKI
PTPμ-/-+Scrb
% maximum response
120
100
80
60
40
20
0
***
***
-11
-10
-9
-8
-7
-6
-5
Log[M]

Figure 7


A
Control subjects
AIS patients
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10
PTPμ
β-actin
B
Normalized mRNA
2.0
1.5
1.0
0.5
0.0
Control Subjects
AIS Patients
C
AIS patients
Control
1075
1282
1012
PTPμ
Na/ K-ATPase

GENERAL DISCUSSION

GENERAL DISCUSSION

The major challenge for orthopaedic surgeons regarding the management of AIS is the identification of patients at risk of progression and to determine, at an early stage, the best suitable therapeutic option. Due to the limited knowledge concerning the etiopathophysiology of AIS, no approach has yet been developed to address those issues with certainty. Nevertheless, through the various studies performed in our laboratory, we have discovered a systemic Gi protein coupled receptor signaling dysfunction as a decisive factor for the pathogenesis of AIS. Our interest in identifying molecular determinants of AIS and to better understand the mechanistic aspect of Gi-mediated signaling defect under high plasma OPN conditions may lead to a more comprehensive molecular concept to explain the pathomechanism implicated in the development and progression of AIS. This may thereby provide a molecular basis for the development of innovative pharmacological options and personalized treatments for AIS.

The CDS method within this project was chosen because it allowed the simultaneous Gi, Gs and Gq-mediated signaling measurement and also allowed for the testing of many receptor agonists in various cell types, all within the same assay. The use of bipedal mouse model for scoliosis helped to discover a direct link between Gi-mediated signaling under high plasma OPN conditions and the development of scoliosis. In the following text, I will discuss the overall results of my Ph.D. projects and present a potential mechanism exacerbating Gi-mediated signaling dysfunction in AIS and the relationship of the disease progression under high OPN conditions.

Methodological considerations

The significance of any experimental results is mainly dependent on the relevance of analytical methods and/or animal models utilized. In this regard, the first question addressed in the context of the present PhD project, was the choice of appropriate techniques to rapidly assess GPCR signaling as well as the suitable animal models.

Choice of analytical methods

Most of the common cell-based assays to assess GPCR signaling primarily rely on the measurement of a specific cellular response, including the generation of a second messenger, the dynamic relocalization of a target tagged with a fluorescent molecule, and the expression of a reporter gene. [226] Generally, these technologies require manipulations or engineering of cells, such as loading of fluorescent molecules into cells or over-expression of a target with or without a fluorescent tag. These manipulations may alter the cellular physiology of the target receptor. Together with the possible interference of many compounds with the detection technology, these common cell-based assays suffer imperative limitations.

To overcome these limitations, a non-invasive cell-based assay technology, termed cellular dielectric spectroscopy, or CDS, that measures whole-cell response in a label-free manner, has been developed. This technology yields information regarding the total integrated response of the cell to external stimuli and is based on applying electrical current to the cells within a microplate and measuring the changes in impedance. Precisely, the

CDS technology employs the application of alternating voltages at set frequencies to cellular components and intact cells and monitors the resulting currents generated from the dipoles within the sample. At each frequency, the difference in magnitude of the reflected signal, is compared to the incident wave, and is measured. In this electrical detection method, cells are cultured on small gold electrodes arrayed onto a substrate, and the system's electrical impedance is followed over a period of time. The impedance is a measure of changes in electrical conductivity of the cell layer. The latest generation of systems, such as the CellKey™ system (MDS Sciex, San Francisco, CA) has been created to overcome high assay variability usually associated with the classical impedance systems, which use small detection electrodes and large reference electrodes. [227]

The CellKey™ system consists of an environmentally controlled impedance measurement system, a 96-well electrode-embedded microliter plate, an onboard 96-well fluidics system, and a custom acquisition and analysis software. [228] The cells are seeded in the culture wells; each well has an integrated electrode array. The system operates using a small amplitude alternating voltage at 24 frequencies, from 1 KHz to 10 MHz. The resultant current is measured at an update rate of 2 seconds. The system is thermally regulated and experiments can be conducted between 28°C and 37°C. A 96-well head fluid delivery device handles fluid additions and exchanges onboard. In a single assay format, the cellkey system monitors the activation of different classes of endogenous GPCR and simultaneously differentiates among subtypes coupled to Gi, Gs and Gq proteins. In this

regard, this system has a large advantage over other universal technologies that do not distinguish among responses to these different GPCR subtype stimulations.

