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The role of transcription factor Pitx1 and its regulation by hypoxia in Adolescent Idiopathic Scoliosis

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Ce mémoire intitulé :

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Résumé

La scoliose idiopathique de l'adolescent (SIA) est définie comme une courbure de la colonne vertébrale supérieure à 10 degrés, qui est de cause inconnue et qui affecte de façon prépondérante les adolescents. Des études précédentes sur des modèles murins ont démontré une inactivation partielle du gène Pitx1. Cette inactivation partielle provoque une déformation spinale sévère lors du développement des souris Pitx1$^{+/-}$, ce qui est grandement similaire au phénotype de la SIA. En se basant sur ces observations, nous postulons que la perte de fonction de Pitx1 pourrait avoir un rôle dans la SIA et pourrait être régulée par des mécanismes moléculaires spécifiques. En effet, des études faites sur l'expression de Pitx1 révèlent une perte de son expression dans les ostéoblastes dérivés de patients SIA au niveau de l'ARNm. Nous émettons l'hypothèse que la perte de Pitx1 dans la SIA pourrait être déclenchée par des facteurs hypoxiques puisqu'il est connu que Pitx1 est réprimé par l'hypoxie et que HIF-2 alpha est surexprimés dans les ostéoblastes des patients SIA même dans des conditions normoxiques. De plus, nous avons découvert une mutation dans le domaine ODD des HIF-1 alpha chez certains patients SIA (3,1%). Une fonction connue de ce domaine est de stabiliser et d'augmenter l'activité transcriptionnelle de HIF-1 alpha dans des conditions normoxiques. Nous avons confirmé, par la technique EMSA, l'existence d'un élément de réponse fonctionnel à l'hypoxie au niveau du promoteur de Pitx1. Cependant, des co-transfections avec des vecteurs d'expression pour HIF-1 alpha et HIF-2 alpha, en présence de leur sous-unité beta ARNT, ont conduit à une activation du promoteur de Pitx1 dans la lignée cellulaire MG-63 ainsi que dans les ostéoblastes des sujets contrôles. Il est intéressant de constater qu'aucune activité du promoteur de Pitx1 dans les ostéoblastes SIA n'a été observée, même après la co-expression de HIF-2 alpha et ARNT, confirmant le fait que l'expression de Pitx1 est abrogée dans la SIA. Dans l'ensemble, nos résultats démontrent un rôle important de Pitx1 dans la SIA et une possible régulation par des facteurs hypoxiques.

Mots clés: SIA, Pitx1, hypoxie, HIF-1α, HIF-2, ARNT, ODDD, osteoblastes, elément de réponse à l'hypoxie.

Abstract

Adolescent Idiopathic Scoliosis is a lateral curvature of the spine greater than 10 degrees, with an unknown cause, affecting primarily adolescents. Previous mouse model studies showed that partial inactivation of Pitx1 gene resulted in the development of severe spinal deformities in Pitx1 $^{+/-}$ mice, which is strikingly similar to the AIS phenotype. Based on this observation, we postulated that loss of Pitx1 function might have a role in AIS and could be regulated through specific molecular mechanisms. Indeed, expression studies revealed a loss of Pitx1 expression in osteoblasts derived from AIS patients, at the mRNA level. We hypothesized that the loss of Pitx1 in AIS could be triggered by hypoxic factors, since Pitx1 is known to be repressed by hypoxia and that HIF-2 alpha was up regulated in AIS osteoblasts even under normoxic conditions. Also, we found a mutation in the ODD domain of HIF-1 alpha in some AIS patients (3.1%), which is known to stabilize and enhance HIF-1 alpha transcriptional activity in normoxic conditions. We confirmed through EMSA the existence of a functional hypoxia response element on Pitx1 promoter. However, co-transfection assays with HIF-1 alpha and HIF-2 alpha expression vectors in the presence of their beta subunit ARNT led to the activation of Pitx1 promoter in human osteoblast cell line MG-63 cells and osteoblasts from control subjects. Interestingly, no Pitx1 promoter activity was observed in AIS osteoblasts, even after the co expression of HIF2 alpha and ARNT, consolidating the fact that Pitx1 expression is abrogated in AIS. Taken together, our findings show an important role for Pitx1 in AIS and hypoxic factors could be one of its regulators.

Keywords: AIS, Pitx1, hypoxia, HIF-1α, HIF-2, ARNT, ODDD, Osteoblasts, hypoxia response element.

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CHAPTER 1: REVIEW OF LITERATURE

CHAPTER 2: RESULTS

List of Abbreviations

4E-BP1	4E binding protein 1
ACTH	Adrenocorticotropin
AIS	Adolescent Idiopathic Scoliosis
ARD1	Arrest defective 1
ARNT	Aryl hydrocarbon receptor translocator
cAMP	Cyclic adenosine monophosphate
EPAS-1	Endothelial PAS protein-1
Epo	Erythropoietin
ERK	Extracellular regulated MAPK
FBT	Forward bend test
GPCR	G protein coupled receptors
Gpp(NH)p	Guanylyl imidodiphosphate
H2AFY	Histone family member Y
HIF	Hypoxia inducible factor
HLF	HIF like factor
HRE	Hypoxia response element
HRF	HIF related factor
MCF-7	Mammary carcinoma cells-7
MNK	MAP kinase interacting kinase
MOP	Member of PAS protein
ODDD	Oxygen dependent degradation domain
PHD	Prolyl hydroxylases domain
PI3K	Phosphatidyl inositol 3 kinase
Pitx1	Pituitary homeobox factor 1
RASAL-1	RAS GTPase activating protein like
RT-PCR	Reverse trancriptase polymerase chain reaction
TAD	Transactivation domain
VEGF	Vascular endothelial growth factor
VHL	Von hippel lindau
OA	Osteoarthritis

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Finally, I would like to thank all the people who were indirectly involved in the successful completion of my thesis.

Chapter 1. Review of Literature

1.1.1 Scoliosis: Definition

According to the Scoliosis Research Society, the disease Scoliosis is defined as a *lateral curvature of the spine greater than 10 degrees when measured on a standing radiograph*. The term Scoliosis originated from its Greek meaning "crookedness". It is characterized by a three dimensional deformity of the spine accompanied by vertebral rotation. A few centuries ago, doctors believed that scoliosis was caused by poor posture, nutritional deficiency of vitamin D, and five decades back, as a consequence of rickets [1].

1.1.2 Prevalence:

Scoliosis greater than 10º affects 3-4% of population in North America [2]. Curves greater than 30º is found in 0.2% in the population, while the percentage of curves greater than 45º affects 0.1% [3]. For minor scoliotic curves of 10º, the girls to boys ratio is 4:1. But for curves greater than 30º, the girls to boys ratio increases to 10:1. These data suggests that scoliotic curves in girls have a greater tendency to progress than in boys [2].

1.1.3 Screening for Scoliosis

Scoliosis screening is done in majority of the schools in North America due to its significant percentage of prevalence. Boys and girls aged 13-14 years and 10-12 years respectively are screened using Adam's Forward Bending Test (FBT) in which the child is made to bend forward with hands pointing to the toes. Any asymmetry in the contour of the

back (rib-humps) can be observed in this position [2]. Once this is detected, a radiograph is taken in order to evaluate the child's condition and determine the severity of the curve. The degree of the curve is measured using the Cobb's angle method, where the most tilted vertebrae above and below the apex of the curve is taken into account and the angle between the intersecting lines drawn perpendicular to these vertebrae gives the Cobb angle. Once the presence of scoliosis is confirmed, the next step is to pinpoint the underlying cause for it, and to determine accurately if there is a chance of progression of the curve. Curve progression is usually dependent on the gender, skeletal maturity, age at onset, and severity of the curve. Girls are ten times more at risk than boys of curve progression, and the risk increases if the patient has larger Cobb angle and greater growth potential at the time of detection [3].

1.1.4 Classification of Scoliosis:

Scoliosis is divided into two classes, structural and non structural scoliosis. Non structural scoliosis is divided into postural and compensatory scoliosis. Postural scoliosis is natural and has a low degree of curvature, while compensatory scoliosis can develop due to leg-length inequality. This is compensated by the pelvis leaning towards the shorter limb. Structural scoliosis is subdivided into congential scoliosis, secondary scoliosis: curves that are secondary to other diseases like syringomyelia, Marfan's syndrome, or trauma. Idiopathic scoliosis; curves without an underlying cause, and is further subdivided based on the age of onset of the disease. It is called infantile scoliosis when it occurs in infants up to the age of three, juvenile scoliosis between age of three and ten (puberty), and adolescent scoliosis when it occurs between puberty and maturity [4].

1.1.5 Cause:

Scoliosis can result from congenital spinal deformities, hereditary connective tissue disorders leading to weakening of skeletal tendons and ligaments, asymmetrical stress due to neuromuscular disorders, and metabolic disorders such as osteogenesis imperfecta [5]. But these factors only account for about 15-20% of scoliosis curvatures. Hence, the remaining 80% is considered to be or idiopathic, without a clear underlying cause.

1.1.6 Treatment:

Treatment of scoliosis mainly depends on the severity of the curve. Observation is usually recommended if the degree of the curve is between 10º and 25º. Bracing is adopted if the curve is between 30 and 40º. Braces like underarm thoracolumbar-sacral orthosis type cannot correct scoliosis, but can prevent the curve progression to a significant level [6]. Curves greater than 40-45º require spinal surgery with instrumentation in order to correct the scoliosis to a significant extent and stop future progression of curve severity [7].

1.2 Etiology of Adolescent Idiopathic Scoliosis

The etiology of AIS remains obscure, and seems to be multi-factorial in origin without a single underlying cause. Extensive research is being done on the possible role of the endocrine system, nervous system, and spinal musculature, basic structural elements of the spine, environmental factors, and genetics in the pathogenesis of AIS. Unfortunately, most of the study findings weren't convincing enough, and some factors seems to be more of a consequence than a cause. In the next section I will talk about factors that have been attributed to the development of AIS, including the hormone melatonin and its signaling, genetics and other chemical, metabolic factors.

1.2.1 Role of Melatonin in AIS etiology:

Melatonin is an indole synthesized from tryptophan, secreted mainly by the pineal gland in mammals and is synchronized to the light-dark cycle, with maximum basal levels in the night and low basal levels in the day. Thillard et al in 1959 demonstrated that pinealectomy (removal of pineal gland) in chickens resulted in the deformation of the vertebral column, which is phenotypically similar to that of human AIS [8]. Since then, experimental induction of scoliosis in chickens has been used to study the potential role of melatonin in the etiology of AIS [9]. Machida et al experimentally induced scoliosis in chickens and rats through pinealectomy, maintaining some rats in bipedal and others in quadrupedal state. Interestingly, only bipedal rats developed scoliosis compared to the quadrupedal ones. This indicates that bipedal condition like in humans facilitate the development of scoliosis after pinealectomy [10]. The importance of melatonin in the etiology of scoliosis is further strengthened by another study by the same group wherein scoliosis was experimentally induced in C57BL/6J mice, a mouse strain naturally deficient in melatonin, through re-sectioning of forelimbs and tail. All the bipedal mice developed scoliosis, whereas the bipedal mice that received intraperitoneal melatonin did not develop scoliosis, suggesting that melatonin deficiency is an important etiologic factor for AIS. Based on this, Machida et al. measured the levels of melatonin in AIS patients with progressive and stable curves and observed that the former group had very low levels of melatonin compared to the latter, whose levels where similar to the control subjects. These results strongly suggested a role for melatonin in the etiology of scoliosis but was highly controversial as a majority of other clinical studies showed that there were no significant difference between the circulating levels of melatonin in AIS when compared to control subjects [11], and experimental pinealectomy

did not give rise to scoliosis [12], in non human primates, suggesting that melatonin deficiency leads to scoliosis only in smaller animals and not in humans [13].

In that context, Moreau et al. postulated that the real culprit was not a deficiency of melatonin but rather a melatonin signaling dysfunction in AIS [14]. They tested the ability of osteoblasts obtained from AIS patients and healthy controls to inhibit accumulation of forskoline induced cAMP. They observed that osteoblasts of AIS patients had higher levels of cAMP compared to controls, which suggested that the melatonin signaling pathway was somehow impaired in AIS patients at varying degrees. The cAMP assay led to the functional classification of AIS patients into three functional groups based on their responsiveness to melatonin and cAMP levels (Figure 1.1). Using a non hydrolyzable GTP analogue Gpp(NH)p, they demonstrated that the melatonin signaling dysfunction can be caused by a reduced functionality of Gi proteins, in AIS patients. They experimentally ruled out the possibility of involvement of melatonin receptors, which was in agreement with previous studies showing no specific association of mutations found in MT1 or MT2 melatonin receptor [15]. By far this is the most convincing evidence for melatonin's role in pathogenesis of AIS and the presence of three distinct functional groups in AIS strongly argues in favor of more than one gene involved in this condition.

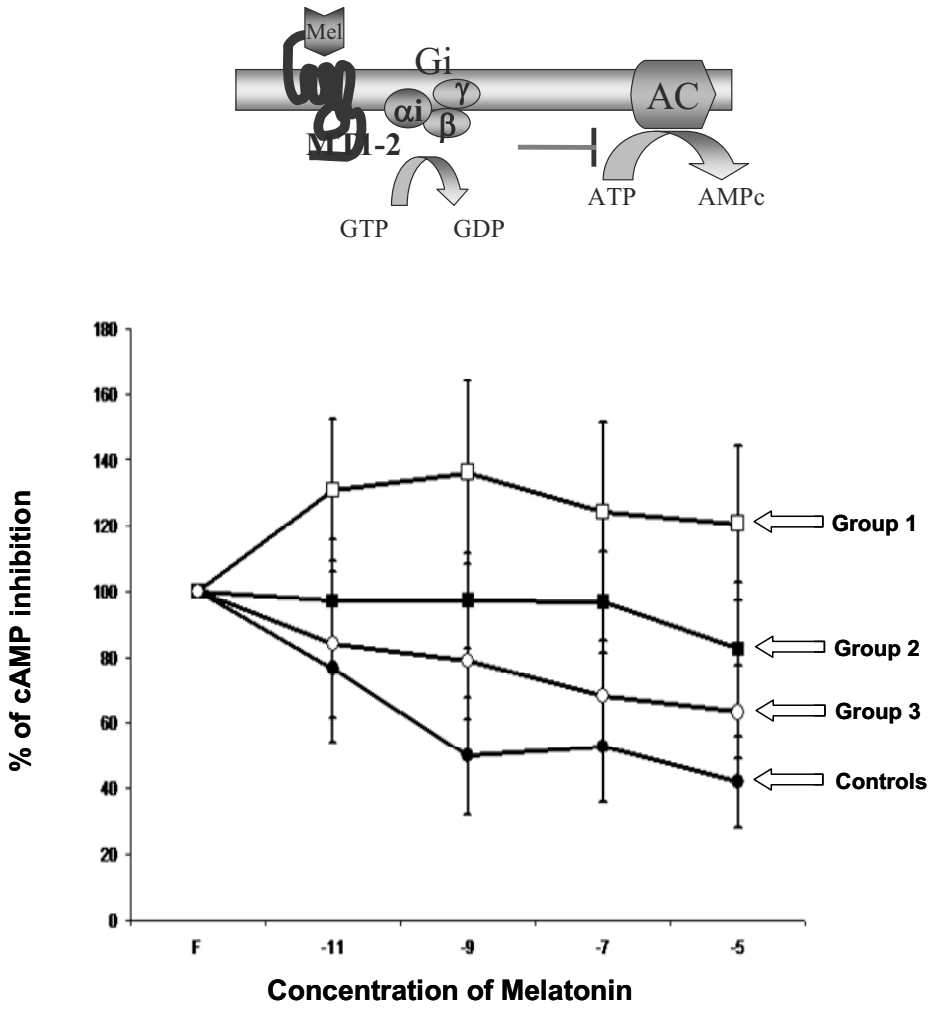


Figure 1.1: A. Melatonin signal transduction. Melatonin binds to its G protein coupled receptors (GPCR) MT1 or MT2 and inhibits the accumulation of cAMP via Gi subunits. **B.** Analysis of melatonin inhibition of adenylyl cyclase activity in human osteoblasts derived from AIS and control subjects. The graph shows the inhibition of forskolin-induced cAMP by increasing melatonin concentrations, from -11M to -5M. The patients are classified into three functional groups based on the percentage of cAMP inhibition by melatonin. Adopted from [16]

1.2.2 Genetic factors

Clinical and population studies demonstrated a greater incidence of scoliosis among individuals who were closely related to scoliotic patients, compared to the general population [17]. Harrington et al found that there was a 27% incidence of scoliosis in daughters of women affected by scoliosis greater than 15º [18]. Studies conducted in twins showed a high concordance rate (75%) of idiopathic scoliosis in monozygotic twins, compared to dizygotic twins (36%) [19]. Taken together, these results show that idiopathic scoliosis can be a genetic disorder.

