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TBX5: THE MISSING CULPRIT GENE IN THALIDOMIDE TOXICITY

by
ATHAR AHMAD KHALIL

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by
ATHAR AHMAD KHALIL

Approved by:

Dr. Georges Nemer, Associate Professor — Advisor
Biochemistry and Molecular Genetics Department

Dr. Mazen Kurban, Associate Professor — Co-advisor
Dermatology Department
Biochemistry and Molecular Genetics Department

Dr. Firas Kobeissy, Assistant Professor — Member of Committee
Biochemistry and Molecular Genetics Department

Dr. Fadi Bitar, Professor — Member of Committee
Pediatrics and Adolescent Medicine Department

Date of thesis defense: April 27, 2015

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AN ABSTRACT OF THE THESIS OF

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Title: *TBX5*: The Missing Culprit Gene in Thalidomide Toxicity

Holt-Oram syndrome (HOS) is a rare autosomal dominant disease associated mainly with upper limb malformation and congenital heart defects (CHD) caused by a haploinsufficiency of T-box transcription factor 5 (TBX5). Congenital heart disease (CHD) is a leading cause of death, with an incidence of approximately 6–8 in 1,000 live births. Only 13 % of all CHD cases are thought to be inherited and the rest are sporadic in nature. Some CHD are caused by environmental factors and teratogens like thalidomide. Thalidomide was synthesized in 1957 and marked as a sedative drug that was used by pregnant women to prevent morning sickness but it caused severe malformations in the newborns similar to those detected in HOS patients, thus it was removed from the market in 1961. Previous studies showed that TBX5 transcription was reduced as a response to thalidomide detected by semi-quantitative RT-PCRs on RNA extracted from wing buds of chicken embryos.

We aimed to investigate the effect of thalidomide on TBX5, suggesting an interaction between them as the in-silico docking prediction showed. We used the electric mobility shift assay (EMSA) to confirm that thalidomide decreases the binding affinity between TBX5 protein and consensus sequence of T-box. While thalidomide didn't affect the cellular localization or the protein stability of TBX5 as indicated by immuno-fluorescence and western blot respectively. Suppressed expression activity of vascular endothelial growth factor (VEGF) and atrial natriuretic factor (ANF) promoter was obtained in the presence of thalidomide assessed by luciferase assay. While thalidomide was neither able to suppress the interaction of TBX5 with GATA4 presented by VEGF promoter expression, nor affected this interaction on the protein level as shown by co-immunoprecipitation assay. Thalidomide could significantly suppress cellular proliferation and migration of embryonal rhabdomyosarcoma cells as indicated by the MTT and wound healing assays respectively, but it did not affect the endogenous expression TBX5 in this cells line.

These results were the first to show that thalidomide bind specifically to TBX5 on its DNA binding domain suppressing its transcriptional properties. Also we were the first to show the antiproliferative and antiangiogenic effect of thalidomide on embryonal rhabdomyosarcoma. Revealing the two faces of thalidomide; one related to its teratogenic mechanism of action and a second one related to its benefits in cancer treatment.

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ABBREVIATIONS

a	Atria
ANF	Atrialnatriuretic factor
ANOVA	Analysis of variance
Ao	Aorta
AS	Aortic stenosis
ASD	Atrial septal defects
AVC	Atrioventricular Canal
AVSD	Atrioventricular septal defect
BAV	Bicuspid aortic valve
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenic Protei
CGH	Comparative genomic hybridization
CHD	Congenital Heart Disease
CNC	Cardiac neural crest
CNV	Copy number variation
CoA	Coarctation of aorta
C-terminal	Carboxy Terminal
Cx40	Connexin 40
DDB1	DNA damage-binding protein 1
DROV	Double outlet right ventricle
E7.5	Embryonic day 7.5
E8	Embryonic Day 8
ECM	Extracellular Matrix
ENL	Erythema nodosum leprosum
ERMS	Embryonal rhabdomyosarcoma
FDA	US Food and Drug administration
FHF	First heart field
FL	Forelimb
HEK293	Human Embryonic Kidney 293 cells
HL	Hindlimb
HLHS	Hypoplastic left heart syndrome
HOS	Holt-Oram syndrome
IAA	Interrupted aortic arch
IFT	Inflow tract
IL-2	Interleukin-2
IMiD	Immune-modulatory drug
La	Left atrium
Luc	Luciferase
LV	Left Ventricle
MEF-2c	Myocyte Enhancing Binding Factor-2c
MLD	Membrane Lipid Desaturase
MM	Multiple Myeloma
NFκB	Nuclear Factor κB
Nkx2.5	Homeobox protein
NLS	Nuclear Localization Signal
Nppa	Natriuretic peptide precursor A
N-terminal	NH2 terminal

OFT	Outflow Tract
Pa	Pulmonary artery
PDA	Patent ductus arteriosus
PPS	Peripheral pulmonic stenosis
PS	Pulmonic stenosis
Ra	Right atrium
RV	Right Ventricle
SHF	Second heart field
SNP	Single nucleotide polymorphisms
TNF-α	Tumor necrosis factor alpha
TOF	Tetralogy of Fallot
VEGF	Vascular Endothelial Growth Factor
VSD	Ventricular Septal Defects

CHAPTER I

INTRODUCTION

The heart is the first organ to form in the embryo and starts to pump blood by the third week of pregnancy when the needed nutrients and oxygen can no longer be accessible by simple diffusion [1]. Cardiogenesis is a complex process at the molecular and cellular levels, making the heart more susceptible to perturbations before birth and into adulthood[2].The molecular and developmental biologic techniques that are newly developed are being used to understand more cardiovascular morphogenesis and giving us an insights into the into the causes of genetically and acquired cardiac diseases.

A. Anatomic Heart Development

The first heart field (FHF) cells are the earliest stem cells that are derived from the anterior lateral plate mesoderm and give rise to the cardiac crescent form. This event occurs at approximately embryonic (E) day 7.5 in the mouse embryo, equivalent approximately to week two of human pregnancy [2,3]. The second heart field (SHF) cells are derived from the dorsal-medial aspect of the cardiac crescent. The early primitive heart tube which is made up of an interior layer of endocardial cells and an exterior layer of myocardial cells, separated by extracellular, observed by mouse E8.0, or third week in human pregnancy[1]. The primary heart field contributes to the left ventricle, right and left atria, while the secondary heart field will contribute to the development of

the right ventricle and outflow tract (OFT). This OFT will give rise to the aorta and pulmonary arteries, while the second end of the heart tube will be the inflow tract (IFT) that will differentiate into the atrioventricular canal. At the molecular level bone morphogenetic protein (Bmp), sonic hedgehog (Shh), fibroblast growth factor (*Fgf*), Wnt, and Notch proteins are the signaling network responsible for the positive and negative regulation of both FHF and SHF [2].

When the cells of the FHF of the heart tube starts to beat, SHF cells migrate from the pharyngeal mesoderm into the anterior part of the tube. Then the potential atria at the posterior end of the tube begin to kink and twist near the left side of the embryo. After heart looping at about the sixth week of human development, the neural crest cells (NCC) migrate from the neural tube into the OFT and pharyngeal arches[1]. These cells will participate in the septation of the cardiac outflow tract into the aorta and pulmonary trunks. The septation will result in the four-chambered heart by day 49 of gestation [1-3]. (Figure-1)

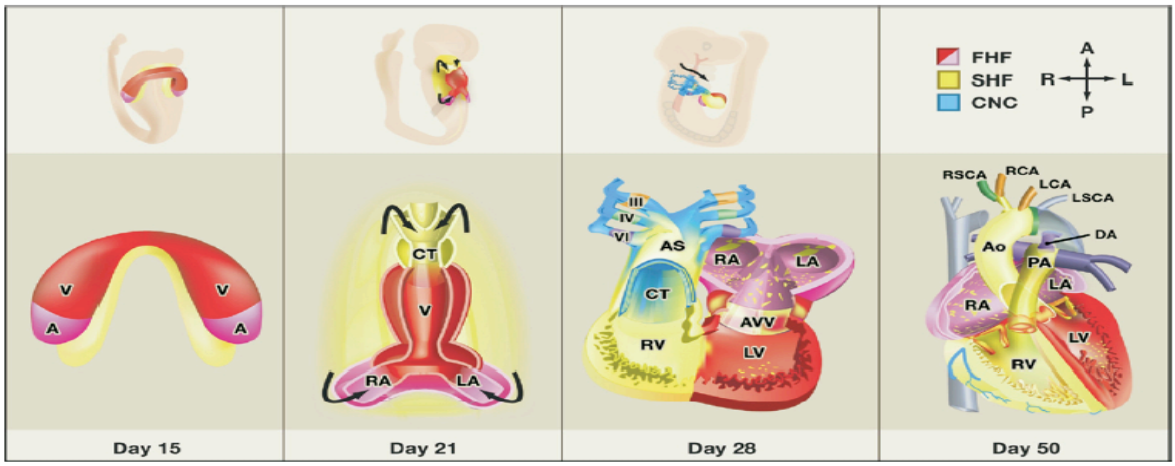


Figure-1: Mammalian heart development. First panel: First heart field (FHF) cells form a crescent shape in the anterior embryo with second heart field (SHF) cells medial and anterior to the FHF. Second panel: SHF cells lie dorsal to the straight heart tube and begin to migrate (arrows) into the anterior and posterior ends of the tube to form the right ventricle (RV), conotruncus (CT), and part of the atria (A). Third panel: Following rightward looping of the heart tube, cardiac neural crest (CNC) cells also migrate (arrow) into the outflow tract from the neural folds to septate the outflow tract and pattern the bilaterally symmetric aortic arch arteries (III, IV, and VI). Fourth panel: Septation of the ventricles, atria, and atrioventricular valves (AVV) results in the 4-chambered heart. [3]

B. Congenital heart disease

Congenital heart disease as defined by by Mitchell et al. is “a gross structural abnormality of the heart or intrathoracic great vessels that is actually or potentially of functional significance" [4,5] . .It is the second leading cause of death in the first year of life after infectious etiologies. Thus CHD is a central cause of childhood morbidity and mortality worldwide[6]. This definition excludes some disorders that are present at birth but that don’t cause functional abnormalities such as congenital arrhythmias (example: Wolf-Parkinson-White syndromes) [4].

1- Congenital Heart Disease Incidence

Congenital heart disease (CHD) is the most common type of birth defects, affecting 1% of all live births, and is the leading noninfectious cause of death in the first year of life [7]. Many studies were interested in the incidence of CHD which is defined as the number of new affected persons per unit of time or population. The incidence of CHD reported in 1968 was about 4 to 5 per 1,000 live births, while this incidence raised to reach 12 to 14/1,000 live births or higher in 2000 as have been reported in the literature[4]. This increased incidence rate doesn't reflect an actual increase in the number of CHD patients, but rather it reflects other factors that changed throughout years. These factors include a previous passive way of collecting information depending on the doctor's referrals, in which it might miss those tiny ventricular septal defects (VSD) or a mild pulmonic stenosis (PS) that could be managed early on. Also missing some lesions that might not be detected until they appear in adult life such as atrial septal defects (ASDs), or missing those with severe critical heart disease that will die in the first few days after birth without cardiologic or autopsy diagnosis [4,8]. Add to these factors, the availability of new good echocardiography and developed diagnosis techniques that were not present before. Significant geographical differences were also found, Asia reported the highest incidence (9.3 per 1,000 live births) while Africa reported the lowest incidence (1.9 per 1,000 live births) [9].

2- Incidence of main congenital heart disease categories

Ventricular septal defects (VSD) are by far the most common form of CHD with an incidence of 2% to 5% but with 85% to 90% of these defects closing spontaneously by one year of age [4]. Functionally, it is characterized by ejection of some blood into the aorta and some across the ventricular septal defect into the right ventricle and pulmonary artery, when the left ventricle contracts[10]. The second common lesion is Patent ductus arteriosus (PDA), accounting for 10 percent of CHD cases, by which the ductus arteriosus does not close spontaneously after birth ending up with continuous flow from the aorta to the pulmonary artery. Atrial septal defects (ADS) account for about one third of the cases of CHD detected in adults, in which blood from the pulmonary veins enters the left atrium and some of it crosses the atrial septal defect into the right atrium and ventricle. Bicuspid aortic valve (BAV) is another frequent form of CHD characterized by being asymptomatic in childhood but can cause major heart disease after age of 40 [1]. BAV and other CHD like aortic arch anomalies, and small atrial or ventricular septal defects (VSD) may go unnoticed all through life due to the absence of symptoms, or may resolve (eg, VSDs), and some have no clinical significance. Thus the incidence rate might miss these anomalies since they do not always require specialized care [3]. One of the most severe CHD is Tetralogy of Fallot, and without surgical intervention most patients diagnosed with this disease will die in childhood[11]. TOF patients appear blue as a result of the mixing of oxygenated and deoxygenated blood[12].