Particularly, the application of the CellKey™ system to any receptor subtype coupled to Gi proteins (GiPCR) is very noteworthy for the benefits it holds over other systems using [^{35}S] GTPγS binding or modulating of cAMP production. [^{35}S] GTPγS binding requires radioactivity and uses isolated plasma membrane fragments. cAMP production is a viable whole-cell assay alternative that we have initially used to achieve our previous studies. [229], [105], [230] However, this classical cAMP assay is limited to marginal precision due to the need to first stimulate adenylate cyclases with forskolin and then measure a reduction in this response by GiPCR agonists.

Based on these considerations, we have introduced the CellKey™ system in our laboratory and developed a cell-based assay to classify AIS patients and asymptomatic children according to the degree of maximum response to melatonin stimulation. [231] Indeed, we have demonstrated that the deficient melatonin signaling in AIS and the disparity of this defect among patients can be detected by the CellKey™ system. Moreover, relatively to the classical cAMP assay, we found that the CellKey™ system is more accurate and more reproducible to classify AIS patients or asymptomatic children by targeting the melatonin receptor. Recently, we have published a procedure for our cell-based assay as currently performed in our laboratory using the CellKey™ system. [231] The highest specificity, reproducibility and accuracy of our assay combined with the

remarkable simplicity of experimental and analytical procedures, the possibility of analysing a large number of samples under various conditions in the same assay, and the fact that this assay is applicable to any receptors coupled to any G proteins within any cell type, including adherent and suspension cell lines and primary cells, make the CellKey^{TM} system an appropriate technology to attend to the goal of the present project.

Choice of animal models

Multiple models in a multitude of animals have been created to understand the pathogenesis of AIS. Given the unclear origin of this disease in humans, various methods have been used. In most cases, these animal models sufficiently mimic scoliotic deformities observed in patients and have contributed to the generation of various hopeful hypotheses which continue to be investigated.

The neuroendocrine hypothesis pointing to a melatonin deficiency as the source for AIS has generated great interest. This hypothesis stems from the fact that experimental pinealectomy in different vertebrate species, [232], [53], [233], [234], [235] rats, mice, [80], [236], [2], [76], [237] rabbits [238] and Atlantic salmon [239] result in scoliosis that closely resembles AIS. However, despite low circulating levels of melatonin, bipedal ambulation appears to be required to generate scoliosis in mammalians. Indeed, Machida and collaborators (1999) have shown that melatonin deficiency secondary to pinealectomy alone does not produce scoliosis in rats if the quadrupedal condition is maintained. [80] Also, mice which are genetically deficient in melatonin develop scoliosis only when they

gain bipedal ambulation following amputation of their forelimbs and tails. [2] In that context, the underlying injury is not to be negated. Indeed, injury may induce production of various factors and enhance their circulating levels including plasma OPN, which is one of the concerns in the project. Therefore, it is possible that one or many factors induced by injury contribute to the development of spinal deformities, in agreement with the multifactorial etiology of this complex disease. In this regard, the genetic modification by either deletion of specific genes to dissect the individual genetic and cellular components that contribute to the mechanisms underlying pathophysiological events in various complex diseases has been very useful in gaining some understandings of their pathogenesis.