Even though there is an increase in evidence of the genetic nature of scoliosis, its mode of inheritance still remains obscure. Studies conducted by Wynne-Davies et al concluded that the disease is hereditary and have a dominant and multiple-gene pattern of inheritance [17]. Riseborough's study on 2869 individuals concluded that a multi-factorial mode of inheritance maybe involved [20]. Work by Cowell et al, suggested that idiopathic scoliosis has an X-linked dominant inheritance pattern [21].

More recently, many researchers have used genome-wide linkage analysis studies to identify specific genetic loci associated with the occurrence of idiopathic scoliosis in a variety of populations. Several putative regions have been identified till to date, including four regions on chromosomes 3, 6, 10, and 19 the latter being the most significant [22, 23]. Genome wide scan based on the mode of inheritance showed that the group with a potential X-linked mode of inheritance showed a link between the disease and a region on the X-chromosome, in a few families [24].

1.2.3 Other Factors:

Apart from melatonin signaling dysfunction and genetic factors, researchers postulated that adolescent idiopathic scoliosis may be caused by abnormalities in the structural elements of the spine, neurological disorders, chemical and metabolic factors, hormones, etc.

The most striking characteristic of AIS is a severe deformity of the spine with wedging of vertebrae and an extensively deformed inter-vertebral disc. Taking this as a cue, several studies investigated the possible role of structural elements of spine and its metabolic aspects in etiology of idiopathic scoliosis (IS). Inter-vertebral disc is made of annulus fibrosus enclosing a nucleus pulposus, which contain collagen and proteoglycans. IS nucleus pulposus showed an increase in collagen and a decrease in glycosaminoglycan levels, which supposedly lead to an increased degradation, [25], but later they were proven to be secondary due to the high pressure exerted on the inter-vertebral disc by the deformed vertebrae [26].

Apart from hormonal, genetic, structural and chemical factors, the central nervous system has also been a target of detailed investigation for its possible role in IS pathogenesis. Earlier studies indicated an incidence of scoliosis in animals exposed to experimental medullary damage [27], excision of dorsal spinal nerve roots [28], destruction of brain stem, posterior hypothalamus and the postural reflex system [29].

However, in spite of all the promising research in a wide variety of fields, the etiology of AIS still evade being unraveled and still remain a true multi-factorial entity.

1.3 Hypothesis 1

The first hypothesis for our study is primarily based on evidence from earlier research using Pitx1 knockout mouse model.

1. Pitx1 +/- mice were phenotypically normal and fertile at birth, but developed severe spinal deformity around two months after birth.
2. The Pitx1+/- phenotype is strikingly similar to that found in AIS patients (Figure 1.2).
3. Moreover, individuals diagnosed with AIS are normal at birth without any congenital malformations, but later develop scoliosis, during the onset of puberty. This somewhat mirrors the onset of scoliosis in Pitx1+/- mice.

Based on the above listed observations, we postulated that Pitx1, a homeo-box containing transcription factor maybe involved in the patho-mechanism leading to the development of AIS in Humans.

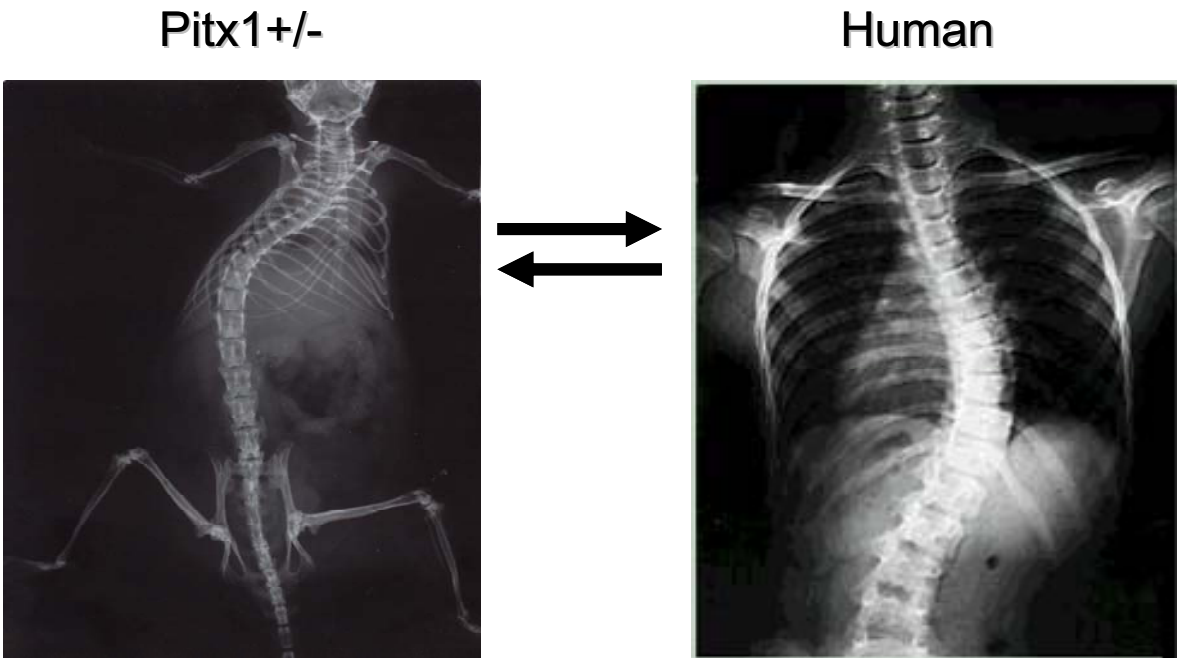


Figure 1.2: Radiographs of Pitx1+/- mouse and a Human with adolescent idiopathic scoliosis. Pitx1$^{+/-}$ mice develop scoliosis after 2-4 months of being born. Humans develop AIS during the onset of puberty.

1.3.1 Pitx1

PITX1 is the pituitary transcription factor, which belongs to the PITX family containing two other members Pitx2 and Pitx3 (Figure 1.3). They are members of a paired class of homeo domain transcription factors. Pitx1 codes for a protein of 315 amino acids long and have a characteristic homeo domain containing three α helices and an N terminal arm involved in protein-protein interactions and influences binding specificity via a lysine 49 residue within the homeodomain [30]. Pitx1 can induce or prevent transcription of target genes by binding as a monomer to a TAATCC sequence within target promoters [31]. This binding site is recognized by its family members and is highly conserved from Drosophila to Humans.

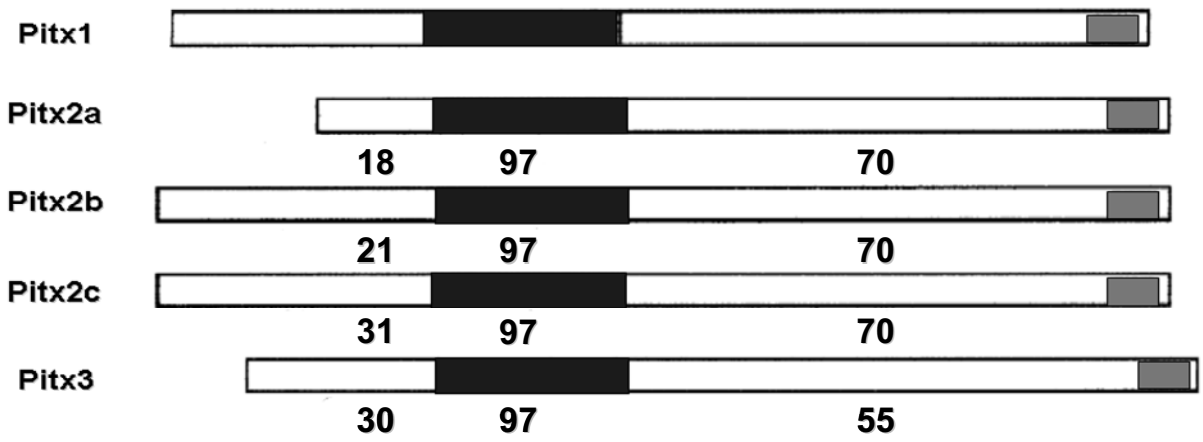


Figure 1.3: Vertebral Pitx1 paralogues. The black region represents the homeodomain, and the grey region represents the OAR region (located next to the C-terminus, with the ability to inhibit the protein's DNA binding activity), which is highly conserved in Drosophila and vertebrates. The numbers show percentage similarity between domains of paralogues with respect to Pitx1. (Adapted from [32]).

1.3.1.1 Role of Pitx1 in development

PITX1 plays an important role in development and function of organs through unique expression pattern. It is expressed in the brachial arches, eye, pituitary, forebrain, midbrain, teeth, heart, forelimb mesenchyme and the hindlimb mesenchyme [33]. In addition, it is expressed in the olfactory system, posterior lateral plate mesoderm, bladder, stomach, pancreas etc. PITX1 shows specific expression in the posterior limbs, and is involved in the patterning of hindlimb and mandible development. In in situ hybridization studies on whole mount mouse embryos, Pitx1 was first detected at E9.5 in the first brachial arches, and at the posterior region corresponding to future hindlimb buds. Its expression intensifies in the hindlimb buds and mandibular arches at E10.5, but is absent in the forelimb buds. Continued expression of Pitx1 in the proximal region of hindlimb and mandibular arches is observed at E11.5 through E13.5 during which it is observed in the Rathke's pouch, tongue and genital eminence [34]. Targeted inactivation of PITX1 in mouse leads to severe hindlimb and craniofacial malformations. Pitx1 $^{-/-}$ mice died at birth and showed severe impairment of bone development in their hindlimbs and mandibles (Figure 1.4), thereby confirming the importance of Pitx1 in skeletal development [35].

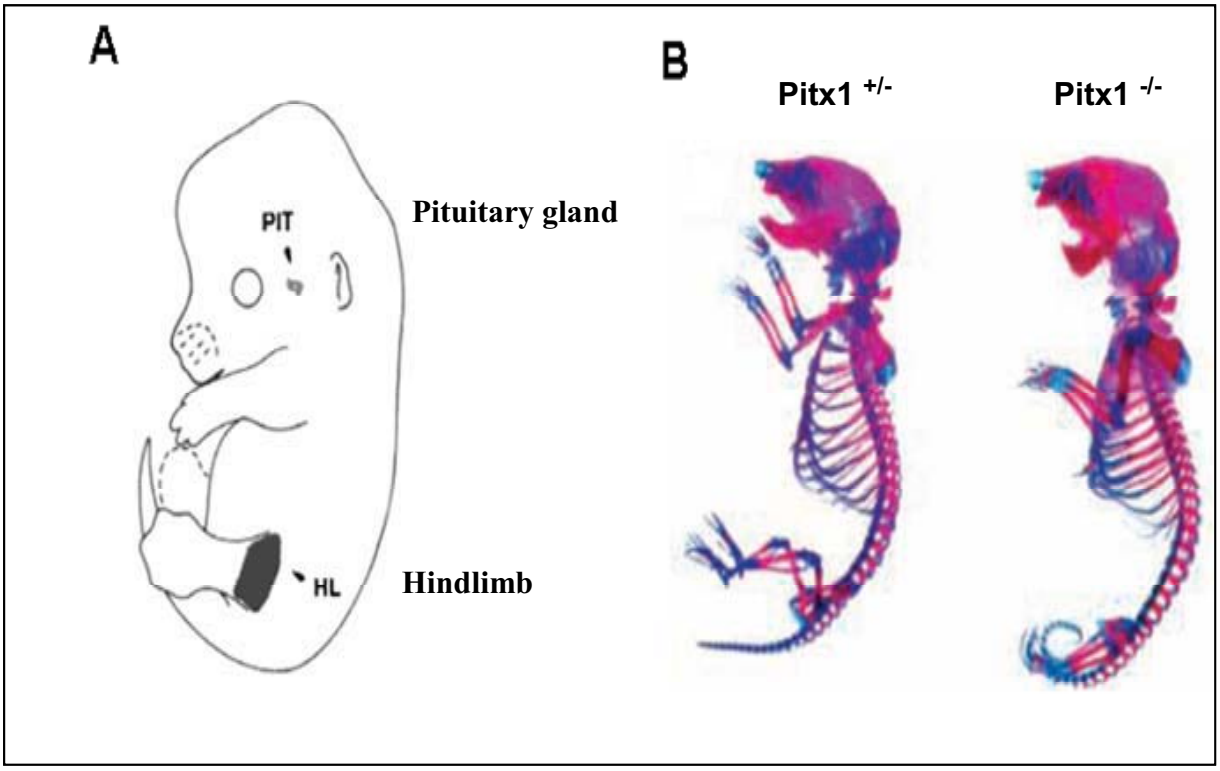


Figure 1.4: The role of Pitx1 in development. A. Important Pitx1 expression sites in a developing embryo include the hindlimb mesenchyme, pituitary, branchial arches, palate and teeth. (Adopted from [32]). **B.** Skeletal preparation of $Pitx1^{+/-}$ and $Pitx1^{-/-}$ new born pups. $Pitx1^{-/-}$ new born (right) exhibits severe craniofacial and limb malformations (Adopted from [35]).

1.3.1.2 Pitx1 in the pituitary:

Involvement of Pitx1 in the function of anterior pituitary gland is well studied. There are six types of hormone producing cells in the pituitary gland. The cell type that expresses the pro-opiomelanocortin (POMC) is the first to differentiate. Lamonerie et al identified Pitx1 as a novel protein that regulated the expression of POMC in the anterior pituitary lobe. Hence, PITX1 binds to the target site within the promoter of POMC and regulates its transcription in corticotroph cells, thereby playing a role in pituitary gland formation [31]. In addition, Pitx1 acts synergistically with other pituitary specific transcription factors (Table 1.1) to promote gene expression, which leads to specific cell phenotypes in the anterior pituitary [36].

Table 1.1: Table shows the various co factors with which Pitx1 acts synergistically to regulate target promoters in the pituitary gland.

Pitx1 cofactors	Target promoter	Organ	Cell type	References
Tpit	POMC	Pituitary gland	Corticotropes	Tremblay 1998
SF-1	β-LH	Pituitary gland	Gonadotropes	Tremblay 1998
Pit1	PRL	Pituitary gland	Somatolactotrophs	Tremblay 1998
NeuroD1	POMC	Pituitary gland	Corticotropes	Gino poulin 2000

1.3.1.3 The role of Pitx1 in limb development:

In the developing mouse embryo, Pitx1 is mainly expressed in the developing hindlimb [33], the first branchial arch and the anterior pituitary [37], and very less expressed in the forelimbs [34]. Pitx1 is required for the establishment of hindlimb and forelimb identity. Szeto et al., studied the importance of Pitx1 in limb development using $Pitx1^{-/-}$ mice. They observed that proper development of hindlimb, first branchial arch and anterior pituitary was compromised in the $Pitx1^{-/-}$ mouse strain. Moreover, the skeletal structures of the hindlimb like tibia and fibula were significantly reduced in length and had morphology similar to the corresponding structures of the forelimb. Furthermore, the expression of Tbx4, a downstream target gene of Pitx1 and marker of hindlimb specificity was reduced in Pitx1-/- hindlimbs.