3- ***Etiology of congenital heart disease***

Over the past couple of decades, gene targeting technologies have been improved tremendously. These improvements of gene targeting technologies helped in generating animal models with different cardiac developmental defects, which allowed researchers to understand the importance of different transcriptional factors, signaling molecules and structural genes in cardiac development[4]. Aneuploidy, microdeletions, single gene mutation, copy number variation (CNV), and single nucleotide polymorphisms (SNPs) are all involved in the genetic causes of CHD[6]. If we search in NCBI gene bank for "cardiac or heart" we can find up to 630 genes that are involved in cardiac development in humans. Although genes are main contributors of CHDs, other factors are known to be responsible for some defects in cardiac development such as environmental exposures, exposure to medications during pregnancy, and maternal diseases [3]. Still 85% to 90% of cases, don't have defined cause, and it is usually considered to be caused by multifactorial inheritance.

5%-10% of patients with a CHD occur as a part of chromosomal abnormality, including abnormal chromosomal number or aneuploidy[13]. Though most patients diagnosed with CHD don't have other birth defects, 30% of patients with a chromosomal abnormality will have CHD[6,14]. Trisomy 21(Down syndrome), trisomy 18, trisomy 13, monosomy X (Turner Syndrome) and 47, XXY (Klinefelter Syndrome) are common syndrome caused by aneuploidy and are associated with different cardiac anomalies (ASD, VSD, BAV…etc) . On the other hand, DiGeorge and Williams-

Beuren Syndromes are caused by microscopic deletions leading to cardiac malformations along with other clinical features. (Table-1)

Syndromes	Cardiac anomalies	% with CHD
Trisomy 13	ASD,VSD,PDA,HLHS	80%
Trisomy 18	ASD, VSD, PDA, TOF, DORV, CoA, BAV	90-100%
Trisomy 21 (Down Syndrome)	ASD,VSD,AVSD,TOF	40-50%
Monosomy X (Turner Syndrome)	CoA, BAV, AS, HLHS	25-35%
22q11.2 deletion (DiGeorge Syndrome)	IAA Type B, aortic arch anomalies, truncus arteriosus, TOF	75%
7q11.23 deletion (Williams-Beuren Syndrome)	Supravalvar AS, PPS	50-85%
47, XXY (klinefelter Syndrome)	PDA, ASD, mitral valve prolapse	50%

Table-1: Common Syndromes Resulting from Anueploidy and Microdeletions associated with CHD[6].

Some syndromes that are associated with congenital heart disease due to single gene defect have been characterized (Table-2). Examples of such syndromes are: Costello Syndrome, Alagille Syndrome, Holt-Oram Syndrome, and Char Syndrome

caused by single gene defect of the following genes *HRAS, JAG1, TBX5* and *TFAP2b* respectively[6].

Syndromes	Cardiac anomalies	Causative Gene (s)
Noonan Syndrome	PS, AVSD, HCM, CoA	PTPN11, KRAS, RAF1,SOS1
Costello Syndrome	PS , HCM, cardiac conduction abnormalities	HRAS
LEOPARD Syndrome	PS, and cardiac conduction abnormalities	PTPN11, RAF1
Alagille Syndrome	PS, TOF, ASD, peripheral pulmonary stenosis	JAG1, NOTCH2
Marfan Syndrome	Aortic root dilatation and dissection, mitral valve prolapsed	FBLN, TGFBR1, TGFBR2
Holt-Oram Syndrome	ASD, VSD, AVSD, progressive AV conduction system disease	TBX5
Char Syndrome	PDA	TFAP2b

Table-2: Common syndromes sssociated with CHD resulting from single gene defects [5].

Copy number variation (CNV) results from intermediate-size duplications and deletions that are responsible for changes in gene dosage in 12% of the human genome detected using Comparative genomic hybridization (CGH). Example on this is CHARGE syndrome characterized by heart defects (conotruncal and aortic arch malformations) and associated with *CHD7* haploinsufficiency detected by array CGH[6].

Besides the genetic factors that cause CHD we have environmental factors that will contribute to minority of these cases. Among these are maternal by exposure to specific substances at some stage in pregnancy, during the baby's heart development. It was shown that taking anti-seizure medication, lithium and some chemical teratogens (such as retinoic acid, amphetamines and thalidomide) increases the risk for having a child with congenital heart disease[15]. Mothers who contract rubella during their pregnancy, or suffer from insulin-dependent diabetes (particularly if the diabetes is not well-controlled) or lupus erythematosus have a significant chance of having a baby with birth defects, including congenital heart disease [13]. But still the most CHDs are caused by the interaction of a genetic predisposition and environmental factors at a critical early period in cardiac embryogenesis.

C. Thalidomide

1- Origin of Thalidomide

Thalidomide (N-phthalimidoglutarimide) (2-(2,6-dioxopiperidin-3-yl)-1H-isoindole-1,3 (2H)-dione) was marketed as a sedative drug, synthesized in the 1957 by a

German pharmaceutical company[16]. It is a racemic mixture with presumed differential activities of the (–)-(S)- and (+)-(R)-isomers, this lipophilic structure made it insoluble in water(figure-2).In 1960, 14.6 tons of Contergan (another name for thalidomide) was sold in Germany with widespread popularity in Europe and Canada[17]. Pregnant women at that time used this drug for morning sickness without need for doctor's prescription, since it helped relieving their nausea. However, this drug was not approved by the US Food and Drug administration (FDA) at that time taking into consideration its potential to cause irreversible neuritis[18]. Shortly after severe malformations were detected in the newborns of women who took thalidomide during pregnancy. Scientists proved that it is the (-)(S)-thalidomide from which was the responsible agent that caused the severe side effects.

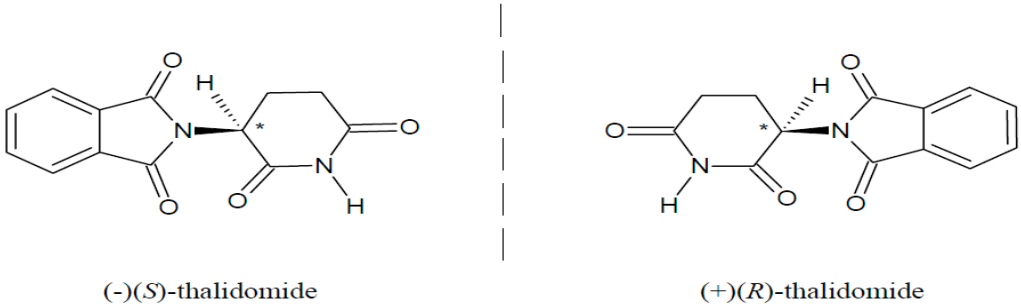


Figure-2: (–)-(S)- and (+)-(R)-isomers of thalidomide.[19]

2- Phenotypical deformities

Dr. McBride on Nov. 18, 1961, considered thalidomide as a possible teratogenic agent while discussing some other potential factors that can cause defects of the extremities[17]. Studies showed that different malformations caused by this drug on the developing embryo depend mainly on the time when thalidomide was utilized. Malformations observed in the newborns are deformities of the arms and legs, anomalies of the ears, and internal malformations such as aplasia of the gall-bladder, appendix, and kidney, defects of the middle lobe of the right lung, malformations of the heart, pyloric stenosis, duodenal stenosis and atresia, rectal stenosis, and anal atresia[20,21]. The most striking among these is phocomelia: malformed or missing limbs (Figure-3). In total, the malformations affected approximately 10,000 children in 46 different countries[22]. Thus thalidomide was removed from the market in late 1961, and became only available for research purpose[23].

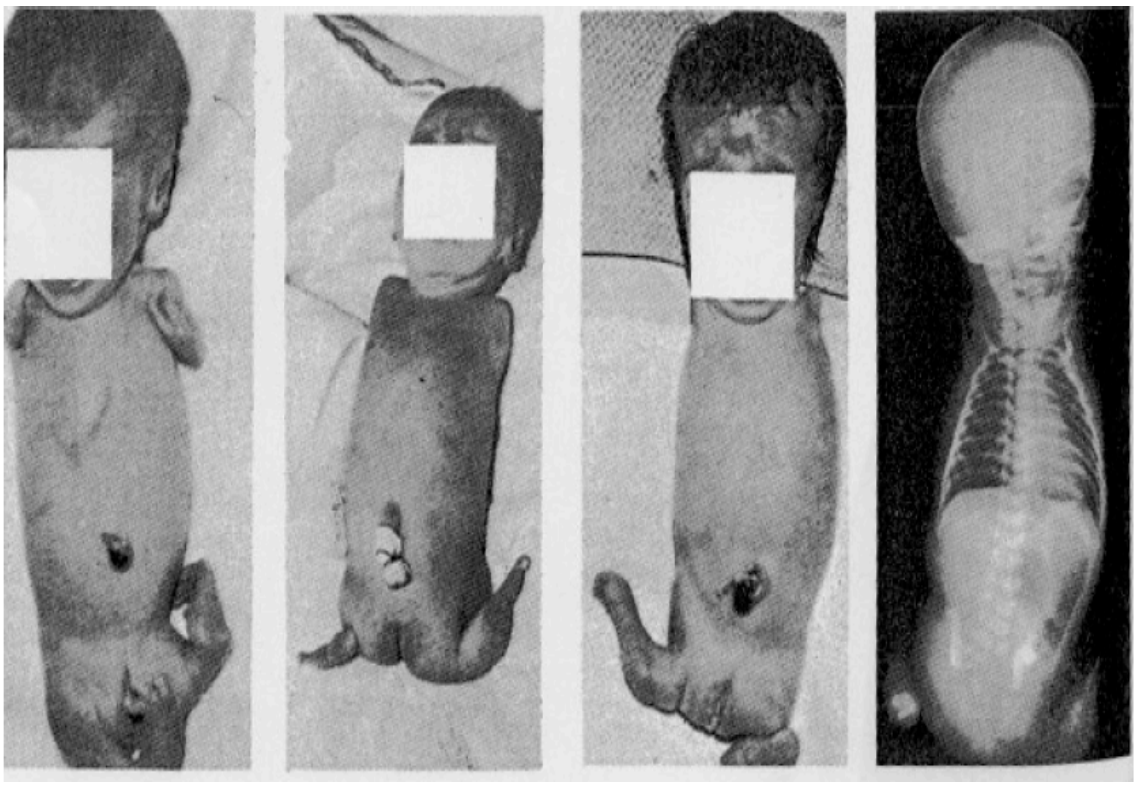


Figure-3: Babies with severe cases of phocomelia. This deformity resulted when thalidomide was taken during early pregnancy. (Wikipedia)

3- Suggested mechanisms of action

Many mechanisms of action were proposed regarding the effect of thalidomide. These mechanisms include the ability of this drug to affect DNA replication or transcription, synthesis and/or function of growth factors, synthesis and/or function of integrins, angiogenesis, chondrogenesis, and cell death[20]. But the exact mechanism is not known yet. Studies that focus on the mechanism of action of thalidomide that is responsible for the limb teratogenicity revealed evidence that thalidomide can cause oxidative stress/damage, DNA intercalation, inhibit angiogenesis, and can bind CRBN protein[17]. To prove its potential in increasing the production of oxygen radicals and

induce oxidative stress, Hansen et al used in vitro whole embryo culture techniques. Rat and rabbit embryos were injected with thalidomide and glutathione was measured, results showed glutathione depletion in the embryo culture.

On the other hand, Koch and Czejka (1986) proved that thalidomide decreases the transcription of insulin-like growth factor (IGF-1) and *FGF*-2 by binding to GC-rich promoter sites by intercalation. These genes are responsible for stimulating transcription of alpha-5 and beta-3-integrin subunit gene that play critical role in angiogenesis. Ito et al. (2010) reported that CRBN is a thalidomide-binding protein, by using zebrafish embryos that are thalidomide sensitive and overexpressing them with zCRBN mutant protein which rescued them form the lethality of this drug [24]. Thus they hypothesized that CRBN directly binds DNA damage-binding protein 1 (DDB1) in a DCX (DDB1-CUL4-X-Box) E3 ubiquitin ligase complex. This interaction between thalidomide and CRBN hinders the function of CRBN associated E3 ubiquitin ligase, which may stop some unidentified proteins from being degraded by the proteasome, and eventually affects downstream molecules linked to teratogenicity, most likely through the down-regulation of *FGF*8. Other studies showed that inhibition of TNF-α synthesis by inducing TNF-α mRNA degradation is achieved by thalidomide. It can also inhibit IkB kinase activity and thus block the activation of nuclear factor (NF)-kB[25].

4- Thalidomide usage now

The immunomodulatory potency of thalidomide gave it a good therapeutic effect in treating erythema nodosum leprosum (ENL) patients, human immunodeficiency virus (HIV) infection, and autoimmune diseases[22]. In July, 1998, the Food and Drug Administration in the USA approved thalidomide as a treatment of erythema nodosum leprosum (ENL) while its antiangiogenic effect was demonstrated by inducing marked and durable responses in some patients with multiple myeloma, together with those who relapse after high-dose chemotherapy [26]. Though, all patients must be registered on the System for Thalidomide Education and Prescribing Safety (STEPS) program before taking this drug [25,27].