It is well known that mice are the main species used in the field of genetic modification and this for many reasons, such as their small size and low cost of housing compared to larger vertebrates, their short generation time and their fairly well defined genetics. Importantly, C57Bl/6 mice deficient in OPN have been created in certain specialised laboratories. To take advantage of this availability, we chose to use mice of C57Bl/6 background and found that genetic deletion of ssp1 gene (encoding OPN) protects these mice against scoliosis induced by the amputation of forelimbs and tails, while scoliotic bipedal wild type mice exhibited high plasma OPN. These findings undoubtedly indicate that this animal model of scoliosis can be a suitable model to examine the importance of defective GiPCR signaling in the development of scoliosis under high circulating levels of OPN.

Physiopathological considerations

Despite intensive research in the field of AIS, physiopathological events responsible for the onset and progression of this disease remain mysterious at this time. Nevertheless, accumulating evidence indicate that melatonin deficiency can be an important factor in pathological processes that drive scoliotic deformities in animals. However, the consideration of this hypothesis for the development of scoliosis in humans has been controversial for many years. Indeed, the lack of a significant decrease in circulating melatonin levels in AIS patients [94], [96], [240] has raised serious doubts around this hypothesis, which has been reconciled in our laboratory by the demonstration of a melatonin signaling dysfunction occurring in osteoblasts from AIS patients. [229] Our data indicate that AIS results from the inability of melatonin to regulate biological functions through its signaling pathway rather than a failure in its bioavailability. Specifically, we have observed that primary osteoblasts cultures prepared from bone specimens obtained intra-operatively during spinal surgeries of AIS patients with severe deformities, exhibited reduced ability to inhibit cAMP production in response to melatonin stimulation, arguing for reduced melatonin signaling through Gi proteins. Importantly, we have suggested that this defect lies at the level of Gi proteins by demonstrating the presence of increased phosphorylation at serine residues, since such post-transcriptional modification is well known to affect the activity of Gi proteins. From these findings has emerged the notion that biological processes driving the pathogenesis of AIS may implicate defective Gi proteins-mediated signaling.

Nature of defective Gi protein-mediated signaling in adolescent idiopathic scoliosis (AIS)

The major difficulty in investigations regarding the pathogenesis of AIS is its heterogeneous nature. [10], [241] Indeed, the heterogeneity of AIS is clearly illustrated by the variability of curve patterns and localisations among patients, even those within the same family. Similarly, we have reported the heterogeneous appearance of AIS on the biochemical aspect by demonstrating the existence of a disparity in the extent of melatonin signaling dysfunction among patients, leading to their classification into three functional groups. [229], [105], [230]

Although melatonin receptors can also couple to Gq proteins, the drastic reduction in response to melatonin receptor agonists observed in the presence of PTX, excludes the possibility that Gq-mediated signaling becomes a predominant or compensatory pathway for melatonin signaling in the absence of functional Gi proteins. Moreover, no evidence for Gq protein dysfunction was noted since response to agonists specific for GqPCR were similar in cells from AIS patients and control subjects. In contrast, we noticed an increased response to agonists specific for GsPCR in cells from AIS patients, supporting the notion that reduced Gi proteins function leads to increased Gs protein activity. It was noteworthy that disparity among AIS patient groups in response to melatonin stimulation was also observed in response to GsPCR agonist stimulation.

Currently, no evidence links this signaling disparity to melatonin receptors. Various studies indicate that expression of melatonin is not affected in AIS, [97], [242], [229] and

polymorphism analysis of genomic DNA has revealed no correlation between gene variants of melatonin receptors and the phenotype of AIS. [97], [242] Moreover, results presented in the present manuscript 1 show that the affinity of melatonin receptor for partial or full agonists was preserved in AIS and remained similar among the three functional groups, definitively excluding the possibility that different extent of melatonin signaling among the three functional groups can be explained by changes in melatonin receptor function. In contrast, competition studies performed in this study suggest that AIS is accompanied by a decreased sensitivity of Gi proteins for melatonin receptors and results have revealed that this decreased sensitivity is at different degrees among the three functional groups. The fact that similar profiles of response were obtained with various agonists operating through distinct receptors coupled to Gi proteins, argues against an abnormality specifically or exclusively related to melatonin receptors. Moreover, we found that this defect was not confined to osteoblasts since myoblasts and lymphocytes from the same AIS patients displayed similar features in response to all tested compounds. Collectively, these data not only indicate that the ability of Gi proteins to transduce extracellular signals is reduced in a systemic manner and generalized to all receptors coupled to Gi proteins, but also suggest that the mechanism triggering Gi proteins impairment could be the same in all cell types.

