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Pitx2 is Overexpressed in Follicular Cell-Derived Thyroid Cancer and Promotes Thyroid Cancer Proliferation by Regulating Cell Cycle

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PITX2 IS OVEREXPRESSED IN FOLLICULAR CELL-DERIVED THYROID CANCER AND PROMOTES THYROID CANCER PROLIFERATION BY REGULATING CELL CYCLE PROGRESSION

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ABSTRACT

Thyroid cancer is the most prevailing malignancy of the endocrine system. Its incidence is rapidly rising at the second fastest rate of all malignancies in the United States, making it a significant health problem.

Although the majority of thyroid cancer is slowly-growing and well-differentiated, available treatment options are very limited, and most of them require complete removal of the thyroid gland and surrounding tissues. Patients who have undergone thyroid removal have to take life-long hormone replacement therapy, which is very inconvenient and costly. Therefore, there is an urgent need to develop new treatments for this disease. As a prerequisite for designing a better therapy for thyroid cancer patients, we now must further our understanding on how thyroid cancer develops, especially its underlying molecular mechanisms.

In this study, we have found that PITX2, a *bicoid* homeodomain transcription factor known to play a critical role in the left-right asymmetry formation as well as the development of multiple organs, is frequently expressed in human follicular cell-derived (papillary, follicular and anaplastic) thyroid cancer tissues but not in normal thyroids. This is the first finding that indicates over-activated PITX2 may contribute to the development of thyroid cancer. Following this exciting discovery, we performed cell-based and biochemical studies to uncover the molecular mechanism of PITX2 action in thyroid tumorigenesis.

Knockdown of PITX2 gene expression in human thyroid cancer cells significantly reduced cell proliferation and soft-agar colony formation. Biochemical analysis of cell

cycle regulators upon PITX2 knockdown revealed downregulation of Cyclin D1, Cyclin D2 and dephosphorylation of Rb. Chromatin immunoprecipitation and promoter reporter assay indicated that Cyclin D2 was a direct target gene of PITX2. Consistently, we observed that high expression levels of Cyclin D2 were frequently associated with PITX2 expression in follicular cell-derived thyroid cancer tissues. To confirm these findings *in vivo*, we took advantage of a mouse model of thyroid cancer (*TRbeta*$^{PV/PV}$ mouse). Consistently, the aberrant elevation of *Pitx2* levels in the thyroid cancer of *TRbeta*$^{PV/PV}$ mice were accompanied by the upregulation of Cyclin D1, Cyclin D2 and increased phosphorylation of Rb. Taken together, these results provide the first evidence implicating an oncogenic role of PITX2 in human cancer.

To better understand the role of PITX2 in the regulation of gene transcription, we aimed to decipher PITX2 regulating and interacting networks by genomic and proteomic approaches. As a result, we identified four novel PITX2-associated protein partners YB-1, hnRNP K, nucleolin and hnRNP U in mass spectrometry analysis. Overexpression of PITX2 resulted in upregulation of 868 genes (two-fold to twenty five-fold) and downregulation of 191 genes (two-fold to fifteen fold) in microarray analysis. Using semi-quantitative RT-PCR, we verified 16 potential PITX2 target genes. Interestingly, Cyclin A1, a male germ cell-specific gene essential for spermatogenesis, is among the most upregulated genes.

We then investigated whether Cyclin A1 was a PITX2 target gene in the context of thyroid cancer cells. Remarkably, we found that Cyclin A1 indeed was expressed in papillary thyroid cancer but not in normal thyroids. Using promoter-driven reporter assays, an evolutionarily conserved DNA element responsible for PITX2-induced gene

transcription was identified in the Cyclin A1 promoter. Intriguingly, further biochemical evidence demonstrated that PITX2 activated Cyclin A1 through a histone H3K4 methylation pathway.

Collectively, our data reveal for the first time that PITX2 may play an oncogenic role in human thyroid tumorigenesis. Aberrant expression of PITX2 in thyroid cancer promotes cell proliferation by facilitating cell cycle progression. This oncogenic effect of PITX2 is at least in part mediated by its transcriptional target genes Cyclin D2 and Cyclin A1. This study furthers our understanding of the molecular mechanisms that govern thyroid carcinogenesis and provides a new perspective on the development of novel therapeutics for thyroid cancer patients.

Keywords: Thyroid cancer, PITX2, Gene Transcription, Cyclin D2, Cyclin A1, Cell Cycle, Protein-Protein Interaction

DEDICATION

I would like to dedicate this work to my family and all of my friends who have supported me during my education.

To my grandmother, who blesses me from heaven. Thank you for giving me the motivation of always looking forward, no matter how hard the life is.

To my parents, who lost the opportunity to receive their education but do everything they can to support my education and my dreams.

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LIST OF SYMBOLS AND NOMENCLATURES

PITX2 -- pituitary homeobox 2

YB-1 -- Y box binding factor-1

hnRNP U -- heterogeneous nuclear ribonucleoprotein U

hnRNP K -- heterogeneous ribonucleoprotein K

NF-1 -- nuclear factor-1

HMG-17 -- high mobility group-17

GcMa -- glial cells missing a

Six1 – six homeobox 1

LEF-1 – lymphoid enhancer factor 1

MEF2a – myocyte-enhancing factor 2A

SRF – serum response factor

Pit-1 – pituitary-specific POU homeodomain factor 1

RET -- rearranged during transfection

RAS – rat sarcoma viral oncogene homolog

BRAF – v-raf murine sarcoma viral oncogene homolog b

PPARγ – peroxisome proliferator-activated receptor γ

PAX8 -- paired box-8

MAPK – mitogen-activated protein kinase

PLOD -- procollagen lysyl hydroxylase

TRβ -- thyroid receptor beta

CDK -- cyclin-dependent kinase

Rb – retinoblastoma

TGFβ -- transforming growth factor β

Wnt – wingless-type MMTV integration site family

GAPDH -- glyceraldehyde-3-phosphate dehydrogenase

NCOA4 -- nuclear receptor coactivator 4

HD – homeodomain

CSD – cold shock domain

OAR -- otx, aristaless, rex

DNA - deoxyribonucleic acid

mRNA -- messenger ribonucleic acid

shRNA -- small interfering ribonucleic acid

GTP -- guanosine triphosphate

IHC – immunohistochemistry

IF -- immunofluorescence

IP – immunoprecipitation

ChIP – chromatin immunoprecipitation

IB – immunoblotting

WB – western blotting

RT-PCR – reverse transcriptase polymerase chain reaction

nM – nanomole

µM – micromole

µm – micrometer

kD – kilodalton

bps -- base pairs

US – United States

H3K4--histone H3 lysine 4

MLL--mixed-lineage leukemia

MLL4-- mixed-lineage leukemia 4

HMT---histone methyltransferase

PREFACE

There are five chapters in this dissertation, three of which contain primary research data. Chapter I is a comprehensive literature review of thyroid cancer development. Chapter II mainly addresses the pathological role of PITX2 in thyroid cancer. Chapter III focuses on our efforts to decipher the regulatory and interacting networks of PITX2. Chapter IV explores the molecular mechanism of PITX2 action. The final chapter includes a general discussion, conclusions and future directions of my study.

CHAPTER I

LITERATURE REVIEW

Thyroid cancer development and progression

Overview

Thyroid cancer is the most common endocrine-related cancer in the US [1]. The case number of newly diagnosed thyroid cancer has been rapidly rising recently, with an average annual growth rate of approximately 10% in the past five years [1]. According to the American Cancer Society, the estimated new thyroid cancer cases were 44,670 in 2010 in the US. The increasing incidence of thyroid cancer has been largely attributed to the advancement of diagnostic techniques, such as ultrasound and fine needle aspiration [3]. There are many risk factors associated with thyroid cancer, such as age, gender, exposure to radioactive substances and predisposed genetic aberrations. Interestingly, women are three times more likely than men to develop thyroid cancer. Although numerous studies have been conducted to unravel the etiology of thyroid cancer, our understanding of the development of thyroid cancer is still largely incomplete.

The thyroid gland is the largest endocrine organ in human and consists of two lobes that attach to the larynx. It secretes various hormones to regulate the balance of metabolism. The functional unit of thyroid is called thyroid follicle, which is surrounded by thyroxine-secreting follicular cells and calcitonin-secreting C cells. Thyroid cancer can be developed from both follicular cells and C cells (Figure 1).

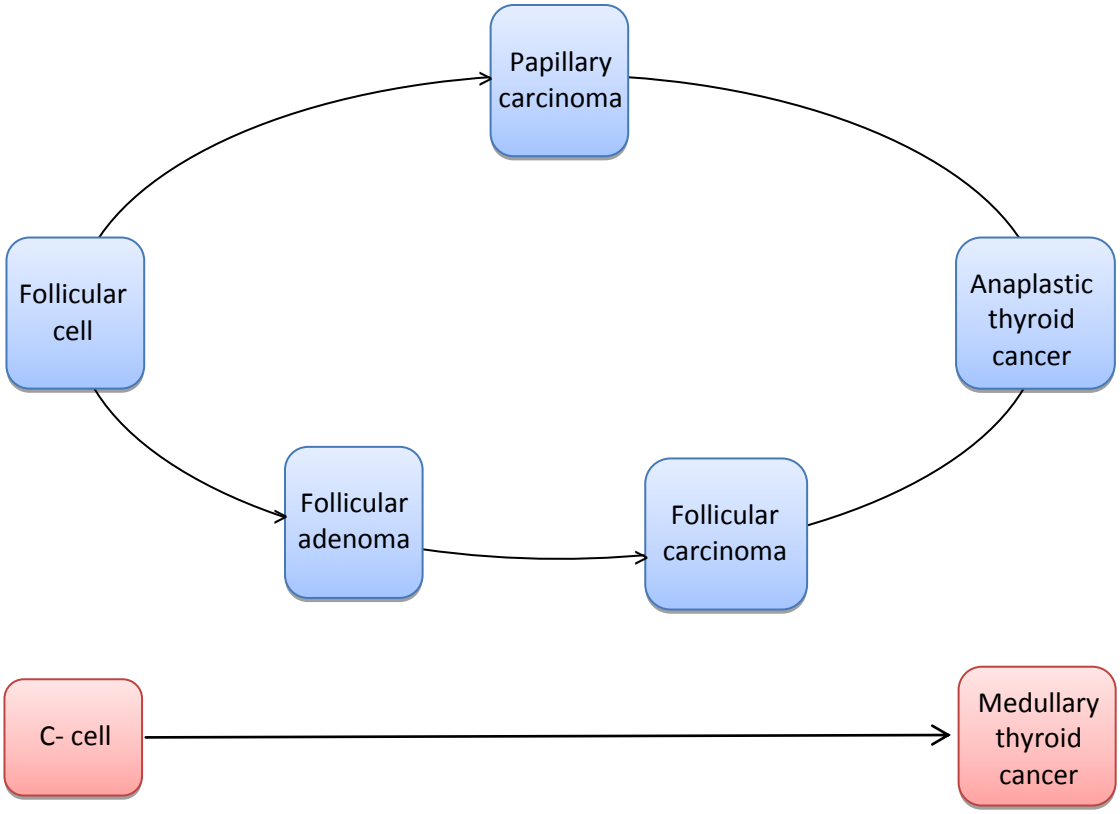

Figure 1. A proposed model of thyroid cancer development. Both papillary and follicular thyroid cancers are originated from T3/T4 secreting thyroid follicular cells. Anaplastic thyroid cancer is developed from either papillary thyroid cancer or follicular thyroid cancer. Medullary thyroid cancer is originated from calcitonin secreting C-cells.

There are four major types of thyroid cancer: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), anaplastic thyroid cancer (ATC) and medullary thyroid cancer (MTC). Both papillary and follicular thyroid cancers are mostly well-differentiated and slow growing. PTC and FTC account for ~95% of total thyroid cancer cases [4]. Metastasis to distant organs is rarely observed in PTC and FTC patients. The 5-year survival rate for well-differentiated thyroid cancer is more than 95% [3]. Anaplastic

thyroid cancer is very aggressive and poorly-differentiated. Although only representing 1% to 2% of total thyroid cancer incidences, ATC contributes to approximately 40% of total thyroid cancer deaths [5]. The mean survival time for ATC patients after diagnosis is merely 6 months [5]. Medullary thyroid cancer is generally more malignant than PTC and FTC, but it is less malignant than ATC. Approximately 3% of thyroid cancer incidences are classified as medullary thyroid cancer [4]. Local vascular and lymph node invasions are frequently observed in MTC patients. However, distal organ metastasis is unusual for MTC. Both PTC and FTC are developed from thyroxine-secreting follicular cells. Anaplastic thyroid cancer is considered to be arisen from well-differentiated thyroid cancers, such as PTC and FTC. Medullary thyroid cancer is originated from calcitonin-secreting C cells (Figure 1).

Diagnosis, prognosis and treatments

The diagnostic techniques for thyroid cancer have been much advanced with the invention and utilization of new technologies, especially ultrasound and fine needle aspiration. In fact, the extensive application of new diagnostic techniques has been considered as the main reason for the rapidly growing rate of newly diagnosed thyroid cancer cases. Although most papillary and follicular thyroid cancer cases have distinguishable characteristics that can help clinicians render accurate diagnosis, there are about 15% of thyroid cancer cases that demonstrate characteristics of both papillary and follicular thyroid cancers [3]. Current diagnostic techniques are not sufficient to make a diagnosis without partial or total thyroidectomy. Similarly, 15-20% of the fine needle aspirations are inconclusive. This typically results in patients undergoing partial or total

removal of the thyroid gland [3]. Therefore, new diagnostic techniques, which can help clinicians to make a diagnosis without partial or total thyroidectomy, are urgently needed.

Currently, several commonly-used prognostic scoring systems, like the tumor node metastasis classification, have been adopted for the prognosis of thyroid cancer patients. However, these prognostic systems are not suitable to predict the local or distant recurrence, which occurs in approximately 20% of thyroid cancer patients [6]. Anaplastic thyroid cancer is the most aggressive thyroid cancer and is considered to be a fatal disease. There is not a prognostic method available right now to predict whether a well-differentiated thyroid cancer will ultimately develop into poorly-differentiated anaplastic thyroid cancer. About 500 to 600 million people around the world suffer from thyroid nodular goiter, and 5% of these thyroid abnormities will eventually develop into malignant thyroid tumors [3, 7]. There is a great need for an effective prognosis system to discriminate high-risk thyroid nodules from the vast amount of nodules that have been diagnosed.

Thyroid cancer management has not been changed much in the past several decades. Partial or total thyroidectomy is still the most common treatment option for primary thyroid tumor. Radioiodine and suppressive treatment are the usual regimen for thyroid cancer patients after undergoing thyroidectomy. Patients who have undergone thyroid removal have to be placed on life-long thyroid hormone replacement to maintain their normal homeostasis rate. Like other types of cancer, the treatment options for metastasized thyroid cancer patients are very limited and mostly ineffective. Hence, there is a constant urgency for clinicians and scientists to develop new therapies for metastasized thyroid cancer. Our knowledge about thyroid cancer has greatly expanded in

the past decade, especially regarding the molecular and genetic aspects. In light of this, many novel therapies, such as those targeting deregulated or mutated molecules, have been designed and developed. A few of them have entered clinical trials. These new therapies will give patients more options and hopefully are more effective and less painful than traditional therapies.

Papillary thyroid cancer

Papillary thyroid cancer is the most frequent type of thyroid cancer. It alone makes up about 80% of thyroid cancer cases [4]. With the improvement of diagnostic methods, the incidence rate for papillary microcarcinoma has experienced a 2.4-fold increase over the past decade [8]. Most papillary thyroid cancer cases are well-differentiated and slow growing. Although metastasis to distant organs are uncommon for papillary thyroid cancer patients, local invasions to surrounding tissues, such as lymph nodes, are relatively frequent when compared to well-differentiated solid tumors originated from other organs. Papillary thyroid cancer is generally considered curable, with a 5-year survival rate about 97% [3].

Papillary thyroid cancer is arisen from thyroxine-secreting thyroid follicular cells. Almost 100% of radiation exposure-induced thyroid cancer is papillary thyroid cancer. With the swift advancement of cancer genetics, we are now able to understand more about the underlying molecular mechanisms of thyroid tumorigenesis. Genetic alterations have been found in the majority of papillary thyroid cancer patients. There are three types of genetic alterations that are frequently detected in papillary thyroid cancer: *BRAF*,

RET/PTC and *RAS* (Table 1). *BRAF* mutation in papillary thyroid cancer is a point mutation that results in a valine (V) substituted by a glutamic acid (E). *BRAF* mutation is the most common genetic alteration in papillary thyroid carcinoma and is found in approximately 45% of papillary thyroid cancer patients [4, 9-10]. *BRAF* V600E mutation is highly prevalent in conventional papillary thyroid cancer and tall-cell variant papillary thyroid cancer [9-10]. The point mutation in papillary thyroid cancer leads to constitutive

Tumor type	Prevalence (%)
Papillary carcinoma	
BRAF	45
RET recombination	20
RAS	15
Follicular carcinoma	
RAS	45
PAX8-PPARγ	20
PIK3CA	<10
PTEN	<10
Anaplastic thyroid carcinoma	
TP53	70
β-catenin	65
RAS	55
BRAF	20
PIK3CA	20
PTEN	10
Medullary thyroid carcinoma	

Inherited RET	>95
Sporadic RET	40

Table 1. Prevalent of common genetic alterations in thyroid cancer

activation of BRAF kinase activity, which phosphorylates downstream effectors and ultimately activates the MAPK (mitogen-activated protein kinase) signaling pathway [11-12]. MAPK signaling is a well-known oncogenic pathway that promotes cancer development and progression [3-4]. *BRAF* mutation has been associated with more aggressive papillary thyroid cancer with characteristics of extra-thyroidal extension, advanced tumor stage at presentation, tumor recurrence and lymph node or distant metastases [13-15]. In light of this, *BRAF* mutation has been used as a prognostic biomarker for tumor recurrence. BRAF is also an excellent target for therapeutic purpose since BRAF mutation is observed in a high frequency of papillary thyroid cancer patients and generally presents in more advanced papillary thyroid cancer. In addition, since BRAF is the downstream effector of RET and RAS signaling, inhibiting BRAF alone may have inhibitory effects on RET and RAS signaling as well. Currently, several small-molecule kinase inhibitors have been specifically designed to inhibit the kinase activity of BRAF. One of the most promising inhibitors is BAY 43-9006, which has demonstrated potent effects on inhibiting thyroid tumor growth in both pre-clinical and early stages of clinical trials [16-17].

The second most frequent genetic alteration in papillary thyroid cancer is the rearrangement of the RET gene, which occurs in about 20% of papillary thyroid cancer

patients [4]. The incidence of *RET* rearrangements for child and young-adult patients, who have been exposed to radiation, is 50%-80% and 40%-70%, respectively [4]. *RET* rearrangements are clearly linked to radiation exposure as evident in many studies, such as those following the well-known Chernobyl accident [18]. In 1986, a nuclear reactor explosion caused fallout of radiation to Belarus, Northern Ukraine and part of the Russian Federation. This accident dramatically increased the incidence of childhood thyroid cancer from 10-fold to 100-fold [18]. Later, histological and genetic analysis revealed that almost 100% of these radiation-induced thyroid cancers were papillary thyroid cancer and ~70% of them presented with *RET* rearrangements [18]. Although 12 different RET rearrangements have been identified, all these rearrangements produce a fusion protein, which consists of the C-terminal (kinase domain) of RET and the N-terminal part of the fusion partner [19-22]. The most frequent fusion partners of RET are histone H4 and NCOA4, which account for almost 100% of RET rearrangement cases [19-22]. These fusion proteins have demonstrated constitutively activated RET kinase activity, which causes constant activation of downstream MAPK signaling [23]. Papillary carcinoma carrying recombinant RET is associated with a high rate of lymph node metastasis [9]. A number of small-molecule kinase inhibitors have been developed to inhibit RET kinase activity. SU12248 (sunitinib) has been shown to effectively inhibit signaling from the recombinant RET kinase in the experimental models and has been tested in Phase II clinical trials in radioiodine-refractory, unresectable differentiated thyroid cancer [24].