These investigations encouraged researchers to see the effect of thalidomide on other types of cancer including prostate cancer, glioblastoma, lung adenocarcinoma, head and neck squamous cell carcinoma[22]. More research was conducted to assess the effect of thalidomide as an anticancer drug. Inhibiting of angiogenesis, cell adhesion and cytokine circuit, the induction of cancer cell apoptosis and oxidative stress, as well as the enhancement of host immune response along with the suppression on the activity of its binding targets made this drug interesting topic when talking about cancer treatment.

5- Thalidomide derivatives

As mentioned above thalidomide is an immune-modulatory drug (IMiD) used against multiple myeloma, by which it can improves the response rate and survival of patients as compared with conventional chemotherapy. Multiple Myeloma accounts for about 1% of all cancers and 10% of all hematological malignancies with estimated 22,350 new cases of MM were detected in the United States in 2013 [28].

Multiple myeloma is a mature B-cell neoplasm marked by proliferation and clonal expansion of plasma cells and excessive production of monoclonal immunoglobulin that cause hemocytopenias, immunodeficiency, osteolytic lesions, hypercalcemia and renal failure [29]. The drawbacks of thalidomide associated with dose-limiting toxicities including somnolence, constipation, and neuropathy that led to the production of second generation of IMiDs. Patients treated with 100 mg of thalidomide daily experienced considerably fewer thalidomide-related side effects compared with patients receiving 400 mg daily[30]. Lenalidomide (CC-5013) and pomalidomide are thalidomide derivatives that proved more potent anti-MM, anti-inflammatory and immunomodulatory activities compared to thalidomide [31] (Figure-4). Though the exact mechanism of action of the IMiDs is not completely understood, lenalidomide and pomalidomide showed a direct down-regulation of key functions of the tumor cell, and indirect modulation of the interaction of myeloma cells with their microenvironment[31]. The direct effect exerted by these drugs is by reducing the production of key pro-survival cytokines such as TNF-α, IL-6, IL-8, and VEGF that favor tumor cell survival

and proliferation, inhibition of apoptosis, and resistance to therapy [29,32]. The indirect effect is by blocking the upregulation of adhesion molecules on MM cells and BMSC. The newest IMiD is pomalidomide, this derivative aimed to be more effective and less toxic than thalidomide and lenalidomide. Though thalidomide and lenalidomide proved to be beneficial in treating both newly diagnosed and relapsed MM, once patients are no longer responsive to these drugs, pomalidomide will be used [33]. In 2013, the US Food and Drug Administration (FDA) approved the using pomalidomide for MM patients who have received two or more prior therapies, including lenalidomide and bortezomib, and have revealed disease progression on or within 60 days of completion of their last therapy (FDA, 2013).(Table-3)

The only difference between the chemical structure of pomalidomide and thalidomide is an amino group added to the fourth carbon of the phthaloyl ring of thalidomide. This additional amino group increased potency of both anti-inflammatory and anti-angiogenic properties with reduced toxicities of the drug [33,34].The toxicity described in pomalidomide myeloma trials are neutropenia, thrombocytopenia, anemia, and fatigue but it induces less constipation, asthenia, skin rash and neuropathy than thalidomide. pomalidomide showed greater inhibition of tumor necrosis factor–alpha (TNF-α) in vitro, and more potency in T-cell co-stimulation.

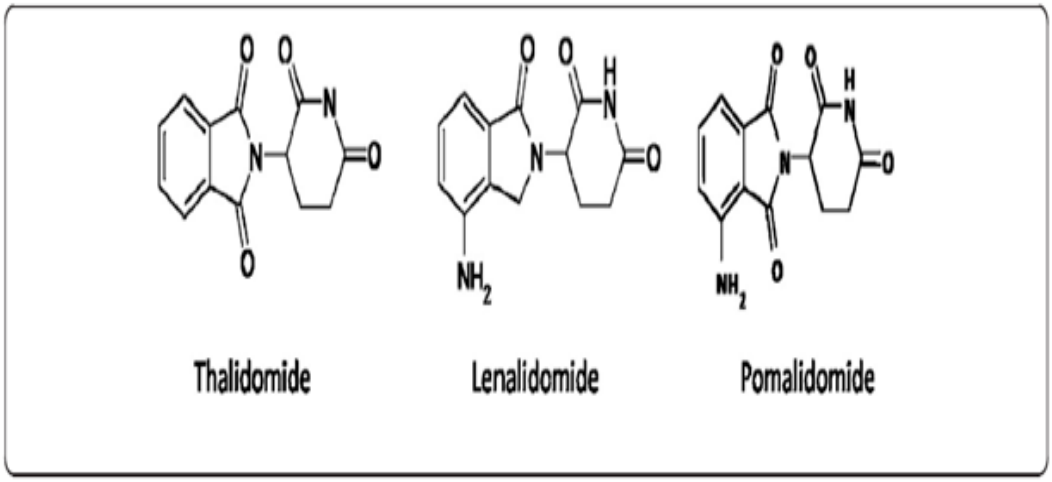


Figure-4: The molecular structure of thalidomide derivatives[33].

Drug name	Route of administration	Dosage	Metabolism	Half life	FDA approval date
Thalidomide	Oral	50-100 mg	Hepatic	4-9 hours	2006
Lenalidomide (CC-5013)	Oral	15-25 mg	Renal	3.1-4.5 hours	2006
Pomalidomide (CC-4047)	Oral	1-5 mg	Renal	6.2-7.9 hours	2013

Table-3: Pharmacokinetics properties of thalidomide derivatives in multiple myeloma treatment.

D. T-box gene family

1- History of the T-box gene family

The Brachyury (T) locus was introduced to the world in 1927, reported as the responsible gene for the short tail phenotype in the mutant mouse line[35]. It took more than 60 years to identify the gene and to classify it as a transcriptional factor, but at that time it didn't belong to any known transcription factor families[3,35]. In 1992, sequence homology between the mouse T gene and a newly cloned Drosophila gene called optomotor-blind (omb) was discovered. After two years Bollag et al. proved that there is a family of T-related genes in the mouse genome, and it was given the name T-box gene family [36]. Researchers' interest in this gene family allowed the discovery and characterization of T-box genes from different species as divergent as Homo sapiens and C. Elegans, as well as zebrafish, frogs, newts, etc.[36,37].

2- T-box gene family features

The common feature among the T-box gene family is the conserved homology domain referred to as the T-box which serves as DNA-binding motif[37]. This was revealed from studying the gene product of three distantly linked members of the T-box gene family (T, omb, and VegT) from three distantly related species (mouse, Drosophila, and Xenopus, respectively)[36]. From that time, over 50 proteins have been recognized with sequence similarity to the DNA-binding domain of Brachyury and Omb[38].

Phylogenetic analysis showed that the T-box family is an ancient gene family and its first expansion appeared at the outset of metazoan evolution[39] (Figure-5). This phylogenetic analysis allowed the creation of phylogenetic tree suggesting that the genome of most animal species—from nematodes to mammals—have at least five T-box genes. Most of the T-box gene family show direct homology, high degree of sequence similarity, expression pattern, and function between a variety of vertebrates, including fish, frogs, dogs, and mice [37]. On the other hand some T-box gene like VegT is unique to endoderm formation in Xenopus, with no known ortholog in mice or humans[38].

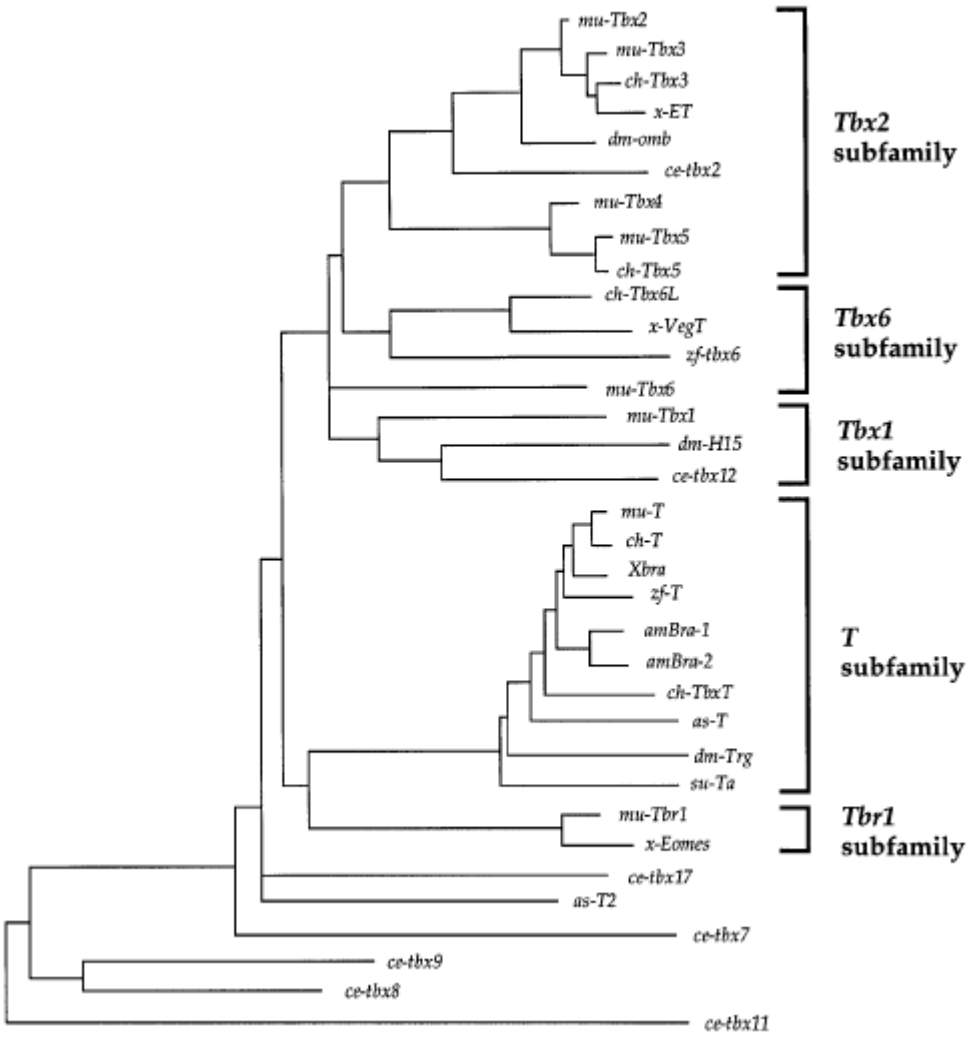


Figure-5: A phylogenetic tree of the T-box gene family. The phylogenetic tree was constructed by using the neighbor-joining algorithm of Saitou and Nei59 based on Poisson-corrected distances between amino acid sequences [36]

The genes containing T-box in mouse and human are randomly localized throughout chordate genomes, with few example of clustering like in human (*TBX2* and *TBX4)* and (*TBX3* and *TBX5*), having a similar arrangement on chromosomes 17 and 12, respectively (Table-4). Multiple exons are responsible for coding the T-box genes, with

at least five exons needed to code for the T-box domain. These exons are dispersed over a relatively large distance but with conserved intron-exon boundaries whereby only the lengths of the introns will vary between species[38,40].

Subfamily	Gene	Human Chromosome	Expression	Heterozygous phenotype in human (null phenotype of mouse homolog)
Brachyury	*BRACHYURY*	6	Primitive streak, tail bud, and notochord	Spinal cord defects (anteroposterior axis defects)
	TBX19 (TPIT)	1	Pituitary	
T-brain1	*T-BRAIN1*	2	Cerebral cortex	
	EOMESODERMIN/			
	(?T-BRAIN2)	3	Trophoblast, early primitive streak, and cerebral cortex	(Early postimplantation failure)
	TBX21 (T-BET)	17	Th1 lineage, lung, and spleen (adult)	
Tbx1	*TBX1*	22	Heart and pharyngeal arges	DiGeorge syndrome
	TBX10	11		
	Tbx13 (MmTbx7)*			
	Tbx14 (MmTbx8)*			
	TBX15	1	Craniofacial region and limbs	
	TBX18	6	Heart, somites, and limbs	
	TBX20 (TBX12)	7	Heart, eye, ventral neural tube, and limb	
	TBX22	X	Fetus	
Tbx2	*TBX2*	17	Limbs and heart	X-linked cleft palate
	TBX3 (including an alternative splice form)	12	Limbs and heart	Ulnar-mammary syndrome
	TBX4	17	Allantois, hindlimb	
	TBX5 (including an alternative splice form)	12	Forelimb	Holt-Oram syndrome (failure of heart development)
Tbx6	*TBX6*	16	Primitive streak and tail bud	(Respecification of posterior paraxial mesoderm as neurectoderm)

*These sequences have been reported in mouse but not human; the human genes are hypothetical.

Table-4: Mouse and human T-box-containing genes [38]

3- Structural features

T-box genes encode for proteins that range between ~400 to 900 amino acids in which conserved residues in the C terminus are involved in transcriptional activation or repression. On the other hand residues in the N terminus may interact with cofactors[41]. In this large family of transcription factors; 20 members were identified in human including TBr, TBX2, TBX3, TBX4, TBX5, and TBX6. Other members have been established in vertebrates (e.g., Xbra and Eomesodermin in Xenopus laevis) and invertebrates (e.g., optomotor blind (omb) in Drosophila melanogaster). T-box proteins usually range in size from 50 kDa to 78 kDa. Studying the different T-box protein members revealed that each of these members should at least have two structural and functional domains: a sequence-specific DNA-binding domain (known as the T-box) and a transcriptional activator or repressor domain[36].