In this regard, results obtained in the present project are consistent with previous studies and confirm that AIS is not associated with changes in expression of Gi proteins but involves increased phosphorylation of Gi-alpha-subunits. It was striking to observe that the Gi protein isoforms were differentially phosphorylated among the three functional groups

of AIS patients and that the phosphorylation pattern as well as the kinases potentially implicated in this phenomenon, were specific to each functional group. However, further investigations are necessary to gain better understanding into the pathological processes associated with these disparities between the functional groups of AIS patients.

Indeed, it is well known that AIS is a multifactorial disease with a genetic component. Therefore, we cannot exclude the possibility that mutations in the gene encoding for Gi proteins themselves or for proteins implicated in the regulation of their function may be responsible for the reported abnormalities. In this regard, arrestin domain containing 4 (ARRDC4) gene, a negative regulator of G proteins signaling cascade, has been identified as a potential candidate gene on chromosome 15q25-26 in patients affected with AIS. [243] Moreover, mutations in G proteins have been reported in human diseases. [244] In each case, the mutation has been found on the alpha subunit of G proteins. However, because the beta-gamma dimmers are also known to play a decisive role in the ability of Gi proteins to mediate signal transduction, mutations or alterations in posttranslational processing of any beta or gamma subunits cannot be excluded yet.

The possibility of a genetic component in the Gi protein impairment in AIS, was further supported by the observation that all affected members of the same family displayed the similar response to GiPCR stimulation and were classified into the same functional subgroup, suggestive of an hereditary transmission of this defect. The expression of defective GiPCR signaling at the similar extent in the monozygotic twins affected with AIS

lends more credit to the notion that this condition may be inherited. This contrasts with other factors such as gender, age at disease onset, curve severity and curve pattern that are not conserved among affected twins and family members.

Nevertheless, we detected the functional reduction of Gi proteins in certain family members who have not developed spine deformity. This appears ideally suited to fit in the framework of the current concept regarding the contribution of genes versus environment in the process leading to AIS. Thus, functional reduction of Gi proteins would provide the inherited susceptibility for AIS, yet not sufficient to establish AIS by itself. We propose that, for each functional subgroup, there is a threshold of reduction. The genetic background may become important only when the functional reduction of Gi proteins reaches this threshold.

One can easily envisage that estrogens could contribute to the reduced functionality of Gi proteins, and thus initiate the decisive or aggravating step in the development of AIS. Indeed, it is well known that puberty is a crucial period for estrogen production, and certain reports indicate that estrogen interferes with G protein activities in various cells. [245], [246] This effect could be explained by the fact that estrogens have been shown to reduce Gi protein levels inside the cells by interfering with their transcription and synthesis. [246] On this basis, and because a large amount of Gi proteins are in cells before puberty, it could be speculated that functional reduction of Gi proteins is present at birth but has no significant impact prior to puberty, this pre-existing condition is however exacerbated by the increase in estrogens at puberty. This hypothesis is supported at least in part by our findings that estrogen treatments

exacerbate Gi protein impairment in cells from AIS patients (Akoume et al 2013, in preparation). This may explain the greater prevalence of AIS in girls than in boys and its greater incidence around the time of puberty.

Although the genetic determinants remain to be defined, results of the first manuscript provide solid evidence for a systemic and generalized reduction of Gi protein function in AIS, which could be conferred by hereditary and regarded as a prominent factor in etiopathology of this disease.