The ectopic expression of Pitx1 in the chicken wing resulted in its abnormal growth and patterning leading to the straightening out of the curved wing structure, alterations in length and number of digits, characteristic of hindlimb patterning [38]. All these data suggests an evolutionarily conserved role of Pitx1 gene in limb specificity among vertebrates.

1.3.2 Importance of Pitx1 in human disease:

1.3.2.1 Pitx1 and osteoarthritis:

Pitx1 plays an important role in chondrogenesis during development. Partial inactivation of Pitx1 in mouse ($Pitx1^{+/-}$) leads to the formation of osteoarthritis like phenotype, characterized by abnormal thickening of subchondral, trabecular and cortical bone, and increased calcification of the cartilage. Based on this evidence, Picard et al. investigated the role of Pitx1 in human osteoarthritis [39]. In human OA articular chondrocytes, Pitx1 was hardly detected at the mRNA levels, compared to that of human control subjects. In addition, histological sections of human OA cartilage showed a decrease in Pitx1 proteins, while the normal cartilage still retained the expression. Picard et al. demonstrated a loss of Pitx1 in human primary knee joint in OA, suggesting a role for this transcription factor in the pathogenesis of osteoarthritis in humans.

1.3.2.2 Role of Pitx1 in the etiology of Clubfoot:

Clubfoot is an autosomal dominant, congenital limb malformation, with an incomplete penetrance that affects 1 child in 1000 births with a male to female ratio of 2:1 [40],[41]. 80% of clubfeet cases are idiopathic [42]. Gurnett et al identified Pitx1 as the gene responsible for clubfoot through genome-wide linkage analysis of 13 members of a family affected with the malformation using Affymetrix 10K mapping data. A 12Mb candidate region on chromosome 5q23.3-q31.2 was identified. Pitx1 lies within this candidate region, and a sequence analysis showed a G to A substitution, which resulted in the replacement of lysine with glutamic acid at position 130 (E130K) within the homeodomain, which is highly conserved. This mutation was detected in all the affected individuals tested, compared to five hundred test subjects. The glutamic acid is conserved in Pitx1, Pitx2 and Pitx3, and its substitution leads to reduced transcriptional activity at least in Pitx1, proven using transfection assays. Moreover, the mutant Pitx1 acts a dominant negative inhibitor of Pitx1 transactivation [43]. Since Pitx1 is an important transcription factor for hindlimb development, the E130K mutation could be one of the links in the Pitx1 pathway leading to Clubfoot.

1.3.2.3 Role of Pitx1 in autism:

Autism represents a spectrum of behavioral disorders characterized by reduced social interaction and communication skills, and abnormal behavior. It is known to affect more boys than girls, a ratio of 4:1 [44]. To date, no specific cause for autism has been described. It is believed to be a genetic disorder, and studies conducted in monozygotic twins [45] as well as discovery of nucleotide polymorphisms in genes like neuroligin 3 and 4 suggests that it's a complex, multi-gene disorder [46].

Through genome-wide linkage analysis in 276 affected families, Philippi et al identified a 1.2 megabase region on chromosome 5q31, which had a strong association. This region harbors three genes, Pitx1, neurogenin 1 (involved in neurogenesis) and histone family member Y (H2AFY). Single point association analyses ruled out the last two, and showed a significant association of Pitx1 with autism, Haplotype analysis revealed that individuals with an A-C haplotype risk allele were 2.54 and 1.59 fold respectively, susceptible to autism. This might explain the abnormal levels of POMC (pro-opio-melanocortin) and adrenocorticotropin (ACTH) in autistic patients suggesting a gain of Pitx1 expression, since these hormonal genes lie downstream of Pitx1 [31]. More studies need to be done to understand the exact mechanism through which Pitx1 contributes to autism etiology.

1.3.2.4 Pitx1 and Cancers:

Activation of RAS pathway is a significant contribution to tumor pathogenesis. RAS is a small GTPase which is activated by GTP exchange factors and attenuated by GTPase activating proteins (GAP). Although mutations leading to the gain of function of RAS are often attributed to tumorigenesis, wild type RAS genotypes also can enhance the phenomenon due to the activation of their pathway via the inhibition of a tumor suppressor.

Pitx1 is known to be often downregulated in various cancers (Table 1.2) including colon cancer where it is associated with levels of wild type RAS expression. Kolfschoten et al. used RNAi screening strategy on immortalized fibroblast cell line which only require knockdown of a single tumor suppressor for its transformation. They identified Pitx1 to be knocked down resulting in the transformation of cells. The phenotype of $Pitx1^{kd}$ cells was found to be strikingly similar to those over-expressing RAS. They also looked at potential RAS GTP-activating factors that could inhibit RAS activity, and found RASAL1 to be transcriptionally activated by Pitx1 (Figure 1.5). Inhibition of RASAL1 resulted in cell phenotype similar to that of $Pitx1^{kd}$ cells. Hence, they concluded that the role of Pitx1 as a tumor suppressor comprises of activating RASAL1, a RAS-GTPase activating protein which inhibits RAS activity and tumorigenicity [47].

Further evidence for the tumor suppressor activity of Pitx1 is gradually surfacing in other types of cancer. DX Liu et al. analyzed its influence on the transcriptional activity of p53, a potent tumor suppressor that influences programmed cell death and apoptosis. Forced expression of hPitx1 in human mammary carcinoma cells (MCF-7) increased the mRNA and protein levels of p53, resulting in 91% apoptosis after third day of transfection [48]. Two Pitx1

consensus elements were found on the promoter of p53, and transient over-expression of Pitx1 showed an increase in expression levels of p53 and its direct downstream targets p21 (G1 and G2/M phase arrest) [49] and PTGFβ (G1 cell cycle arrest and apoptosis) [50]. This was further confirmed by siRNA depletion of Pitx1, which led to a decrease in their basal expression. Taken together, these data suggest an emerging role for Pitx1 as a tumor suppressor.

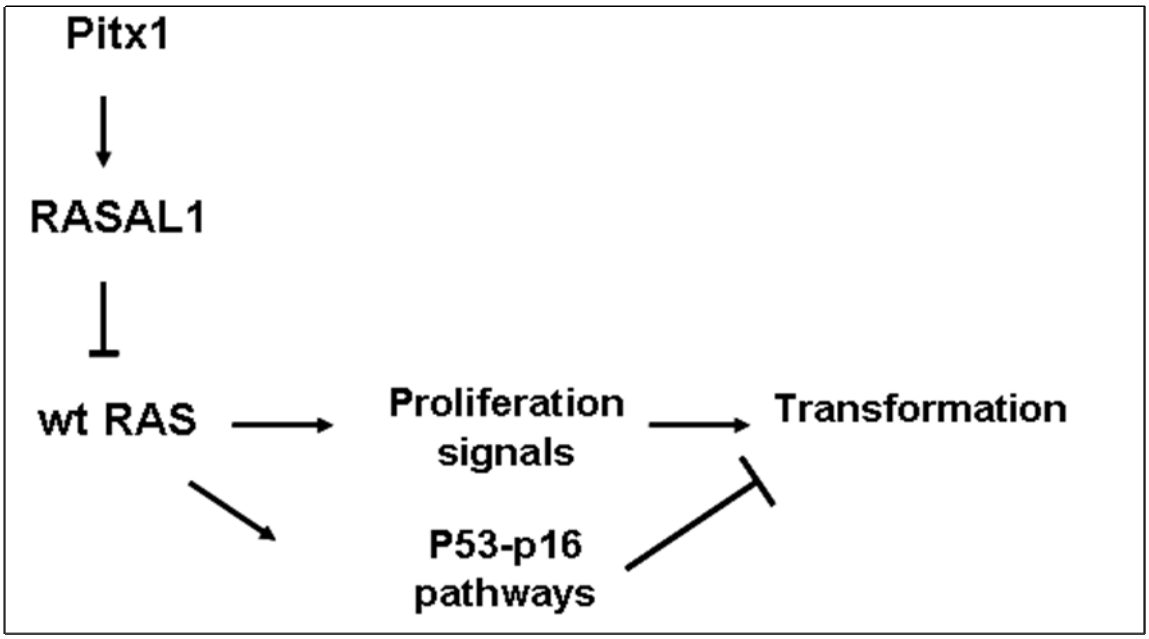


Figure 1.5: Pitx1 mediated inhibition of RAS. A pathway showing how pitx1 mediates the repression of RAS through the activation of RASAL1, a RAS-GTPase activating protein, thereby controlling the transformation activity by RAS.

Table 1.2: List of various types of cancers where downregulation of Pitx1 occurs.

Cancer Type	Study
Human lung cancer	Chen,Y. et al., 2007
Human gastric cancer	Chen, Y.N. et al., 2008
Prostrate cancer	Singh, D. et al., 2002
Barrett's esophagus and Barett's associated adenocarcinoma	Lord, R.V. et al., 2005
Lingual squamous cell carcinoma	Huang, Y.D. et al., 2007
Bladder cells carcinoma	Ramaswamy, S. et al., 2001

1.4 Hypothesis 2

We postulate that hypoxia, is involved in the regulation of transcription factor Pitx1, which could contribute towards AIS pathogenesis. Our hypothesis is based on the following studies:

1. Sonna et al used the powerful DNA micro array technology to study the effect of hypoxia exposure on human hepatocytes (HepG2) [53]. They analyzed the mRNA expression levels using Affymetrix Gene Chip arrays consisting of approximately 12600 sequences. Apart from the known hypoxic responsive genes being up regulated, they found a down regulation of a number of genes involved in cell adhesion, cell growth, differentiation and proliferation, membrane transport, metabolism and biosynthesis. Interestingly, their experiments showed a significant down regulation of our gene of interest Pitx1.
2. Rivard et al., in 1986 conducted a study wherein pregnant mice were exposed to hypoxia for 5 hours at 9.5 days of pregnancy. The 9.5^{th} day is significant for the proper development of the somites to form the precursor of spine. They were able to induce spinal deformity in 90% of the pups that underwent maternal hypoxic treatment, which is similar to the phenotype found in human. This study showed that hypoxia is a powerful agent that can influence normal segmentation and vertebrae structure [51].
3. In a similar study, Loder et al., exposed pregnant mice at 9.5^{th} day of pregnancy to 7 hours of hypoxia (660 ppm CO), which induced congenital deformities in the cervical, thoracic and lumbar regions of the spine in 77% of progeny. They found that deformities were directly proportional to increase in hypoxia [52].

Points 2 and 3 clearly show that hypoxia is a potent inducer of severe spinal deformities in mice, which raises our curiosity in analyzing whether this stress can contribute towards the development of AIS in Humans. Moreover, the fact that hypoxia can down regulate Pitx1 expression in human cells led us to hypothetically establish a link between hypoxia, Pitx1 and AIS pathogenesis.

1.5 Hypoxia and Hypoxia inducible factors

Oxygen is very important for the survival of most living organisms. Since a sharp decrease in oxygen levels (hypoxia) would prove fatal, there evolved a complex oxygen sensing mechanism that triggers activation/deactivation of various genes to survive and adapt to the hypoxic stress. The hypoxia response pathway is well studied as it plays crucial role in development and cancer. A well-studied example for this would be the activation of Erythropoietin gene by hypoxia in humans and mice [54-56]. Erythropoietin is a hypoxia-inducible cytokine made of 165 amino acids. During hypoxic stress, erythropoietin gene gets activated, which induces erythropoiesis by stimulating proliferation and differentiation of erythroid progenitor cells leading to increased oxygenation of the hypoxic tissue. Thus, erythropoietin induction is a defensive response, which promotes oxygen supply to areas affected by hypoxia [57].

1.5.1 Hypoxia Inducible Factors (HIFs):

It was while investigating the molecular mechanisms responsible for the regulation of Erythropoietin gene (EPO) by hypoxia that Beck et al. discovered a cis-acting hypoxia response element (HRE) in the 3' flanking region to the EPO gene [56]. Subsequently, Semenza et al. identified the proteins that bound the HRE on EPO gene using DNA affinity chromatography [58]. One of them was a novel protein subunit designated Hypoxia Inducible Factor-1α (HIF-1α) and a previously known β subunit called HIF-1β [59]. HIF-α family consists of three α subunits: HIF-1α, HIF-2α and HIF-3α (Figure 1.6). The expression of these α subunits are elevated during hypoxia and tightly controlled during normoxia. The β subunit HIF-β is a constitutively expressed protein in the nucleus independent of oxygen levels and is

also known as aryl hydrocarbon receptor nuclear translocator, or ARNT. It exists in three isoforms, ARNT1, ARNT2 and MOP3 (Member of Pas Protein 3).

Hypoxia Inducible Factor α is a heterodimeric transcription factor consisting of a α and β subunits, which are two basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain proteins [60]. The HIF-α subunit is highly regulated via a proteosomal degradation pathway under normoxia conditions, whereas it exhibits higher stability and transcriptional activity under hypoxia. It is 826 amino acids long, contains bHLH and PAS domains for dimerization with the β subunit and DNA binding. The Oxygen Dependent Degradation Domain (ODDD) regulates the stability of HIF-1α [61]. It also contains two Transactivation Domains (TAD), N-TAD and C-TAD.

HIF-2 α, widely known as EPAS-1 (Endothelial PAS protein-1) was first identified through a homology search against HIF-1 α. It is also known as HLF (HIF-like factor), HRF (HIF related factor) and MOP2 (Member of PAS super family 2). Like HIF-1α, it contains an ODDD, and is induced by hypoxia and regulates target genes by binding to their hypoxia response elements as a heterodimer. It is also degraded by the same proteosomal degradation pathway. Even though it shares high homology in the DNA binding domain with HIF-1α, there is less redundancy in their functions or target genes, which explains why HIF-1$\alpha^{-/-}$ and HIF-$2^{-/-}$ mouse knockout models showed completely different types of phenotype. HIF-1$\alpha^{-/-}$ mice show poor vascular and cardiac development and embryonic lethality, whereas HIF-$2^{-/-}$ embryos exhibit more refined vascular defects, lethality due to multi-organ failure and aberrant mitochondrial function. In addition, in human cancers, poor prognostic outcomes are more associated with over expression of HIF-2α than HIF-1α.

The third member of the HIF-α family is **HIF-3α**, also known as MOP7. There are multiple variants namely, HIF-3 alpha 1 to 6. Interestingly, the HIF-3α subunit is known to

inhibit HIF-1α by binding to it, resulting in the inhibition of HIF-1 target genes like VEGF. The splice variants HIF-3 α 1 to 3 are regulated and degraded by the same pathways of HIF-1 and HIF-2α [62].