The third most common genetic alteration is *RAS* mutation. Point mutations in the *N-RAS*, *H-RAS* or *K-RAS* genes have been identified in about 15% of papillary thyroid cancer

patients [4]. Papillary thyroid cancer with *RAS* mutation presents histological characteristics of follicular thyroid cancer. Less frequent lymph node metastasis, more frequent distant metastasis and more frequent encapsulation are also the characteristics of *RAS*-mutated papillary thyroid cancer [9, 25-26]. Binding of GTP switches RAS from an inactive state to an active state, which allows RAS to activate downstream effectors and thus turn on the MAPK signaling pathway [4]. Point mutations of *RAS* either increase the affinity of RAS with GTP, or decrease the dissociation of GTP from RAS. Consequently, RAS mutations lead to constitutively activated MAPK signaling in papillary thyroid cancer [4].

Follicular thyroid cancer

Follicular thyroid cancer is the second most frequent thyroid cancer and represents about 15% of total thyroid cancer cases [4]. Follicular thyroid cancer is also developed from thyroxine-secreting follicular cells. Most follicular thyroid cancer is well-differentiated and slow growing, with a 5-year survival rate of more than 90% [3-4]. Thyroid follicular carcinoma has been known to be developed from thyroid follicular adenoma, a precancerous stage not seen in papillary thyroid cancer. There are three types of genetic alterations that are frequently found in follicular thyroid cancer: *RAS* point mutation, *PAX8-PPARγ* rearrangement and alterations in the PI3K signaling pathway (Table 1).

Point mutations of *RAS* are the most common genetic alteration in follicular thyroid cancer with a frequency ranging from 40% to 50% [4]. *RAS* mutations in follicular thyroid cancer, as in papillary thyroid cancer, lead to constitutive activation of the

oncogenic MAPK signaling pathway [3-4]. Follicular thyroid cancer with *RAS* mutation has been associated to more advanced and malignant characteristics, such as dedifferentiation and metastasis to distant organs [27-29]. Follicular thyroid cancer patients with mutated *RAS* are more likely to have a poor prognosis [27-29]. However, *RAS* mutations may not be useful as a diagnostic biomarker because *RAS* mutations are also present in follicular adenoma and papillary thyroid cancer. Nonetheless, *RAS* mutations may still be used in combination with other molecular markers to improve diagnostic and prognostic accuracy for follicular thyroid cancer patients.

The second most frequent genetic alteration is *PAX8-PPARγ* fusion, which occurs in ~35% of follicular thyroid patients [4]. *PAX8-PPARγ* fusion is produced from chromosome rearrangement between t(2;3)(q13;p25) [30]. *PAX8-PPARγ* fusion is more common in younger-age thyroid follicular cancer patients and often associated with small tumor size [31-32]. The mechanism of *PAX8-PPARγ* rearrangement, as well as its contribution to thyroid tumorigenesis, is still elusive. However, *PAX8-PPARγ* rearrangement can be used as a diagnostic biomarker since this type of rearrangement is almost exclusively found in follicular thyroid cancer.

There are other rarely-found genetic alterations in papillary thyroid cancer [3-4]. Most papillary thyroid cancer patients possess one genetic alteration resulting in the constitutively activated MAPK signaling pathway, indicating that oncogenic MAPK signaling is the major oncogenic event that drives the development of papillary thyroid cancer.

Anaplastic thyroid cancer

Anaplastic thyroid cancer is a very aggressive and poorly-differentiated. It is considered to be a fatal disease. The mean survival time after diagnosis is merely 6 months [5]. Although anaplastic thyroid cancer only accounts for 1% to 2% of total thyroid cancer cases, it contributes to ~40% of thyroid cancer deaths [5]. Anaplastic thyroid cancer is arisen from follicular cell-derived, well-differentiated thyroid cancer, such as papillary and follicular thyroid cancer. Since many anaplastic thyroid cancer samples possess the characteristics of well-differentiated thyroid cancer both histologically and genetically, it has been proposed that anaplastic thyroid cancer represents a more advanced and malignant stage of thyroid cancer development. Many genetic alterations have been found in anaplastic thyroid cancer (Table 1). With no surprise, many tumor-initiating genetic alterations found in papillary and follicular thyroid cancer, such as *RAS* and *BRAF* mutations, are also frequently detected in anaplastic thyroid cancer patients. In addition, several genetic mutations occurring in late malignant events, such as vascular invasion and metastasis, are found in anaplastic thyroid cancer [4].

The most frequent genetic alteration in anaplastic thyroid cancer is p53 mutation, which is found in about 70% of anaplastic thyroid cancer samples [4]. Mutations in p53 inhibit the DNA binding ability of p53, and thus inactivate the tumor repressive effect of p53 [4]. Mutation of p53 is considered as a late event because it is not found in well-differentiated thyroid cancer. Inactivation of p53 has also been proposed to be a necessary event for thyroid cancer cell dedifferentiation, since restoration of p53 in thyroid cancer cell lines

leads to re-expression of terminally-differentiated molecular markers, including TPO and PAX8 [32-33]. Restoration of p53 also inhibits anaplastic thyroid cancer cell proliferation. Therefore, re-expressing p53 is an attractive therapeutic approach and is under evaluation for anaplastic thyroid cancer [34-35].

The second most common genetic alteration in anaplastic thyroid cancer is β-catenin mutation, which is found in approximately 65% of anaplastic thyroid cancer patients [36-37]. Mutation in β-catenin is also considered as a late event since this mutation is not found in well-differentiated thyroid cancer. The pathological significance of β-catenin mutation has not been established. Although nuclear accumulation of β-catenin has been found in the majority of anaplastic thyroid cancer, there is no clear evidence to support that mutation of β-catenin causes its aberrant subcellular location [38-39].

Other mutations, such as RAS, BRAF, PI3K and PTEN, have also been found in anaplastic thyroid cancer [4]. Because these mutations present in well-differentiated thyroid cancer, they are considered as tumor-initiating genetic mutations. Whether or not these tumor-initiating mutations are necessary for late tumor progression and metastasis are still unclear.

Medullary thyroid cancer

Medullary thyroid cancer makes up about 3% of thyroid cancer cases [4]. In contrast to other types of thyroid cancer that are arisen from thyroid follicular cells, medullary

thyroid cancer is originated from calcitonin-secreting thyroid C cells. Medullary thyroid cancer is generally more malignant than well-differentiated thyroid cancer, but it is still curable if the patient is diagnosed at an early stage and has thyroid removed.

Almost 100% of medullary thyroid cancer is caused by germline-transmitted mutation on the *RET* gene (Table 2). Unlike *RET* rearrangements in papillary thyroid cancer, *RET* is point-mutated in medullary thyroid cancer. Nonetheless, the effects of RET genetic alterations are virtually same, with the constitutively activated kinase domain and downstream effectors including the oncogenic MAPK signaling pathway [40-41]. Since *RET* mutation is inherited, it is considered as a tumor-initiation event in medullary thyroid cancer. Several studies using transgenic mice have demonstrated that mice with *RET* knock-in mutation develop various degrees of medullary thyroid tumor that is histologically similar to human medullary thyroid tumor [40-41]. In fact, *RET* point mutation is one of very few mutations that are transmitted by germline. Since *RET* point mutation causes aberrantly activated RET kinase activity, a range of small-molecule kinase inhibitors are being tested for medullary thyroid cancer patients [40-41].

Mouse models of thyroid cancer

Genetically-engineered mice have provided a useful model for researchers to investigate a gene's function in an *in vivo* setting close to human. To examine how genetic alterations contribute to thyroid tumorigenesis, researchers have established several genetically-modified mouse models that develop thyroid cancer spontaneously. *RAS* point mutations are frequently detected in papillary and follicular thyroid cancer. Interestingly,

mice with *Nras* E61K knock-in mutation develop thyroid cancer that is histologically similar to follicular thyroid cancer [42]. About 40% of *Nras* mutant mice develop invasive follicular thyroid cancer [42]. In contrast, mice with *Kras* G12D mutation do not show any abnormality, but develop aggressive follicular-like thyroid cancer when crossed with *Pten* knockout mice [43-44]. Deletion of mouse *Pten* alone causes the enlargement of thyroid follicules, but does not induce thyroid cancer [44]. Mice with either *Ret* rearrangement or *Braf* knock-in mutation develop slow-growing, well-differentiated thyroid cancer that hardly metastasizes to distant organs [45-48]. These studies demonstrate that *Nras* G61K, *Ret* rearrangement and *Braf* mutation are all capable of initiating thyroid cancer in mammalian animal models. *Kras* G12D mutation or loss of *Pten* are not sufficient to induce thyroid cancer alone, but when combined, they can initiate thyroid tumorigenesis, suggesting that initiation of some types of thyroid cancer may require normal thyroid cells to acquire multiple genetic alterations.

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CHAPTER II

PITX2 IS OVEREXPRESSED IN THYROID CANCER AND PROMOTES THRYOID CANCER CELL PROLIFERATION BY ACTIVATION OF CYCLIN D2

Abstract

Pituitary homeobox 2 (PITX2), a Paired-like homeodomain transcription factor and a downstream effector of β-catenin signaling, plays substantial roles in normal embryonic development but its possible involvement in tumorigenesis was unknown. In this study, we extend its function in human cancer. Remarkably, we found that PITX2 was frequently expressed in human follicular cell-derived (papillary, follicular, and anaplastic) thyroid cancer tissues but not in normal thyroids, indicating for the first time that overactivated PITX2 may contribute to thyroid cancer. Cell-based and biochemical studies were performed to uncover the molecular mechanism of PITX2 action in thyroid cancer. Knockdown of PITX2 gene expression in human thyroid cancer cells significantly reduced cell proliferation and soft-agar colony formation. Biochemical analysis of cell cycle regulators upon PITX2 knockdown revealed down-regulation of Cyclin D1, Cyclin D2, and dephosphorylation of Rb. Chromatin immunoprecipitation and promoter reporter assay indicated that Cyclin D2 was a direct target gene of PITX2. Consistently, we observed that high expression levels of Cyclin D2 were frequently associated with PITX2 expression in follicular cell-derived thyroid cancer tissues. To confirm our results in vivo, we took advantage of a mouse model of thyroid cancer ($TR\beta^{PV/PV}$ mouse). Consistently, the aberrant elevation of Pitx2 levels in the thyroid cancer of $TR\beta^{PV/PV}$ mice was accompanied by up-regulation of Cyclin D1, Cyclin D2,

and increased phosphorylation of Rb. Collectively, our findings demonstrate that the over-activated PITX2-Cyclin D2 pathway promotes thyroid tumorigenesis, and they provide the first evidence implicating an oncogenic role of PITX2 in human cancer.

Key words: thyroid cancer, PITX2, homeodomain transcription factor, Cyclin D2, β-catenin signaling

Introduction

Thyroid cancer is the most prevalent endocrine cancer and constitutes approximately 1% of all newly diagnosed cancer cases. Its incidence has increased significantly worldwide in the past years [1-2]. There are four major types of thyroid cancer, including papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC) and anaplastic thyroid cancer (ATC). Of these, PTC and FTC account for approximately 80% and 15%, respectively, of total thyroid cancer cases. MTC is relatively rare (~3%), but more malignant than PTC and FTC. ATC (~2%) is mostly undifferentiated and highly aggressive. The mean survival period for ATC is less than 8 months after diagnosis. Both PTC and FTC are originated from thyroxin-secreting follicular cells, whereas MTC is developed from calcitonin-secreting C-cells. ATC is believed to arise from PTC and FTC. Recent studies have considerably expanded our understanding of the aberrant genetic events underlying these thyroid carcinomas. Recombinant *RET* gene products (*RET/PTC*), as well as the mutation of *BRAF(V600E)* [3-4], are commonly found in PTC. In FTC patients, mutations in *RAS* genes and *PAX8-PPARγ* rearrangement are frequently

observed [3]. *RET* point mutations are common in MTC [5]. Despite these progresses, the molecular mechanisms involved in thyroid tumorigenesis are still elusive.

Recently, aberrant stabilization and mislocalization of β-catenin has been shown to contribute to thyroid tumorigenesis in humans and in a mouse model of follicular thyroid cancer (thyroid hormone receptor beta PV mouse) [6-8]. β-catenin is an ubiquitously expressed multifunctional protein that has important roles in cell adhesion and signal transduction [6]. Upon activation, β-catenin is translocated from the plasma membrane to the nucleus, where it interacts with transcription factors, such as PITX2 (Pituitary homeobox 2), to activate expression of genes required for cell proliferation (e.g., *c-Myc, Cyclin D1*) [9-10].

Given the important role of PITX2 in mediating beta-catenin signaling, it was tempting to speculate that aberration in PITX2 signaling could also contribute to thyroid cancer, but this question remained to be addressed. This transcription factor, which belongs to the *Paired*-like (*bicoid*) class of homeobox proteins, plays important roles during embryonic life for the determination of left-right asymmetry and development of multiple organs by serving as a critical downstream effector of Nodal, TGFβ and Wnt signaling [9. 11-15]. *Pitx2* knockout mice die by embryonic day 15 due to severe developmental defects [9]. Mutations in the *PITX2* gene have been linked to several human disorders, including Axenfeld-Rieger syndrome, iridogoniodysgenesis syndrome, and sporadic cases of Peters anomaly [16-17].

Here, we report for the first time that PITX2 is overexpressed in human papillary, follicular and anaplastic thyroid cancers and in the follicular thyroid cancer of TRbeta$^{PV/PV}$ mice. Importantly, our mechanistic studies indicate that Cyclin D2 is a

transcriptional target of PITX2 and may mediate PITX2's stimulating role on thyroid cancer cell growth. In line with this, our data also reveal overexpression of Cyclin D2 in human papillary and follicular thyroid cancers. Altogether, our results demonstrate that aberrant PITX2-Cyclin D2 signaling leads to increased thyroid cancer cell proliferation and thereby promotes thyroid carcinogenesis.

Materials and Methods

Cell culture

Human papillary thyroid cancer cell line (TPC-1), human follicular thyroid cancer cell line (WRO), human anaplastic thyroid cancer cell line (FRO) and human medullary thyroid cancer cell line (TT) were kindly provided by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center, NY). TPC-1 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 10% fetal bovine serum, and penicillin-streptomycin (100 IU/ml). WRO and FRO cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and penicillin-streptomycin (100 IU/ml). TT cells were cultured in F-12K medium with 10% fetal bovine serum and penicillin-streptomycin (100 IU/ml). Cell culture media and supplements were purchased from ATCC. Cells were incubated at 37^0C in a humidified atmosphere with 5% CO_2.

RT-PCR analysis

Total RNA was extracted from cultured cells and thyroid glands of wild-type or thyroid hormone receptor beta ($TRbeta^{PV/PV}$) mutant mice using TRI Reagent (Ambion, TX) as

previously described [30]. RT-PCR was performed to examine the expression of PITX2. The primers used in this study are listed in Table 2. PCR parameters: 94^0C for 2 min, 1 cycle; 94^0C for 20 sec, 56^0C for 20 sec, 72^0C for 2 min, 32 cycles; followed by a 6 min extension at 72^0C.

Immunohistochemistry

Tissue microarray slides, which included normal and malignant human thyroid tissues, were purchased from US Biomax (Ijamsville, MD). Formalin-fixed, paraffin-embedded tissue sections (5 μm thickness) were de-paraffinized by xylene and ethanol. Antigen retrieval was performed by heating tissues samples in a microwave. Anti-PITX2 (Abcam, MA) or anti-Cyclin D2 (Santa Cruz Biotechnology, CA) was incubated with tissue sections overnight at 4^0C. Immunostaining was done by the IHC Select Immunophophatase Secondary Detection System (Chemicon, CA) according to manufacturer's instruction. Tissue microarray slides and their staining intensity were evaluated and classified by two individuals independently. A tissue sample with a minimum of 20% cells showed staining was counted as a positive case for consistent expression of PITX2 or Cyclin D2.

RNA interference

The lentiviral pGIPZ non-silencing control plasmid (pGIPZ-shRNA-NS) and the plasmids targeting PITX2 (pGIPZ-shRNA-PITX2) were purchased from Open Biosystems (Huntsville, AL). The shRNA sequence targeting PITX2 was

AGCCGACTCCTCCGTATGTTTAtagtgaagccacagatgtaTAAACATACGGAGGAGTCG GCG (uppercase indicates sense and antisense sequences and lowercase indicates loop sequence). Second generation of lentiviral packaging system (Addgene, MA) was used to produce lentivirus particles. To generate stable cell lines, 1μg/ml puromycin (Sigma, MO) was added to TPC-1 cells 48 hours after transfection. All stable cell lines were maintained in the medium containing 0.1μg/ml puromycin.

Cell proliferation assay by MTT

Cell proliferation activity was examined by the MTT assay. $1x10^4$ TPC-1 parental cells and TPC-1 cells stably transfected pGIPZ-shRNA-NS or pGIPZ-shRNA-PITX2 were seeded in 24-well plates in quadruplicate, respectively. Each subsequent day, MTT was added into each well to a final concentration of 0.5mg/ml and incubated with cells for 3 hours at 37^0C. A microplate spectrophotometer (Bio-Rad, CA) was used to measure the MTT absorbance at 570 nm. The experiment was repeated three times independently.

Soft agar colony formation assay

$5x10^3$ TPC-1 cells stably expressing pGIPZ-shRNA-NS or pGIPZ-shRNA-PITX2 were mixed with 0.7% agar in DMEM. The cell/agar mixtures were placed in 24-well plates coated with 0.3% agar in quadruplicate. The cells were incubated at 37^0C for 10 days. Colonies with a diameter greater than 100μm were counted. The experiment was repeated three times independently.

Reporter assay

The Cyclin D2 luciferase reporter construct contains 798 bps (-1 to -798) of the cyclin D2 promoter. Cyclin D2 promoter was cloned (the primers are listed in Table 1) from human blood cell genomic DNA and inserted into *Kpn*I/*Nhe*I sites of pGL4.24 luciferase vector (Promega, MI). The pEGFP-NFLAG-PITX2c plasmid (expressing N-terminal FLAG tagged PITX2C) has been previously described [26]. The pEGFP-NFLAG-PITX2C-ΔHD plasmid, which expressed N-terminal FLAG tagged, homeodomain (HD)-deleted PITX2C (amino acid 1-131), was inserted into *HindIII*/*Kpn*I sites of pEGFP vector.

For reporter assay, $2x10^3$ TPC-1 cells were seeded in 96 wells. A total of 100ng of various plasmid mixes were transfected into TPC-1 cells by Fugene HD transfection reagent (Roche, IN). Renilla vector was included in all transfections and served as internal control. Luciferase and renilla levels were measured by the Dual-Luciferase Reporter Assay System 48 hours after transfection according to manufacturer's instruction (Promega, WI). Each experiment were performed in triplicate and repeated twice.