Contribution of OPN in the development of scoliosis in bipedal mice

For many years, it is has been recognized that OPN is implicated in a variety of human diseases. Recently, this chemokine has been suggested to be a potential contributor in the pathogenesis of AIS following the observation of its high circulating levels in individuals affected with this disease. Therefore, the role of OPN in the development of scoliosis was addressed for the first time in the present study using mice in which scoliosis was induced by forced bipedal ambulation after amputation of forelimbs and tails. Results revealed that these bipedal scoliotic mice exhibited higher plasma OPN and lower response to GiPCR stimulation when compared to non-scoliotic quadrupedal mice, suggesting a cause-and-effect relationship between these phenomenons. Consistent with this, we found that genetic removal of OPN prevented spinal deformities in bipedal mice and improved

the response to GiPCR stimulation in their osteoblasts. Furthermore, we demonstrated that OPN reduces response to GPCR stimulation, using *in vitro* assays.

OPN is well-known to induce various physiologic and pathologic effects by binding to CD44 or integrin receptors. Our *in vitro* assay revealed that OPN interferes with GiPCR signaling by interacting with RGD-dependent integrin. We identified $\alpha_5\beta_1$ integrin as the main receptor responsible for the inhibitory effect of OPN on GiPCR signaling. It was particularly interesting to observe that the treatment of cells with OPN caused decrease in the amount of Gi proteins coupled with their cognate receptors, while this treatment enhanced their interaction with $\alpha_5\beta_1$ integrin. These findings were interpreted to indicate that OPN reduces GiPCR signaling by increasing the sequestration of Gi protein by $\alpha_5\beta_1$ integrin. Moreover, we found that this OPN treatment also enhanced the phosphorylation of Gi proteins, a modification that reduces their activity. Interestingly, these effects of OPN on Gi proteins were prevented by the inhibition of various molecular intermediates engaged in the integrin signaling pathway.

In parallel, the examination of the functional status of Gs protein has revealed that OPN enhances its phosphorylation state and the interaction with its cognate receptors. This suggests that OPN has a reverse effect on Gs and Gi proteins-mediated signaling. Indeed, while GiPCRs are decreased, GsPCRs appear to be increased by OPN. This may cause an imbalance between bone mass and muscular strength around the spine due to an imbalance between myoblast and osteoblast proliferation, and/ or differentiation, and thus offers an

explanation for the apparition of the lateral curvature. Although this notion remains strictly speculative, our collective findings suggest that OPN is a relevant contributor in the pathogenesis of scoliosis in bipedal mice.

Impact of PTPμ deficiency on spinal deformity progression

Our demonstration that OPN contributes to the development of scoliosis by interfering with GiPCR signaling via a dual mechanism involving $\alpha_5\beta_1$ integrin engagement, allowed us to suggest that factors that regulate the interaction of OPN with integrin may influence the nature of scoliosis under high circulating OPN conditions. Since a recent study has revealed that PTPμ is a key player in the regulation of the interaction between integrin and its ligands, [247] we took advantage of the availability of PTPμ deficient mice on a C57Bl/6 background to test our hypothesis. Like the wild type phenotype, amputation of forelimbs and tails caused increased OPN levels in PTPμ deficient mice and this increase was to the same extent in both phenotypes. However, bipedal PTPμ deficient mice displayed greater incidence and severity of scoliosis when compared to bipedal wild type mice. Moreover, we found that osteoblasts from these mice were also less responsive to GiPCR stimulation relative to those from bipedal wild type mice. The regression analysis of their respective concentration-curves has revealed that the lack of PTPμ influences the response to GiPCR stimulation in a receptor-independent manner. In contrast, we found that osteoblasts depleted of PTPμ were more sensitive to the inhibitory effect of OPN on GiPCR signaling. This is strongly indicative of an impact of PTPμ deficiency which lies at the level of the interaction of OPN with its receptors. The

lack of PTPμ increases the interaction of OPN with $\alpha_5\beta_1$ integrin which further supports the idea this integrin is the receptor responsible for the inhibitory effect of OPN on GiPCR signaling. We further provided the mechanistic insights by demonstrating that the lack of PTPμ favours the interaction of OPN with $\alpha_5\beta_1$ integrin via PIPKIγ action. These observations suggest that the lack of PTPμ influences the nature of scoliosis by amplifying the inhibitory effect of OPN on GiPCR signaling.