4- The T-box

It is the minimal needed region in the T-box protein for sequence-specific DNA binding [37]. This highly conserved DNA binding motif (T-box or T-domain) is composed of approximately 180 amino acid residues found to bind the DNA consensus sequence TCACACCT [37]. This common DNA consensus sequence was discovered by examining the downstream targets and binding-site selection experiments for a number of T-box proteins. It composes about one third of the entire protein (17-26 kDa), with varying homology degree among this gene family [36]. Some T-box residues are highly

conserved in all the family members, while others vary providing the basis for subdivision of the family[38]. (Figure-6) The ability to bind the sequence is protein-specific, were the specificity of several T-box proteins for their target sites lies mainly within the T-box[38,42].

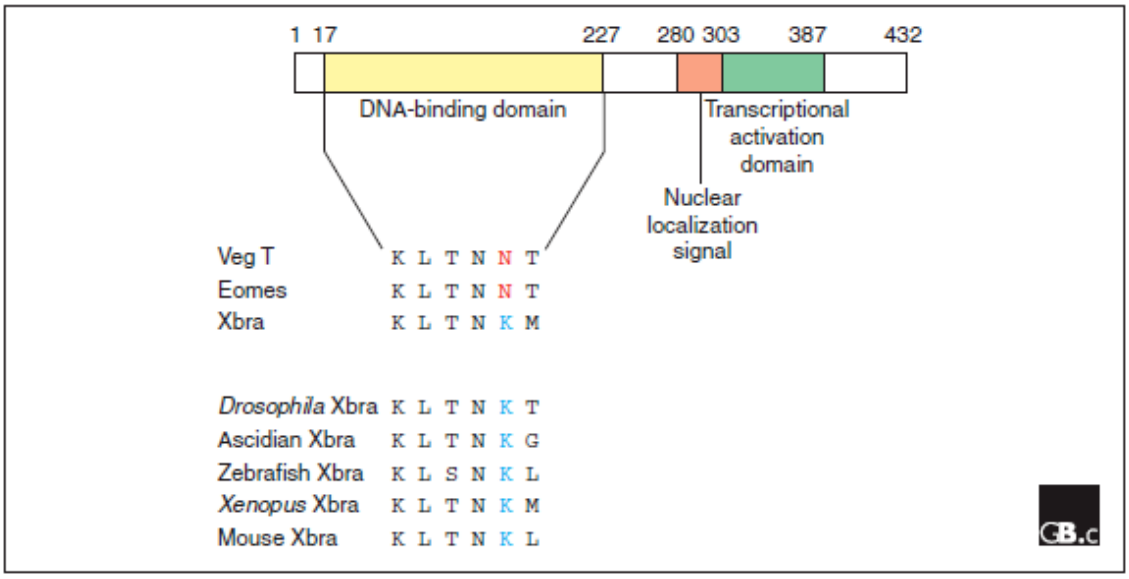


Figure-6: Conservation of selected T-box residues and the presence of diagnostic residues for different members of the family.

The initial binding selection experiment was done on Brachyury, were it showed binding to the palindromic sequence"AATTTCA**C**A**C**CTAG**G**T**G**TGAAATT" as a dimer. Each monomer of Brachyury can bind half of the sequence, or T-half site (5_-AGGTGTGAAATT-3_) in which the protein contacts the DNA in the major and the minor grooves [43].

The crystallographic structure of the T-box domain of TBX3 and Brachyury illustrates that T-box protein make the same DNA contacts with the same amino acids. However TBX3 binds the DNA as a monomer and the binding is due to the N-terminal

domain of 229 amino acid residues (Figure-7). On the contrary, human TBX22 has been found to contain truncated T-box missing residues present in the amino-terminal portion of all other family members and would be expected not to bind DNA[38]. Thus TBX22 might have T-box that functions away from DNA binding[44].

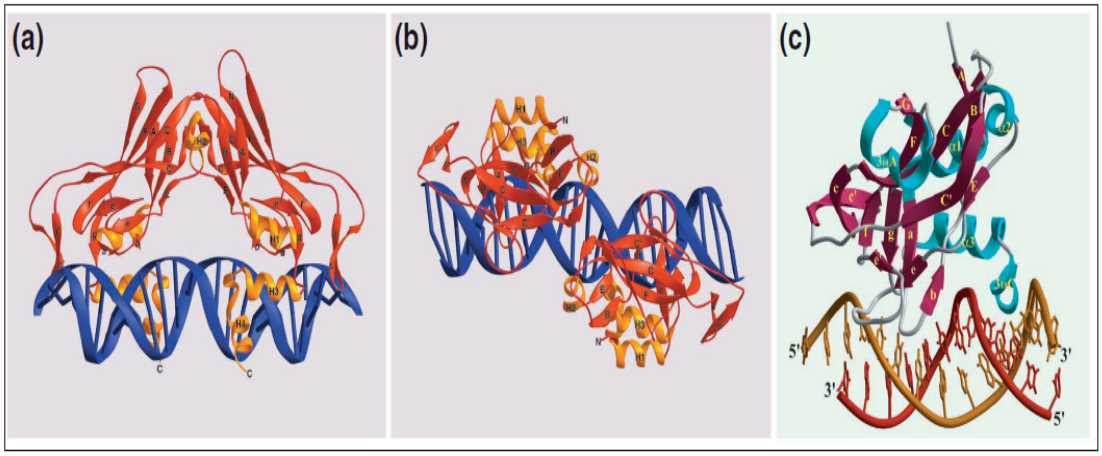


Figure-7: Ribbon diagram of crystal structures of (a,b) *Xenopus* Xbra and (c) human TBX3 bound to DNA. Beta strands are depicted in red and alpha helices in (a,b) orange or (c) turquoise [38].

5- Transcriptional regulatory domains

These transcriptional factors proved to have different functions throughout the developmental or molecular contexts due to the presence of both activator and repressor activity. The activation domain of this family is present in the C-terminal domain of several T-box proteins (ex: TBX1) needed to positively regulate transcription. The mechanism of activation differs between the family members, few of these mechanisms are known. For example: TBX19 activates transcription by recruiting SRC/p160 coactivators to the promoter[43,45]. Other T-box proteins have also conserved

transcriptional repression domains, for example TBX2 and TBX3. These repression domains are located in the amino and carboxy-termini, and are activated depending on promoter context[43].

6- Localization and function

T-box genes are expressed in specific organs and cell types, particularly during development, and thus they are required for the development of those tissues (Table-5)[37]. The presence of T-box proteins exclusively in the nucleus is an emphasis on its function in DNA-binding and transcriptional activation/repression capacity. A defined nuclear localization signals NLS is present for all members on the T-box family. For example in the C-terminal of TBX5 member there is nuclear localization signal NLS2 within the transactivation domain. Another localization signal is present in the N terminal and called NLS1. While in the binding domain of Tbx5, nuclear export signals (NES) was discovered that is made up of hydrophobic amino acids that are conserved among T-box family members. The function of this NES is to export the protein to the cytoplasm after its interaction with CRM1 protein [46].

E9.5	Tbx1	Tbx2	Tbx3	Tbx5	Tbx18	Tbx20	Cx40	Nppa
2nd HF, posterior	−	+	+	+	−	+	−	−
Caudal progenitors	−	−	−	−	++	−	−	−
Inflow tract	−	+/−	+	++	−	+	−	−
Atria	−	−	−	+	−	+	+	+
AVC myocard.	−	+	+	+	−	+	−	−
AVC mesench.	−	+	+	+/−	+/−	+	−	−
Left ventricle	−	−	−	+	−	+	+	+
Right ventricle	−	−	−	+/−	−	+	+/−	+/−
OFT myocard.	−	+	−	−	−	+	−	−
OFT mesench.	−	+	+/−	−	+	+	−	−
2nd HF, anterior	+	+	+	−	−	+	−	−
Pro epicardium	−	−	−	+	++	+/−	−	−
E12.5								
Sinus horns	−	−	−	+	++	+	−	−
Atria	−	−	−	+	−	+	+	+
Mediastinal myocard.	−	−	−	+	−	+	+	−
Pulm. vein myocard.	−	−	−	+	−	+	+	+
Pulm. vein mesench.	−	−	−	+	−	+	−	−
Sinoatrial node	−	−	+	+	+	+/−	−	−
Venous valves	−	−	+/− [1]	+	−	−	+	+
Primary atrial septum	−	−	−	+	−	+	+	−
AV canal myocard.	−	+	+	+	−	+	−	−
AVC mesench.	−	+ [2]	+ [2]	+ [2]	+/− [3]	++ [2]	−	−
Left ventricle	−	−	−	+ [4]	+ [5]	+ [6]	+ [4]	+ [4]
Interventr. septum	−	−	−	+/− [7]	+	+	+/−	+
AV bundle	−	−	+	+	−	+/−	−	−
Right ventricle	−	−	−	+ [8]	−	+ [6]	+ [8]	+ [4]
OFT myocard.	−	+	−	−	−	+	−	−
OFT mesench.	−	+/−	+/−	−	+	++	−	−
Intraper. art. trunk	+	−	−	−	−	+	−	−
Epicardium	−	− [9]	− [9]	− [9]	++	+ [10]	−	−

Table-5: Cardiac expression patterns of T-box factors during recruitment and chamber formation (E9.5) and during septation (E12.5). [31]

E. Transcriptional factors involvement in heart development

Heart development is a complex process that needs well regulated genetic program to achieve the morphogenetic and functional requirements of the heart. Among the important transcription factors required are Nkx2-5 and members of the Mef, Gata, Hand, Iroquois, Forkhead and T-box families of factors [47] (Figure-8)

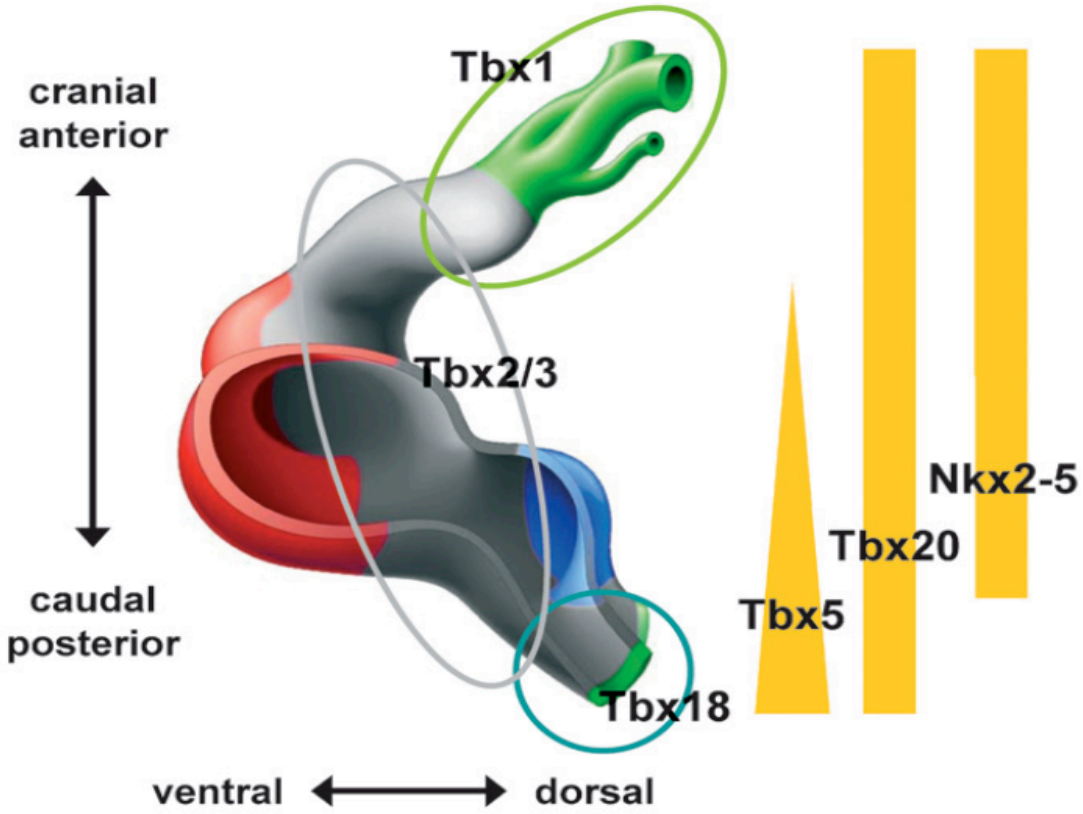


Figure-8: Role of T-box factors in early heart development. Schematic representation of an E9.5–10.5 heart showing T-box patterning in the different emerging structures. *Tbx2* and *Tbx3* exert their function in the non-chamber myocardium, *Tbx1* in the outflow tract and Tbx18 in the sinus horns. Yellow bars indicate expression patterns of *Tbx5, Tbx20* and *Nkx2-5*. *Tbx5* is required for antero-posterior patterning and, along with *Tbx20* and *Nkx2-5*, for chamber differentiation[47]

1- Involvement of T-box transcriptional factors in heart development

Members of the TBX1 subfamily (TBX1, TBX18 and TBX20) and of the TBX2 subfamily (TBX2, TBX3 and TBX5) are key regulators in heart development [47]. The importance of this family is revealed by its role in heart cell maturation and morphogenesis, cardiac lineage determination, chamber specification, epicardial development and specialization of the conduction system[48].