Immunoblotting

Total cell lysates from cultured cells were extracted in the cell lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). Immediately before use, the lysis buffer was supplemented with a protease inhibitor cocktail tablet (Roche, IN). Preparation of whole cell lysates from thyroid glands has been described previously [23, 31]. Immunoblotting was performed as reported previously [30]. The following primary

Table 1

	5′	3′
PITX2	AATCGCTGTGTGGACCAACC	TCCACTGCATACTGGCAAGC
GAPDH	TGAAGGTCGGAGTCAACGG ATTTGGT	CATGTGGGCCATGAGGTCCACCAC
Beta-actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC
cyclinD2-promoter	TGCAGGTACCGAAACGCCACCAGATCGTAT	TGCAGCTAGCTTCTCCGCTCTGAAGCGGTGA
CCND2-ChIP	AGAAACACGATGGTTTCTGCT	ATACCCAGAGATAAAGGTCAT
FLAG-PITX2C-HDΔ	ACTGAAGCTTGCCACCATGGATTACAAGGATGACGACGATAAGAACTGCATGAAAGGCCCGCTTCAC	AGCTGGTACCTTAGTTGGTCCACACAGCGATTT

Table 1. The sequences of the primers used in this study.

antibodies were used (1:1000 dilution): PITX2 (3D2, GeneTex, TX); Cyclin D2 (Santa Cruz Biotechnology, CA); Cyclin D1, Rb, phospho-Rb^{780}, phospho-Rb$^{807/811}$ (Cell Signaling, MA); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), actin (Ambion, TX). The loading controls were obtained from the same blot after being stripped and reprobed with rabbit polyclonal antibodies to GAPDH or actin. ImageJ was used for densitometry analysis.

Chromatin immunoprecipitation (ChIP) assay

TPC-1 cells were transiently transfected with pEGFP-NFLAG-PITX2C or pEGFP-NFLAG-PITX2C-ΔHD by FugeneHD transfection reagent. After 48 hours of initial transfection, cells were cross-linked by 1% formaldehyde for 15 minutes. Cell genomic

DNA was then sheared into fragments by sonication. Cell lysates were pre-cleared by protein G beads (Pierce, IL) for 2 h at 4^0C. Anti-FLAG M2 conjugated agarose beads (Sigma, MO) were then incubated with pre-cleared cell lysates at 4^0C overnight. Beads were washed by the high salt and LiCl washing solution for eight times as previously described [32]. Samples were reverse cross-linked in the high salt solution at 65^0C overnight. DNA was purified by phenol-chloroform extraction and precipitated by isopropanol. PCR was then used to analyze precipitated DNA samples. ChIP primers (listed in Table 2) covering the conservative *bicoid* homeodomain binding site in the promoter of Cyclin D2 (-516 to -715) was used in PCR analysis. PCR parameters: 94^0C for 2 min, 1 cycle; 94^0C for 20 sec, 56^0C for 20 sec, 72^0C for 50 sec, 32 cycles; followed by a 6 min extension at 72^0C.

Statistical analysis

Data were presented as mean ±S.D. and analyzed by Student's *t* test. Chi-square test was used to analyze immunohistochemistry studies. Statistical significant differences were defined as $p<0.05$.

Results

Expression of PITX2 in human follicular cell-derived thyroid cancers

Previously, PITX2 has been proposed to act downstream of β-catenin signaling to promote cell proliferation during development [9]. However, whether PITX2 contributes to cancer progression remained to be determined. To address this question, we carried out immunohistochemistry studies on four major types of thyroid cancers (papillary, follicular, anaplastic and medullary thyroid cancer), and compared PITX2 staining in these tissues to those in normal thyroids. As shown in Fig. 1 and Table 2, a consistent expression of PITX2 was frequently observed in the cancer samples of papillary and follicular types (PITX2 positive cells are exemplified by arrows), while it was absent in normal thyroid tissues and in medullary thyroid cancer. Expression of PITX2 was also noticed in anaplastic thyroid cancer, although the frequency was significantly lower than that in papillary and follicular cancers (Fig. 1 and Table 2).

Figure 1

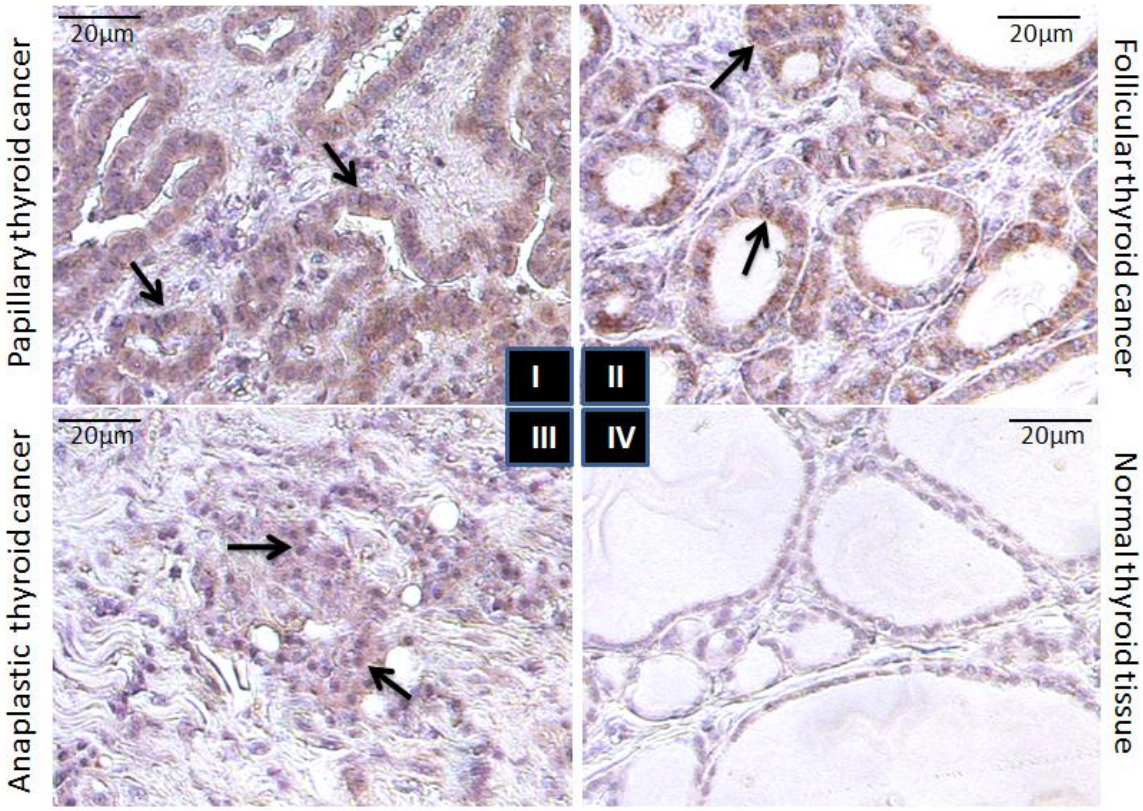


Figure 1. Overexpression of PITX2 in human thyroid cancer tissues. Tissue microarrays containing various thyroid cancer tissues and normal thyroid tissues were subjected to the immunohistochemistry assay by using a rabbit polyclonal anti-PITX2 as the primary antibody. One representative image (I-IV) of each type of human thyroid cancer and normal thyroid tissue was shown (original magnification 100X). Arrows exemplify the PITX2 staining cells. Scale bar indicates 20μm length.

Table 2

	-	+	Incidence
Normal	21	0	0\21(0%)
Papillary	18	13	13\31(42%)
Follicular	17	13	13\30(43%)
Anaplastic	7	2	2/9(22%)
Medullary	21	0	0\21(0%)

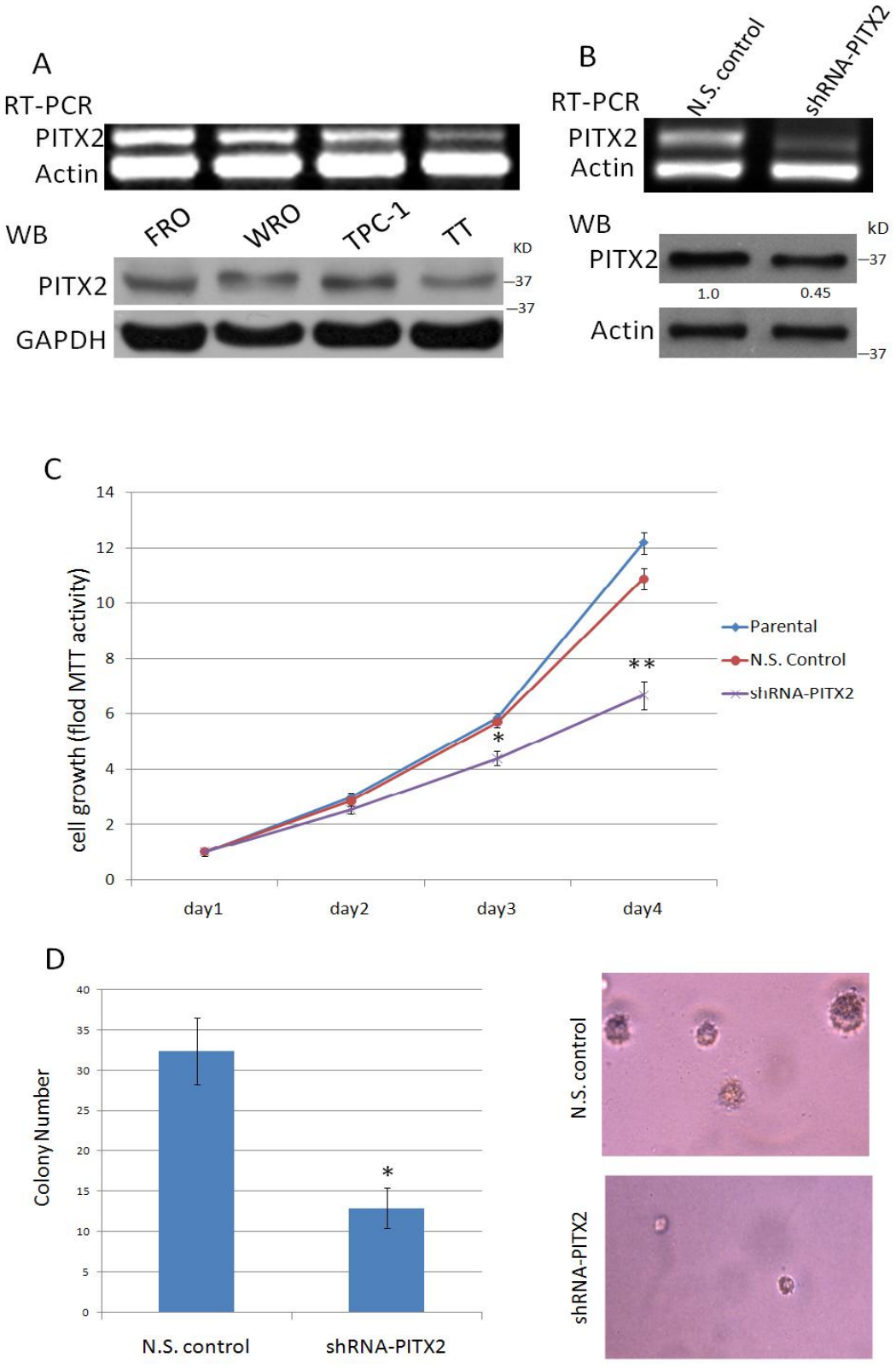
Table 2. The symbol "-", and "+" represents no expression and consistent expression of PITX2, respectively (see Materials and Methods for categorization). The number indicates the sample size of each category, and incidence is defined as the percentage of consistent PITX2 expression of total tissue samples examined. As compared to normal thyroids, the incidence of PITX2 expression is significantly higher in papillary, follicular and anaplastic thyroid cancers (Chi-square test, $p < 0.05$), but not in medullary thyroid cancer (Chi-square test, $p = 1$). Moreover, the frequency of PITX2 expression in papillary and follicular thyroid cancers is significantly higher than that in anaplastic thyroid cancer (Chi-square test, $p < 0.05$).

Knockdown of PITX2 gene expression inhibited cell growth and soft-agar colony formation

The finding that PITX2 is frequently expressed in follicular cell-derived thyroid cancers (Fig. 1 and Table 2) suggests that it may contribute to the tumorigenesis of the thyroid. In order to test this hypothesis, we used a cell culture system to knock-down *PITX2* gene expression in the papillary thyroid cancer cell line TPC-1. Indeed, as shown in Fig. 2A, PITX2 was expressed in this cell line as well as in human follicular thyroid cancer cell line (WRO), human anaplastic thyroid cancer cell line (FRO) and human medullary thyroid cancer cell line (TT).

The knock-down of *PITX2* gene expression by short hairpin RNA (shRNA) yielded a 45% reduction of PITX2 protein expression as compared to non-silencing control shRNA (Fig. 2B). Then, we determined any changes in the tumorigenic ability, i.e., cell proliferation and soft-agar colony formation, of PITX2-knockdown cells. Interestingly, as compared to non-silencing control cells, cell growth of PITX2-knockdown cells was significantly reduced by 40% on day 4 ($p<0.005$) (Fig. 2C). Moreover, we observed that, upon PITX2 knockdown, the cell soft-agar colony formation ability was strikingly impaired. The number of colonies derived from PITX2-knockdown cells was significantly decreased to 38% of that from non-silencing cells ($p<0.01$) (Fig. 2D), and the colony size was noticeably reduced. These results suggest that PITX2 may promote the tumorigenic ability of thyroid cancer cells.

Figure 2


A
RT-PCR
PITX2
Actin
WB
FRO
WRO
TPC-1
TT
KD
PITX2
37
37
GAPDH
B
N.S. control
shRNA-PITX2
RT-PCR
PITX2
Actin
WB
kD
PITX2
37
1.0
0.45
Actin
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cell growth (flod MTT activity)
14
12
10
8
6
4
2
0
day1
day2
day3
day4
Parental
N.S. Control
shRNA-PITX2
*
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D
Colony Number
40
35
30
25
20
15
10
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N.S. control
shRNA-PITX2

Figure 2. Knockdown of PITX2 in TPC-1 cells inhibits cell proliferation and soft agar colony formation. (A) RT-PCR and Western blot were used to analyze the expression of PITX2 in four human thyroid cancer cell lines: TPC-1 (papillary thyroid cancer), WRO (follicular thyroid cancer), FRO (anaplastic thyroid cancer) and TT (medullary thyroid cancer). Actin was used as an internal control in RT-PCR. No PCR products were obtained without RT. GAPDH was used as an internal control in Western blot. (B) The expression of PITX2 in TPC-1 cells was knocked down by PITX2-shRNAs, as assessed by RT-PCR and Western blot. Actin was used as an internal control. The Western blot result demonstrates that expression of PITX2 in TPC-1 cells was reduced to ~45% by shRNAs. (C) Proliferation activity of parental TPC-1 cells and TPC-1 cells stably expressing non-silencing (N.S.) control or shRNA-PITX2 constructs was measured in 24-well plates in quadruplicate. MTT absorption was used to measure cell's viability. Data were presented as mean ±S.D. *, $P<0.05$; **, $P<0.005$. (D) $5X10^3$ N.S. control and shRNA-PITX2 TPC-1 cells were seeded in 24-well plates in quadruplicate. Colony number was counted after 10 days of incubation. *, $P<0.05$. One representative image from N.S. control and shRNA-PITX2 soft agar plates was shown at right.

PITX2 knockdown results in down-regulation of Cyclin Ds and dephosphorylation of Rb

Based on the above evidence linking PITX2 to thyroid cancer, we next investigated whether PITX2 might regulate the protein levels and/or activity of cell cycle regulators. As shown in Fig. 3A, knockdown of PITX2 gene expression in TPC-1 cells resulted in clear down-regulation of Cyclin D1 and Cyclin D2. The D-type Cyclins complex with cyclin-dependent kinases (CDK) to phosphorylate the retinoblastoma gene product (pRb) in proliferating cells. Consistent with these results, the levels of phosphorylated Rb (serine residues 780, and 807/811) were considerably reduced after *PITX2* knockdown (Fig. 3B). Since it is known that decreased levels of Cyclin Ds and phosphorylated Rb could result in slowed cell cycle progression and thus reduced cell proliferation activity, these data (Fig. 3) may mechanistically explain the abovementioned phenotypic effects of decreased cell proliferation upon PITX2 knockdown (Fig. 2). Taken together, our results suggest that PITX2, directly or indirectly, regulates several cell cycle modulators to promote thyroid cancer cell growth.

Figure 3

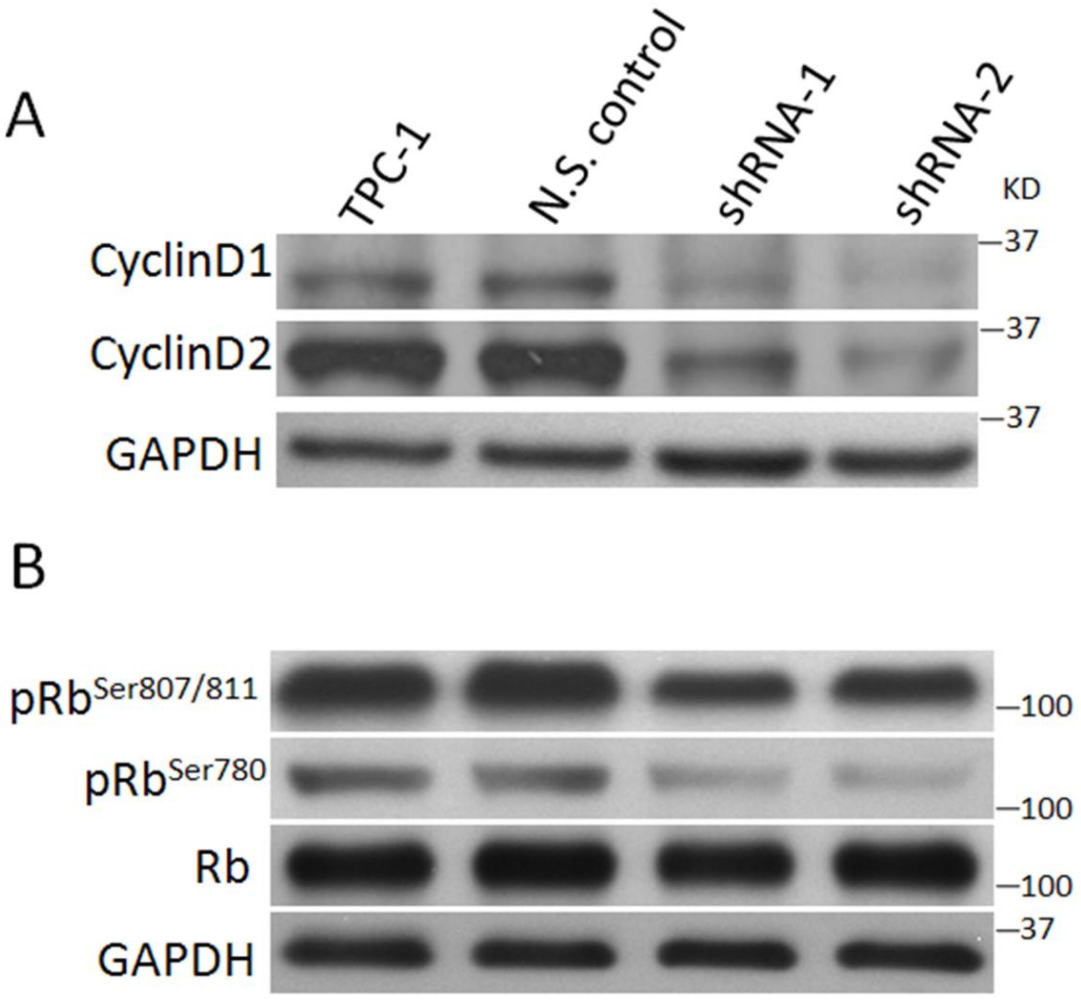

Figure 3. Effects of PITX2 knockdown on cell cycle regulators. Protein samples were prepared from parental TPC-1 cells, non-silencing control cells and two stable clones of PITX2-knockdown cells, and then subjected to Western blot analysis. Panel A indicates downregulation of Cyclin D1 and D2. Panel B reveals explicit dephosphorylation of Rb protein. GAPDH was used as an internal control.