Pathomechanism leading to the development of scoliosis in bipedal mice

Results presented in this thesis support the notion that a defective GiPCR signaling occurs in AIS and reveal a causative link between this defect and the circulating OPN levels. Our collective findings allowed us to elaborate the hypothetic scenario illustrated at figure 9 to explain the mechanism by which OPN reduces GiPCR signaling and how this effect is further exacerbated in absence of PTPμ.

Indeed, it is well known that integrins switch from low affinity to high affinity form to interact with their ligands and that integrins switching is favoured by phosphorylated PIPK1γ, which is normally dephosphorylated by PTPμ. We propose that the interaction of OPN with $\alpha_5\beta_1$ integrin in its high affinity form simultaneously causes a depletion of the pool of unphosphorylated Gi proteins and the phosphorylation of different Gi protein isoforms, leading to the reduction in the quantity of functional Gi proteins necessary for efficient GiPCR signaling. In the absence of PTPμ, PIPK1γ is not deactivated and

maintains $\alpha_5\beta_1$ integrin in its high affinity form. This favours its interaction with OPN and amplifies the inhibitory effect of OPN on GiPCR signaling, as well as the resulting consequences underlying spinal deformities.

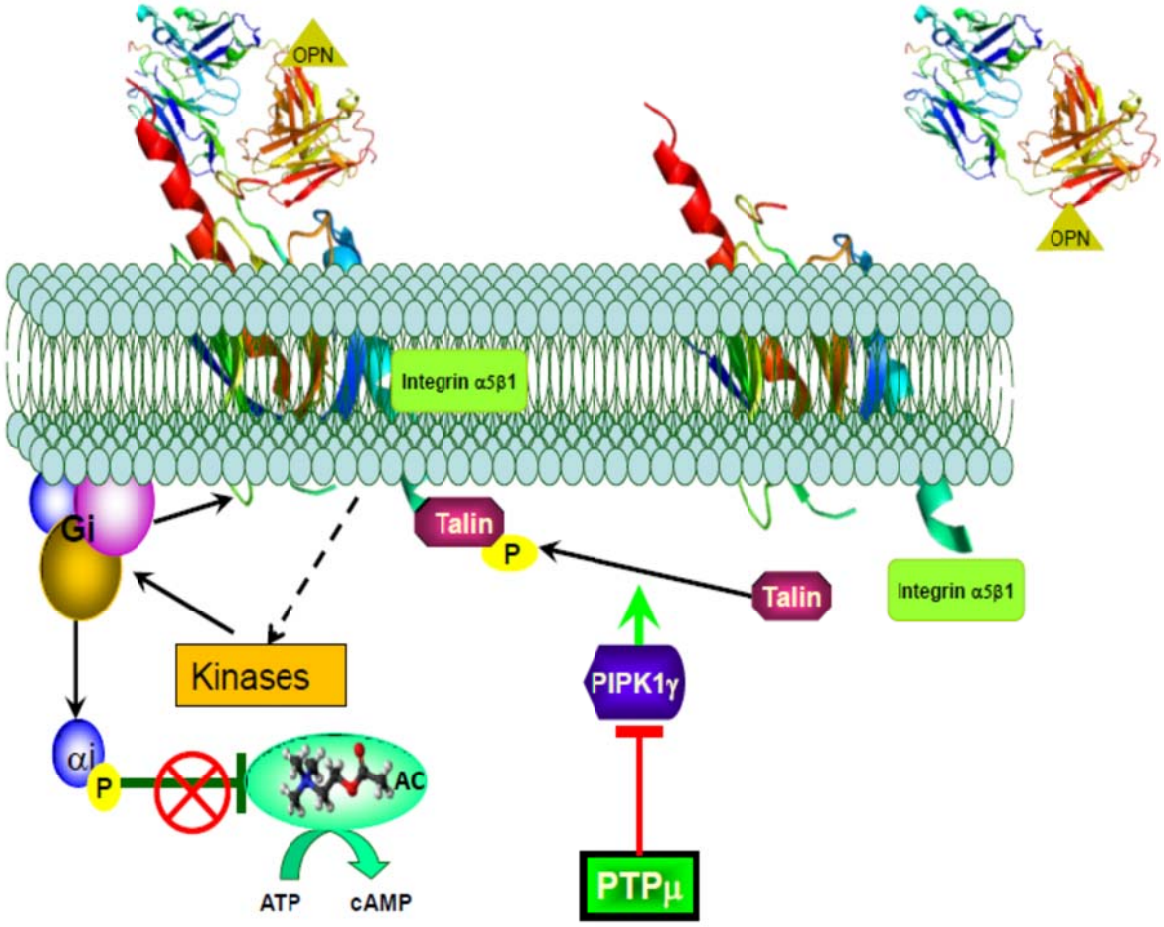


Figure 9: PTPμ deficiency exacerbates the harmful role of OPN in AIS.

PTPμ regulates the interaction between OPN and $\alpha_5\beta_1$ integrin through the regulation of PIPK1γ. PTPμ = protein tyrosine phosphatase mu, ATP= Adenosine triphosphate, cAMP= cyclic Adenosine monophosphate, AC= adenylate cyclase, p= phosphate, OPN= OPN and PIPK1γ= Phosphatidylinositol-phosphate kinase type 1 γ.

GENERAL CONCLUSION

General conclusion

The collective findings presented in this thesis support the notion that the defective GiPCR signaling occurring in AIS should be regarded as a key event in the pathologic process leading to idiopathic scoliosis. These findings are the first evidence pointing to the defective GiPCR signaling as a systemic and generalized defect, likely conferred by heredity. The realisation that OPN acts as a strong inhibitor of GiPCR signaling, has served to highlight the role of this cytokine as a potential target for future therapeutic interventions. Moreover, this study has introduced PTPμ deficiency as a risk factor for the development of severe lateral curvature. However, many issues remain to be