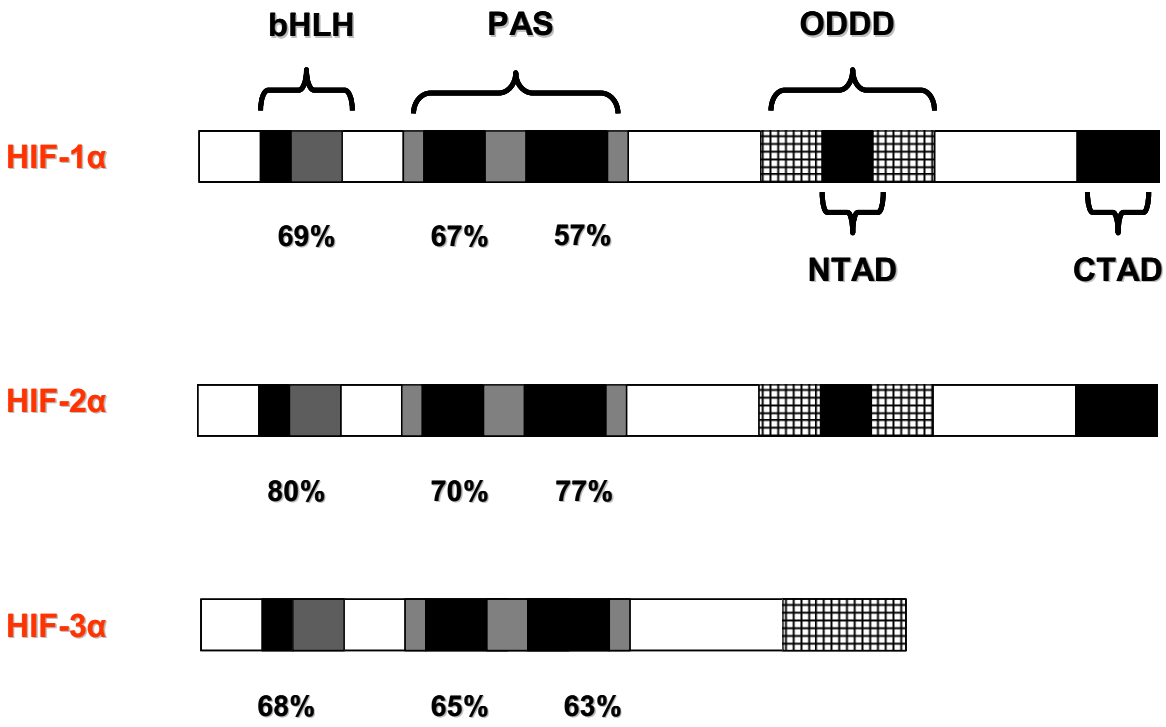


Figure 1.6: Comparison of various domains between the three hypoxia inducible transcription factors, HIF-1, HIF-2 and HIF-3α. The numbers show the percentage of similarity between the domains. bHLH: basic helix loop helix, PAS: Per-Arnt-SIM domain, ODDD: oxygen dependent degradation domain, NTAD: N terminal transactivation domain, CTAD: C terminal transactivation domain.

1.5.2 Regulation of HIFs:

HIF-1α is sometimes called the *master commander* of cellular oxygen response pathway. Its induction is very important for the viability of cells faced with hypoxia. Both HIF-1α and HIF-1β are expressed at the mRNA levels in normoxia conditions, but at the protein level, HIF-1α becomes undetectable in cells. This is because of an oxygen dependent degradation of HIF-1α protein in the cytoplasm through a proteosomal degradation pathway. This degradation is made possible by oxygen sensing enzymes called HIF Prolyl Hydroxylases Domain Proteins (PHDs) which requires oxygen, Fe2+, 2-oxoglutarate and ascorbate for their activity [63]. There are three isoforms of PHDs, namely PHD1, PHD2 and PHD3 capable of hydroxylating HIF-1α, but PHD2 shows the highest activity [64].

1.5.2.1 Oxygen dependant regulation of HIF-1α and HIF-2 α protein:

Under normoxia, PHD hydroxylates two proline residues Pro402 and Pro564 in the Oxygen Dependent Degradation Domain (ODDD) of HIF-1α [65]. The modified residues are then recognized and bound by von Hippel-Lindau (VHL) tumor suppressor protein, a component of the E3 ubiquitin protein ligase, which makes HIF-1α a target for proteosomal degradation. Inactivating mutations in VHL was shown to result in the accumulation of HIF-1α, even under normoxia [66].

Another oxygen dependant hydroxylase called Factor Inhibiting HIF-1 (FIH-1) provides a second-level of “security” if any HIF-1α somehow escapes degradation and accumulates in the cell [67]. It is an asparaginyl hydroxylase that modifies ASN803 in the C-terminus TAD of HIF-1α. This disrupts the hydrophobic interface dependent interaction

between C-TAD of HIF-1α and the CH1 domain of its co- activator P300/CBP, leading to inhibition of its transactivation [68].

Another post transcriptional modification apart from hydroxylation brings about degradation of HIF-α under normoxia. The Arrest-Defective-1 protein (ARD1), member of the super family of acetyltransferases, acetylates Lys532 in the ODDD of HIF-1α. This results in the binding of VHL to HIF-1α and its subsequent degradation by the proteosomal pathway [69].

1.5.2.2 Oxygen Independent regulation of HIFs

Apart from being regulated via oxygen dependent mechanisms like hypoxia, HIF-α has been shown to be activated by growth factor signaling, loss of tumor suppressor genes like VHL and PTEN, as well as gain of function mutations in oncogenes that can trigger the Mitogen-activated Protein Kinase (MAPK), Phosphatidylinositol 3-Kinase (PI3K) and the SRC signal transduction pathways. Recent studies have shown that HIF-1α can be activated in normoxic cells by Insulin [70] and Insulin-like growth factors [71], Tumor Necrosis Factor α (TNF-α) and Interleukin-1 (IL-1β) [72], angiotensin II [73] , and thrombin [74].

An excellent example for the oxygen independent regulation of HIF-α would be the process of its stimulation by growth factors via the activation of MAPK pathway [75]. Binding of a growth factor to its specific membrane receptor results in the activation of ERK1/2 by an upstream MAP/ERK kinase, MEK. ERK then activates MNK (MAP kinase-interacting kinases). Meanwhile, ERK also phosphorylates the P70 S6 kinase (S6K), which then phosphorylates the ribosomal S6 protein and the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1). Normally, binding of 4E-BP1 to eIF-4E prevents cap-dependent translation of mRNAs in cells, but its phosphorylation by ERK inhibits this activity.

Meanwhile, MNK phosphorylates and activates eIF-4E, which results in cap-dependent translation of mRNAs into proteins including HIF-α, in response to growth factor signaling.

In addition, current studies have demonstrated that MAPK signal transduction pathway up regulates HIF-1 activity, by encouraging the formation HIF-p300/CBP transcription complex, by modulating the transactivation of p300/CBP [75]. While oxygen dependent activation and regulation of HIF-1α happens in all cell types, growth factor mediated (oxygen-independent) regulation of HIF-1α is cell-type specific.

1.5.3 HIF-α activation: The classic hypoxia response pathway:

As mentioned earlier, under normoxia, two prolyl residues (Pro 402 and Pro 564) in the ODDD of HIF-1α are modified and leads to VHL-mediated proteosomal degradation of the protein. Hydroxylation of HIF-α requires oxygen, 2-oxoglutarate and Fe (II) as substrates. Hence during hypoxia, there would be a considerable shortage of these substrates, which would limit the activity of these hydroxylases. Thus, prolyl and asparaginyl (by oxygen dependent FIH-1 hydroxylase) hydroxylation is inhibited, and VHL cannot recognize and bind to HIF-α for its proteosomal degradation. This leads to the accumulation of HIF-α protein, which translocates into the nucleus. It forms a heterodimer with its β-subunit HIF-β and along with its transcriptional co-activators p300/CBP [76], binds to hypoxia response elements (HRE) within promoters of its target genes and induces their expression.

1.5.3.1 Targets of HIF-Hypoxia Response pathway.

Hypoxia and HIFs are known to control important genes involved in transcriptional regulation, angiogenesis, erythropoiesis, vasodilation, glucose metabolism, cell proliferation, cell survival, apoptosis, epithelial homeostasis and many more (Figure 1.8). Here is a well studied example.

Vascular endothelial growth factor (VEGF) is a cytokine whose expression is induced by HIF-1α during hypoxia. It signals to hematopoietic stem cells and endothelial progenitor cells to form new blood vessels at the site of an injury or stress thereby promoting angiogenesis. The induction of VEGF by HIF-1α/HIF-1β occurs through the HRE (TACGTGGG) within the VEGF promoter in response to hypoxic stress. Hypoxia-induced expression of VEGF is critical for the development of new blood vessels during embryonic development and enhanced blood supply to solid tumors. VEGF can be directly activated by hypoxia, or by certain growth factor stimuli which triggers MAPK or PI3K signaling pathway, activating HIF-1α leading to VEGF transcription.

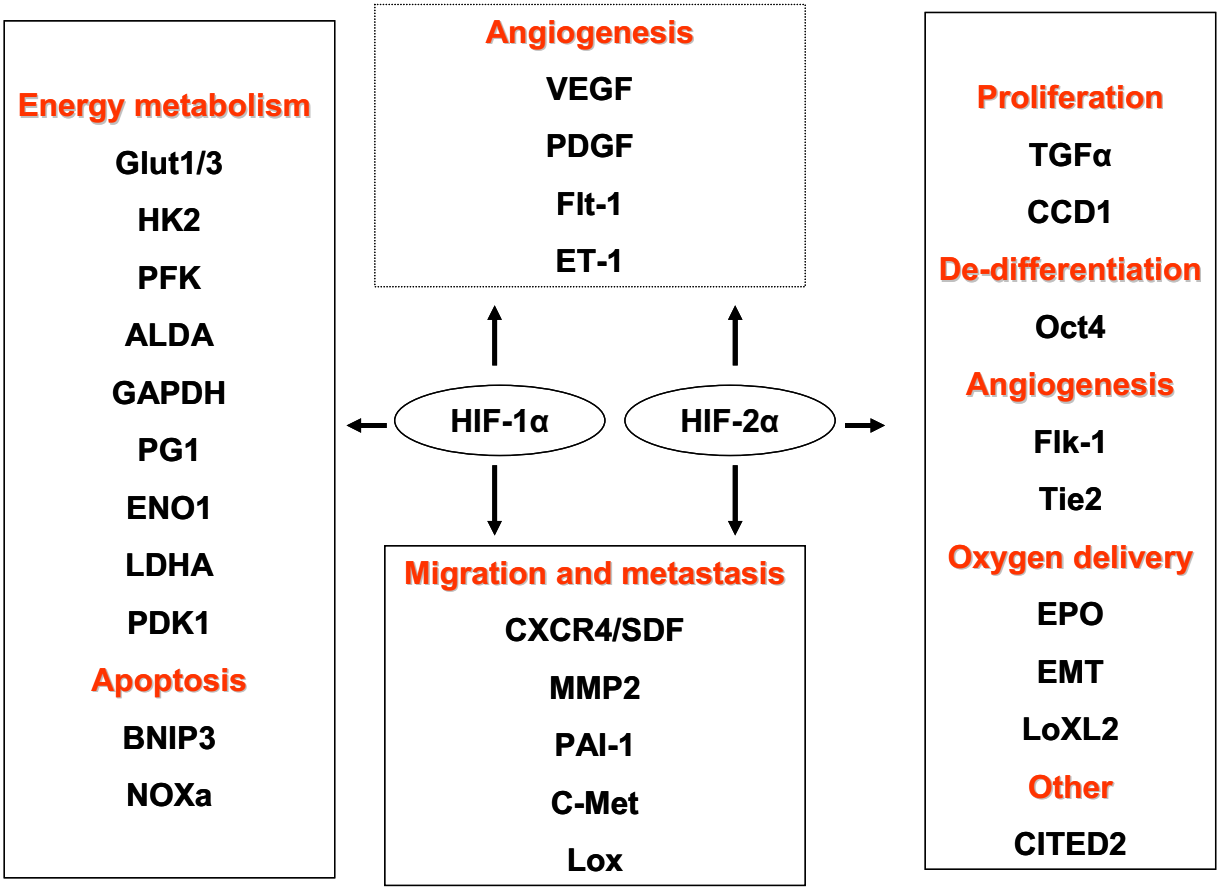


Figure 1.7: A schematic diagram showing the various targets of the hypoxia inducible factors HIF-1 and HIF-2α. Both hypoxic factors are involved in the regulation of important physiological processes including angiogenesis, apoptosis and energy metabolism.

1.5.4 HIFs and Cancer

Hypoxia is one of the well studied characteristics of most common human cancers [77]. Occurrence of hypoxia results in release of cytokines which promote vascularization, leading to progression of tumor growth and metastasis. Growing primary tumors need to establish new blood vessels to sustain their growth, hence a combination of hypoxic conditions and loss and gain of function of various tumor suppressor genes and oncogenes respectively promote over-expression of VEGF in a HIF-α dependent manner.

A relevant example for the involvement of HIF-1α in tumor severity would be Glioblastoma Multiforme (GBM), the most malignant of cancers with a patient survival rate less than 12 months and highly resistant to radiotherapy and chemotherapy [78]. The tumor cells rapidly proliferate exceeding their limited blood supply, resulting in necrosis. The surviving tumor cells surrounding the region of necrosis over express HIF-1α protein and VEGF mRNA [77]. This example shows a correlation between hypoxic condition within the tumor, induction of HIF-1α and VEGF over expression, and vascularization, and ultimately enhanced tumor growth and progression.

In certain forms of cancer, the over expression of HIF-1α protein and VEGF mRNA is not due to hypoxic conditions, but due to loss of function of important tumor-suppressor genes. Hemangioblastoma, a form of brain tumor is well characterized by extensive vascularization. As expected, it showed very high levels of VEGF mRNA and HIF-1α protein, but surprisingly not induced by hypoxic condition. Further analysis revealed a loss of function of the von-Hippel Lindau (VHL) tumor suppressor [79]. Under normal circumstances, VHL targets HIF-1α protein for proteosomal degradation, but its inactivation in this type of cancer

leads to the over-expression of its target, resulting in extremely high levels of VEGF mRNA, extensive vascularization and increased tumor survival.

Gain of function mutations in oncogenes is another deciding factor in tumor incidence and progression. Activation of RAS in human cancers switches on the MAPK pathway, resulting in the phosphorylation of HIF-1α. This increases its transcriptional activity, by encouraging the formation of HIF-1α/p300 transcriptional complex which then activate genes like VEGF, to promote vascularization in tumors and their subsequent progression.

Enhanced expression levels of HIF-2α are a characteristic feature of colo-rectal, non-small cell lung, and head and neck cancer [80]. It is also an indicator of poor prognosis outcome.

Chapter 2. Materials and Methods

2.1 Materials:

All AIS bone biopsies were collected during spinal surgery after obtaining parents' signature on a consent form approved by the ethics committee of Sainte-Justine University Hospital. Each AIS case was clinically assessed by orthopedic surgeons at the hospital. Table 2.1 and 2.1.1 contains clinical data for each patient. Details about heredity were obtained by questioning patients and their family.