PITX2 activates the expression of Cyclin D2 in human papillary and follicular thyroid cancers

Previous studies have shown that PITX2 can transcriptionally regulate Cyclin D2 in a cell type-specific manner [9]. As Cyclin D2 is down-regulated in TPC-1 cells upon PITX2 knockdown (Fig. 3A), we wanted to evaluate whether that regulation occurs at the transcriptional level in this cell line. Indeed, by chromatin immunoprecipitation analysis with primers that span the conserved PITX2 binding sites, we detected the binding of PITX2 on the promoter of Cyclin D2 in TPC-1 cells (Fig. 4A). Furthermore, we found that PITX2 significantly stimulated the activity of the Cyclin D2 promoter-driven reporter in TPC-1 cells by 11.3 fold ($p<0.05$), as compared to control (Fig. 4B). Therefore, in thyroid cancer cells, PITX2 transcriptionally regulate Cyclin D2, and this direct regulation may mediate the stimulatory role of PITX2 in promoting thyroid cancer cell proliferation.

Based on the above results showing the expression of PITX2 in follicular cell-derived thyroid cancer tissues and the transcriptional regulation of Cyclin D2 by PITX2, we predicted that Cyclin D2 was expressed in follicular cell-derived thyroid cancer tissues as well. Indeed, the immunohistochemistry assay revealed that Cyclin D2 was significantly overexpressed in papillary and follicular thyroid cancers, as compared to normal thyroids (Fig. 4C and Table 3). In contrast, no overexpression of Cyclin D2 was observed in medullary thyroid cancer (Fig. 4C and Table 3). Moreover, the expression pattern of PITX2 and Cyclin D2 was well correlated in papillary and follicular thyroid cancers, i.e., 92% of PITX2-positive tissues also expressed Cyclin D2 (Table 4). Hence, our data support the existence of a PITX2-Cyclin D2 pathway in papillary and follicular thyroid

cancers. We also noticed that 43% of PITX2-negative thyroid cancer tissues expressed Cyclin D2, indicating that PITX2 is not the sole upstream regulator for Cyclin D2 expression in thyroid cancer.

Figure 4

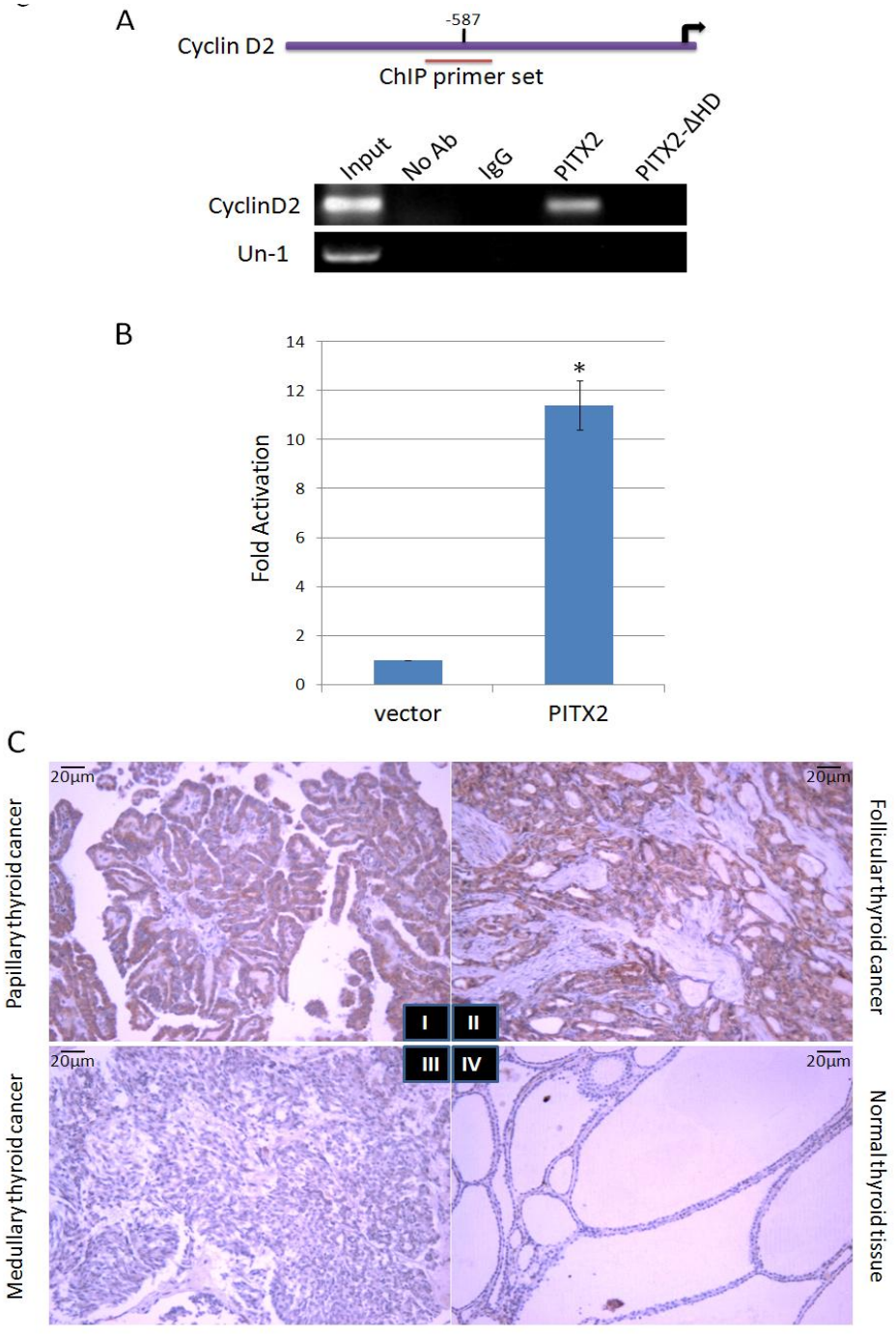

Figure 4. Cyclin D2 is a transcriptional target of PITX2 in thyroid cancer. (A) ChIP analysis was performed using anti-FLAG M2 on TPC-1 cells transiently expressing FLAG-tagged PITX2 (PITX2) or FLAG-tagged homeodomain-deleted PITX2 (PITX2-ΔHD). No Ab and IgG indicate no primary antibody and mouse IgG control, respectively. The upper scheme indicates the conserved PITX2 binding site on the Cyclin D2 promoter (-1 to -798) and the location of the primers used in ChIP. The primers of an unrelated gene were included as control. (B) A luciferase reporter vector containing the Cyclin D2 promoter, which included the conserved bicoid-homeodomain binding site, was transiently transfected into TPC-1 cells along with the PITX2 expression vector or empty vector. A renilla vector was included in each transfection to normalize transfection efficiency in all experiments. Data were presented as mean ±S.D. *, $P<0.05$. (C) Cyclin D2 is overexpressed in thyroid cancer tissues. Thyroid cancer tissue microarrays were examined by immunohistochemistry with a polyclonal anti-Cyclin D2. One representative image (I-IV) of each type of human thyroid cancer and normal thyroid tissue was shown (original magnification 40X). Scale bar indicates 20μm length.

Table 3

	-	+	Incidence
Normal	16	4	4\20(20%)
Papillary	5	19	19\24(79%)
Follicular	7	10	10\17(59%)
Medullary	5	1	1/6(17%)

Table 3. Immunohistochemistry assay of Cyclin D2 in human thyroid cancer tissues. The symbol "-", and "+" represents no expression and consistent expression of Cyclin D2, respectively (see Materials and Methods for categorization). The number indicates the sample size of each category, and incidence is defined as the percentage of consistent Cyclin D2 expression of total tissue samples examined. Cyclin D2 is significantly overexpressed in papillary and follicular thyroid cancers (Chi-square test, $p<0.05$).

Table 4

PITX2 / cyclin D2	+	-
+	11/12(92%)	16/34(43%)
-	1/12(8%)	18/34(57%)

Table 4. Each of two consecutive tissue sections was used to examine expression of PITX2 and Cyclin D2, respectively, in the immunohistochemistry assay. Consistent expression "+" or no expression "-" was scored for each tissue sample and the percentage was calculated.

Overexpression of PITX2 and Cyclin D2 in a mouse model of thyroid cancer

To further confirm the existence of a PITX2-Cyclin D2 pathway *in vivo*, we took advantage of a mouse model of thyroid cancer that was created by a targeted mutation of the thyroid hormone β receptor (*TRβPV*) [18]. As $TR\beta^{PV/PV}$ mice age, they spontaneously develop follicular thyroid carcinoma similar to human thyroid cancer with pathological progression from hyperplasia to vascular invasion, capsular invasion, anaplasia, and eventually metastasis [18-19].

We observed an evident increase in *Pitx2* mRNA levels in the thyroid cancer of $TR\beta^{PV/PV}$ mice as compared with wild-type mice (Fig. 5A). Consistently, the protein abundance of Cyclin D2, along that of Cyclin D1, was also greatly increased in the thyroid of $TR\beta^{PV/PV}$ mice (Fig. 5B). In line with these data, the phosphorylation levels of Rb (Ser807/811 and Ser 780) were considerably increased in $TR\beta^{PV/PV}$ mice (Fig. 5C).

These mouse model data are consistent with the abovementioned results and, together, they strongly demonstrate that in papillary and follicular thyroid cancer cells there exists a PITX2-Cyclin D2 pathway, in which PITX2 directly regulates Cyclin D2 expression to promote thyroid carcinogenesis.

Figure 5

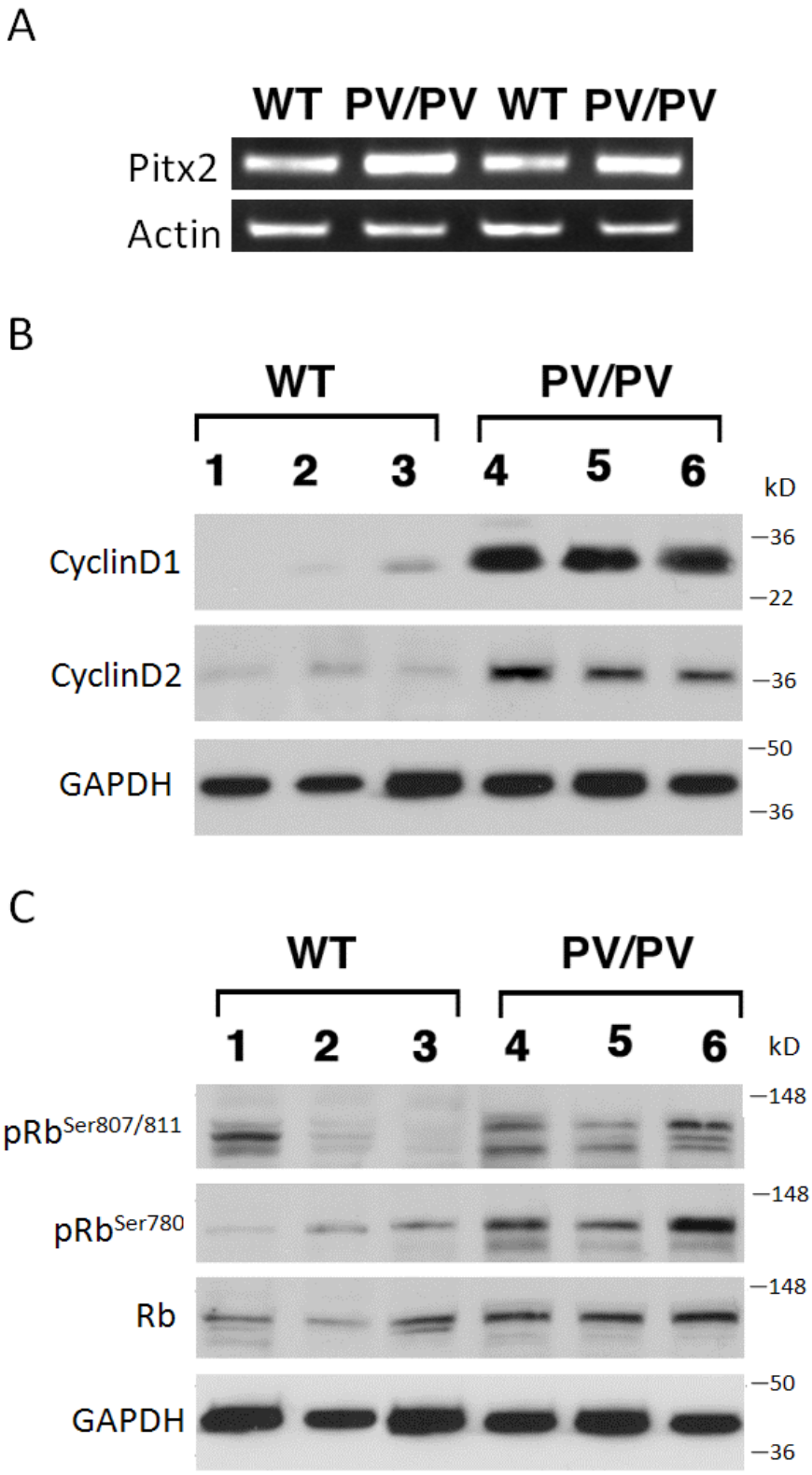
A
WT PV/PV WT PV/PV
Pitx2
Actin
B
WT
PV/PV
1 2 3 4 5 6
kD
CyclinD1
CyclinD2
GAPDH
36
22
36
50
36
C
WT
PV/PV
1 2 3 4 5 6
kD
pRb Ser807/811
pRb Ser780
Rb
GAPDH
148
148
148
50
36

Figure 5. Overexpression of PITX2 and Cyclin D2 in a mouse model of thyroid cancer. Total RNA and protein extracts were prepared from normal thyroid glands of wild-type mice (WT) and from thyroid tumors of $TR\beta^{PV/PV}$ mice aged 8 months, as described in Materials and Methods. The littermates with different genotypes were used in the analysis. Representative results from 2 (panel A) or 3 (panel B-D) mice are shown and the genotypes marked. (A) The RT-PCR result indicates the upregulation of Pitx2 in thyroid tumors. (B) Protein abundance of Cyclin D1, Cyclin D2 and the loading control GAPDH. (C) Protein levels of Rb, phosphorylated either on serine 807 and 811 (p-$Rb^{Ser807/811}$) or on serine 780 (p-Rb^{Ser780}), total levels of Rb, and GAPDH.

Discussion

Previous studies have clearly demonstrated the importance of PITX2 in embryonic development, e.g., organogenesis of the heart, eye, pituitary and tooth [9, 12-15]; however, whether it plays a significant role in human cancer, a disrupted tissue homeostasis after birth, was unknown.

Here, we demonstrate for the first time that the expression of PITX2 was significantly upregulated in human thyroid cancer. Interestingly, we found that frequent expression of PITX2 was solely observed in follicular cell-derived thyroid cancers (papillary, follicular and anaplastic types), but not in C-cell-derived medullary thyroid cancer. This finding indicates that PITX2 is upregulated in thyroid cancer in a cell type-specific manner. Furthermore, we observed that the frequency of PITX2 expression in well differentiated

papillary and follicular thyroid cancers was significantly higher than that in poorly differentiated anaplastic thyroid cancer. Since anaplastic thyroid cancer is a highly invasive cancer developed from papillary and follicular thyroid cancers, it is tempting to speculate that PITX2 might function to promote primary tumor growth during the early stage of thyroid tumorigenesis, but is down-regulated in the late stage of cancer development. To validate this speculation, PITX2 expression should be analyzed in a large scale of thyroid tumor tissues, especially those arising from thyroid follicular cells, ranging from early stage microtumors to more advanced locally invasive tumors and late stage metastasized cases.

Since PITX2 has been shown to promote cell proliferation in a cell type-specific manner during embryonic development [9], our observation prompted us to hypothesize that upregulated PITX2 may play a similar proliferative role to stimulate thyroid cell growth and, consequently, promotes thyroid tumorigenesis. This hypothesis was robustly supported by our findings. Our cell-based studies showed that PITX2 knockdown remarkably reduced cell growth and soft-agar colony formation. Our biochemical studies led to the finding that knockdown of PITX2 caused apparent changes in the expression or activity of key cell cycle regulators, such as Cyclin Ds and pRb. Notably, our *in vivo* studies using a unique mouse model of thyroid cancer showed that overexpression of PITX2 in the thyroid was associated with the upregulated Cyclin Ds and with the increased activity of pRb. Thus, our results strongly suggest that PITX2 promotes thyroid cancer cell growth by modulating the expression or the activity of cell cycle controlling genes.

Notably, our data of chromatin immunoprecipitation and reporter assays clearly showed that PITX2 regulates Cyclin D2 expression at the transcriptional level in thyroid cancer cells, by binding to the Cyclin D2 promoter. This transcriptional regulation of Cyclin D2 by PITX2 has also been previously demonstrated in murine C2C12 myoblast cells [9-10]. Thus, it appears that PITX2-Cyclin D2 might well represent a broad-spectrum cell cycle regulation pathway. However, whether the PITX2-Cyclin D2 pathway functions as a general mechanism in other cancers remains to be determined.

At present, we cannot conclude that Cyclin D2 is the only mediator of overactivated PITX2 in follicular cell-derived thyroid cancers. In fact, other cell cycle regulators, including c-Myc and Cyclin D1, have also been previously reported as PITX2 transcriptional targets [10]. On the other hand, we cannot rule out the existence of other upstream regulators besides PITX2 for regulating Cyclin D2 expression in thyroid cancer, since the expression of Cyclin D2 was observed in the PITX2-negative cancer tissues. Indeed, previous studies have shown that Cyclin D2 can be regulated by multiple mitogenic signals in a number of human cancers, such as leukemia, testicular cancer and ovarian cancer [20-22]. Furthermore, whether PITX2 cooperates with other oncogenic signals to upregulate Cyclin D2 in thyroid tumorigenesis remains unclear and warrants further study.

Dysregulated (stabilization and translocation of) β-catenin plays a critical role in oncogenesis [6-7]. In papillary thyroid cancer, β-catenin is frequently mislocated to the cytoplasm and the nucleus [3, 6-7]. Recent studies have shown that the aberrant localization or over-expression of β-catenin can result from genetic mutations, such as *RET* mutations and *TRβ*PV mutation, both of which are capable of driving thyrocyte

neoplastic transformation [6, 8, 23]. Since PITX2 has been shown as a target and an effector of β-catenin signaling in development [9, 24, 25], it is tempting to speculate that the β-catenin-PITX2 pathway might be functional in thyroid tumorigenesis as well and the aforementioned PITX2-Cyclin D2 pathway might be expanded into the β-catenin-PITX2-Cyclin D2 pathway. This speculation apparently deserves further investigation since it would offer a mechanistic explanation for those thyroid tumors derived from dysregulated β-catenin, which itself could arise from genetic alterations in *RET*, *RAS*, *RAF*, *PPARγ* and *PIK3CA*. With this regard, it will be interesting to finely map upstream activators and downstream effectors of the PITX2-Cyclin D2 pathway.

Recently, we reported a study on PITX2-interacting and regulating networks [26]. Y-box binding protein-1 (YB-1) was identified as a novel PITX2-associated partner. YB-1 belongs to a family of evolutionarily conserved, multifunctional Y-box proteins that function as regulators of cell proliferation, drug resistance, DNA damage repair and RNA stability [27-28]. Interestingly, overexpression of YB-1 in thyroid cancer has also been reported [29]. Thus, whether PITX2 and YB-1 has a functional linkage in thyroid cancer would deserve additional studies.

In summary, our data have shown that PITX2 is frequently expressed in follicular cell-derived thyroid cancers and functions to promote thyroid tumorigenesis by regulating critical cell cycle regulators, and in particular Cyclin D2 by acting at the transcriptional level. To our knowledge, this is the first evidence to demonstrate an oncogenic role of PITX2 in thyroid cancer. Since PITX2 may function to promote cancer cell growth, therapeutic approaches targeting PITX2 could have clinical benefits for some thyroid cancer patients.