addressed regarding the role of OPN and the impact of PTPμ deficiency in the pathogenesis of AIS. Understanding whether these factors contribute to the functional disparity of GiPCR signaling among the three functional subgroups of AIS patients, and provide the mechanism of action in each functional group are important research goals. These questions must be answered before we can fully understand the role of these factors in the disease's processes within in each functional group, and elucidate how they can become targets for drug development.

Perspective

The work undertaken in this thesis demonstrates the importance of the differential Gi protein signaling defect occurring in AIS. Evaluation of this defect may give orthopedic surgeons a diagnostic tool which may help them to indentify at an early stage, patients at risk of scoliosis whom may benefit from early and minimally invasive surgical interventions. The possibility of using PBMCs rather than osteoblasts will undoubtedly facilitate the process of classification of AIS patients. We showed how OPN plays an essential role in the etiology of AIS by attenuation of the Gi protein signaling through α5β1 integrin. We then demonstrated that PTPμ deficiency further exacerbates the harmful effect of OPN in AIS. PTPμ plays a role in all three groups though differently, and the next step will be to identify which proteins control PTPμ function within these different groups.

It is important to study the cause of PTPμ downregulation in the three functional AIS groups. Among others, nectin 3 protein is known to control PTPμ activity. Nectin 3 interacts with other nectins and nectin-like family members. It was also demonstrated that there is a physical interaction between nectins and nectins-like proteins with integrins. [213] The next step should focus on the expression of these nectins in our AIS functional groups and the association between these nectins with nectin-likes and PTPμ or integrin α5β1.

Another protein of interest is tetraspan CD151, which is a cell surface glycoprotein. It was shown that CD151 physically associates with integrins and integrin α5β1. [248] The reduction of the expression of integrin β1 was demonstrated in CD151 knockdown

podocytes. [249] It was also shown that integrin β1 and CD151 regulate PTPμ expression. [214] Taken together these results lead to the hypothesis that CD151 could play a role in AIS through the control of α5β1 integrin.

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