Table 2.1 Clinical data of Adolescent Idiopathic Scoliosis patients

Case	Gender	Age	Curve type	Cobb angle	Heredity
1137	f	20.5	Double scoliosis	65-42	No
1167	m	14.5	Left thoracic	49	No
1045	f	19.4	Left thoracolumbar	38	No
1007	m	18.6	Right thoracolumbar	61	Mother
1006	f	12.6	Double scoliosis	61-46	No
1325	f	16.1	Left thoracic	44	Paternal grandmother
1322	f	13.1	Double scoliosis	51	No
1311	f	14.6	Double scoliosis	78	Mother
1263	f	13.3	Double scoliosis	53	No
1349	f	11.6	Left thoracolumbar	74	No
1274	f	13.2	Double scoliosis	42	No
1277	f	12.7	Double scoliosis	57-48	No
1276	f	15,2	Left thoracolumbar	42	No
1266	m	15.5	Double scoliosis	52	No
1280	f	14.4	Double scoliosis	56-46	No
1402	f	15.8	Right thoracic	51	No
1066	f	17.0	Right thoracic	53	Aunt
1439	f	nd	Right thoracic	69	Mother
1425	f	13.4	Right thoracic	68	Mother, maternal grandmother
1395	f	17.7	Left thoracolumbar	84	Aunt
1385	f	16.0	Double scoliosis	42-23	No
1391	f	15.0	Left lumbar	54	No
1409	f	13.6	Right thoracic	40	No
1417	f	13.2	Right thoracic	59	Maternal grand mother, son of cousin from father's side.
1410	f	13.7	Right thoracic	56	nd
1337	f	14.1	Double scoliosis	57-48	Father's side
1390	f	15.6	Left thoracolumbar	53	No
1406	f	14.8	Double scoliosis	62-60	No
1352	f	7.8	Right thoracic	51	No
1420	f	13.4	Double scoliosis	60-48	Maternal aunt and younger sister
1418	f	13.0	Right thoracic	41	No
1422	f	12.4	Double scoliosis	60-50	Sister
1360	f	9.9	Double scoliosis	53-46	Father, maternal aunt
1442	f	13.0	Right thoracic	60	No
1310	f	15.5	Double scoliosis	55-42	No
1318	f	13.7	Left thoracolumbar	49	No
1308	f	15.3	Double scoliosis	77-20	No
1294	f	16.9	Left thoracic	nd	Mother
1306	f	13.1	Double scoliosis	77-48	Mother, maternal grand uncle
1315	f	14.6	Right thoracic	91	No
1317	f	13.9	Right thoracic	53	Older sister
1329	m	14.0	Right thoracolumbar	61	Mother
1335	f	17.6	Double scoliosis	47-50	No
1339	f	14.2	Right thoracic	31	No
1347	f	18.5	Double scoliosis	56-45	No
1346	f	13.5	Double scoliosis	50-34	Mother

nd = no data available

Table 2.2 Clinical data of control subjects

Case	Gender	Age	Curve type	cobb angle	Heredity
1293*	m	11.7	Left lumbar	38	No
1375*	f	13.7	Right thoracolumbar	53	Cousin from father's side
1285*	f	15.8	nd	72	Cousins, mother side
1436*	f	13.9	Cyphose	120	No
1341*	f	11.1	Double scoliosis	61-65	No
1434*	f	12.4	Double scoliosis	79-77	No
1431*	m	19.1	Double thoracic	90-90	No
C100	f	nd	none	none	none
C101	nd	nd	none	none	none
C102	f	18.0	none	none	none
C103	nd	Nd	none	none	none
C104	f	14.0	none	none	none
C105	f	11.0	none	none	none
C106	m	12.0	none	none	none
C107	f	13.0	none	none	none
C108	f	12.0	none	none	none
C109	m	14.0	none	none	none
C110	m	15.0	none	none	none
C111	m	14.0	none	none	none
C112	f	11.0	none	none	none
C113	m	14.0	none	none	none
C114	nd	nd	none	none	none
C115	m	17.0	none	none	none
C116	m	12.0	none	none	none
C117	m	16.0	none	none	none
C118	m	12.0	none	none	none
C119	f	15.0	none	none	none
C120	f	15.0	none	none	none
C121	f	8.0	none	none	none

*** Non AIS: samples from patients with other types of scoliosis; C100 to C121: samples from non scoliotic, healthy subjects who underwent treatment for trauma. nd= no data available.**

2.2 Methods:

2.2.1 Cell Culture of human osteoblasts

AIS bone specimens obtained from spinal surgery (Table 2.1) were reduced to smaller pieces and incubated in a 10 cm culture dish (Corning, Life science) containing α-MEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin at 37ºC in 5% CO_2 for 30 days. Control bone specimens were similarly processed. Once the new osteoblasts derived from the bone fragments reached confluence after a proliferation period of around 15 days, they were treated with Trypsin, separated from the bone fragments and a primary osteoblast culture established. The cells were passed according to requirements, and harvested for RNA extraction and nuclear protein extraction.

2.2.2 Reverse Transcriptase-PCR

Total RNA from osteoblasts of patients and control subjects was extracted using Trizol (Invitrogen, CA, USA). 4µg of RNA was taken to synthesize cDNA using the Thermoscript RT-PCR kit (Invitrogen, CA, USA) according to the manufacturer's protocol. The PCR reaction was carried out in a final volume of 50µl containing 0.5µM of forward and reverse primers each, 0.2mM dNTP mix, 2mM $MgCl_2$, 1X Taq buffer, 0.5µg cDNA, and 1U of DNA Taq polymerase (Invitrogen, Burlington, ON, Canada). The genes Pitx1, and β-actin were amplified from the cDNA using the following primers and conditions. Pitx1 (501bp) Forward: 5'-GACCCAGCCAAGAAGAAGAA-3' Reverse:5'-GAGGTTGTTGATGTTGTTGAGG-3'; Primer annealing at 69ºC for 45 seconds, and elongation at 72ºC for 1 minute, 35 cycles. B-actin (233bp) Forward 5'-GGAAATCGTGCGTGACAT-3', Reverse: 5'-TCATGATGGAGTTGAATGTAGTT-3. Primer annealing at 55ºC for 1 minute, elongation at 72ºC for 1 minute, 30 cycles.

2.2.3 Preparation of Nuclear Extracts:

Three 10 cm dishes containing approximately $1X10^7$ osteoblast cells each were washed twice, scraped with cold PBS (10.1mM Na_2HPO_4, 1.8mM KH_2P0_4, 2.7mM KCl, and 137mM NaCl, adjusted to pH 7.4). Cells were spun at 0.1xg for five minutes at 4ºC and the resulting cell pellets re-suspended in 300µl of hypotonic buffer A (10mM Hepes pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 1% NP40, 0.5mM DTT, and 1X complete EDTA free protease inhibitor cocktail ((Roche, IN, USA)). This was incubated on ice for 25 minutes, vortexing at regular intervals. Homogenates were then spun at 0.9xg for five minutes at 4ºC to pellet the crude nuclear fraction. Both centrifugations were done using Heraeus Fresco 17 centrifuge (Thermo Scientific, MA, USA). The supernatant containing the cytosolic proteins was transferred into new tubes and spun using the above criteria, to remove residual contaminants from nuclear fraction. The crude nuclear fraction was re-suspended in 8ml of Buffer containing 50mM Tris-HCl pH 7.6, 2mM EDTA, 2mM EGTA, 1mM DTT, 0.1% Triton X-100 and 1X complete EDTA free protease inhibitor cocktail (Roche, IN, USA) and layered over a 30% sucrose solution made in the above buffer, without triton in 15ml tubes. Samples were centrifuged at 3716xg for fifty minutes at 4ºC using a Sorvall legend RT plus easy-set centrifuge with a swing out rotor (Thermo Scientific, MA, USA). The supernatant was removed, and the pellet of pure nuclear proteins was re-suspended in 50-150µl of double distilled water. The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA).

2.2.4 EMSA:

Double stranded oligo nucleotide 5'-CGACAAACTTCACCTACGTGCAAG-3' containing HIF binding site of Pitx1 promoter was labeled with ^{32}P dCTP and incubated with nuclear extracts from patients or controls in a final volume of 20µl reaction mixture containing 25ng poly(dI-dC) and a suitable 1X binding buffer (5% Glycerol, 20mM HEPES, 0.2mM EDTA, 0.2mM EGTA, 100mM KCl, 2mM Dithiothreitol). The whole mixture was incubated at room temperature for 45 minutes. For super-shifts, 2µg of appropriate antibodies were incubated with the reaction mixture on ice for 1 hour. For competitions, 10 fold, 20 fold, 40 fold and 80 fold of cold double stranded oligo nucleotide containing the consensus HIF binding site 5'-TCTGTACGTGACCACACTCACCTC-3' was added to the mixture. The DNA-protein complex was separated from the free probe on a 7% non-denaturing polyacrylamide gel at 180 V for approximately 4 hour. The gel was dried and exposed to an auto radiographic film to visualize the complexes.

2.2.5 Plasmids:

4 kb fragment corresponding to the proximal Pitx1 promoter was amplified from human genomic DNA and cloned into pGL4 reporter vector (Invitrogen). The HA tagged HIF1 α pcDNA3 expression vector and ARNT (HIF1 β) pcDNA3 expression vector were kind gifts from Dr. Eric Huang (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA).

2.2.6 Cell Culture and Transient transfection:

MG-63 human osteosarcoma cells were cultured in DMEM containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (Invitrogen) till they were confluent. Osteoblasts derived from AIS and normal subjects were cultured in α-MEM with 10% FBS

and 1% Penicillin/Streptomycin. Around 150,000 cells/well were plated in 12 well plates with DMEM or α-MEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (Invitrogen), 16 hours prior to transfection. Transfection of cells were done in Opti-MEM I reduced serum medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to standard protocol, with 250ng of reporter plasmid, 250ng of expression plasmids, and 150ng of β-Galactosidase as an internal control. 2 hours after transfection, the Opti-MEM™ I reduced serum medium was replaced by 1 ml/well serum-free DMEM or α-MEM containing 1% Penicillin/Streptomycin (Invitrogen), and the cells were incubated overnight at 37ºC. Next day the media was replaced with DMEM or α-MEM with 10% FBS and incubated for 24 hours at 37ºC.

2.2.7 Luciferase and β- Galactosidase Assays

After 24 hours of incubation, the cells were washed with PBS and lysed in 200µl of 1X Glo Lysis Buffer (Promega). The cells were collected in eppendorf tubes and spun quickly. 30µl aliquots of the supernatant were used for Luciferase and β-Galactosidase assays. Each reaction was measured in triplicate. The total Luciferase activity for control was normalized against β-Galactosidase activity and taken as 1. The results were expressed as fold-activity with respect to the control.

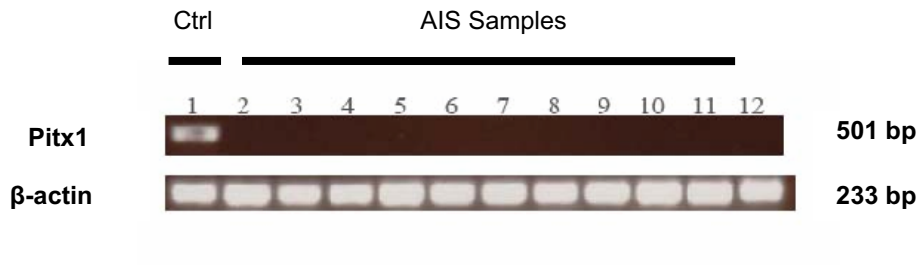
Chapter 3. Results:

3.1 Loss of Pitx1 is observed in osteoblasts from AIS subjects:

Previous studies have demonstrated that targeted inactivation of one allele of Pitx1 in mice gave rise to severe spinal deformities, which is similar to the phenotype found in patients with AIS (Figure 1.2). The Pitx1+/- mice were initially generated in Dr. Jacques Drouin's laboratory, at IRCM, Montreal.

To investigate if Pitx1 have a role in the aetiology of AIS, we analyzed its expression in osteoblasts obtained from severely affected AIS patients and non-scoliotic patients (trauma cases) using Reverse-Transcriptase PCR. All AIS osteoblasts showed a complete loss of Pitx1 expression (n=46), while the control subjects still expressed the gene (n=29) (Figure 3.1.A). In a related experiment, Pitx1 mRNA levels in AIS and control subjects were quantified using Real time PCR. Again, we observed negative levels of Pitx1 expression in AIS while the control subjects showed higher levels. (Figure 3.1.B)

A



B

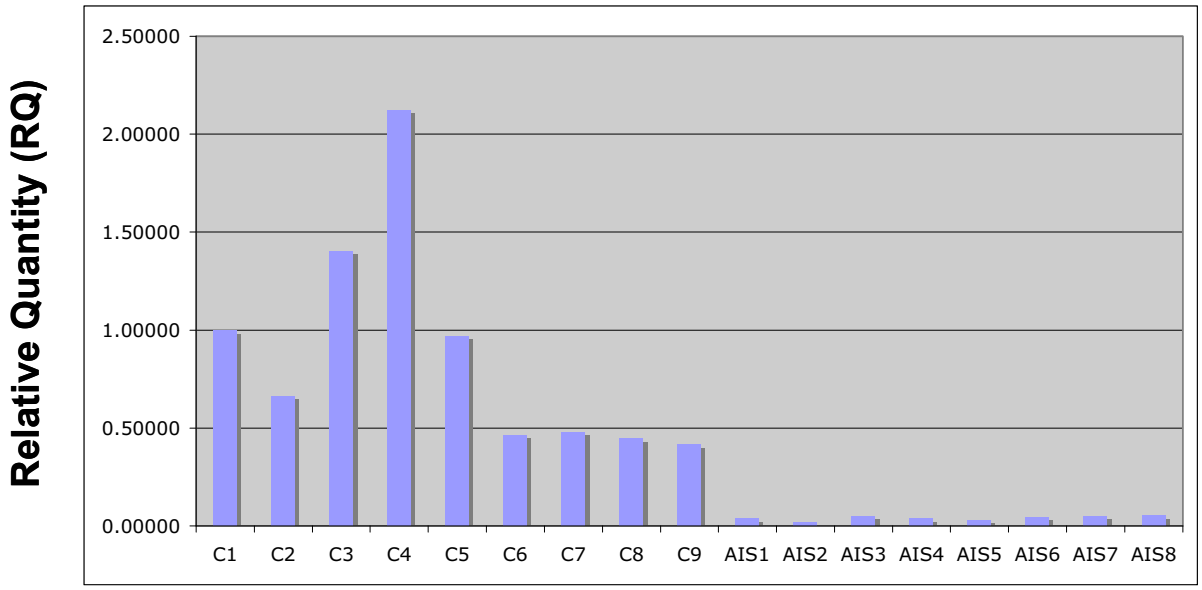


Figure 3.1: Comparison of Pitx1 expression in AIS and normal osteoblasts. A. A representative RT-PCR showing the loss of Pitx1 expression in AIS (n=46) when compared to control subjects (n=29) osteoblasts. All AIS samples showed a loss of Pitx1 expression, compared to all controls that still retained the expression. β-actin expression was taken as an internal control. **B.** Real time PCR showing the levels of Pitx1 obtained with respect to the levels of internal control GAPDH. The figure clearly shows a complete loss of Pitx1 expression in AIS compared to the control. Statistical analysis using unpaired t-test gave a value $P<0.0048$ (Control vs AIS) and $P<0.0001$ using F test to compare variances.

3.2 Identification of a single nucleotide polymorphism in the Pitx1 promoter

Once we confirmed the loss of Pitx1 expression in AIS patients, we wanted to determine if this occurs due to inactivating mutations or other genetic alterations within the regulatory regions of the gene. Approximately 10 kb of the human Pitx1 promoter was analyzed in genomic DNA obtained from AIS (n=92) and control subjects (n=88) using Fluorescence Polarization–Single Base Extension (FP-SBE) technology, at McGill University and Genome Quebec Innovation Centre. We identified a G to A polymorphism (rs6867012) located at nucleotide position -3727, upstream of the transcriptional initiation site. Statistical analysis revealed that the heterozygous variant AG had a significant association with AIS (30.6%) than controls (18.4%), p=0.057 (Table 3.1). The occurrence of the homozygous variant AA was rare in AIS (3.4%) and controls (1.2%), with p=0.61. Interestingly, the SNP is located within an E2F-like binding site, which is of interest since E2Fs can stimulate Pitx1 expression [81]. E2F is a family of transcription factors.

Table 3.1 A. Table shows the percentage of association of the homozygous wild type, heterozygous and homozygous mutant Pitx1 polymorphisms with controls (n=92) and AIS (n=88) subjects.

	Controls		AIS		P
Genotype	N	%	N	%	
GG	73/92	79.34	58/88	66	0.043
AG	17/92	18.47	27/88	30.6	0.057
AA	1/92	1.2	3/88	3.4	0.61

Table 3.1 B. Table shows the association of the Pitx1 polymorphism with clinical variables age and Cobb's angle.