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CHAPTER III

PROTEOMIC AND GENOMIC ANALYSIS OF PITX2 INTERACTING AND REGULATING NETWORKS

Abstract

PITX2 is a homeodomain transcription factor that has a substantial role in cell proliferation and differentiation in various tissues. In this report, we have conducted a systematic study, using proteomic and genomic approaches, to characterize PITX2-interacting proteins and PITX2-regulating genes. We identified four novel PITX2-associated protein partners YB-1, hnRNP K, nucleolin and hnRNP U in mass spectrometry analysis. We also found that overexpression of PITX2 upregulated 868 genes (two-fold to twenty five-fold) and downregulated 191 genes (two-fold to fifteen fold) in DNA microarray analysis. These data provide an insightful perspective for further studying PITX2 function and mechanism of action.

Introduction

Transcription factor PITX2 is a member of the homeobox gene family. A number of studies have demonstrated that PITX2 has a diverse role in cell proliferation, differentiation, hematopoiesis and organogenesis [1-4]. During early embryogenesis, PITX2 is a key regulator in the establishment of embryo left-right asymmetry[5]. In response to Wnt and other growth factors, PITX2 regulates cell-type specific cell

proliferation during the development of cardiac outflow tract[3]. Mutations of PITX2 have been identified in several human disorders, such as Axenfeld-Rieger syndrome, iridogoniodysgenesis syndrome and sporadic Peter syndrome[6,7]. *Pitx2*-deficient mice are embryonic lethal and show severe defects in heart, eye, pituitary gland and tooth organogenesis[3]. Previous studies have shown that PITX2 cooperates with β-catenin and LEF/TCF and thus regulates cell proliferation by directly activating transcription of cyclin Ds and c-myc[3,8,9]. Besides β-catenin and LEF/TCF, other functional binding partners of PITX2, such as NF-1, HMG-17, MEF2A, Pit-1 and GcMa, have also been reported [1,10-13].

In this study, we analyzed the co-immunoprecipitated protein complex of PITX2 by mass spectrometry and successfully identified four proteins, YB-1, nucleolin, hnRNP K and hnRNP U, as novel PITX2-interacting partners. We also investigated the immediate regulatory effects of PITX2 by examining gene expression profile of HEK293 cells with transient overexpression of PITX2. Our result indicated that 868 genes and 191 genes were upregulated and downregulated more than two-fold, respectively. Many of these regulated genes have previously been linked to cell proliferation, cell differentiation, and organogenesis of muscle and eye. Taken together, our findings provide an insightful perspective on PITX2 function and related molecular mechanism.

Materials and Methods

Cell culture

HEK293 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 10% fetal bovine serum, and penicillin-streptomycin (100 IU/ml) at 37^0C in a humidified atmosphere with 5% CO_2.

Immunoprecipitation and immunoblotting

The open reading frame of human PITX2c, along with a FLAG epitope inserted between Met1 and Asn2, was amplified by PCR with the following primers: forward primer (5′-ACTGaagcttgccaccATGGATTACAAGGATGACGACGATAAGAACTGCATGAAAG GCCCGCTTCAC -3′, HindIII and Kozak site in lowercase) and reverse primer (5′-AGCTggtacctcaCACGGGCCGGTCCACTG-3′, KpnI site and stop codon in lowercase). The PCR product was cloned into the HindIII/KpnI sites of the vector pEGFP-N1 (Clontech). The resulting construct pEGFP-NFLAG-PITX2c, along with the control vector pEGFP-N1, was transfected into HEK293 cells using FugeneHD transfection reagent (Roche). The cells were observed 24 and 48 hours post-transfection, and no significant difference of cell growth and cell viability was noticed between the test and control groups. Cells were then lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) after 48 hours of initial transfection. For nuclear protein extraction, cells were incubated in hypotonic buffer (10 mM HEPES, 10 mM KCl, 1.5mM $MgCl_2$, 0.5 mM DTT) for 10 minutes before adding of 0.5% CA-630. Cell nuclei were then isolated by centrifugation. Nuclear proteins were

extracted by the immunoprecipitation buffer. Cell lysates were pre-cleared by protein G beads (Pierce) for 2 h at 4^0C, and then incubated with anti-FLAG M2 conjugated-agarose beads (Sigma) or rabbit anti-YB-1 (Epitomics) for 2 h or overnight at 4^0C. The beads were washed five times using the immunoprecipitation buffer. To eliminate indirect protein-protein interactions, protein complexes were washed by consecutive addition of 2% and 4% Triton X-100 solutions. Washed beads were boiled in reducing SDS loading buffer for 10 min to elute proteins, which were then subjected to mass spectrometry and immunoblotting. Immunoblotting was performed as described previously [16]. Primary antibodies were mouse anti-PITX2 (Abnova), rabbit anti-β catenin and anti-YB-1 (Cell Signaling), rabbit anti-hnRNP K (Epitomics), rabbit anti-nucleolin and anti-hnRNP U (Abcam).

Peptide mass fingerprinting and LC-MALDI mass spectrometry

Proteins in the PITX2-immunoprecipitated complex were separated by SDS-PAGE and silver stained. Protein bands with high abundance were excised and analyzed by LC-MALDI (ABI Tempo LC MALDI) and MALDI MS/MS (ABI 4800 MALDI TOF/TOF) mass spectrometry analysis by Protea Bioscience (WV, USA). Peptides were identified by performing Mascot MS/MS Ion search (maximum missed cleavages: 2; peptide mass tolerance: ± 1-2 Da; fragment mass tolerance: ± 0.8-1 Da) on the non-redundant fasta database obtained from the National Center for Biotechnology Information (NCBI) and Swissprot database. The peptides and proteins with significant Mowse score ($p<0.05$), except trypsins, were reported.

RNA preparation

HEK293 cells were transiently transfected with pEGFP-NFLAG-PITX2c or pEGFP-N1. Forty eight hours after transfection, cells were harvested for total RNA extraction by using TRI Reagent (Ambion). RNA quality was assessed by electrophoretic analysis on an Agilent Model 2100 Bioanalyzer. All RNA samples used in this study had RNA Integrity Numbers greater than 8.0.

Microarray analysis

We employed a balanced block design with dye swap using four biological replicates from HEK293 cells transfected with either pEGFP-N1 or pEGFP-NFLAG-PITX2c. For microarray probes, total RNA (250 ng) was used as the template for synthesis of internally labeled cRNAs using Agilent QuickAmp Labeling kit and cyanine 3-CTP and cyanine 5-CTP (Perkin Elmer, Waltham, MA) and a modified QuickAmp protocol [14]. A total of 825 ng of cyanine 3- and cyanine5-labeled cRNAs was combined and hybridized onto Agilent Whole Human Genome 4 x 44 K microarrays at 65^{o}C for 17 hours and then washed according to the manufacturer's protocol. Slides were scanned on Agilent DNA Microarray Scanner.

Statistical analysis of microarray data

Data were lowess-normalized and extracted from arrays using Agilent Feature Extraction v9.5. Features for which both channels had low signal were excluded. Log2 expression ratios of PITX2-overexpressed to control samples were imported to the Multiple Experiment Viewer (MeV) v4.0 to perform statistical analysis[15]. Values were

compared for significant deviation from zero using one-class Significance Analysis of Microarrays (SAM)[16]. Only probes for which at least three replicates passed the low signal filter were included. SAM was performed with the maximum number of unique permutation, and delta was chosen to give 0% reported 90^{th} percentile False Discovery Rate (FDR). Raw data from the microarray experiment are available at the NCBI Gene Expression Omnibus with accession number GSE13216.

Semi-quantitative RT-PCR analysis

To validate microarray data, we performed semi-quantitative RT-PCR. The cDNAs were reverse transcribed from total RNA in the presence of random primers (Clontech), and used as templates in the PCR under the following parameters: 94^0C for 2 min, 1cycle; 94^0C for 20 sec, 56^0C for 20 sec (58^0C for FOXJ1 and H19), 72^0C for 2 min, 32 cycles; followed by a 6-min extension at 72^0C. The primers used for this study are listed in Table 1.

Table 1

Gene	Forward primer	Reverse primer	Product size (bp)
DHRS2	AGTGAGCAGATCTGGGACAA	GAGAATGCCGAAGCGTTTTT	621
RSPO3	GCCCCACTTCGCTTGCCATCA	ACCCGTGTTTCAGTCCCTCTT	695
CCNA1	GTCCCGATGCTTGTCAGATA	CCAACCTCCACCAGCCAGTC	579
BAIAP3	ACGGCTTAAGTGACCCCTTT	TTCACAGCACACACCAGACA	621
RET	GTGTGAGTGGAGGCAAGGAG	AGGCGTTCTCTTTCAGCATC	673
FOXJ1	GCTTCCCCAGGTCTCTATCC	CACCAAACCCAAACTTCCAG	335
H19	AAAGACACCATCGGAACAGC	GCTCACACTCACGCACACTC	349
PDE6B	CACCGACACCTACGACAAGA	GGGTTCTGGGACTTCATCTG	551
BRIP3	ACACGCACACACAAGCAGAT	GAGCATTTCATTTCCACTCCA	675
OLFM1	GGACGGCTATCACAACAACC	GGCAGCAGTTTCACAGGAG	729
CDH15	AGAGCCTCTGCCTGTCTTTG	ACTGTGCGGATCTCTCCTGT	600
DGKG	GGAGGGAGACAAGGAGAAAGA	TGCTGGGAATGTTGAGAAT	657
β-actin	GGGTCAGAAGGATTCCTATG	ATGAGGTAGTCAGTCAGGTC	428

Table 1. List of primers used in RT-PCR.

Results

Identification of novel PITX2-associated proteins

Despite the substantial role of PITX2 in cell proliferation and differentiation, the molecular mechanism underlying PITX2 action is still limited. Identifying novel PITX2-interacting protein partners would further our understanding of how PITX2 works. To do so, we first transiently overexpressed PITX2 in HEK293 cells (Figure 1A) and then co-immunoprecipitated PITX2 and its associated protein complex from the cell lysates. After the protein complex was separated by SDS-PAGE, five protein bands with high abundance, which did not appear in the pEGFP-N1 control sample (data not shown), were excised and subjected to mass spectrometry analysis. In addition to PITX2, four novel PITX2-associated proteins, YB-1, hnRNP K, nucleolin and hnRNP U, were identified from each protein band, respectively (Table 2). These four proteins achieved the highest significant scores among other candidates and their molecular weights matched the PAGE-determined molecular weights. To further confirm the association of these four proteins with PITX2, we performed immunoblotting on PITX2-immunoprecipitated proteins. As shown in Figure 1B, we indeed detected YB-1, hnRNP K, nucleolin and hnRNP U in the PITX2-immunoprecipitated protein complex. As a positive control for the PITX2 complex, we also demonstrated the presence of β-catenin, a previously reported binding partner of PITX2 (Figure 1B). Using an YB-1 antibody, we also detected the interaction of PITX2 and YB-1 at endogenous levels (Figure 1C). We were not able to test for interactions between PITX2 and other novel binding partners since immunoprecipitation-suitable antibodies for these proteins are not currently available.

Figure 1

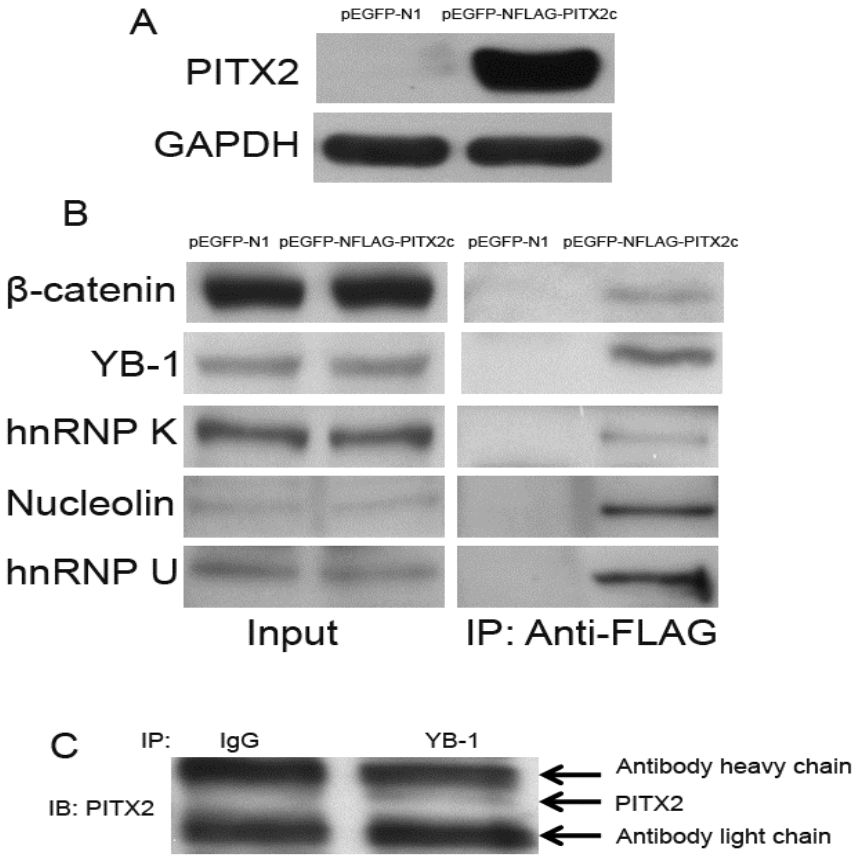

Figure 1. Verification of PITX2 association partners. (A) PITX2 was transiently overexpressed in HEK293 cells. The cell lysates from pEGFP-N1 and pEGFP-NFLAG-PITX2c were analyzed by immunoblotting. (B) YB-1, hnRNP K, nucleolin, hnRNP U and β-catenin are PITX2-interacting proteins. The PITX2-immunoprecipitated complex, along with the protein samples equivalent to 10% input, was probed with respective antibodies. (C) The endogenous protein complex of YB-1 and PITX2 was immunoprecipitated from HEK293 nuclear lysates by an YB-1 antibody and then detected by a PITX2 antibody.

Table 2

Protein	Peptides (ions score)	RefSeq No.	Gel region
Heterogeneous nuclear ribonucleoprotein U (hnRNP U)	(R) NFILDQTNVSAAAQR (123) (R) GYFEYIEENKYSR (16) (K) SSGPTSLFAVTVAPPGAR (50)	NP_114032.2	100-120 kDa
Nucleolin	(K) EVFEDAAEIR (27) (K) GFGFVDFNSEEDAK (16) (K) GLSEDTTEETLKESFDGS (17)	NP_005372.2	80-100 kDa
Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	(R) NLPLPPPPPPR (43)	NP_002131.2	60-70 kDa
Y box binding protein-1 (YB-1)	(R) NEGSESAPEGQAQQR (49) (R) NGYGFINR (59)	NP_004550.2	40-50 kDa
Pituitary homeobox 2 (PITX2)	(R) EEIAVWTNLTEAR (58) (R) THFTSQQLQELEATFQR (53)	NP_700476.1	30-40 kDa

Table 2. List of proteins identified from the PITX2-immunoprecipitated complex, as described in Materials and methods. Proteins with ions scores > 32 indicate identity or extensive homology ($p<0.05$).

Identification of PITX2-regulated genes

Since PITX2 has been shown to participate in a number of physiological processes, we hypothesized that PITX2 may have numerous target genes. To explore this idea, we performed DNA microarray to examine the gene expression profile of HEK293 cells transiently overexpressing PITX2 compared to cells with normal PITX2 levels. Remarkably, we found that 868 genes and 191 genes were upregulated and downregulated, respectively, by more than two-fold. Many of these regulated genes can be clustered into different biological processes, such as cell proliferation, cell differentiation, and organogenesis of muscle and eye, as exemplified in Table 3. A more comprehensive Gene Ontology (GO) analysis is provided in Table 4. To verify microarray data, we used semi-quantitative RT-PCR to measure relative changes in the mRNA abundance of twelve genes. The RT-PCR results were very consistent with the microarray results (Figure 2).

Table 3

Accession No.	Gene name	Fold	Description
Proliferation			
NR_002196	H19	12.0	imprinted maternally expressed untranslated mRNA
NM_003914	CCNA1	11.6	cyclin A1, cell cycle regulator
NM_003933	BAIAP3	7.7	BAI1-associated protein 3
NM_001007139	IGF2	5.5	insulin-like growth factor 2 (somatomedin A)
NM_020975	RET	5.4	ret proto-oncogene
NM_032043	BRIP1	0.17	BRCA1 interacting protein C-terminal helicase 1
NM_004407	DMP1	0.16	dentin matrix acidic phosphoprotein
Muscle			
NM_004933	CDH15	9.5	cadherin 15, M-cadherin (myotubule)
NM_000257	MYH7	6.0	myosin, heavy chain 7, cardiac muscle, beta
AB002384	C6orf32	4.6	mRNA for KIAA0386 gene
Eye			
NM_014279	OLFM	9.1	olfactomedin 1
NM_000283	PDE6B	8.9	phosphodiesterase 6B, cGMP-specific, rod, beta (congenital stationary night blindness 3, autosomal dominant
NM_014421	DKK2	6.1	dickkopf homolog 2
NM_000327	ROM1	3.9	retinal outer segment membrane protein 1
Development/differ			
NM_032784	RSPO3	20.5	R-spondin 3 homolog
NM_000582	SPP1	9.5	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
NM_001454	FOXJ1	7.5	forkhead box J1
NM_030761	WNT4	6.8	wingless-type MMTV integration site family, member 4
Others			
NM_001346	DGKG	9.0	diacylglycerol kinase, gamma 90kDa
NM_182908	DHRS2	25.2	dehydrogenase/reductase (SDR family) member 2

Table 3. Exemplified genes regulated by PITX2.

Table 4

Biological Process	GO ID#	# of Entities	Overlap	Holm-Bonferroni adjusted p-value
multicellular organismal development	61990	1100	78	1.44404E-10
potassium ion transport	62621	190	25	2.23621E-07
cell-cell signaling	59266	334	32	2.71767E-06
cell differentiation	60383	691	47	4.37307E-05
Wnt receptor signaling pathway	59323	127	16	0.000710167
ion transport	62490	619	40	0.001614313
muscle contraction	63107	107	14	0.002207541
cell adhesion	56848	741	43	0.010103486
peripheral nervous system development	62382	32	7	0.027104815
transport	62462	1962	87	0.027471747
blood circulation	63101	57	9	0.031548558

Table 4. Biological processes enriched for PITX2-regulated genes.

In order to discover biological processes that were enriched for PITX2-regulated genes, we entered expression data from the 1059 member gene set into Ariadne Pathway Studio software (Rockville, MD) and used Fisher's Exact Test as implemented by Pathway Studio to determine the p-value associated with the biological processes, and subsequently adjusted for multiple hypothesis testing using the Holm-Bonferroni method with an overall type-1 error rate of 0.05. Since 1015 biological processes were represented by the list of genes, Holm-Bonferroni was used to adjust for 1015 different hypotheses."Entities" refers to the total number of genes within a given process while "Overlap" refers to the number of PITX2-regulated genes within a given process. Only processes which met the criterion for statistical significance ($p < 0.05$) are listed.