TBX1: Shown to be expressed in pharyngeal endoderm, the mesodermal core of the pharyngeal arches and the second heart field[47,49]. The presence of TBX1 in SHF provides broad assistance to the outflow tract myocardium, endocardium and mesenchymal cushions between E8.5 and E9.5[50,51]. By its binding site, TBX1 can regulate the transcriptional activity of *Fgf8* and *Fgf10*, by acting on their promoter sequences, these three transcriptional factors are co-expressed in the second heart field, suggesting a common acting pathway[52]. Mutations in the *TBX1* gene is shown to cause DiGeorge syndrome[40].

TBX2 and TBX3: As mentioned previously, the division of T-box gene family is according to conservation of protein structure and function resulting is different subfamilies. Among these subfamilies is TBX2 that contains TBX2, TBX3 and TBX5. Co-expression of the transcriptional repressors TBX2 and TBX3 in heart primordia, primitive myocardium and AV canal of chick and mouse embryos play a critical role in cardiogenesis[48]. The repression activity on both TBX2 and TBX3 is critical for *Bmp* a cardiac chamber-specific gene required for chamber growth and maturation in the AV canal, and contributes to cardiac conduction system development[53,54]. Other genes that are downregulated by TBX2 are chamber specific genes, *Nppa*, *Cx40* and *Chisel* and thus cause repression in the differentiation and formation of the cardiac chambers. While TBX3 critical role is in regulating the formation, maturation and function of the sinoatrial node (SAN). The repression activity of TBX3 is also represented on the transcription of the chamber myocardial-specific genes, *Cx40*, *Cx43* and *Nppa.* Thus it

can suppress both atrial and ventricular myocardial gene expression in the conduction system.

TBX18: This member of T-box family is expressed in a small subpopulation of cells ventral to the developing heart tube in the E8 period. Its importance is in its presence in the area that gives rise to the pro-epicardium and the mesenchyme that borders the myocardial inflow tract of the heart [48,55,56].Also TBX18 is necessary for sustaining antero-posterior polarity in somites. The *Tbx18* deficient mice die as soon as they are born due to severe skeletal malformations, but no known genetic mutations in *TBX18* is isolated in humans. The expressions of TBX18 and TBX3 during SAN formation represent a co-expression and the modulation of multiple T-box transcription factors of different subfamilies[53,57].

TBX20: is present in the first heart field, in a subset of second heart field progenitors, and in the endocardium and derived mesenchyme of the atrioventricular and outflow tract cushions [47,58-60]. Underdeveloped short heart tube is the result of deficient TBX20 in mice, which is responsible for the early death[58]. The short heart tube feature is due to failure in recruiting SHF which is essential for the elongation process. Mice deprived from *Tbx20* by RNAi knock-down process showed hypoplasia of the outflow tract and right ventricle[58,61].

TBX5: This transcription factor contributes for many aspects of cardiovascular development where mutations in human *TBX5* cause Holt-Oram syndrome [48,62,63].TBX5 is expressed in all chambers of the heart and higher levels of protein are expressed in

the atrial chambers or what we call caudal-high antero-posterior gradient. This gradient pattern of expression is vital since the forced expression of TBX5 in the entire heart will cause the arrest in heart development and loss of *Mlc2v* expression, an anterior marker gene not normally expressed in the sinuatrial region[64] . Also the deficiency in this factor will cause cardiac developmental arrest, by which the formed but un-looped heart tube will be characterized by a hypoplastic caudal end. This indicates that TBX5 plays a pivotal role in development (recruitmentor expansion) of the sinuatrial precursor population. One of TBX5 targets is connexin 40 that plays important role in the conduction of electrical impulses throughout the heart and this is responsible for arrhythmias in patients with HOS[65,66].

TBX5 one of this large family of transcription factors that is located on 12q24.1, and is mainly expressed in the embryonic heart and upper-limb tissues [67]. This gene is coded by eight exons distributed over 53 kilobases (kb) of chromosome 12. The T-box domain of TBX5 is made up of a seven-stranded β-barrel domain that is closed by a smaller β-pleated sheet [68]. The general structure is very similar to TBX3 and the two T-box domains have 62% identical residues. The interface between the T-box domain of TBX5 and its target DNA has a total size of 821 Å by which only a few residues of TBX5 are responsible for direct interaction with the DNA, with most of the interacting residues located in α-helix 3 and 310-helix C (Figures-9 and 10). Normally TBX5 localizes in the nucleus of cardiomyocytes[65,66]. Protein-producing mutations of this gene can impair nuclear translocation, and alter DNA binding affinity or the synergy with accessory transcription factors. On the other hand minor fluctuations in

TBX5 dosage are appropriate to cause significant variations in gene expression within cardiomyocytes. Thus studies suggest that mutations in TBX5 proteins might mimic its haploinsufficiency [63,67].

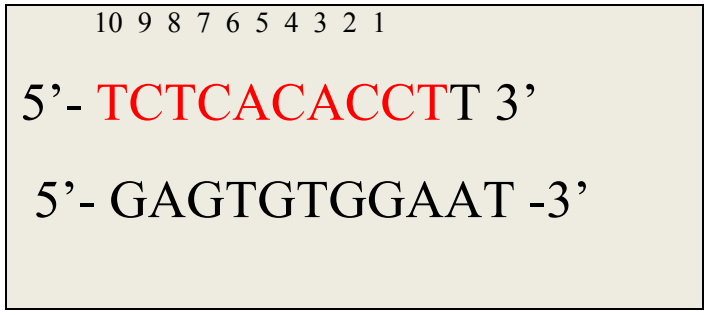


Figure-9: DNA oligonucleotides used for the TBX5–DNA complex (natural TBE from the ANF promoter)[68]

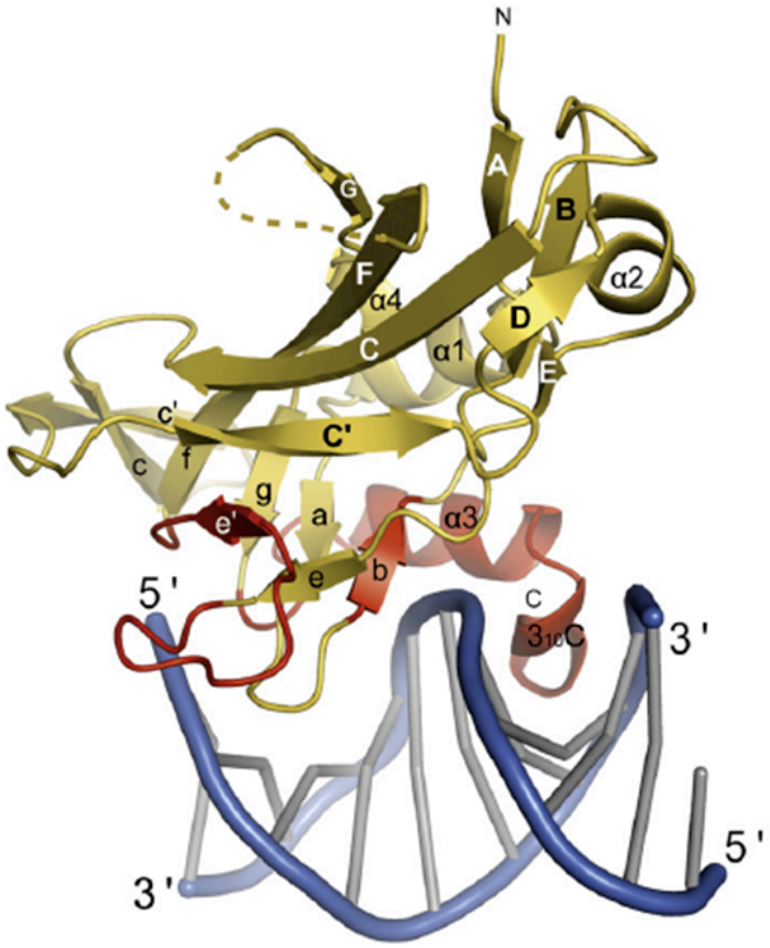


Figure 10: Overview of the TBX5–DNA complex. TBX5 is depicted in yellow, parts interacting with DNA are in red, and the DNA is in marine and gray. The unstructured loop is depicted as yellow broken line. β-Strands involved in the β-barrel are labeled with capital letters (A–G), and β-strands from the β- pleated sheets are in lowercase letters (a–g).[68]

2- The Heart Trio

The relationship between GATA4, TBX5 and NKX2 was investigated based on the common phenotype observed in humans with mutations in either of these factors [3]. The three transcription factors TBX5, GATA4 and NKX2-5 function together to activate genes. The overlapping expression patterns and complex interactions of these

factors permit the regulation of cardiac gene expression and morphogenesis[48,69]. Studies showed that this interaction will direct cardiac chamber differentiation by upregulating the expression of *Nppa* (Natriuretic Precursor Peptide type A, also known as *ANF*) [47]. By its binding site; TBX5 binds to the target genes in order to activate transcription. TBX5 can also combine with other cardiac transcription factors such as Nkx2-5 or GATA4 for synergistic activation of target gene. GATA4 interacts directly with NKX2.5 via its zinc finger domain with the homeodomain of NKX2.5[70,71]. These three genes *TBX5, NKX2-5* and *GATA4* function in a complex to control a subset of genes required for cardiac septal formation and can lead to upregulation of cardiogenesis in cells under differentiation[72]. GATA4 and NKX2.5 are central transcription factors in the primary heart field and the secondary heart field while T-box 5 is only expressed in the primary heart field[12]. Studies that showed disruption in the interaction between these factors in CHD cases either due to mutations in *GATA4* (G296S mutation) or due to mutations in Tbx5, stress on the possibility that TBX5, NKX2-5 and GATA4 function in a complex to regulate a subset of genes required for cardiac septal formation[73]. Myosin heavy chain 6 (MyHC6) is the known target of all three-transcription factors, in which its mutation cause ASD.

NKX2.5: The *NKX2.5* gene is located on chromosome 5q34 consisting of two exons, which encode a 324 amino acid protein[74,75].This transcription factor starts it expression at embryonic day (E) 7.5 in the developing mouse's pericardiac mesoderm and the adjacent endoderm until adulthood [74]. At least five members of the *Nkx2* gene family are expressed during vertebrate heart development. Studies on Nkx2.5 ortholog

in flies (called tinman) expressed in its dorsal vessel showed that any disorder in this transcription factor will result in absolute loss of cardiac cells. Less sever results are obtained in mice that are lacking Nkx2.5, with lethality incidence at E9.5 after initial formation of the heart tube but prior to the heart looping stage[76]. By using the linkage analysis and positional cloning, Schott et al proved that in human: mutation of Nkx2.5 follow Mendelian inheritance of familial atrial septal defects[75,77]. Researchers have proved the proportional relationship between Nkx2.5 dosage and the number of cells in the cardiac conduction system, in which the mRNA and protein expression of this factor are higher during formation of conduction fibers. Furthermore, genetic screening for patients with structural cardiac anomalies associated with conduction system disease, especially conotruncal disorders revealed several mutations in the Nkx2.5 gene.

GATA 4: GATA1, GATA2, GATA3, GATA4, GATA5 and GATA6 belong to a family of transcription factors, in which the first three are expressed predominantly in hematopoietic cells while the last three are expressed in the developing heart and in several endodermal lineages. Studies have shown that *GATA1* and *GATA3* if mutated they can cause human blood disorders and organ malformations, respectively. While mutations in the zinc-finger transcription-factor-encoding gene GATA4 cause inherited septation defects, GATA4 binds a consensus HGATAR DNA motif and comprises two class IV zinc finger domains with expression mainly restricted to the heart and gonad tissues[78]. Preceding the expression of most primitive cardiac differentiation markers, protein GATA4 was found in the developing heart as one of the first proteins to be expressed [79-81]. Mice that are heterozygous for *Gata4* do not have apparent cardiac

anomalies, but additional reduction in *Gata4* dosage from a hypomorphic *Gata4* allele will results in cardiac septal and other congenital heart defects. On the other hand, homozygosis in the Drosophila *Gata4* orthologue, pannier or mouse *Gata4* results in early defects in cardiogenesis [73]. Mutations in the GATA4 gene have been identified in patients with CHD but not in patients with upper-limb malformations [82-85]

F. Overview of the association of some diseases and T-box genes

1- DiGeorge syndrome and TBX1

It is also known as Velocardiofacial syndrome affecting about 1 in 4000 live births [3]. DiGeorge syndrome is caused by a chromosomal deletion of 22q11 where *TBX1* gene is present within this commonly deleted region, and it is implicated as a major contributor to the craniofacial and cardiac phenotypesd[86,87]. This syndrome characterized by hypoplastic or aplastic thymus and parathyroids, causing defects in cell-mediated immunity, and hypoparathyroidism. Also it is associated with some facial dysmorphism, learning difficulties and behavioral problems, but with no known defects in the limbs. However these characteristics are highly variable between patients with the same syndrome[40].