	Age			Cobb's Angle		
Genotype	N	%	Mean	N	%	Mean
GG	56	67.4	14.8±2.5	53	65.4	53.7±14.3
AG	24	29.0	14.8±1.6	25	30.9	58.2±13.0
AA	3	3.6	15.9±2.0	3	3.7	56.0±11.4

Cobb's Angle (Two sample t-test) P = 0.17
Age (Two sample t-test) P = 0.88

Figure 3.2: A cartoon depicting the E2F like site on the Pitx1 promoter. The base change is at position -3727**.**

3.3 Presence of putative Hypoxia Response Element (HRE) on the promoter of human Pitx1 gene:

The hypoxia response element (HRE) consists of a core sequence 5'-RCGTG-3' and is found within promoter and enhancer regions.

Based on literature evidence that exposure to hypoxia in cells can down regulate a great number of genes, including Pitx1, we assumed it could be a direct target for the HIF transcription factors and may harbour functional hypoxia response elements within its promoter region. Using a web based Transcription Factors Binding Site Prediction tool [82], [83], we identified a potential 16bp HRE located between nucleotide -1113 and -1097 with respect to the transcription initiation site (figure 3.3)

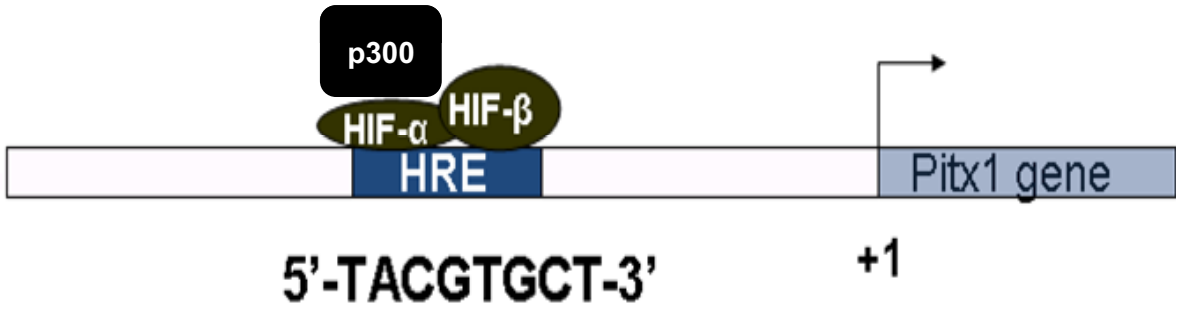


Figure 3.3: Pitx1 hypoxia response element. A cartoon showing the position of the hypoxia response element within the Pitx1 promoter, which is bound by HIF-α and β subunits along with their co-activator p300.

Table 3.2: List of a few known HREs that share sequence similarity (in uppercase) with the HRE found within the pitx1 promoter.

Hypoxia response elements	Gene	Species
gccctACGTGctgtctca	Erythropoietin	Human
gagctACGTGcgcccgta	GAPDH	Human
ggagtACGTGacggagcc	Enolase 1	Human
gctgtACGTGcattggaa	CAIX	Human
caactACGTGctctggtt	IGFBP-1	Human
cagctACGTGcccacctc	Cited2/p35srj	Human
cacatACGTGccacagtg	ID2	Human
gccagACGTGcctggagt	DEC1	Human
agctcACGTGcggaacgt	DEC2	Human
tcgctACGTGcgctcagt	Collagen prolyl 4-hydroxylase	Rat
ggtgtACGTGcagagcgc	PHD2	Human
gggctACGTGcgctgcgt	PHD3	Human

3.4 Hypoxia Inducible Factors (HIF) bind to HRE in the Pitx1 promoter:

To investigate whether Hypoxia inducible factors bound the HRE on the promoter region of Pitx1, we conducted an Electrophoretic Mobility Shift Assay (EMSA) using a radio-labelled 24bp long double stranded probe 5'-CGACAAACTTCACCTACGTGCAAG-3', (HRE-Pitx1 probe) and a cold probe containing the consensus HIF binding site 5'-TCTGTACGTGACCACACTCAC-3' (HIF CS probe) (Santa Cruz Biotechnology) as mentioned in Materials and Methods. ^{32}P labeled DNA and protein complex were visualized after electrophoresis by autoradiography (Figure 3.4). In the absence of cold HIF CS probe, two complexes were detected, showing a strong association between the labeled HIF-Pitx1 probe and proteins bound to it. The binding of both complexes were gradually reduced (Figure 3.4, lane 3-5) using AIS nuclear extracts from competition experiments with molecular excess of consensus HRE binding sequences, while such an effect was less efficient with control nuclear extracts. Hence we conclude that the potential HRE present in the promoter of human Pitx1 is indeed bound by Hypoxia Inducible Factors. To further confirm this result, we competed the HRE-Pitx1 probe with an unrelated probe, which did not contain any HIF binding site (URP-Pitx1). As expected, there was no dissociation of the two radio-labeled HRE-Pitx1-protein complexes in the presence of molar excess of cold URP-Pitx1 probe. These findings confirm the fact that there is a functional HRE on the promoter of human Pitx1 and that it is specific to Hypoxia Inducible factors.

Moreover, we observed almost no DNA-protein complex dissociation with nuclear extract from control subjects compared to AIS nuclear extract, which is intriguing.

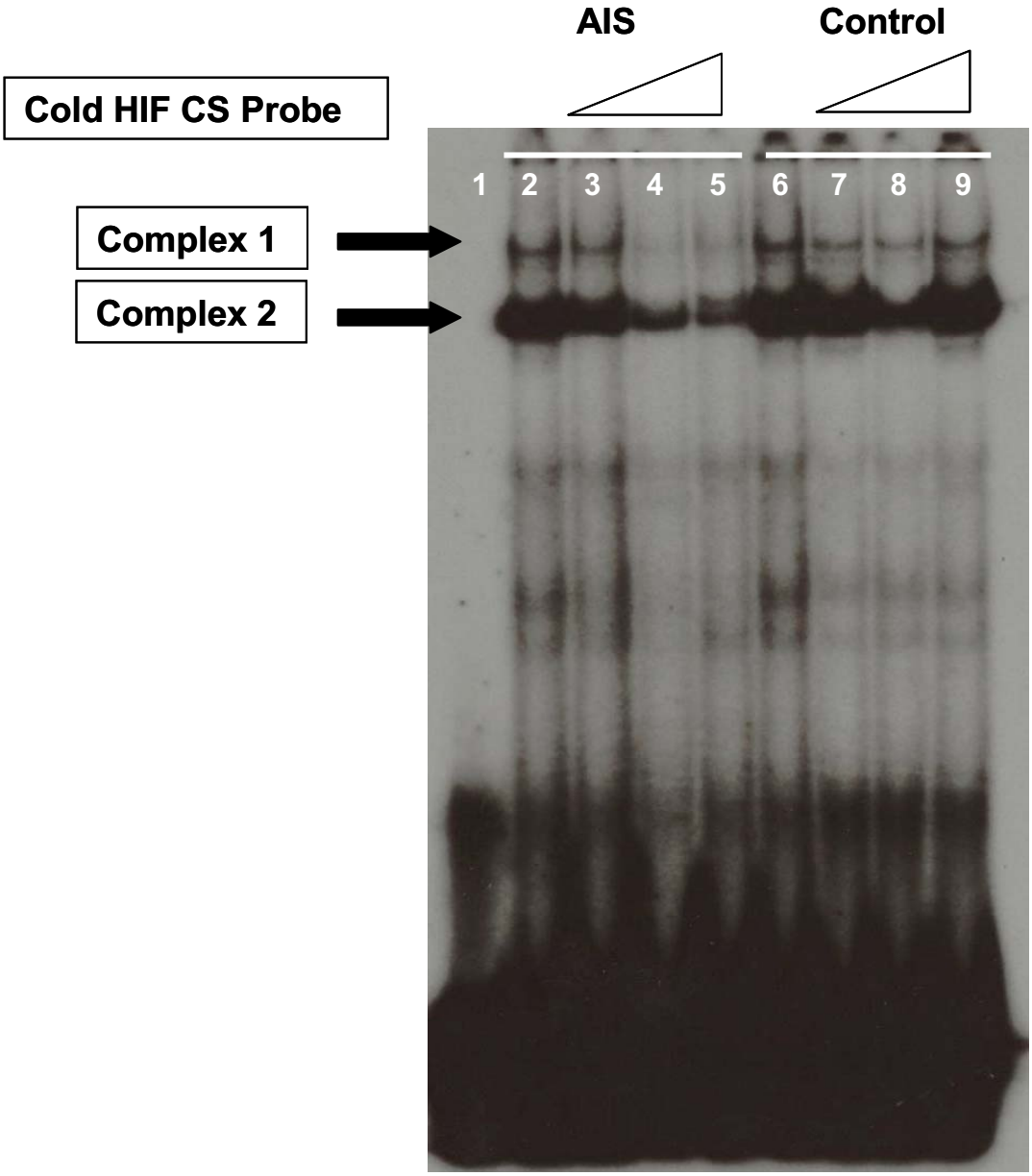


Figure 3.4: A. Determination of the putative HRE on Pitx1 promoter. Nuclear extracts from AIS osteoblasts (Lane 2-5) and control osteoblasts (Lane 6-9) were incubated with hot HRE-Pitx1 probe and molar excess of cold HIF CS probe for competition to analyze the specificity of the binding (Lane 3-5 and Lane 7-9). Lane 1 contains the free probe. Slower dissociation of the complex was observed with controls (Lanes 7-9) compared to AIS osteoblast nuclear extracts (Lane 3-5).

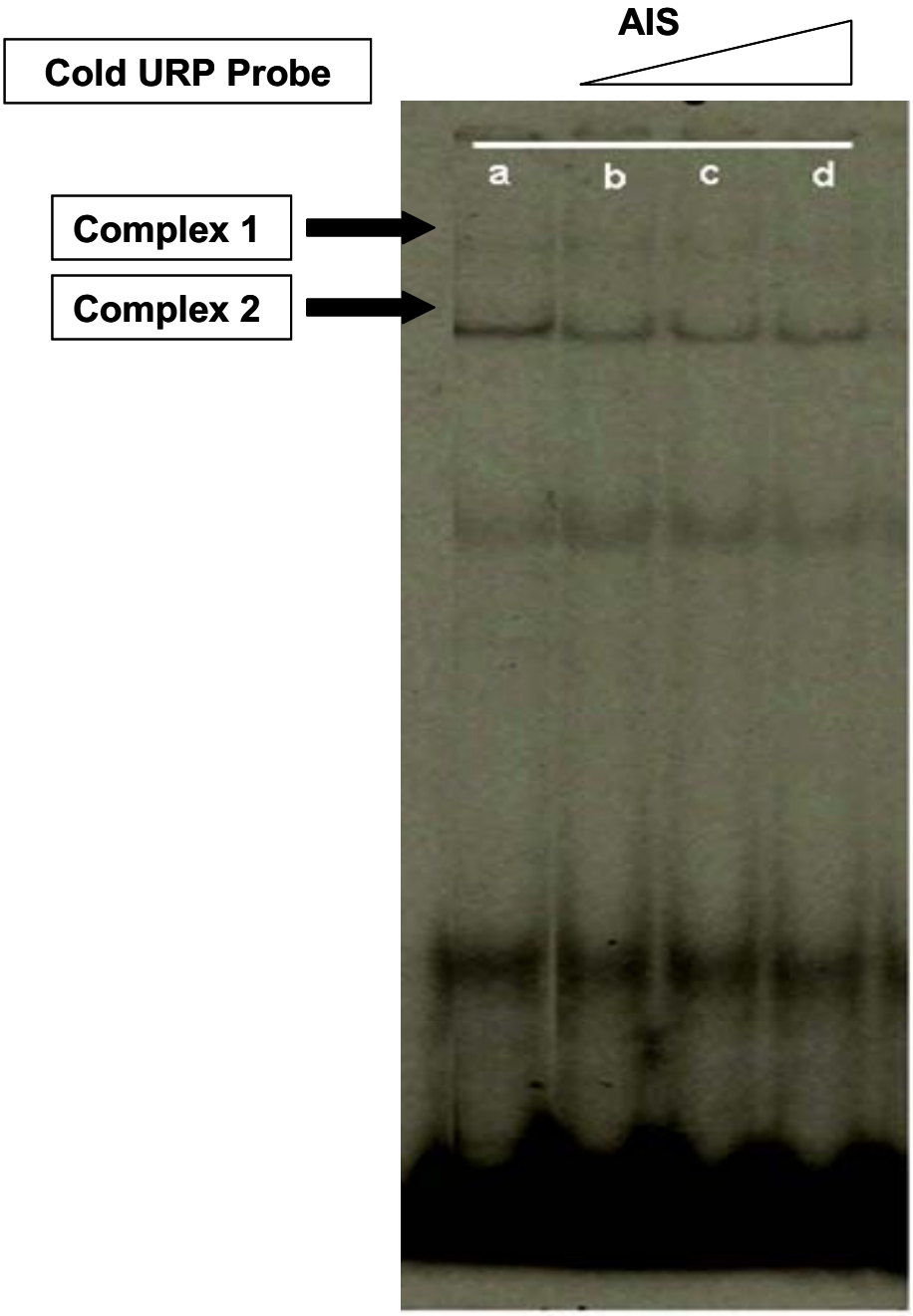


Figure 3.4 B: Determination of the specificity of HRE on Pitx1 promoter. Nuclear extract from AIS osteoblasts was incubated with hot HRE-Pitx1 probe (Lane a-d) and molar excess of cold unrelated probe URP-Pitx1 without any HIF binding site (Lane b-d) for competition. The HRE-protein complex did not dissociate with the competition, confirming the specificity of the Pitx1 HRE.

3.5 HIF-2α levels are significantly higher in AIS:

To quantify and compare HIF-2α expression levels in AIS and controls, osteoblasts were cultured in 5cm petri dishes with α MEM under normoxia. First, cells were starved with serum free media for 24 hours, and harvested for RNA extraction after 6 hours, 16 hours, 24 hours within the starvation period. Next, the serum free media was removed and cells incubated with media containing 10% FBS for a period of 24 hours. Within this time period, cells were harvested at 6 hours, 16 hours, 20 hours and 24 hours for RNA extraction.

After RNA extraction, cDNA was generated using RT-PCR for the quantification of HIF-2α expression levels using qPCR. The levels of HIF-2α were significantly higher in AIS than controls at every corresponding interval of time (Figure 3.5). For example, HIF-2α levels were higher in AIS at 6 hours after starvation than in controls. Hence, our findings suggest that HIF-2α is significantly up regulated in AIS osteoblasts even in normoxic conditions, which is quite atypical.