Figure 2

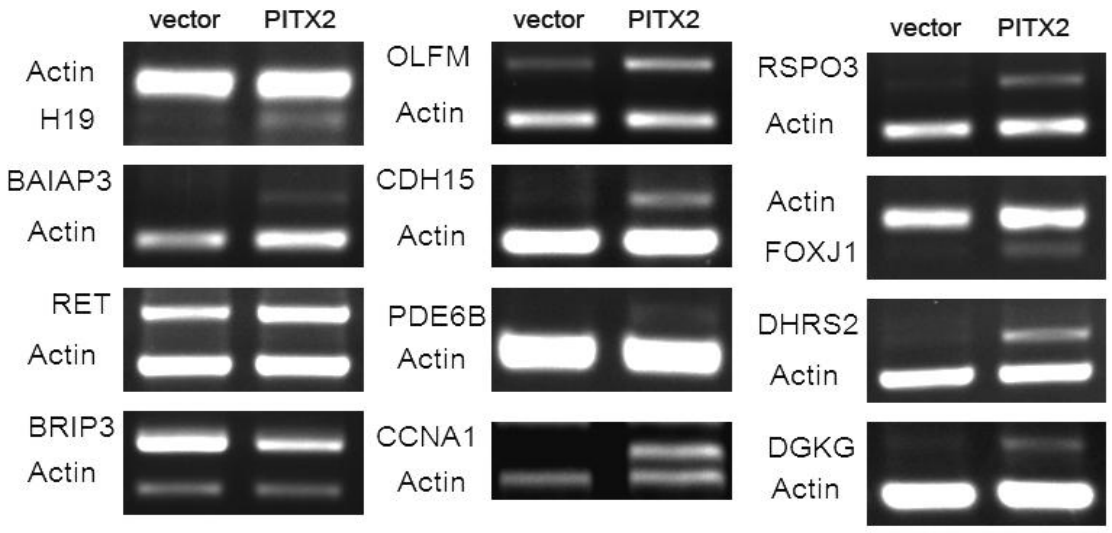

Figure 2. Semi-quantitative RT-PCR analysis of PITX2-regulating genes. Actin was used as an internal control. Vector=pEGFP-N1; PITX2=pEGFP-NFLAG-PITX2c.

Discussion

In this study, we have identified YB-1, nucleolin, hnRNP K and hnRNP U as the novel PITX2-interacting partners. The four proteins show consistency in terms of their functions in that all of them play roles in regulating cell proliferation and RNA processing [17-20]. YB-1 is a multifunctional protein that functions in the regulation of cell proliferation and drug resistance[21]. Nuclear YB-1 acts as a transcription factor which controls transcription of genes involved in cell proliferation, such as cyclin A [17,22]. In this regard, our two observations, upregulation of cyclin A upon overexpression of PITX2 and the association of YB-1 and PITX2, may well echo each other. YB-1 also can bind to mRNA and become part of messenger ribonucleoprotein particels (mRNPs), thus controls gene translation[21]. Nucleolin is a ubiquitously expressed protein and a major component of the nucleolus[18]. Nucleolin functions include chromatin-remodeling, regulation of mRNA processing, ribosome assembly and nucleo-cytoplasmic transport[18]. Nucleolin, hnRNP K and YB-1 have been previously reported to be assembled into a macromolecular complex regulating mRNA stability[23,24]. In this respect, it is tempting to postulate that PITX2, through association with YB-1, may regulate mRNA stability as well. Indeed, such function has been previously assigned to PITX2 [25] . The identification of novel PITX2-interacting partners provides a new look at the mechanistic aspect of PITX2 function. It also suggests that PITX2 may regulate its downstream targets at both transcription and translation levels. Hence, the functional implication of this novel PITX2-interacting network will be a valuable topic in future studies.

Previously, only a few PITX2 transcription targets have been identified, such as cyclin Ds, LEF and PLOD[3,9,26,27]. However, these target genes may not be capable of mediating all functions of PITX2. In this study, we have found a remarkable set of potential target genes of PITX2. Of these candidates, FOXJ1 and DKK2 have been reported as direct targets of PITX2 transcriptional regulation in two recently published studies[28,29], consistent with our findings. To this end, we cannot rule out the possibility that some of the genes detected in our expression profile study are not directly regulated by PITX2. Nonetheless, our map of the PITX2-regulated gene network opens new avenues for studying biological processes involving PITX2.

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CHAPTER IV

PITX2 ACTIVATES CYCLIN A1 THROUGH MLL4-MEDIATED H3K4 METHYLATION IN THYROID CANCER CELLS

Abstract

Pituitary transcription factor 2 (PITX2) is a homeodomain transcription factor that regulates cell proliferation and differentiation in a variety of organs such as eye, tooth, heart, pituitary and smooth muscle. Although the importance of PITX2 in many biological events has been well-known, the transcriptional targets and underlying regulatory mechanism of PITX2 action are still elusive. Here we show that Cyclin A1, a testis-specific member of Cyclin As, is a novel PITX2 transcriptional target. Overexpression of PITX2 increased Cyclin A1 expression, whereas knockdown of PITX2 reduced Cyclin A1 expression at the mRNA and protein levels in human papillary thyroid cancer cells. Using promoter-driven reporter assays, we identified in the Cyclin A1 promoter an evolutionarily conserved PITX2 response element (PRE) that was critical for PITX2-induced Cyclin A1 gene transcription. Chromatin immunoprecipitation indicated that ectopically expressed PITX2 bound to the wild-type but not the PRE-mutated Cyclin A1 promoter. Interestingly, our data further revealed that PITX2 associated with MLL4 histone H3K4 methyltransferase complex and facilitated recruitment of the latter to the Cyclin A1 promoter. Consistently, we observed increased H3K4 methylation and dissociation of HDAC1 in the Cyclin A1 promoter. Moreover, we found that Cyclin A1 was expressed in 40% of human papillary thyroid cancer tissues but not in normal thyroids, a result similar to our previous report on PITX2 expression in

thyroid cancer. Taken together, our data suggest that PITX2 transcriptionally activates Cyclin A1 through a H3K4 methylation pathway.

Keywords: PITX2, Cyclin A1, histone methylation, H3K4, PTIP, MLL4, thyroid cancer

Introduction

Pituitary transcription factor 2 (PITX2) is a member of the bicoid homeodomain transcription factor family. Previous studies have reported that PITX2 is critical for organogenesis of eye, tooth, heart, pituitary and smooth muscle, by regulating cell proliferation and differentiation in a tissue-specific manner [1-4]. In addition, PITX2 has a pivotal role in controlling left-right asymmetry during early embryogenesis [5]. Pitx2 knockout mice are embryonic lethal and exhibit failures in the development and positioning of multiple organs [1]. Mutations in human PITX2 have been linked to several diseases such as Axenfeld-Rieger syndrome, iridogoniodysgenesis syndrome, and sporadic cases of Peter's anomaly [6, 7]. At the molecular level, PITX2 has been shown to function as a downstream effector of Nodal, TGFβ, retinoic acid and Wnt signaling during embryonic development [1,4,5,8]. When binding to β-catenin, PITX2 transcriptionally activates Cyclin D1, Cyclin D2 and c-Myc to regulate cell proliferation [1, 9]. However, the precise role of PITX2 during various biological events is still largely unclear.

To map out the regulatory and interacting networks of PITX2, we previously have used microarray and mass spectrometry to search for novel PITX2 transcriptional targets and binding partners [10]. Cyclin A1 is one of the most up-regulated genes induced by ectopically expressed PITX2 in HEK293 cells. The Cyclin A gene family consists of two members, Cyclin A1 and Cyclin A2. Cyclin A2 is ubiquitously expressed and critical for cell cycle progression in both S-phase and G2/M phase [11]. Cyclin A2 knockout mice are embryonic lethal [11]. Unlike Cyclin A2, Cyclin A1 displays a tissue-specific expression pattern with high levels of expression restricted in testis, more specifically in the meiosis I stage of spermatocytes [12]. Cyclin A1 knockout mice are developmentally normal except that male mutants are infertile [12]. Cyclin A1-deleted spermatocytes are arrested at the end of meiotic prophase I because of reduced Cdc2 kinase activity [12]. In addition to its function in spermatogenesis, an oncogenic role of Cyclin A1 has been proposed in acute myeloid leukemia, prostate cancer and breast cancer [13-15]. When mouse Cyclin A1 is overexpressed in the myeloid lineage of transgenic mice, a small percentage of mice spontaneously develop acute myeloid leukemia [13]. In prostate cancer, elevated level of Cyclin A1 has been associated with advanced, invasive prostate cancer [14]. Overexpression of Cyclin A1 in a prostate cancer cell line enhances cell invasion and metastasis [14]. Moreover, Six1, a homeobox gene, reactivates Cyclin A1 in breast cancer cells and thus promotes breast cancer cell proliferation [15]. However, whether Cyclin A1 is expressed in thyroid cancer tissues has not yet been studied.

In this study, we investigate the role of PITX2 in regulating Cyclin A1 gene expression. We report here that Cyclin A1 is a novel PITX2 transcriptional target. We have also identified the promoter element responsible for PITX2-induced Cyclin A1 transcription.

In addition, we show that PITX2 activates Cyclin A1 transcription through a histone H3K4 methylation pathway. Our data provide an insightful perspective on the regulatory network of PITX2. Significantly, the finding that PITX2 associates with the H3K4 methyltransferase complex to remodel target genes' chromatin structure extends the role of PITX2 in gene regulation and highlights the versatility of PITX2 as a transcription factor.

Materials and methods

Cell culture

Human papillary thyroid cancer cell line (TPC-1) was kindly provided by Dr. James Fagin (Memorial Sloan-Kettering Cancer Center, NY). HEK293 cell line was purchased from ATCC (Manassas, VA). Both of TPC-1 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 10% fetal bovine serum, and penicillin-streptomycin (100 IU/ml). Cell culture media and supplements were purchased from ATCC. Cells were incubated at 37^0C in a humidified atmosphere with 5% CO2.

RT-PCR analysis

Total RNA was extracted from cultured cells using TRI Reagent (Ambion, TX) as described previously. PITX2-knockdown TPC-1 cells have been described previously. RT-PCR was performed to examine Cyclin A1 expression. The primers used in this study are listed in Table 1. PCR parameters: 94^0C for 2 min, 1 cycle; 94^0C for 20 sec, 56^0C for 20 sec, 72^0C for 2 min, 32 cycles; followed by a 6 min extension at 72^0C.

Table 1

CCNA1-5': GTCCCGATGCTTGTCAGATA
CCNA1-3': CCAACCTCCACCAGCCAGTC
CCNA1-2027-BglII-5': AGTCAGACTCGTGCTGGGACTACAGGCGTA
CCNA1-2027-HindIII-3': AGTCAAGCTTGGCGGGAAGGACCAAGTGTC
CCNA1-765-KpnI-5': AGTCGGTACCCTCGAGCACGACGTGCGACCCT
CCNA1-658-XhoI-5': ACTGCTCGAGATGGAGACGCAACACTGCCG
CCNA1-201-XhoI-5': ACTGCTCGAGAGCGAGTCAGGTGAGCAGGT
CCNA1-102-XhoI-5': ACTGCTCGAGGCTGATTGGCCGATTCAACA
CCNA1-96-XhoI-5': ACTGCTCGAGTGGCCGATTCAACAGACG
PITX2C-FLAG-HindIII-5': ACTGAAGCTTGCCACCATGGATTACAAGGATGACGACGATAAGAACTGCATG AAAGGCCCGCTTCAC
PITX2C-324-KpnI-3': AGCTGGTACCTCACACGGGCCGGTCCACTG
PITX2C-180-KpnI-3': AGCTGGTACCTTAGCCTGGGTACATGTCGTCGT
PITX2C-161-KpnI-3': AGCTGGTACCTTAATTCTTGCATAGCTCGGCCT
PITX2C-HDdel-KpnI-3': AGCTGGTACCTTAGTTGGTCCACACAGCGATTT
PITX2C-FLAG-91-XhoI-5': ACTGCTCGAGCGGCAAAGGCGGCAGCGGACT
PITX2C-152-NheI-5': ACTGGCTAGCGCCACCATGGATTACAAGGATGACGACGATAAGCGCAACCA GCAGGCCGAGCTAT
CCNA1-CHIP-5': ATGGAAACGCTCCCGCTAGGT
CCNA1-CHIP-3': AGGACCAAGTGTCGAGGGATT
CCNA-CHIP1-5': CTGGAATCCCTCGACACTT
CCNA-CHIP-3': TATTCAGCCCATAGCGCTT

Table 1. List of primers used in this study

Immunoblotting and Co-immunoprecipitation assay

Total cell lysates from cultured cells were extracted in the cell lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). Immediately before use, the lysis buffer was supplemented with a protease inhibitor cocktail tablet (Roche, IN). Immunoblotting was performed as reported previously [16]. The following primary antibodies were used: Cyclin A1 (BD Bioscience, CA); FLAG M2 (Sigma, MO); PITX2 (Abnova), GAPDH (Ambion); PTIP, RBBP5 and ASH2L (Bethyl laboratory), hnRNP U (Abcam). The same blot was stripped and reprobed with a GAPDH antibody to obtain loading control.

For co-immunoprecipitation assay, full length FLAG-tagged PITX2 was transfected into HEK293 cells that had been cultured overnight in 6-well plates by Fugene HD transfection reagent (Roche, IN). Transfection efficiency was monitored by green fluorescence 48 hours later to ensure the consistency of experiments. Transfected cells were then lysed by the cell lysis buffer and incubated with primary antibodies at 4^0C overnight before being precipitated by protein A beads (Pierce). Antibodies used for immunoprecipitation are: PTIP, RBBP5 and ASH2L (Bethyl laboratory); MLL4 (a gift from Dr. Ge Kai, NIH). Immunoprecipitated samples were boiled in the SDS loading buffer and subjected to immunoblotting analysis as described above.

Plasmids and Reporter assay

All the primers used in this study are listed in Table 1. The CyclinA1 luciferase reporter constructs contained various lengths of the human Cyclin A1 promoter ranging from -1 to -2027 bps. The Cyclin A1 promoter fragments were cloned from human blood cell genomic DNA and inserted into BglII/HindIII sites of pGL4.24 luciferase vector (Promega, MI) for CCNA2027, KpnI/HindIII sites for CCNA765 and XhoI/HindIII for the others. The pEGFP-NFLAG-PITX2c plasmid (expressing N-terminal FLAG tagged PITX2C) has been previously described [10]. The pEGFP-NFLAG-PITX2C-ΔHD plasmid, which expressed N-terminal FLAG tagged, homeodomain (HD, amino acid 1-131)-deleted PITX2C, was inserted into HindIII/KpnI sites of pEGFP vector. The various truncated human PITX2C genes containing N-terminal FLAG tag were cloned from pEGFP-NFLAG-PITX2C plasmid and inserted into NheI/KpnI sites of pEGFP vector for PITX2C 152-324 and HindIII/KpnI for the others. The EGFP in the parental vector was not incorporated in the same open reading frame with the insert DNA fragments and was solely served as a marker for monitoring transfection efficiency. The pGL4-CCNA658mut plasmid, generated by site-directed mutagenesis (Stratagene), contained the mutated PITX2 response element (-96 to -101 bps) in the Cyclin A1 promoter.

For reporter assay, $2x10^3$ TPC-1 cells were seeded in 96 wells. A total of 100ng of various plasmid mixes were transfected into TPC-1 cells by Fugene HD transfection reagent. Renilla vector was included in all transfections and served as an internal control. Forty-eight hours after transfection, luciferase and renilla signals were measured by the Dual-Luciferase Reporter Assay System according to the manufacturer's instruction (Promega, WI). Each experiment was performed in triplicate and repeated twice.

Chromatin immunoprecipitation (ChIP) assay

HEK293 cells were transiently transfected with pEGFP-NFLAG-PITX2C or pEGFP-NFLAG-PITX2C-ΔHD by Fugene HD transfection reagent. After 48 hours of initial transfection, cells were cross-linked by 1% formaldehyde for 15 minutes. Cell genomic DNA was then sheared into fragments by sonication. Cell lysates were pre-cleared by protein A beads (Pierce, IL) for 2 h at 4^0C. Anti-FLAG M2 conjugated agarose beads (Sigma), anti-PTIP (Bethyl laboratory), anti-H3K4me2, anti-HDAC1 (Cell signaling) or IgG antibody was then incubated with pre-cleared cell lysates at 4^0C overnight. Beads were washed by the high salt and LiCl washing solution for eight times as previously described. Samples were reverse cross-linked in the high salt solution at 65^0C overnight. DNA was purified by phenol-chloroform extraction and precipitated by isopropanol. PCR was then used to analyze precipitated DNA samples. One pair of ChIP primers CCNA-CHIP (listed in Table 1), covering the evolutionarily conserved PITX2 response element (PRE) in the CyclinA1 promoter (-7 to -133), was used to detect the endogenous Cyclin A1 promoter in PCR analysis. Another pair of primers CCNA-CHIP1, which covered both the PRE and part of the pGL4.24 vector, was used to detect the Cyclin A1 promoter inserted into the pGL4.24 vector. PCR parameters: 94^0C for 2 min, 1cycle; 94^0C for 20 sec, 58^0C for 20 sec, 72^0C for 50 sec, 36 cycles; followed by a 6 min extension at 72^0C.

Immunohistochemistry

Tissue microarray slides, which included both normal and malignant human thyroid tissues, were purchased from US Biomax (Ijamsville, MD). Formalin-fixed, paraffin-embedded tissue sections were de-paraffinized by xylene and ethanol. Antigen retrieval

was performed by heating tissue samples in a microwave. Anti-Cyclin A1 (BD Bioscience) was incubated with tissue sections at 4^0C overnight. Immunostaining was done by the IHC Select Immunophophatase Secondary Detection System (Chemicon, CA) according to the manufacturer's instruction. Tissue microarray slides and their staining intensity were evaluated and classified by two individuals independently. A tissue sample with a minimum of 20% cells showing staining was counted as a positive case for consistent expression of Cyclin A1.

Statistical analysis

Data were presented as mean ±S.D. and analyzed by Student's t test. Chi-square test was used to analyze immunohistochemistry studies. Statistical significant differences were defined as $p<0.05$.

Results

PITX2 regulates Cyclin A1 expression in thyroid cancer cells

To decipher the regulatory and interacting networks of PITX2, we previously used microarray and mass spectrometry to search for novel PITX2 transcriptional targets and binding protein partners [10]. As a result, Cyclin A1 was identified as one of the most up-regulated genes induced by PITX2 overexpression. Since we have reported that PITX2 regulates thyroid cancer cell proliferation [17], we speculate that Cyclin A1 is a PITX2 transcriptional target in thyroid cancer cells. To examine this hypothesis, we first knocked down PITX2 by shRNA in a papillary thyroid cancer cell line TPC-1. Both Cyclin A1 mRNA and protein levels were down-regulated upon PITX2 knockdown

(Figure 1A and 1B). Consistently, the Cyclin A1 protein level was up-regulated when PITX2 was overexpressed in TPC-1 cells (Figure 1C). These data suggest that the expression of Cyclin A1 in thyroid cancer cells is regulated by PITX2.