2- Ulnar-mammary syndrome and TBX3

As the name indicates, this syndrome affects the ulnar ray of the limb. The phenotype varies between the patients ranging from hypoplasia of the terminal phalanx of the fifth digit, to the complete absence of forearm and hand [40,88,89].

Other features like abnormal developed breasts, teeth and genitalia are commonly detected as well. Haploinsufficiency of TBX3 is thought to be the responsible cause of this syndrome; here it plays a critical role in specification of posterior limb mesoderm and in setting up the dorso/ventral limb axis.

3- Cleft palate and TBX22

This syndrome affect approximately 1 in 1500 births, in which semi-dominant X-linked mutation mapping to Xq21 Is detected. Cleft palate causes problems with feeding, speech, hearing, dental and also psychological development, and requires corrective surgery, while there is no known heart related problems in these patients [40]. Human genome project detected *TBX22* on that region and screening cleft palate patients revealed point mutations in *TBX22* including splice site, missense and nonsense mutations[40].

4- Holt-Oram Syndrome (HOS) and Tbx5

Holt–Oram syndrome (HOS) or atriodigital dysplasia is an autosomal dominant disease which is highly heterogeneous in its representation and expression causing both cardiac and skeletal congenital abnormalities[90]. This disease was first described by Holt

and Oram in 1960 as autosomal dominant disease affecting one in 100 000 live birth in which 40% of cases are thought to be sporadic. Skeletal abnormalities affecting the forelimb in HOS patient mainly include clinodactyly, limited supination, sloping shoulders and phocomelia(figure-11). They disturb the radial ray and are bilateral and asymmetrical, affecting the left side more cruelly than the right. While the cardiac defects are detected in the conduction system, atrial and ventricular septation and tetralogy of Fallot[91]. Holt-Oram syndrome (HOS) is associated with single-gene mutations in the T-box transcription factor TBX5 by which more than 70% of individuals who are well diagnosed have an identifiable mutation in TBX5[91]. The location of missenses mutation of *TBX5* will dictate the severity of cardiac and skeletal abnormalities, if the missense is at the amino terminus of the DNA binding domain the cardiac abnormalities are more severe than the skeletal ones. While if this missense is at the C-terminal end of the T-box we will have only mild cardiac defects but severe skeletal ones [63]. Screening for patient diagnosed with HOS disease revealed more than 60 mutations in *TBX5* gene along with other environmental and stochastic modifiers that are responsible for the great inconsistency in severity between these patients. The majority of these mutations are found within the T-box DNA-binding domain, they include frameshift mutations, missense mutations, nonsense mutations, splice acceptor site mutations, and small microdeletions (table-6). All these mutations were believed to be responsible for nonfunctional TBX5 proteins that lack an active DNA binding domain or for the total absence of TBX5 protein.

Since previous studies showed that TBX5 haploinsufficiency causes congenital heart and limb abnormalities, producing mice that are deficient in TBX5 was a sufficient tool to investigate the underlying mechanisms [66,92]. Mice that lack *Tbx5* (*Tbx5*del/del) died early in embryogenesis which emphasize on the early and essential role of *Tbx5* in cardiac development. While heterozygous mice (*Tbx5*del/-) exhibit the forelimb and congenital heart malformations (ASD, VSDs, and conduction disease) seen in Holt-Oram syndrome. Also altered *ANF* and *cx40* gene transcription levels were detected. This study showed that both genes *ANF* and *cx40* are regulated by TBX5, and a deletion of one allele would cause repression of both gene promoters that might be the cause of the cardiac defects.

The hindlimb (HL) develops from outgrowths of the lateral plate mesoderm (LPM) at precise positions along the body axis. Knocking out *Tbx5* allele in a range of vertebrate model systems has shown that *Tbx5* is essential for forelimb (FL) and heart development. Results showed that FL will not initiates if this gene is deleted prior to, or during, limb initiation, thus *Tbx5* also adjusts later FL outgrowth and might be important in arrangement of the diverse patterning events within the growing limb [93].

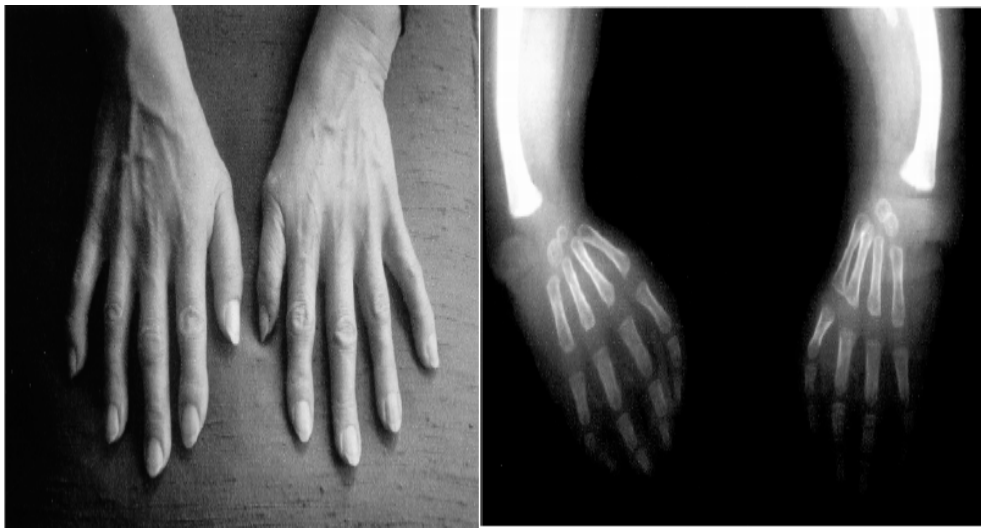


Figure-11: Characteristic forelimb abnormalities in two Holt–Oram patients. (A) An X-ray showing the absence of both thumbs and radial hypoplasia; (B) a photograph of hands showing abnormal thumb development.

Study	Mutation	Proposed mechanism of mutation
	Frameshift	
Brassington *et al.* [12••]	100delG	Nonfunctional protein/nonsense-mediated mRNA degradation causing haploinsufficiency*
Brassington *et al.* [12••]	100-101insG	
Basson *et al.* [11]	246insAA	
Brassington *et al.* [12••]	400-401insC	
Hatcher *et al.* [24]	416delC	
Brassington *et al.* [12••]	426-427insC	
Brassington *et al.* [12••]	456delC	
Hatcher *et al.* [24]	593-594insA	
Basson *et al.* [11]	727delG	
Brassington *et al.* [12••]	798delA	
Cross *et al.* [14]	805delT	
Hatcher *et al.* [24]	1159-1160insA	
	Missense	
Basson *et al.* [11]	G80R	Protein-producing mutations. Aberrant proteins show drastically reduced nuclear between localization and diminished DNA-binding ability. There is also a loss of physical interaction mutant TBX5 and NKX2-5. The G80R and R237W mutations also disrupt TBX5 interactions with GATA4.
Basson *et al.* [5]	R237Q	
Basson *et al.* [11]	R237W	
Hatcher *et al.* [24]	Q49K	Protein-producing mutations. Aberrant proteins show drastically reduced nuclear interaction localization but bind DNA normally. These mutations may lead to loss of physical between TBX5 and NKX2-5 but do not disrupt TBX5 interactions with GATA4.
Hatcher *et al.* [24]	I54T	
Cross *et al.* [14]	G169R	
Cross *et al.* [14]	S251I	
Brassington *et al.* [12••]	W121G	Unknown mechanism.
Brassington *et al.* [12••]	G195A	
Brassington *et al.* [12••]	T223M	
Brassington *et al.* [12••]	S261C	
	Nonsense	
Fan *et al.* [21]	W64Ter	Nonfunctional protein/nonsense-mediated mRNA degradation causing haploinsufficiency.*
Basson *et al.* [5]	E69Ter	
Gruenauer-Kloevekorn and Froster [25]	Y136Ter	
Cross *et al.* [14]	E190Ter	
Li *et al.* [6]	S196Ter	
Brassington *et al.* [12••]	R279Ter	
Cross *et al.* [14]	E316Ter	
	Splicing Mutations	
Basson *et al.* [11]	t(5;12(q15;q24	Nonfunctional protein/nonsense-mediated mRNA degradation causing haploinsufficiency.*
Basson *et al.* [11]	Int2 + 1G → C	
Basson *et al.* [11]	Int2-2C → A	
Cross *et al.* [14]	Int4 + 2T → C	
	Large deletion	
Akrami *et al.* [26]	Ex3_9del	Nonfunctional protein/nonsense-mediated mRNA degradation causing haploinsufficiency.*
	Microdeletions	
Fan *et al.* [21]	381-408del	In-frame deletion. Protein-producing mutation. Aberrant protein shows drastically reduced nuclear localization and diminished DNA-binding ability.
Basson *et al.* [11]	420-432del	Frameshift mutation. Nonfunctional protein/nonsense-mediated mRNA degradation causing haploinsufficiency.*

*Mechanism has not been shown experimentally but rather is proposed based on biochemical/molecular considerations.

Table-6: Mutations in *TBX5* identified in patients with HOS to date [91]

G. Aim of the study

The fact that thalidomide is useful in treatment of serious diseases, this implies that it is likely to continue to be used. Thus understanding the exact mechanism of

action of this drug is of a great value. The similar features observed in thalidomide toxicity and Holt Oram syndrome patients were the base for this project. We aimed to investigate whether thalidomide act directly on Tbx5; underlying its teratogenic effect. Since the exact mechanism of action of thalidomide is not known yet, our project promised for a first and novel explanation of how this drug causes CHD and limb deformities by interacting with specific amino acids located on the DNA binding site of Tbx5. The preliminary result was based on the in-silco prediction tool that suggested that thalidomide have the ability to bind to TBX5 and interfere with its function. For confirmation we studied the effect of this drug on the binding affinity between TBX5 and its T-box by gel shift assay and on its transcriptional activity by luciferase assay. We studied also the effect of thalidomide on the functional properties of TBX5 by western blot analysis and immunofluorescence assays, and on its interaction with it partner GATA 4 by the co-immunoprecipitation and co-transfections assays.

To correlate our findings to the emerged clinical use of thalidomide in cancer treatment, further experiments were conducted. Based on previous results from a work done in our lab that showed an expression of *TBX5* in embryonal rhabdomyosarcoma cells, we investigated the effect of thalidomide in inhibiting cellular proliferation and migration in this cell line as a result of an interaction between the drug and the expression of Tbx5. We studied also the effect of thalidomide on *TBX5* RNA expression in embryonal rhabdomyosarcoma cells.

CHAPTER II

METHODS

A. Chemicals

Thalidomide used in our project is from Sigma. This drug is water insolouble, it was dissolved with DMSO. All the experiments were compared to DMSO to rule out any effect attributed to it alone. The stock concentration of thalidomide used in this study was 20mg/ml.

B. Cell Lines

1-HEK293 cell line

Human Embryonic Kidney 293 cells referred to as HEK 293 cells. HEK293 cells grow rapidly and they are easily transfected. These cells were cultured and maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (PAA) (FBS), 1% Penicillin/ Streptomycin and 1% Sodium pyruvate and incubated in a humid atmosphere 5% CO2 at 37ºC.

2-JR1 cells

The embryonal rhabdomyosarcoma (JR1) cell line is a gift from Dr. Raya Saab. These cells were derived from a lung metastasis of a 7-year-old female with a primary uterine mass and they showed poorly differentiated embryonal histology of the biopsy specimen (14t). This cell line was maintained in DMEM culture media supplemented with 10% FBS and Penicillin streptomycin (P/S) and 1% Sodium pyruvate and incubated in a humid atmosphere 5% CO2 at 37ºC.

C. Transfections

1-HEK293 cells

a- Transient over expression

For transient overexpression experiments, transfection was done using Polyethyleneimine (PEI) transfecting agent, a synthetic polycation for gene delivery inside the cells. 20µg of the plasmid DNA was mixed with 1ml of serum free media (SFM) and vortexed for 20 seconds followed the addition of 35µl of PEI. After that, 20 minutes incubation at room temperature. This mixture is added gently to HEK293T cells that were plated in 100mm culture plates (Corning) with 60% to 70% confluency. After 3 hours of transfection, the media was changed and the cells were incubated for 24 hours before changing media for the second time. Nuclear extraction was done 36-48 hours post transfection.

b- Luciferase Assay

To study the transcriptional regulation of the *VEGF* or *ANF* promoters by TBX5 and/or GATA4 in three different conditions. First one with no treatment, second one with DMSO and third one with thalidomide. HEK293 cells were plated in 12 well culture plates (Costar) with 50,000cells/well. Transfections were done on the second day of seeding using 5µl PEI/ well. HEK239 cells were transfected with VEGF /Luc or ANF/luc as well as TBX5 and/or GATA4. Controls were transfected with either VEGF/Luc or *ANF*/Luc only. After 3 hours of transfection, media is changed and incubated for 24 hours before adding the appropriate treatment. For luciferase assay we added 10µl of thalidomide (20mg/ml) and 10µl of DMSO per well and incubated for 12 to 16 hours.