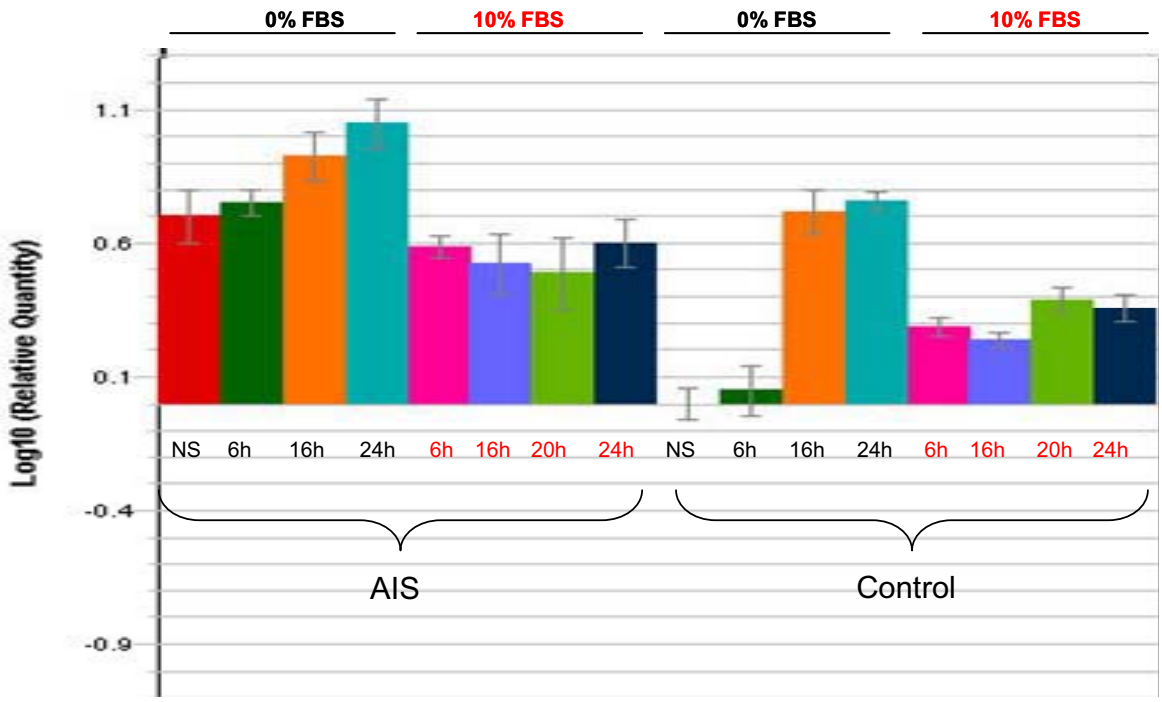
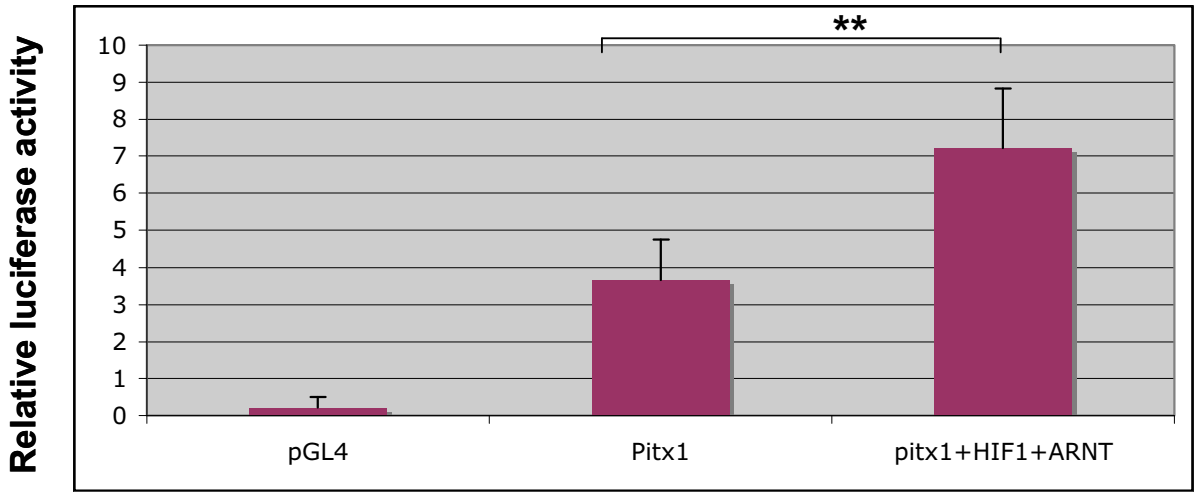


Figure 3.5: Real time PCR showing the expression pattern of HIF-2α in AIS and control osteoblasts after periods of serum starvation and supplementation. Normalization was done against the expression of β-actin.

3.6 Regulation of Pitx1 promoter activity by hypoxia inducible factors in MG63 cells:

To study the influence of hypoxia inducible factors on Pitx1, a proximal 4kb of Pitx1 promoter containing the HRE was cloned into a Luciferase reporter vector (pGL4-Pitx1). This plasmid was co-transfected into human osteoblastic cell lineMG-63 with expression vectors pCDNA-HIF-1 and HIF-2, and their β subunit pCDNA- ARNT and luciferase activity was determined. We used MG-63 cell line for this experiment since they are osteoblast-like cells and were easier to transfect than the primary cells. The luciferase activity of pGL4-Pitx1 was enhanced 250 fold due to the co-expression of HIF-2α and ARNT, and 12.5 fold by HIF-1α (Figure 3.6). This was obtained after normalization using a co-transfected β-galactosidase vector. This result suggests that HIF-2α has greater transcription activity than HIF-1 with respect to Pitx1-HRE.

A



B

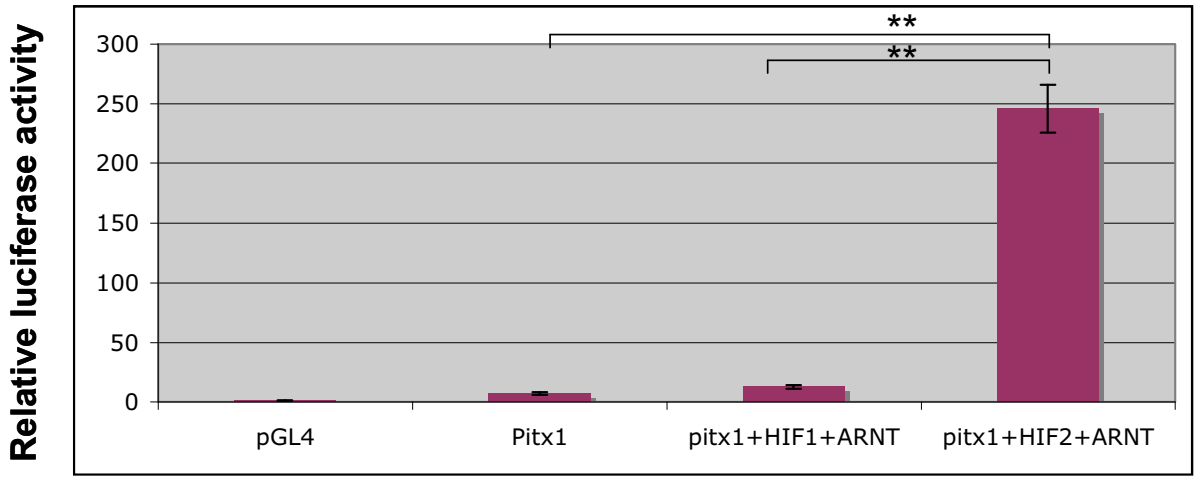
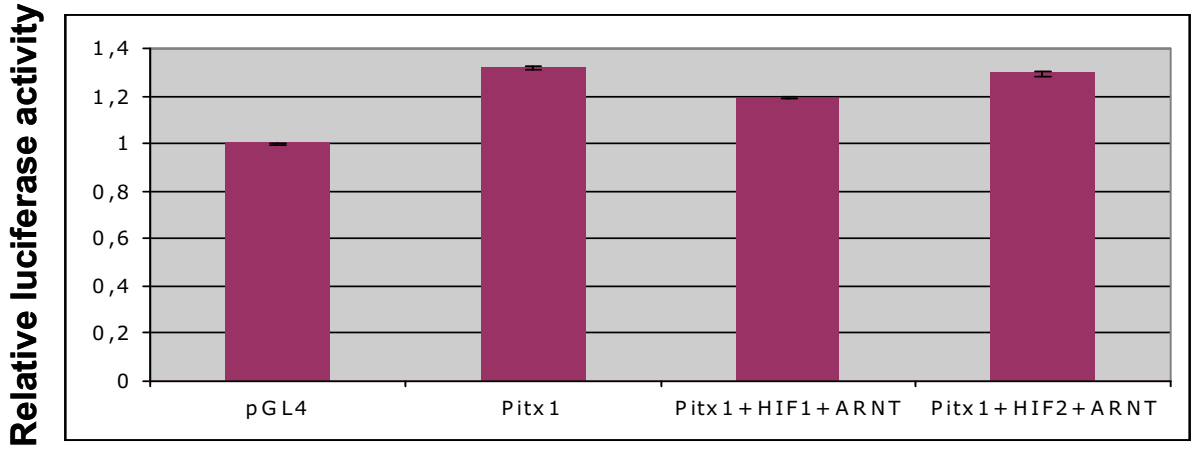


Figure 3.6 Hypoxia inducible factors up regulate Pitx1 promoter activity. A. MG-63 cells were co-transfected with expression vectors pCDNA-HIF-1α, pCDNA-ARNT and luciferase reporter vector containing Pitx1 promoter with the HRE pGL4-pitx1. **B.** Co-transfection of MG-63 cells with pCDNA-HIF-2α, pCDNA-ARNT and pGL4-Pitx1. Luciferase values were normalized using β-galactosidase values. Statistical significance was determined using one way Anova test ($P<0.0043$) followed by Tukey's multiple comparison test (P=**)

3.7 Regulation of Pitx1 promoter activity in osteoblasts derived from AIS and control subjects by hypoxia inducible factors:

Osteoblasts from AIS and controls were co-transfected with the same set of plasmids and combinations mentioned in the previous section, to analyze the activity and regulation of Pitx1 promoter. Interestingly, we found no significant Pitx1 promoter activity in AIS Osteoblasts, even after the addition of HIF2-alpha and ARNT. Whereas, Pitx1 promoter activity was enhanced by the over expression of HIF2-α and ARNT (figure 3.7). The β-galactosidase values from AIS osteoblasts were very similar to those obtained from control cells, confirming similar transfection efficiency in both the samples.

A



B.

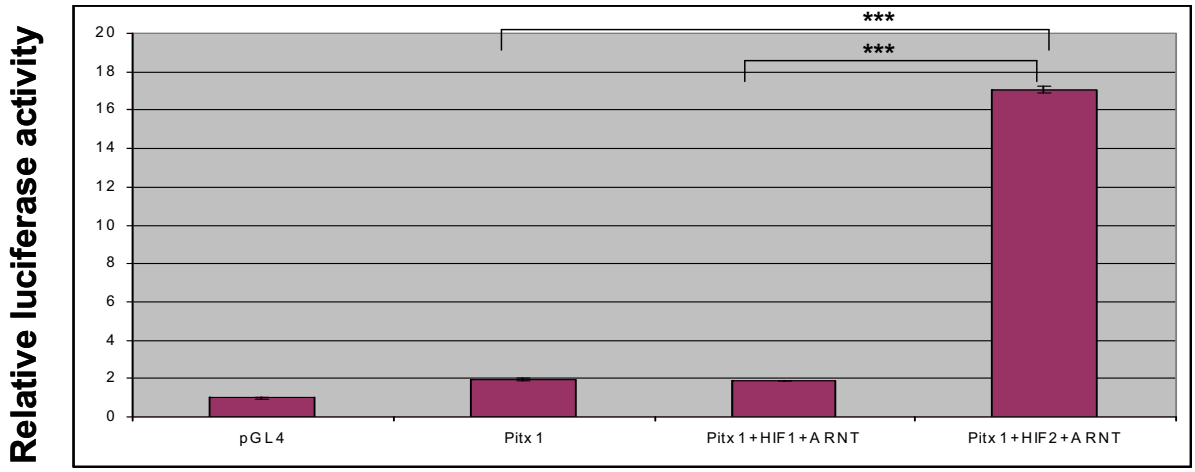


Figure 3.7: HIF-2α enhances Pitx1 promoter activity in controls, but not in AIS. AIS (**A**) and control osteoblasts (**B**) were transfected with expression vectors for HIF-1α, HIF-2α and ARNT, along with pGL4-Pitx1 reporter vector. Luciferase values were normalized against co-transfected β-galactosidase values. Statistical significance was determined using one way Anova test. *** represents $p<0.001$.

C

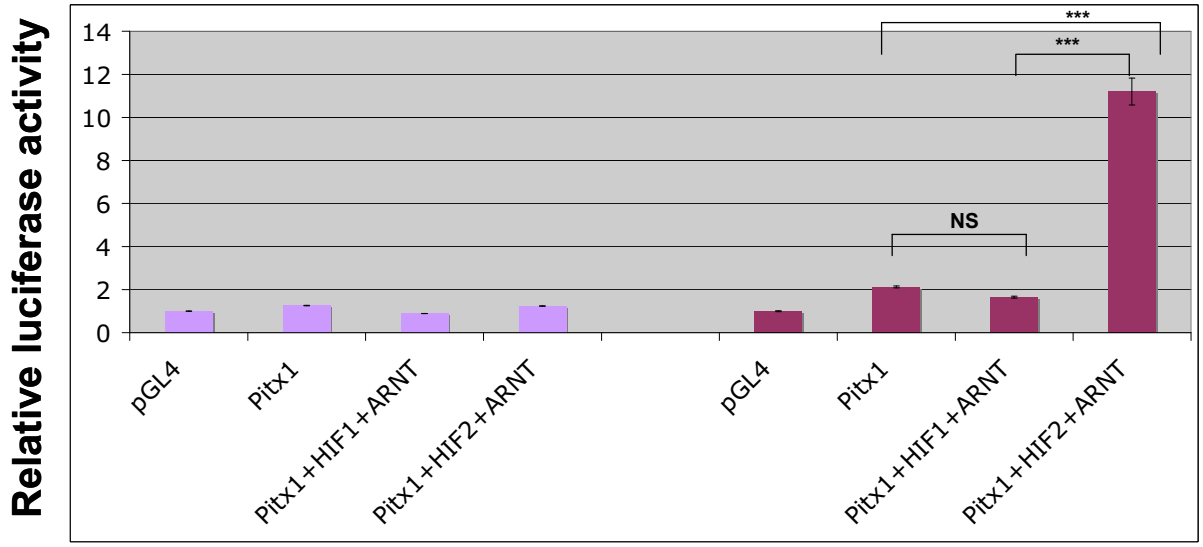


Figure 3.7: C. Pitx1 promoter activity in AIS and control osteoblasts: Values obtained from two different experiments (n=2) including the ones above (figure 3.7 A and B) in AIS (violet histograms) and control (maroon histograms) were used to construct the graph. The *** represents $P< 0.001$, analyzed using one way Anova test.

3.8: Detection of SNP in the ODDD region of HIF-1α in AIS

The existence of mutations or SNPs in the HIF-1α gene have been reported in different types of diseases, including prostrate cancer [84], colorectal cancer [85], cell carcinoma of the bladder [86] etc. The most significant polymorphism seems to be the rare C to T switch at position +1772, which corresponds to a Proline to a Serine change at position 582, within the oxygen dependant domain of HIF-1α. This mutation increased the stability of the HIF-1α protein even under normoxic conditions [84]. Due to its significance, we selected this polymorphism to be screened in AIS.

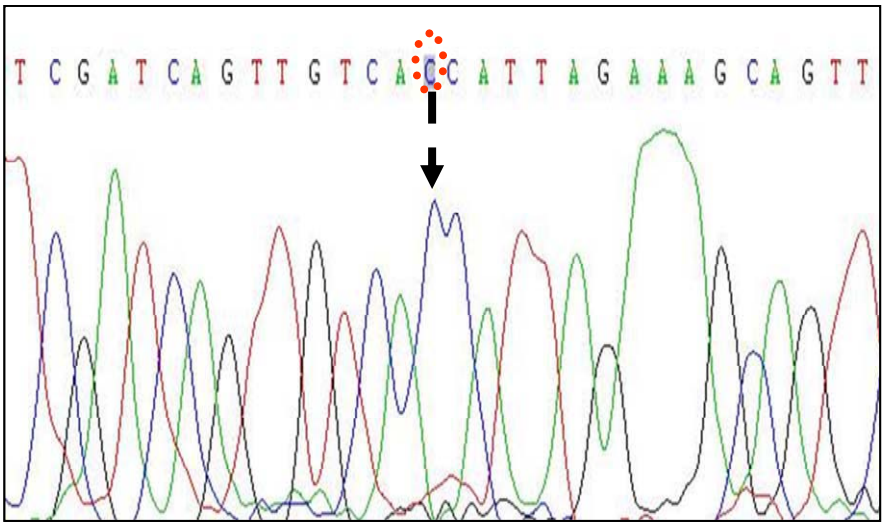
Genomic DNA extracted from AIS (n=160) and control (n=64) osteoblasts using standard protocol was sent to McGill University and Genome Quebec Innovation centre to determine the existence of P582S polymorphism in the HIF-1α gene. The technique used was Fluorescence Polarization – Single Base Extension (FP-SBE) technology.