Figures 1

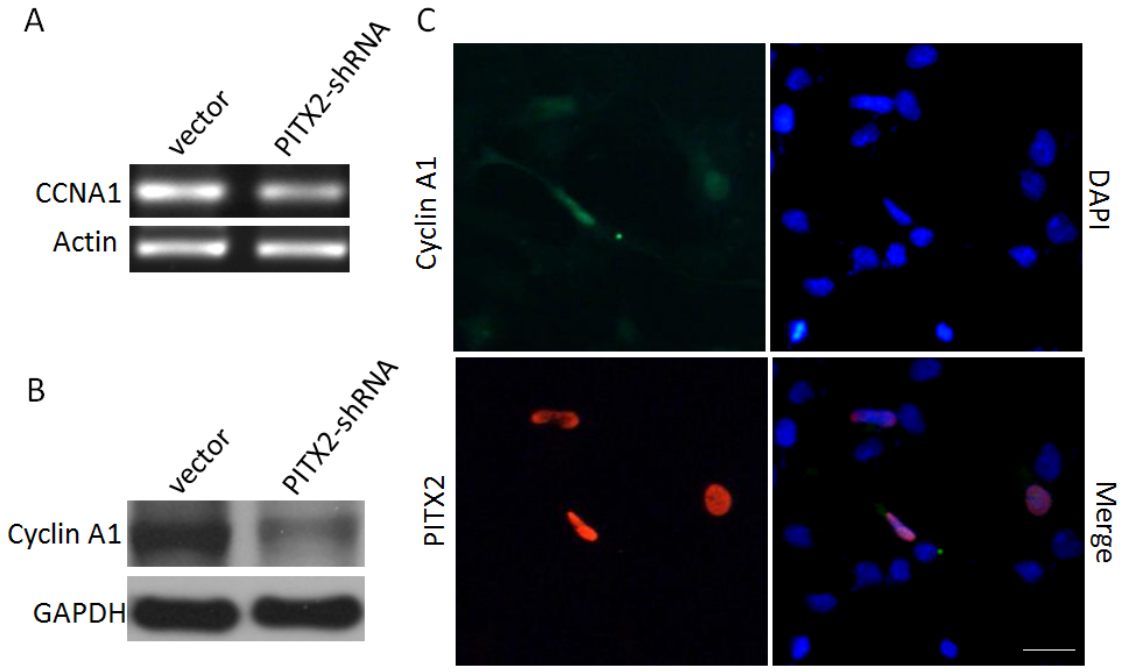


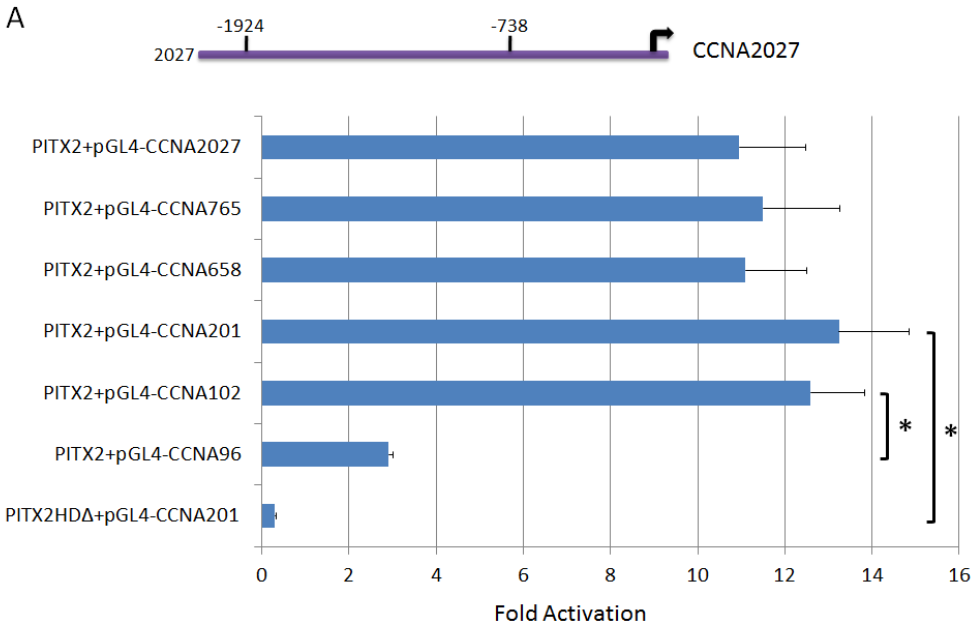
Figure 1. PITX2 regulates Cyclin A1 expression in TPC-1 cells. Cyclin A1 mRNA (A) and protein (B) levels were examined by RT-PCR and WB, respectively. Actin was used as RT-PCR internal control. GAPDH was used as WB internal control. (C) FLAG-PITX2 was transiently expressed in TPC-1 cells. Cells were immunostained by anti-Cyclin A1 and anti-FLAG. Scale bar indicates 50μm.

An evolutionarily conserved element is critical for PITX2-induced Cyclin A1 transcription

To investigate how PITX2 regulates Cyclin A1 gene expression, we constructed a series of reporter plasmids that covered various lengths of the Cyclin A1 promoter (upstream of the transcription initiation site). Two conserved bicoid homeodomain binding sites were found at -738 and -1924 bps of the Cyclin A1 promoter. Surprisingly, PITX2-induced Cyclin A1 transcription was not compromised in reporter assays when the Cyclin A1 promoter was gradually chopped from -2027 bps to -102 bps (Figure 2A). However, reporter signal was dramatically reduced when the Cyclin A1 promoter was further chopped from -102 bps to -96 bps (Figure 2A), indicating that there exists a PITX2 response element (named as PRE herein) from -102 bps to -96 bps. Further DNA sequence alignment suggests that an evolutionarily conserved PRE is located between -93 bps and -101 bps of the Cyclin A1 promoter (Figure 2B and 2C). To test whether PITX2 binds with the PRE, chromatin immunoprecipitation (ChIP) was performed in FLAG-PITX2 transfected HEK293 cells. Cells expressing the homeodomain-deleted FLAG-PITX2 were used as negative control. Using the primers flanking the PRE, we indeed detected the binding of PITX2 on the Cyclin A1 promoter (Figure 4A). To examine whether or not the binding of PITX2 to the Cyclin A1 promoter is PRE-dependent, we performed ChIP using HEK293 cells co-transfected with FLAG-PITX2 and the plasmid containing either the wild-type or mutated PRE. As shown in Figure 4B, the binding of PITX2 on the Cyclin A1 promoter is abolished when the PRE is mutated. Taken together, these data clearly demonstrate that the evolutionarily conserved PRE is responsible for PITX2-induced Cyclin A1 gene transcription.

Figure 2

A



B

-201 AGCGAGTCAGGTGAGCAGGTCGCCATGGCGATGCGGCCCCGGAGAGCGCACGC

-102 -96

-148 CTGCCGCGGTCGGCATGGAAACGCTCCCGCTAGGTCCGGGGGCGCC/GCTGAT/T

-95 GGCCGATTCAACAGACGCGGGTGGGCAGCTCAGCCGCATCGCTAAGCCCGGCC

-42 GCCTCCCAGGCTGGAATCCCTCGACACTTGGTCCTTCCCGCC

C

Human: CGCTAGGTCCGGGGGCGCCGCTGATTGGCCGATTCAACAGACGCGGGTGGGCAGCTC

Mouse: TGCTGGGCTCGGGGTGGCCGCTGATTGGCTGATTCAACAGACGCGGCTGGGTCGAAA

Rat: TGCTGGGCTCGGGGTGGCTGCTGATTGGCTGATTCAACAGACGCGGCTGGGTCGAAA

Pig: CGGGGACCCTGGGGGCGTCGCTGATTGGCCGACTCAACAGACGCGGGTGGGTTGAAA

Zebrafish: AAACCATCAACTAGCCTCATGTTGATTGGCCACCTGCAAGTATAAATGAGTTAGCGTG

Figure 2. An evolutionarily conserved PITX2 response element (PRE) is critical for PITX2-induced Cyclin A1 transcription. (A) Two conventional bicoid homeodomain binding sites are located at -738bp and -1924bp of the Cyclin A1 promoter. Full-length FLAG-PITX2 was co-transfected into TPC-1 cells with the reporter constructs containing various lengths of the Cyclin A1 promoter. Reporter signals were measured 48 hours after initial transfection. The renilla construct was included in every transfection for internal quality control. The relative fold increase was calculated by first comparing the reporter signal of cells transfected with FLAG-PITX2 and the Cyclin A1 reporter construct to that of cells transfected with the empty vector and the same Cyclin A1 reporter construct and then normalized by renilla signals. (B) The first 200-bps DNA sequence of the Cyclin A1 promoter is shown. The 6-bps DNA between two dash lines was deleted in pGL4-CCNA101. (C) The Cyclin A1 promoter sequences of different species were aligned. The red-colored nucleotides are conserved among species.

PITX2 associates with the MLL4 histone H3K4 methyltransferase complex

Since Cyclin A1 normally displays a testis-specific expression pattern, we speculate that PITX2-induced Cyclin A1 expression in thyroid cancer cells and HEK293 cells might require intense chromatin remodeling in the Cyclin A1 promoter. To test this hypothesis, we examined the PITX2-associated protein complex for known chromatin remodeling factors. Reciprocal co-immunoprecipitation revealed that PITX2 associated with the MLL4 histone H3K4 methyltransferase (HMT) complex (Figure 3). The anti-FLAG antibody readily precipitated ectopically-expressed PITX2 along with the endogenous MLL4 HMT subunits, including PTIP, RBBP5 and ASH2L (Figure 3A). Conversely, FLAG-PITX2 was detected in the respective protein complex of endogenous MLL4, PTIP, RBBP5 and ASH2L. Notably, hnRNP U, a PITX2-associated protein identified in our previous study, did not interact with the MLL4 HMT complex (Figure 3A), indicating that PITX2-MLL4/HMT interaction may be functionally distinct from other PITX2-interacting protein complexes. To inspect the PITX2 protein region mediating the interaction with the MLL4 HMT complex in vivo, we expressed the truncated forms of FLAG-PITX2 protein in HEK293 cells and then test these proteins' ability to interact with the MLL4 HMT complex. In this experiment, RBBP5 was chosen as the interaction indicator since it is one of the HMT core subunits. Interestingly, deletion of the homeodomain (HD) and the C-terminal region adjacent to the HD abolished the interaction between PITX2 and the MLL4 HMT complex (Figure 3B), suggesting that the PITX2 HD region mediates PITX2-MLL4/HMT interaction.

Figure 3

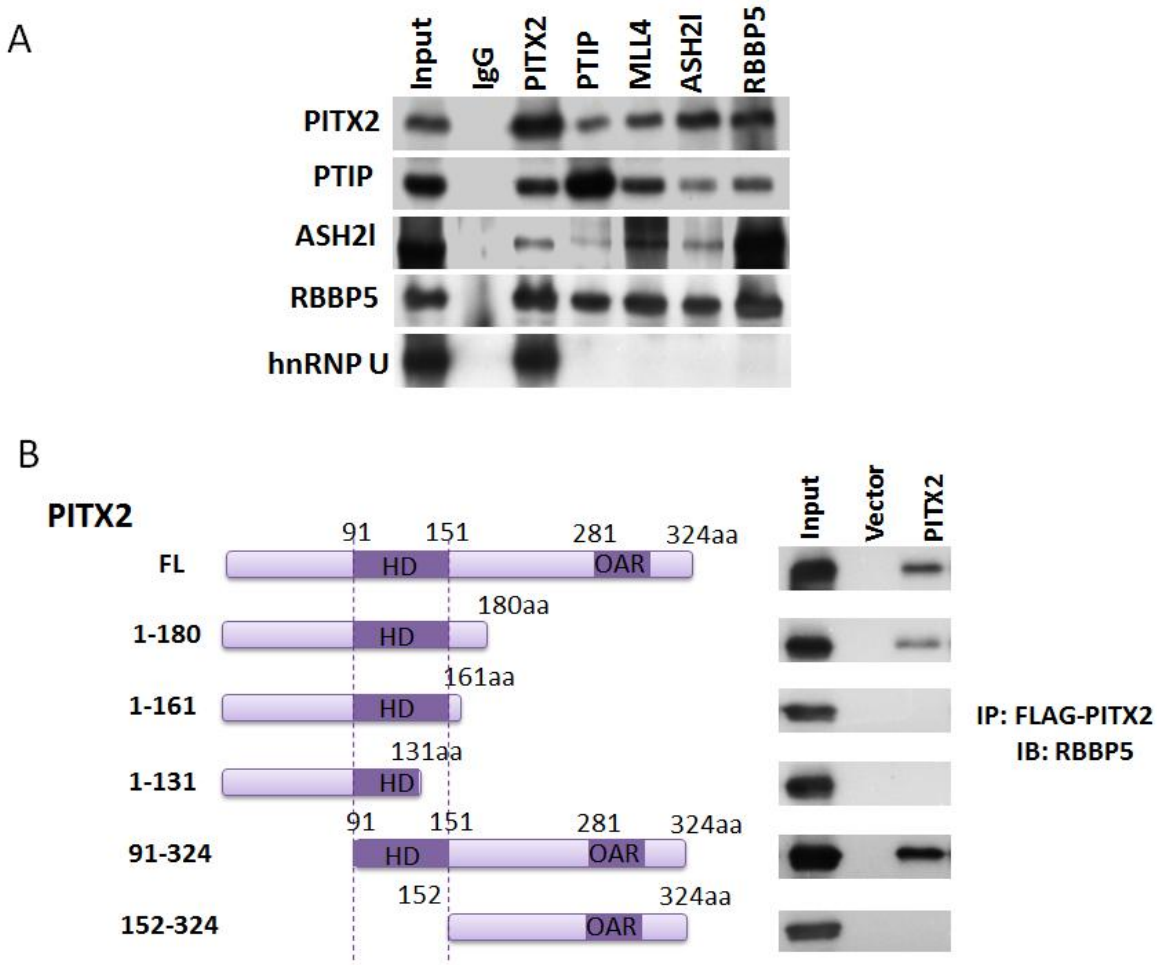


Figure 3. PITX2 associates with the MLL4 HMT complex. (A) Antibodies for FLAG-PITX2, PTIP, MLL4, ASH2L and RBBP5 were used to precipitate respective antigens and their associated proteins from HEK293 cells expressing FLAG-PITX2. WB was used to examine the components of respective protein complex. (B) Plasmids containing various truncated forms of FLAG-PITX2 were transiently expressed in HEK293 cells. Anti-FLAG was used to co-immunoprecipitate PITX2-associated protein complex.

RBBP5 was used as an indicator of the histone H3K4 methyltransferase complex binding with PITX2.

Overexpression of PITX2 promotes H3K4 methylation in the Cyclin A1 promoter

To investigate the functional significance of PITX2-MLL4/HMT interaction on Cyclin A1 gene transcription, we determined H3K4 methylation status in the Cyclin A1 promoter in HEK293 cells expressing either full-length FLAG-PITX2 or homeodomain-deleted FLAG-PITX2. As expected, the anti-H3K4me2 (H3K4 dimethylation) ChIP assay detected the Cyclin A1 promoter in the cells transfected with full-length FLAG-PITX2 but not with homeodomain-deleted FLAG-PITX2 (Figure 4C). This ChIP result was also observed by an anti-PTIP (which is the specific subunit of the MLL4 HMT) antibody, indicating that PITX2 facilitates recruitment of the MLL4 HMT complex to the Cyclin A1 promoter (Figure 4C). On the other hand, HDAC1, a histone deacetylase known for gene repression, dissociated from the Cyclin A1 promoter upon PITX2 overexpression (Figure 4C). Collectively, these results support that PITX2 epigenetically regulate Cyclin A1 gene transcription through MLL4-mediated chromatin remodeling in the Cyclin A1 promoter.

Figure 4

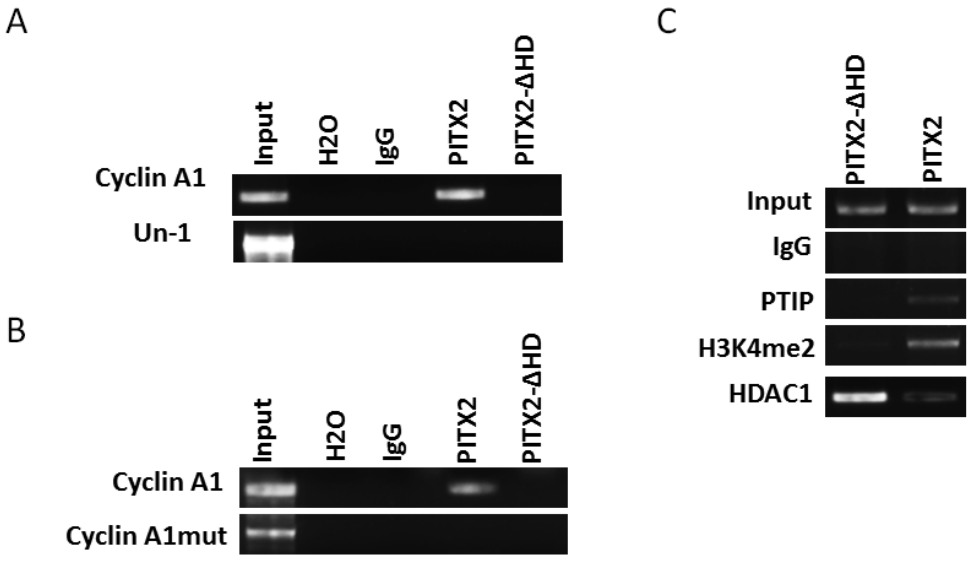


Figure 4. PITX2 recruits the MLL4 HMT to the Cyclin A1 promoter. Input: cell genomic DNA was used as template in PCR; H2O: no DNA template was used for PCR; IgG: mouse IgG was used in ChIP. PITX2: FLAG-PITX2; PITX2-ΔHD: homeodomain-deleted FLAG-PITX2. (A) ChIP analysis was performed using anti-FLAG on HEK293 cells transiently expressing FLAG-PITX2 or homeodomain-deleted FLAG-PITX2. The PCR of an unrelated gene (Un-1) was included as control. (B) FLAG-PITX2 or homeodomain-deleted FLAG-PITX2 was co-transfected to HEK293 cells with pGL4-CCNA658 or pGL4-CCNA658mut (PITX2-response element was mutated). ChIP assays were performed using anti-FLAG. In this experiment, the primers that cover both the Cyclin A1 promoter and the pGL4 vector were used to distinguish the exogenous Cyclin A1 promoter from the endogenous Cyclin A1 promoter. (C) Antibodies for FLAG-PITX2, PTIP, H3K4me2 and HDAC1were used for ChIP. Signals from cells expressing FLAG-

PITX2 were compared with those from cells expressing homeodomain-deleted FLAG-PITX2.

Cyclin A1 is overexpressed in human papillary thyroid cancer

We have previously reported that PITX2 is overexpressed in follicular cell-derived thyroid cancer and promotes thyroid cancer cell proliferation [17]. Since our aforementioned findings reveal that PITX2 can regulate Cyclin A1 expression in thyroid cancer cells, it is tempting to speculate that Cyclin A1 is also up-regulated in thyroid cancer. To address this, we immunostained Cyclin A1 in cancer tissues from three major types of human thyroid cancer (papillary, follicular and medullary thyroid cancer) as well as in normal thyroids. We detected Cyclin A1 in 40% and 14% of papillary and follicular thyroid cancer tissues respectively, but not in medullary thyroid cancer and normal thyroid tissues (Figure 5, Table 2).