2-JR1 cells

a- For Immunofluorescences assay

JR1 cells were plated onto 12-well Costar culture plates on cover slips with 100,000 cells per well. Transfection was done on the second day of the seeding by polyethylenimine (PEI). 3µg of DNA per well were mixed with 150µl of serum free media in an eppendorf tube, vortexed and then 8µl of PEI (ratio 1:4) were added and incubated for 20 minutes at room temperature. The mixture was poured gently on the cells of 70% confluency and medium was switched after 3 hours.

D. Luciferase assay:

After 12 hours of the treatment, cells were lysed with 150µl/well 1X lysis buffer(1mM Tris pH 8, NP40 10%) and left on the shaker for 20 minutes at RT. 100µl of cell lysate is then transferred into a 96 well plate (Costar) to which 100µl of luciferin is added. Luciferin (Promega, Cat # E 1501) was prepared according to the manufacturer's protocol. The signal is read immediately using the Ascent Fluoroscan in the Molecular Biology Core Facility at AUB.

Fold activation was calculated by dividing the relative values of each into that of the reporter alone. The presented result values are the mean +/- standard deviation of at least three independent experiments carried out in duplicates. The data was statistically analyzed with a Student's t-test and one way ANOVA.

E. Proliferation assay:

To assess the effect of thalidomide on JR1 cells we used 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cleavage assay. A total of 5 × 1000 cells in 100µl of medium per well were plated in a 96-well plate. Following incubation overnight, 5 and 10µl DMSO or thalidomide (20mg/mL) was added. The different conditions were held in triplicates, wells with no treatment were considered as the control. Then we exposed the cells to MTT at a final concentration of 1 mg/mL in culture for 4 h. 100µl of the stop solution (10% SDS containing 0.02 N HCl) is then added and incubated overnight at 37 °C. The absorbance was measured at 595 nm. This experiment was repeated three times.

F. Wound healing assay:

JR-1 cells were seeded in a six well plate with 100% confluency. After 24hours of incubation, each confluent monolayer was scratched using a 200-µL plastic pipette tip to form a wounded cell-free area. Treatment with 10µl of thalidomide (20mg/ml) and equivalent amount of DMSO (as control) was applied. Images were taken using inverted microscope equipped with a digital camera at different time points. This experiment was repeated four times.

G. Nuclear protein Extraction

Nuclear protein extracts from HEK293 cells that were transfected with the appropriate plasmids were obtained according to the following protocol. The cells were first washed with 2mL 1X PBS per plate. Then 2 mL of 1X PBS and 40µL of Ethylenediaminetetraacetic acid EDTA 0.1mM (chelating agent) were added to each petri dish to detach the cells. The petri dishes were then placed on the shaker for 20 minutes, to allow the detachment of the cells. Using the scrapers the cells are totally detached and collected in eppendorf tubes followed by 90 seconds centrifugation at 11000rpm (fixed-angle rotor).The supernatant is aspirated gently, and the pellet is resuspended in 800µL of buffer A (0.5 M extraction buffer) (10mM Tris pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.5mM PMSF, 0.5mM DTT and 0.5mM protease inhibitors cocktail). The tubes are placed on ice for 15 minutes. 50µL of NP40 10% is added for each tube, the tubes are vortexed for 15 seconds, and then centrifuged for 90

seconds at maximum speed. The supernatant is carefully discarded, and the transparent pellet is resuspended in 150µL buffer C (20mM Tris pH 7.9,400mM NaCl, 1mM EDTA, 1mM EGTA, 0.5mM PMSF, 0.5mM DTT and 0.5mM protease inhibitors cocktail) and the tubes are placed on the shaker in a cold room (4 ºC) for 20 minutes. After that, the tubes are centrifuged for 90 minutes at maximum speed, the pellet is discarded, and 100µL aliquots of the obtained supernatant are prepared, and stored at -80 ºC for future use.

H. Protein Quantification

Protein concentration was measured using a colorimetric assay is based on Lowry protocol (Bio-Rad DC protein assay kit), according to the manufacturer's protocol.

I. Western blot:

This assay was used both to assure for overexpression of specific proteins, and to study the effect of thalidomide on TBX5 protein. Equal amounts of nuclear protein extracts (20µg) from HEK293 cells over expressing either TBX5 or GATA4 were re-suspended in 5x lamelli buffer (1mL glycerol, 0.5mL β-mercapto ethanol, 3mL 10% SDS, 1.25mL 1M Tris pH6.7 and 2mg bromophenol blue). The samples were boiled for 3min and were resolved under reducing conditions, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (12%) for 1 hour. The proteins were

transferred to a Polyvinylidenedifluoride membrane (PVDF) membrane (Amersham). Membrane soaked in methanol and dried three times before blocking with 5% non-fat dry milk (Nido) solution in Tris-buffered saline (TBS) 1X for 45 minutes with shaking at room temperature. After blocking, the membrane is incubated overnight on the shaker at 4ºC with primary antibody (depending on the target protein) diluted 1:1000 in 1 % non-fat dry milk. The membrane is then washed in TBST (TBS, 0.05% Tween 20) for 8 min each, and incubated for 1 hour at room temperature on the shaker with the corresponding secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase-conjugated) with a dilution of 1:40000. Another 3 consecutive washes were done before revelation that was done using the Western Lightening Chemiluminescence Kit (Perkin Elmer, Cat # NEL 103). The protein bands were visualized by autoradiography.

To study the effect of thalidomide on TBX5 protein, same procedure was done with the addition of either 5 or 10µL from 20mg/ml Thalidomide or same amount of DMSO (5 or 10µL) to the protein prior the loading to the SDS-gel and incubated in ice for 20 minutes. No boiling was done before loading.

J. Co-immunoprecipitation assays:

After detecting TBX5 and GATA4 proteins by western blot assay, co-immunoprecipitaion was done to assess the effect of thalidomide on the interaction between TBX5 (HA-tagged) and GATA4 (Flag-tagged). Beads (Dynabeads® Co- IP Kit (Invitrogen, Oslo, Norway) were captured on a magnetic stand (Invitrogen) and resuspended gently with PBS (1x + 0.001% of Tween 20). Then the beads were

incubated with rabbit anti-HA (Santa Cruz) for 1 hour at 4ºC on a rotating platform. 100µg of TBX5 is mixed with same amount of GATA4 and incubated with the antibody complexed beads at 3 different conditions at room temperature on a rotating platform. First Eppendorf contained only the two proteins (control) the second contained in addition 10µl of DMSO and the third with 10µl of thalidomide (20mg/ml). After 2 hours the mixtures were washed for 2 times with PBS (1x) and proteins were eluted with (1x) SDS. Western blotting was performed with mouse anti- Flag (Santa Cruz), essentially as previously described. Membranes were stripped using a stripping buffer (1M Tris, SDS (10%), β- mercaptoethanol) for 30 minutes at 55 ºC in a thermo-rotator followed by 3 times wash with TBT and blocked with 5% non-fatty milk for 45 min. After that, membranes were incubated overnight with antibody rabbit anti-HA (Santa Cruz) at 4 ºC, washed 3 times with TBT and then incubated with anti-rabbit horseradish peroxidase-conjugated antibody for 1 hour at room temperature. Another 3 consecutive washes were done before revelation that was done using the Western Lightening Chemiluminescence Kit (Perkin Elmer, Cat # NEL 103). The protein bands were visualized by autoradiography.

K. Electrophoretic Mobility Shift Assays (EMSA)

In order to assess the binding affinity of TBX5 on its consensus region in the presence of thalidomide, electrophoretic mobility shift assay (EMSA) was done. The probe harboring T-half site on *ANF* promoter was synthesized using the primers shown in table-7. The single strand primers were first annealed then phosphorylated with T4

polynucleotide kinase and γ-32P-ATP at the 5'-end. A radioactive labeled probe was obtained and migrated on a non-denaturing 12% Bis-Acrylamide gel (Acrylamide: Bis (38:2), 1.6% APS, TEMED, water and 1x TBE) for 45 minutes at 125 volts. The gel was exposed to a XOMAT film and the bands corresponding to a double stranded probe were cut accordingly and purified using Costar Spin-X columns (Costar, Cat # 8161) according to the manufacturer's protocol.

Probe	Sequence
T-box binding element (TBE) half site	Fw: 5'TACCTTCACACCTTATTC Rev: 5'GAATAAGGTGTGAAGGTA

Table-7: The primers used to generate probes harboring half T-box site.

The control binding reaction consisted of 10µg of TBX5 extract, 4µl binding buffer (20mM Tris pH 7.9, 120mM KCl, 2mM EDTA, 25mM MgCl2 and 25% glycerol), 1µl poly dI/dC (Amersham) and 1µl of the probe. 5 or 10 µl of thalidomide (20mg/ml) and 5 or 10 µl of DMSO were incubated on ice with the above mixture. The reaction was completed to 20µl with water. After incubation for 20 minutes the samples were loaded on the 6% non-denaturing polyacrylamide gel (Acrylamide: Bis (29:1), 1.6% APS, TEMED, water and 0.25X TBE) and run for 2.5 hours in 0.25 X TBE buffer at 200 volts. BioRad gel dryer (Model 583) was used for 2 hours at 80ºCto dry the gel.

Once dried,it is exposed to a phosphor imager screen. The screen is then scanned using the STORM (Molecular Dynamics) scanner in the Molecular Core Facility at AUB.

L. Immunofluorescence:

Immunofluorescence was performed on transfected JR1 cells. The cells were first washed for 3 times with PBS 1X (phosphate buffered saline). Followed by fixation with 4 % paraformaldehyde for 20 minutes; after washing with PBS, the cells were blocked with 3% BSA/PBT (bovine serum albumin/ phosphate buffer saline Tween 0.2%) for 1 hour. The primary antibody the primary antibody rabbit anti-HA (santa Cruz) were used for assessment of subcellular localization of TBX5. The primary antibodies were diluted (1:500) in BSA/PBT and added to the cells with an overnight incubation at 4ºC. The cells were then washed in PBT 3 times, and the secondary antibody donkey anti-rabbit biotinylated (General electric) were diluted 1:500 in BSA/PBT. They were added to the cells for 1 hour at RT with shaking. After washing 3 times with PBT, cells were incubated with Alexa fluor (anti-rabbit) for 1 hour at RT with shaking. Hoechst staining for the nucleus was also performed by applying Hoechst, diluted 1:30 in water, to the cells for 30 minutes. The cells were then washed with PBT and mounted on a circular slide containing an antifading agent (DABCO). The slides were examined using the Olympus BH-2 microscope.

M. Transformation and cloning of constructs in bacteria:

This transformation process is performed in aseptic conditions i.e. close to the flame of a Bunsen burner. The previously obtained constructs are then transformed into E.coli, XL1 blue strain bacteria initially stored at -80 oC. In an eppendorf tube 100 µL of bacteria is mixed with 1µg of the plasmids containing our DNA constructs. After inverting the tube up and down several times, the mixture is then placed for 2 minutes on ice followed by 5 minutes at 37OC (in the water bath), then 2 minutes on ice. The transformed bacteria are placed and streaked on agar plate, and then incubated at 37OC overnight. Only bacterial colonies incorporating the desired plasmid will grow on the agar ampicillin selective medium since the undigested plasmid contains an ampicillin-resistance gene. By the pipette tips the bacterial colonies observed to grow on the agar were removed and transferred into 15-mL falcon tubes containing 3 mL liquid broth with 3 µl ampicillin. These tubes were then incubated overnight in the shaker, at 37ºC, at 150 rpm. Miniprep and maxiprep are performed using illustra™ plasmidPrep Midi Flow Kit (GE Healthcare) according to the enclosed manufacturer's protocol. The plasmids obtained were verified by sequencing.

N. Transcriptional expression

1. RNA extraction

We had three different conditions of JR1 cells; first plate with no treatment, second with 10µl DMSO, and the third with 10µL thalidomide (20mg/ml). RNA was

extracted from JR1 cells using Tripure reagent (Roche, Cat# 11 667 157 001) according to the manufacturer's instructions. Briefly, after decanting the media and washing the cells with 1.5 ml PBS, cells were scraped in 1ml Tripure and left for 7 minutes at RT to dissociate nuclear proteins. Chloroform was then added and the samples were incubated for 10 minutes at RT for nucleic acid and protein separation. The RNA was collected from the upper phase after centrifugation; precipitation was carried out using isopropanol 100% and the pellet of RNA was washed with ethanol. After centrifugation the cell was then resuspended in nuclease free water.

2. Polymerase Chain Reaction (PCR)

Tbx5 cDNA (containing the open reading frame of Tbx5) were amplified by PCR from proliferating of JR1 cells using the following primers summarized in the table (Table-8).