Data analysis revealed that occurrence of this rare polymorphism was higher in AIS samples compared to controls (Table 3.3).

Table 3.3: Table shows the percentage of association of homozygous wild type, heterozygous and mutant HIF-1α polymorphisms with AIS (n=160) and control subjects (n=64).

		AIS				**Control**	
Genotype	**N**		**%**		**N**		**%**
CC	**118/160**		**73.75**		**47/64**		**73.43**
CT	**37/160**		**23.1**		**16/64**		**25**
TT	**5/160**		**3.1**		**1/64**		**1.56**

A.



B.

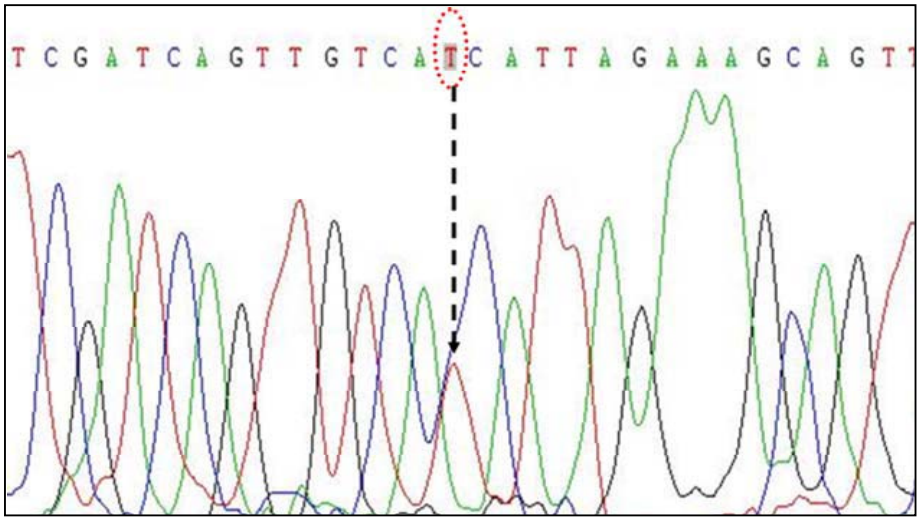


Figure 3.8: Sequence chromatograms obtained after sequencing AIS and control genomic DNA. The bases involved are circled in red, and dotted arrows indicate the peak representing them. Panel A represents the wild type (1772C) and panel B represents the mutant condition (1772T).

Chapter 4: Discussion:

Research to understand the etiology of AIS is ongoing, because observations from previous studies weren't conclusive enough, only proving that this disease is highly complex and involves multiple genes. Hence, our goal was to elucidate a specific molecular mechanism leading to the onset of scoliosis in adolescents.

4.1 Pitx1 expression in AIS:

As previously mentioned, the onset of scoliosis in Pitx1 transgenic mice prompted us to select Pitx1 as a candidate gene for the study. We observed a total loss of pitx1 expression in osteoblasts obtained from AIS subjects, compared to the controls. This was confirmed by real time PCR (Figure 3.1). Our finding is novel, and we conclude that there is a loss of Pitx1 in AIS osteoblasts. The association of Pitx1 with AIS is further strengthened by the fact that even quadrupedal Pitx1+/- mice develop scoliosis, indicating the degree of severity and penetrance of the partial loss of Pitx1 expression to acquire such a phenotype.

Our goal was to determine the cause of pitx1 repression in AIS. We explored mainly two possibilities. First, we screened approximately 10kb of pitx1 promoter region for mutations/single nucleotide polymorphisms affecting possibly its regulation, and found a known SNP (rs6867012) at position -3727 with respect to transcription initiation site. This SNP is of interest because it lies within an E2F-like site. E2Fs are known to activate pitx1 [81]. However, we ruled out the possibility that this SNP has a major contribution towards inactivation of Pitx1 by abrogating E2F binding, because there are several other E2Fs sites that could activate Pitx1 expression, and it is highly unlikely that alteration of one single site

could completely prevent the activation of Pitx1 by E2Fs. So, we didn't test the nature of the complex formed at the E2F-like site. However, we found a weak association of the SNP with AIS through statistical analysis. We propose that this SNP could be used as a genetic marker for AIS.

4.2 Hypoxia inducible factors regulate Pitx1 promoter activity

Second, we tested several possible factors that could induce the repression of Pitx1, based on literature knowledge. One of the candidates was hypoxia and hypoxia inducible factors HIF-1α and HIF-2 α. Rivard et al [51] demonstrated a correlation between hypoxia and scoliosis in mice. Pregnant mice exposed to hypoxia on E 9.5 gave birth to pups with congenital scoliosis. Adolescent scoliosis is not congenital, but they share a lot of similar characteristics. [53].

We identified the presence of a potential HRE within the Pitx1 promoter approximately 1kb upstream of the site of initiation of transcription. Using consensus HRE probe as competition probe against the radio-labeled Pitx1-HRE probe in gel shift assay, we demonstrate for the first time that the Pitx1 gene contains the core HIF binding sequence 5'-CGTGC- 3', which makes Pitx1 a target gene of HIF factors. The gel shift assays also showed that competition with a cold consensus HRE probe resulted in dissociation of the DNA-protein complex in AIS sample, while no dissociation was observed in control sample. This could be possible due to the fact that HIF factors are expressed even under normoxia in AIS, resulting in their binding to Pitx1 HRE and dissociating in the presence of competitive probe.

Although there is a high percentage of homology in the DNA binding domain of HIF-1 and HIF-2 proteins, their other domains show very less similarity which may contribute to interaction with different co-factors and co-repressors, or selective binding of either factor to HREs within target promoters. Our future goal is to examine the mechanism behind the

preferential binding of HIF-2 alpha to the Pitx1-HRE in AIS by studying the nature of the transcription complex, using gel shift assay supplemented by mass spectrometry. Also, when we compared Pitx1 HRE to HREs found on other genes (table 3.2) we found that the former is strikingly similar to the HRE found on the human Erythropoietin gene. And recent work by researchers has shown that the Erythropoietin HRE is bound by HIF-2α, and not HIF-1α, as previously thought [87]. So we assume that HIF-2α might be the important one. Hence, it will be interesting to analyze the role of HIF-2α in AIS.

4.2.1 Expression of HIF transcription factors:

We tested the expression pattern of HIF-1α and HIF-2 α using semi-quantitative PCR and quantitative PCR and observed elevated levels of both transcription factors in AIS even under normoxia, compared to controls. This finding is significant because over expression of HIFs are strongly associated with cancer pathogenesis [88] [77]. This has not been demonstrated before in the context of AIS, and we propose that over expression of HIFs could be an indicator of the severity and progression of idiopathic scoliosis. At the protein level, we expect to see a higher degree of stabilization of HIF-1 and HIF-2α proteins in AIS subjects. This will be eventually tested using Western blot and immuno- fluorescence methods.

4.2.2 Identification of Proline to Serine mutation in the ODD of HIF-1α:

Since HIF-1α is the most well studied hypoxia transcription factor, we first thought that it was the key player in the regulation of Pitx1. SNP detection analysis using genomic DNA of AIS and control subjects identified a C to T change at base 1772 from the start codon resulting in the replacement of amino acid Proline at position 582 to a Serine in 3.1% of the AIS samples compared to 1.5% in controls tested (Figure 3.8). This rare mutation has been previously reported in prostrate cancer and colorectal cancer [84] [89]. The addition of Serine is known to

increase the stability and transcriptional activity of HIF-1α even under normoxia. The discovery of P582S mutation in some of the AIS subjects is intriguing. But one should keep in mind that AIS is highly heterogenous, so it not necessary to explain everything via HIF-1. Still, it will be interesting to test the functional consequence of this mutation in AIS and to determine whether or not those female patients are at risk of developing colorectal cancer during their adult life.

4.2.3 Hypoxia inducible factors regulate Pitx1 promoter activity

We demonstrated for the first time the presence of a putative HRE within the Pitx1 promoter shown to be bound by HIF-2α. Transfection assay experiments showed a 250 fold increase in pitx1 promoter activity in MG-63 cells when co-transfected with HIF-2α and ARNT expression vectors. HIF-1α and ARNT induced only a 12-fold increase, suggesting that Pitx1 HRE can be bound by either of the HIF factors in vitro and enhance pitx1 promoter function. The 250-fold increase of promoter activity induced by HIF-2α could mean that it is the dominant HIF with respect to Pitx1 hypoxia response element. Hence, our results suggest that HIF-2α is more potent than HIF-1α in activating Pitx1 transcription, which makes sense since the HRE found in human Pitx1 promoter is similar to other HRE activated by HIF-2α [87] [90].

This result raises several questions. Pitx1 down regulation in cells treated with hypoxia have been previously reported. HIF-1 and HIF-2α are the two major players in the hypoxic response pathway. So we assumed that hypoxic down regulation of Pitx1 could be mediated through either of these factors. But transient transfection assays showed that Pitx1 promoter activity was being positively regulated by HIF-2α and ARNT in both MG63 and osteoblast cells from normal subjects (figures 3.6 A and 3.7 B). Interestingly, pitx1 promoter showed no

apparent activity in AIS osteoblasts, even with the addition of HIF-2α and ARNT. This leads us to two possible assumptions that: the presence of a repressor protein that competes with HIF-2α for the HRE binding site in AIS osteoblasts, thereby preventing HIF-2α mediated activation of Pitx1 promoter, or there maybe a co-repressor protein/complex in AIS Osteoblasts that can interact with HIF-2α and change its activity towards repression.

4.3 Hypothetical models

4.3.1 Inhibition of Pitx1 via DEC1/DEC2; Two hypoxia inducible transcription factors.

It has been previously demonstrated that hypoxia negatively regulated Pitx1 expression. So we postulated that this down-regulation maybe mediated through HIF-1 or HIF-2α. On the contrary, they exhibited a positive influence on Pitx1 promoter activity, raising the possibility of involvement of other hypoxia induced transcription factors.

Previous studies reported hypoxia mediated repression of genes like PPARγ [91], human mismatch repair gene MLH1 [92] and STAT1 [93] by hypoxia induced transcription factors DEC1 (Differentially Expressed in Chondrocytes 1) and DEC2 (Differentially Expressed in Chondrocytes 2). They are members of the bHLH subfamily and play major roles in the regulation of adipogenesis, carcinogenesis and circadian rhythm. DEC1 is also known as split and hairy related protein (SHARP2) or stimulated with retinoic acid (STRA13), and DEC2 as bHLHB3 and SHARP1. They bind to E box or E box like motifs on the promoters of their targets, which mostly results in repression. An example for this is the repression of Human mismatch repair gene MLH1 by DEC1 and DEC2 via binding to a DEC response element. Other targets of these hypoxia induced transcriptional repressors include the sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS) [94], and vascular endothelial growth factor (VEGF) [95]. Based on these studies, we assume that DEC1 or DEC2 might negatively regulate Pitx1 in AIS.

Interestingly, DEC1 and DEC2 contain hypoxia response elements and are regulated by HIF-1α [96]. So we postulate that, in AIS, HIF-1α or HIF-2α could activate DEC1/DEC2 transcription factors, which could bind to Pitx1 promoter and repress gene expression.

Moreover, DEC2 has been reported to be negatively regulating VEGF, a HIF-1α target. The inhibition occurs by the binding of DEC2 to HIF-1α, which inhibits the binding of the latter to VEGF HRE, thereby, down regulating VEGF expression [95]. So we think that a similar mechanism might be responsible for the down regulation of Pitx1 in AIS osteoblasts. Hence, we will search for putative DEC1/DEC2 binding sites on Pitx1 promoter and study their effect on the latter's activity through transient transfection assays.

4.3.2 eIF3e/Int6 deregulation could lead to hyper activity of HIF-2 alpha in AIS

eIF3e/Int6 is a tumor suppressor, which is also an e regulatory subunit of the eukaryotic translation initiation factor 3. It is a 48kDa protein with a PCI domain in the carboxyl terminal. Int6 was independently discovered during a study involving the insertion of mouse mammary tumor virus (MMTV) as a mutagen into the mouse Int6 gene. This insertion resulted in a dominant-negative C-terminal truncated Int6 protein, whose over-expression could transform cells. Injection of these Int6- transformed cells led to the development of tumors in nude mice.

Chen et al., identified Int6/eIF3e to be regulating HIF-2 α in a hypoxia and pVHL independent manner, using yeast two-hybrid analysis with HIF-2 α C-terminal as bait. Moreover, Int6/eIF3e co-precipitated with HIF-2 α and was found to co-localize together into the nucleus even under normoxia. Int6/eIF3e bound to HIF2, resulting in latter's destabilization. Interestingly, yeast two hybrid analysis showed that Int6/eIF3e did not bind to HIF-1 or HIF-3 α. Moreover, dominant negative mutant of Int6 resulted in an increase in the endogenous levels of HIF-2α. Their results showed that interaction between Int6/eIF3e and HIF-2α results in the proteosomal degradation of the latter, even in cell lines that lacked endogenous pVHL expression [97].

We think it could be possible that de-regulation Int6/eIF3e could result in the over expression and stabilization of HIF-2α in AIS, thereby playing a critical role in the onset of disease. Our initial step will be to test the expression levels of Int6/eIF3e at the mRNA and protein level in AIS and controls, HIF-2α protein expression in AIS and control osteoblasts co-transfected with Int6/eIF3e expression vector through western blot.

Chapter 5: Summary

The main goal of this project was to study factors contributing to the etiopathogenesis of AIS. Using candidate gene driven approach, we chose Pitx1 as our gene of interest. We demonstrate for the first time a complete loss of Pitx1 in the osteoblasts of AIS patients, extracted from bone fragments obtained during spinal surgery. This result validates the first part of our hypothesis: that the expression of Pitx1 is affected in AIS.

Researchers studying human diseases look for single nucleotide polymorphisms mostly exclusive to affected individuals and not to the general population. SNPs can be used as a biomarker and also in finding a susceptible genetic loci associated with a disease. We discovered a SNP within the Pitx1 promoter which had more association with AIS than controls, suggesting its role as a potential biomarker. We will conduct studies to see if this SNP have any functional consequence on Pitx1 transcription by facilitating binding of repressor protein complexes to the modified site using gel shift assays, and DNA pulldown followed by mass spectrometry.

We demonstrated for the first time the existence of a functional hypoxia response element in the Pitx1 promoter. We expected to see a repression of pitx1 promoter activity by HIF-1 and HIF-2, but we were surprised to see them induce a 12 fold and 250 fold increases in luciferase activity respectively. We assume that there might be either other hypoxia inducible factors mediating the hypoxic down regulation of Pitx1 or co-repressors. However, there was no Pitx1 promoter activity induction by HIF-2α in AIS osteoblasts. These observations raise several questions, which we will try to answer as part of an upcoming PhD project.

6. Relevance

Our above finding is novel and is significant to ours and to other fields of study. Our work identifies Pitx1 as a major player in the etiology o AIS, therefore providing a more specific candidate for further studies. We hope that understanding the molecular mechanisms regulating Pitx1 will help us to explain the onset of scoliosis in adolescents.

We also demonstrated that HIF factors bind to a hypoxia response element on Pitx1 promoter and regulate its expression. Our observation linking Pitx1 and HIF factors can be of interest also in the field of cancer research, because Pitx1 is a well studied tumor suppressor usually down regulated in various cancers (Table 1.2). It would be interesting to look at the relationship between Pitx1 and HIF-2α in different types of cancer cell lines, and how it can influence tumor formation and severity.

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