Figure 5

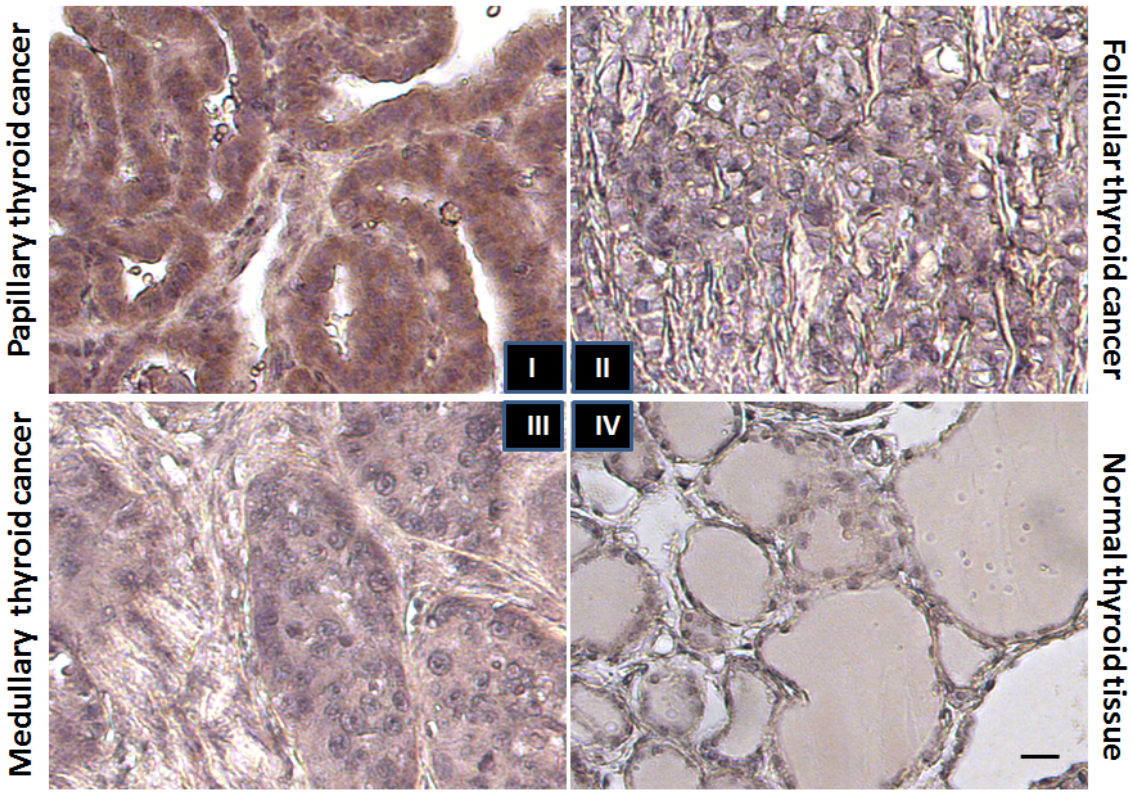


Figure 5. Cyclin A1 is overexpressed in thyroid cancer tissues. Thyroid cancer tissue microarrays were examined by immunohistochemistry with an anti-Cyclin A1. One representative image (I-IV) of each type of human thyroid cancer and normal thyroid tissue was shown (original magnification 40X). Scale bar indicates 20 μm.

Table 2

	-	+	Incidence
Normal	29	0	0\29(0%)
Papillary	21	14	14\35(40%)
Follicular	6	1	1\7(14%)
Medullary	4	0	0/4(0%)

Table 2. Immunohistochemistry assay of Cyclin A1 in human thyroid cancer tissues. The symbol "-", and "+" represents no expression and consistent expression of Cyclin A1, respectively (see Materials and Methods for categorization). The number indicates the sample size of each category, and incidence is defined as the percentage of consistent Cyclin A1 expression of total tissue samples examined.

Discussion

Transcription factors exhibit their biological influence mainly by controlling the expression of their target genes. Identification of transcriptional targets, therefore, is a fundamental step for us to understand the function of a transcription factor. In this study, we aim to discover novel target genes for a homeodomain transcription factor PITX2 and to decipher the regulatory mechanism of PITX2 action. In this report, we demonstrate that Cyclin A1, a spermatogenesis-essential gene, is a novel PITX2 transcriptional target. In an ardent effort to understand how PITX2 regulates Cyclin A1 expression, we fruitfully discover that the MLL4 histone H3K4 methyltransferase complex interacts with PITX2 and mediates PITX2-induced Cyclin A1 gene transcription.

Cell cycle contains distinct phases that are precisely regulated by the protein complexes formed between respective pairs of Cyclins and Cyclin-Dependent Kinases (CDKs) [18]. Therefore, many genes regulate cell proliferation by controlling the expression of Cyclins. Consistent with PITX2's ability to regulate cell proliferation, several PITX2 transcriptional targets, such as Cyclin D1, Cyclin D2 and c-Myc, are directly involved in the regulation of cell cycle progression, [1, 9]. Since the D-type Cyclins are the key regulators for G1/S phase transition, PITX2 is thought to regulate cell cycle progression by facilitating cells to progress through G1 phase. The discovery of Cyclin A1 as a transcriptional target of PITX2 in this study appreciably expands the realm of PITX2 on cell cycle, since the Cyclin A1-CDK2 complex functions during S phase and M phase [11]. Both Cyclin D1 and D2 are widely expressed in a variety of tissues. However, Cyclin A1 is only expressed in male germ cells and essential for spermatogenesis [12]. Hence, Cyclin Ds may be more general targets for PITX2 to control cell cycle

progression, while Cyclin A1 may be a tissue-specific target of PITX2 in spermatogenesis. If PITX2 indeed is a major regulator of Cyclin A1 expression in spermatocytes, it will then be very interesting to know whether PITX2 has an essential role in controlling spermatogenesis.

Although PITX2 may not control both Cyclin Ds and Cyclin A1 at the same time in normal physiological settings, PITX2 may demonstrate its power of cell cycle regulation in pathological settings, such as cancer development. The oncogenic role of D-type Cyclins has been well-known in a number of cancers [18]. Meanwhile, the contribution of Cyclin A1 in cancer development and progression has also been evidenced in several types of cancer, including acute myeloid leukemia, testicular cancer, prostate cancer and breast cancer [13-15]. However, our understanding of Cyclin A1 in tumorigenesis is still limited. In a previous study, we have discovered that PITX2 is overexpressed in follicular cell-derived thyroid cancer and promotes thyroid cancer cell proliferation by activating Cyclin D2 [17]. In this study, we show that PITX2 regulates Cyclin A1 transcription in a papillary thyroid cancer cell line. Consistently, we have found that Cyclin A1 is expressed in 40% of papillary thyroid cancer tissues but not in normal thyroids. Collectively, our findings strongly imply that PITX2 might activate Cyclin A1 and thus promotes papillary thyroid tumorigenesis. Hence, further functional analysis of Cyclin A1 in papillary thyroid cancer is necessary for us to understand the pathological role of PITX2-Cyclin A1 signaling. Because Cyclin A1 has a very restricted expression pattern in male germ cells, it may be an excellent therapeutic target for designing new thyroid cancer treatment.

In the last several years, many studies have attempted to elucidate the upstream regulators that govern Cyclin A1 expression. Although several transcriptional factors, such as Sp1 and Six1, have been proposed to regulate Cyclin A1 gene transcription [15,19], the conclusive evidence is still lacking. Deletion of four GC boxes, where Sp1 family members often bind to, abolished the transcriptional activity of the Cyclin A1 promoter [19]. However, there is very little evidence to support that Sp1 family members are indeed responsible for the regulation of Cyclin A1 expression, since the GC box is the relatively short and unspecific DNA sequence where many other transcription factors may bind to. Furthermore, there is no evidence to show that Sp1 family proteins are able to activate the Cyclin A1 promoter. Six1, a homeodomain transcription factor, has been shown to regulate Cyclin A1 expression in breast cancer cells [15]. Reporter assay and ChIP suggest that Six1 binds to the Cyclin A1 promoter and activates Cyclin A1 transcription [15]. However, the promoter region responsible for Six1-induced Cyclin A1 transcription contains no Six1 binding sites. Therefore, how Six1 activates Cyclin A1 transcription is still unknown. Similarly, PITX2-induced Cyclin A1 transcription is also independent of a conventional homeodomain binding site, even though our ChIP data show that PITX2 physically binds to the Cyclin A1 promoter. Interestingly, all the transcription factors, including PITX2 in this report, that are shown to be capable of activating the human Cyclin A1 promoter, have their response elements located in a narrow region ranging from -100 bps to 100 bps of the Cyclin A1 promoter. Hence, the promoter region flanking the transcription initiation site may be the key regulatory element for transcription factors to control Cyclin A1 gene expression. Since 200 bps is a

relatively short DNA fragment, it is possible that PITX2 and other factors activate Cyclin A1 transcription in a cooperative manner.

Several proteins have been previously shown to interact with PITX2, such as β-catenin, LEF-1, HMG-17, WT-1 and MEF2A [1, 20-22]. In addition, we recently have identified four novel PITX2 interacting partners: YB-1, hnRNP K, hnRNP U and nucleolin through mass spectrometry analysis [10]. Some of these binding partners have been shown to modulate the transcriptional activity of PITX2 by interacting with the PITX2 homeodomain. Intriguingly, our data demonstrate that PITX2 associates with the MLL4 histone H3K4 methyltransferase complex and this association also depends on the PITX2 homeodomain. Moreover, PITX2 facilitates the recruitment of the MLL4 HMT complex to the Cyclin A1 promoter, leading to methylation of H3K4 and dissociation of HDAC1 in the Cyclin A1 promoter. These data suggest that PITX2 is able to remodel chromatin architecture in the target promoter and, thus, activate gene transcription. By interacting with histone methylation proteins, PITX2 is equipped with new tools to turn on silenced genes. This mechanism renders PITX2 the ability to reactivate oncogenic genes in cancer cells, such as Cyclin A1 in thyroid cancer cells. However, whether this novel mechanism of PITX2 action is also applied for other PITX2 transcriptional targets deserves further investigation.

PITX2 is a versatile transcription factor that has a critical role in many physiological and pathological events. In this study, we extend our understanding on PITX2 by identifying a new transcriptional target and proposing a novel mechanism of action. More importantly, overexpression of testis-specific Cyclin A1 in papillary thyroid cancer provides a potential therapeutic target for designing new thyroid cancer treatment.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Thyroid cancer is the most frequent cancer originated from the endocrine system. The majority of thyroid cancer is well-differentiated and slow growing. Overall, the 5-year survival rate of thyroid cancer is over 90%. However, there are many challenges in diagnosis, prognosis and treatment. To solve these problems, we need to better understand the etiology of this disease. In this study, we find that PITX2, a homeodomain transcription factor essential in embryonic development, has an oncogenic role in thyroid cancer development.

Immunohistochemistry analysis reveals that PITX2 is frequently expressed in follicular cell-derived, well-differentiated thyroid cancer, such as papillary and follicular thyroid cancer, but is less frequently expressed in poorly-differentiated anaplastic thyroid cancer In contrast, PITX2 is not detected in normal thyroid tissues and C-cell originated medullary thyroid cancer. To examine the pathological meaning of aberrant expression of PITX2, we knocked down PITX2 by shRNA in a papillary thyroid cancer cell line. Both cell proliferation and anchorage-independent cell growth are significantly reduced in PITX2-knockdown cells. By analyzing cell cycle regulatory genes, we find that D-type Cyclins are downregulated and Rb is dephosphorylated. Chromatin immunoprecipitation and reporter assay further demonstrate that Cyclin D2 is a direct transcriptional target of PITX2 in thyroid cancer cells. Consistently, we observe that Cyclin D2 is also

overexpressed in follicular cell-derived thyroid cancer, but not in normal thyroids and other types of thyroid cancer.

Since transcription factors commonly exhibit their influence by regulating the expression of their target genes, it is very important for us to know which genes are regulated by PITX2 and how PITX2 regulates these target genes. As an attempt to gain a full picture of PITX2's interacting and regulating networks, we use mass spectrometry analysis to discover four novel PITX2-associated protein partners YB-1, hnRNP K, nucleolin and hnRNP U. By microarray analysis, we identify 868 upregulated genes and 191 downregulated genes as candidates of PITX2 targets.

One of the most upregulated genes induced by PITX2 is Cyclin A1, which is a testis-specific gene and essential for meiosis of spermatocytes. Through many biochemical approaches, including reporter assay and chromatin immunoprecipitation, we determine that Cyclin A1 indeed is a novel PITX2 transcriptional target in thyroid cancer cells. Our data further demonstrate that an evolutionarily conserved PITX2 response element in the Cyclin A1 promoter is responsible for PITX2-induced Cyclin A1 expression. Intriguingly, we find that PITX2 associates with the MLL4 histone H3K4 methylation complex and this association facilitates the recruitment of the MLL4 HMT complex to the Cyclin A1 promoter. Hence, through the chromatin remodeling ability of the MLL4 HMT complex, PITX2 turns on gene transcription of the originally-silenced Cyclin A1 gene.

In conclusion, our results support that PITX2 is upregulated in follicular cell-derived thyroid cancer and promotes thyroid cancer proliferation by transcriptionally regulating the genes involved in cell cycle progression. Consistently, we discover and confirm that Cyclin D2 and Cyclin A1 are direct transcriptional targets of PITX2 in thyroid cancer

cells. Furthermore, the expression levels of Cyclin D2 and Cyclin A1 are also upregulated in follicular cell-derived thyroid cancer. These findings provide insightful perspectives on the etiology of thyroid cancer and offer candidate targets for future development of novel therapies.

Limitations

There are several limitations in this study. Firstly, the microarray analysis of PITX2 transcriptional targets was conducted in the HEK293 cell line, which is an embryonic kidney cell line. Therefore, the microarray results may not reflect PITX2's regulation network in thyroid cancer cells. In addition, the microarray results did not address the issue of tissue-specific regulation of PITX2. In fact, Cyclin D2 was not identified in our microarray analysis, suggesting that Cyclin D2 is a tissue-specific target gene of PITX2. Secondly, the precise role of PITX2 in thyroid tumorigenesis is still elusive. Further study is necessary to elucidate whether PITX2 has a role in the initiation of thyroid cancer. Lastly, although Cyclin A1 has the potential to become an excellent therapeutic target for papillary thyroid cancer patients, more evidence is needed to support a positive role of Cyclin A1 in thyroid tumor initiation and progression.

Future directions

Although our data support an oncogenic role of PITX2 in follicular cell-derived thyroid cancer, many questions still remain unanswered. One of the most important questions regarding PITX2 in thyroid cancer is: Can PITX2 initiate thyroid cancer? Or, PITX2

solely promotes thyroid cancer proliferation. To answer this question, we can express ectopic PITX2 in primary mouse thyroid follicular cells and then examine if PITX2 is able to transform normal thyroid follicular cells to thyroid cancer cells both *in vitro* and *in vivo*. For *in vitro* examination, we can test the ability of PITX2-overexpressing thyroid cells to grow anchorage-independently in soft agar. For *in vivo* examination, PITX2-overexpressing thyroid cells can be subcutaneously injected into immune system-compromised nude mice and then monitor the growth of thyroid cells in nude mice. There are several obstacles for performing this experiment. First, thyroid follicular cells need to be isolated from mouse thyroid freshly since there is no protocol available to keep primary thyroid cells dividing in the cell culture system. Secondly, isolation of pure thyroid follicular cells is virtually impossible because there are many other types of cells in the thyroid gland, such as thyroid C cells, parathyroid cells, endothelial cells and others. Thirdly, the transfection efficiency for primary thyroid cells is extremely low. Therefore, it is critical to develop a protocol that can improve the transfection efficiency to an acceptable level. Virus-mediated transfection may worth a try in this case. Lastly, transiently-overexpressed PITX2 may not be sufficient to transform normal thyroid cells. If so, it is necessary to establish cell lines that can stably express ectopic PITX2.

Since the *in vitro* cell culture system is different from *in vivo* mammalian animal models in many aspects, *in vitro* transformation experiments may not be satisfactory to make a conclusion regarding the ability of PITX2 in transforming cells. Therefore, it is worthy to generate a transgenic mouse model with thyroid follicular cell-specific overexpression of *Pitx2*. If these transgenic mice develop thyroid cancer, it is likely that PITX2 is involved in the early events that lead to the transformation of normal thyroid cells to thyroid

cancer cells. If transgenic mice do not develop thyroid cancer, there are two possibilities. One is that PITX2 does not have a significant role in transforming normal thyroid cells; the other is that a single genetic aberration is not sufficient to transform normal thyroid cells. Therefore, other genetically-altered mice can be crossed with *Pitx2* transgenic mice to generate a unique "double-hits" mouse model, which may have a bigger chance for thyroid cancer development.

As detailed in Chapter I literature overview, there are many challenges regarding thyroid cancer diagnosis and prognosis due to the inaccuracy of histological evaluations. Therefore, identifying novel molecular markers and developing unique diagnostic and prognostic protocols are necessary to improve the accuracy of diagnosis and prognosis for thyroid cancer patients. Since PITX2 is only aberrantly expressed in follicular cell-derived thyroid cancer, it will be interesting to see if PITX2 can be used as a diagnostic and prognostic marker for thyroid cancer patients. To assess the validity and accuracy of PITX2 as a diagnostic and prognostic marker, large-scale screening of patient samples and vigilant protocols are required.

Another unanswered question regarding the role of PITX2 in thyroid cancer is how PITX2 is upregulated. Many genetic alterations, including mutations and chromosome rearrangements detailed in Chapter I, have been identified and associated to the development of thyroid cancer. Since transcription factors like PITX2 are usually tightly-regulated by upstream regulators, it is very likely that the upregulation of PITX2 is not an isolated event during thyroid tumorigenesis. Hence, it is important to find out if any previously-known genetically-altered genes function as the upstream regulators of PITX2 overexpression. Those genes that have been proven to be able to transform normal

thyroid cells to thyroid cancer cells deserve immediate attention. One approach to answer this question is to identify the promoter regions that are responsible for PITX2 gene transcription. We have successfully cloned a 3000-bps PITX2c promoter (upstream of the transcription initiation site) into a reporter plasmid. This reporter construct showed extremely high transcriptional activity when transfected into thyroid cancer cells (data not shown). In future study, we aim to identify the DNA elements that control PITX2 gene transcription. Another approach to answer the abovementioned question is to knockout PITX2 in genetically-engineered mice that develop thyroid cancer. There are several genetically-engineered thyroid cancer mouse models available now, such as $Braf^{V600E}$ knock-in mice, $Kras^{G12D}$/*Pten* double-mutant mice and $TR\beta^{PV/PV}$ knock-in mice [1-3]. If knockout of PITX2 in those mutant mice inhibits tumor development, it would indicate that PITX2 may be a downstream effector of those genetically-altered genes.

PITX2 has been identified as a key downstream effector of β-catenin signaling to regulate cell proliferation in previous studies. Nuclear β-catenin is also able to modulate the transcriptional activity of PITX2 in target gene promoters [4-5]. Since β-catenin is frequently accumulated in the nucleus of thyroid cancer cells [6-7], it is possible that β-catenin promotes thyroid cancer development through regulating PITX2. To test this hypothesis, we may generate a mouse model with thyroid follicular cell-specific β-catenin gain-of-function.

In this study, we have identified that Cyclin A1 is a direct transcriptional target of PITX2 in thyroid cancer cells. Since Cyclin A1 expression is highly restricted in testes under physiological condition, it is interesting to notice that Cyclin A1 is frequently expressed in papillary thyroid cancer. The future study should aim to find out the pathological

significance of Cyclin A1 in papillary thyroid cancer, including determining the role of Cyclin A1 in thyroid tumorigenesis. Functional studies of Cyclin A1 can be conducted by using shRNA and microRNA to knock down Cyclin A1 in thyroid cancer cells. This research would give us a hint on whether Cyclin A1 contributes to PITX2-induced thyroid cancer proliferation.

Because Cyclin A1 displays a testis-specific expression pattern, epigenetic regulation, such as DNA methylation, is proposed to play a critical role in inactivating Cyclin A1 gene expression in tissues other than testis. Interestingly, our data demonstrate that PITX2 is able to epigenetically reactivate Cyclin A1 expression in thyroid cancer cells, presumably through interacting with the MLL4 histone methylation complex. In the future, it would be interesting to explore if PITX2 synergistically works with the DNA methylation pathway to promote the expression of the originally-silenced Cyclin A1 gene in cancer cells.

The management of thyroid cancer has not been changed much in the past several decades. Currently, available treatment options are very limited and mostly require a partial or total thyroidectomy, which is both inconvenient and expensive to patients. Target therapy has demonstrated its unique advantages and could be used along with conventional treatments to provide a better outcome to thyroid cancer patients. Since PITX2 and Cyclin A1 may be critical for thyroid cancer proliferation, it will be beneficial to design therapeutics targeting PITX2 or Cyclin A1, such as microRNA and small chemical compounds.

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Publications

1. **Huang, Y.**, Fan, J., Yang, J., Zhu, G.Z., (2008) Characterization of GPR56 protein and its expression in human pancreatic cancer cells. *Mol. Cell. Biochem.* 308: 133-9.

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