Gene name	Primer Type	Primer Sequence
TBX5	Forward	5' CATGGAGACATCACCCAGTG 3'
	Reverse	5' GCAGCTGATGTCCTCTAGGC 3'
18S	Forward	5' GTAACCCGTTGAACCCCATT 3'
	Reverse	5' CCATCCAATCGGTAGTAGCG 3'

Table-8: The forward and reverse primers used to amplify 18s and *TBX5* cDNA.

DNA were amplified in a 20 µl reaction mixture using phusion polymerase kit (BIO-RAD) under the following conditions: 98ºC for 10 seconds as an initial denaturing step; 98ºC for 1 second, 55ºC for 30 seconds, 72ºC for 15 seconds for 30 cycles; a final extension step at 72ºC for 1 minute followed by a hold temperature at 4ºC.

3. Gel electrophoresis

PCR products were loaded on 1.5% agarose gel with a loading dye (0.25 % Bromophenol blue, 0.25 % Xylene Cyanol and 15 % glycerol) in a 6 to 1 ratio, along with PSK/ HpaII molecular ladder (20 µl plasmid with 3 µl HpaII, 5 µl one phor all (OPA) buffer and 22 µl sterile water). Agarose gel was prepared in TBE 1X (Tris Boric EDTA, 1L of 10X contain 108g Tris base, 55g Boric acid and 40ml EDTA 0.5 M pH 8) and stained with 1 mg/ml ethidium bromide to visualize nucleic acids.

O. Statistical analysis

All data are presented as mean ± standard error. Two-tailed unpaired Student's t tests and ANOVA were used for statistical evaluation of the data. SP SS 18 statistical tool was used for data analysis. A p-value < 0.05 was considered significant.

CHAPTER III

RESULTS

A. The hypothetical Interaction between TBX5 and Thalidomide

Since thalidomide effect on human embryogenesis is similar to the phenotype observed with Holt Oram syndrome patients; we hypothesized that thalidomide directly affects Tbx5 which is mutated in HOS patients. Using the in-silico prediction tool we visualized an interaction between thalidomide and TBX5 protein in the DNA binding domain.

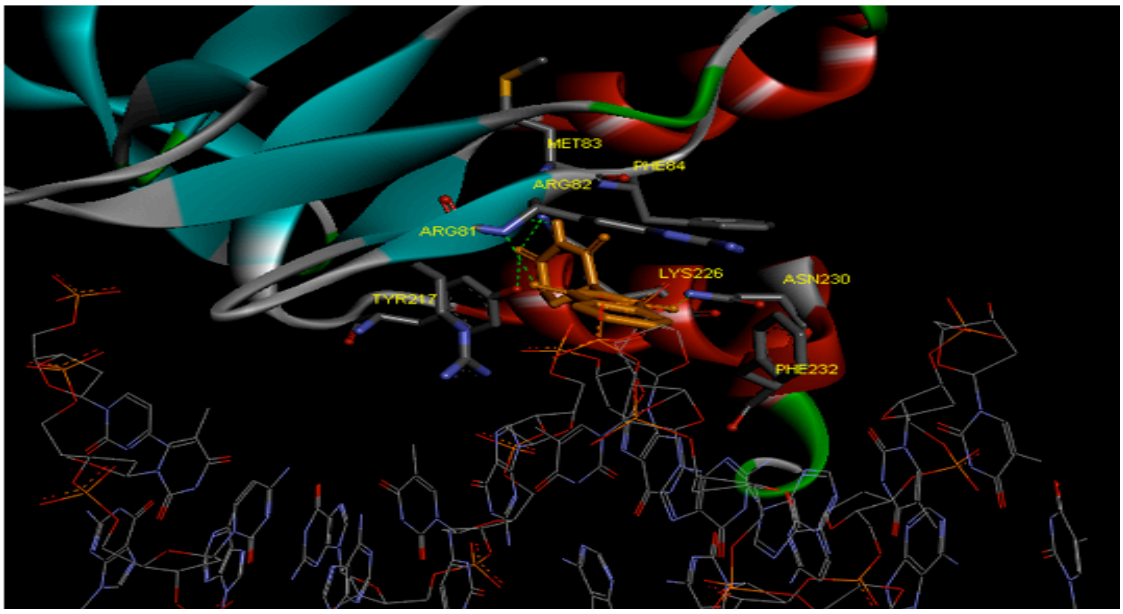
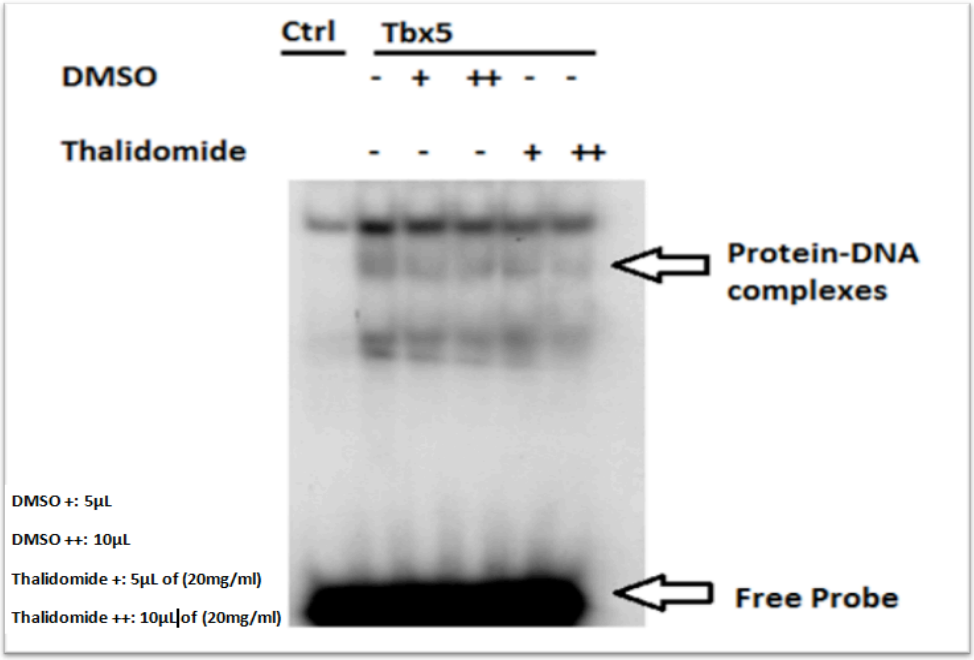


Figure-12: The hypothetical interaction of thalidomide and TBX5. Using the in-silico prediction tool we can see thalidomide (Orange structure) binding to three amino acids (ARG81, ARG82, and LYS226) on TBX5 DNA binding domain.

B. Thalidomide disturbTBX5 physical binding to DNA

Gel shift assay was therefore carried out to assess the effect of thalidomide on the binding affinity of TBX5 protein to a consensus sequence of T-box. Nuclear extract from HEK293 cells overexpressing TBX5 was used after verification with western blot assay. 5µg of TBX5 protein sample of equal concentration was loaded into the gel along with 5µL and 10 µL of thalidomide (20mg/mL) that were compared with equivalent amount of DMSO (depending on the volume added). Thalidomide suppressed the binding affinity of the protein to its DNA binding probe by 35% to 42% compared to the control and depending on the concentration of the added thalidomide (quantified using Image J software). This result confirmed the in-silico prediction that thalidomide will bind on the DNA binding domain of TBX5 suppressing its binding affinity.



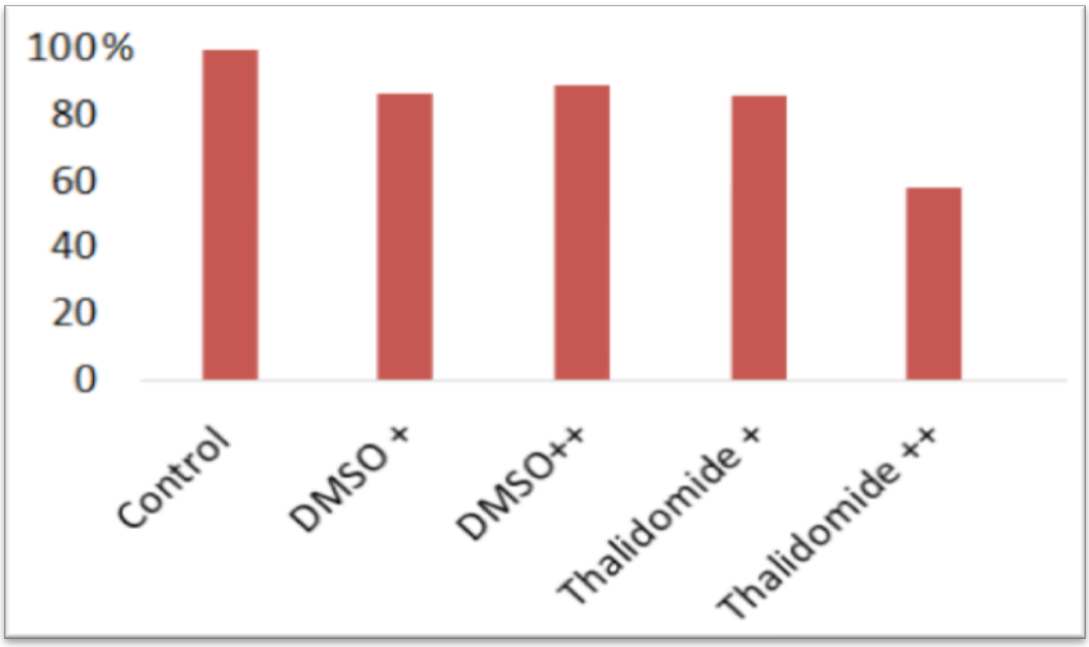


Figure-13: Electro-mobility gel shift revealing the effect of thalidomide on TBX5 binding to DNA. A representative gel shift analysis from nuclear extract overexpressing TBX5 revealed the binding to the radioactive probe harboring T-box DNA binding sites in the control. This binding affinity was decreased in the presence of thalidomide (upper panel). Thalidomide ++ represent 10µL of (20 mg/mL) of this drug that suppressed the binding affinity by 42% while equivalent amount of DMSO ++ that represent 10µL suppressed affinity by less than 10% as shown by Image J software (lower-panel)

C. Effect of thalidomide on Tbx5 physical properties

To generate protein extract for TBX5 protein, we transfected the corresponding plasmid in HEK293 cells. Then, western blot procedure was done on the generated proteins in the presence of DMSO or thalidomide (20mg/ml). Western blot analysis showed no significant physical change in the TBX5 protein, and no evidence of degradation.

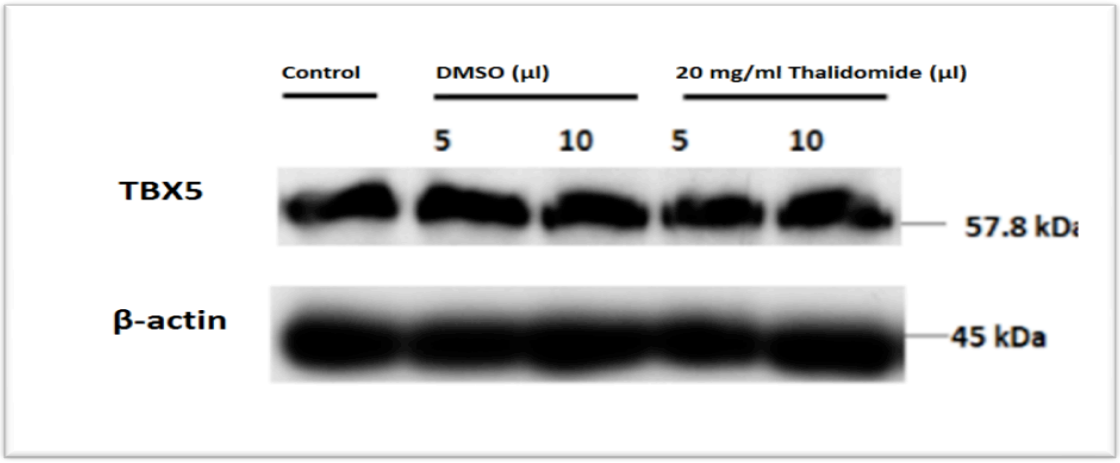


Figure-14: Western blot analysis was used to assess the effect of thalidomide on TBX5 protein. Results showed that there is no effect of DMSO or thalidomide (20mg/ml) on the protein level. Beta actin was used to assure an equal loading.

D. Cellular Localization of TBX5 Protein in the presence of thalidomide

In order to investigate the effect of thalidomide on the cellular localization of TBX5 protein, immunofluorescence assay was done. JR1 cells were transfected with generated plasmids of *TBX5*, and treated with either 10 µL of thalidomide (20mg/mL) or equivalent amount of DMSO. Immunostaining of transfected cells showed that TBX5 proteins are localized in the nucleus in the presence and absence of thalidomide. Thus, thalidomide did not affect the entry of the transcription factor TBX5 to the nucleus or its export to the cytoplasm.

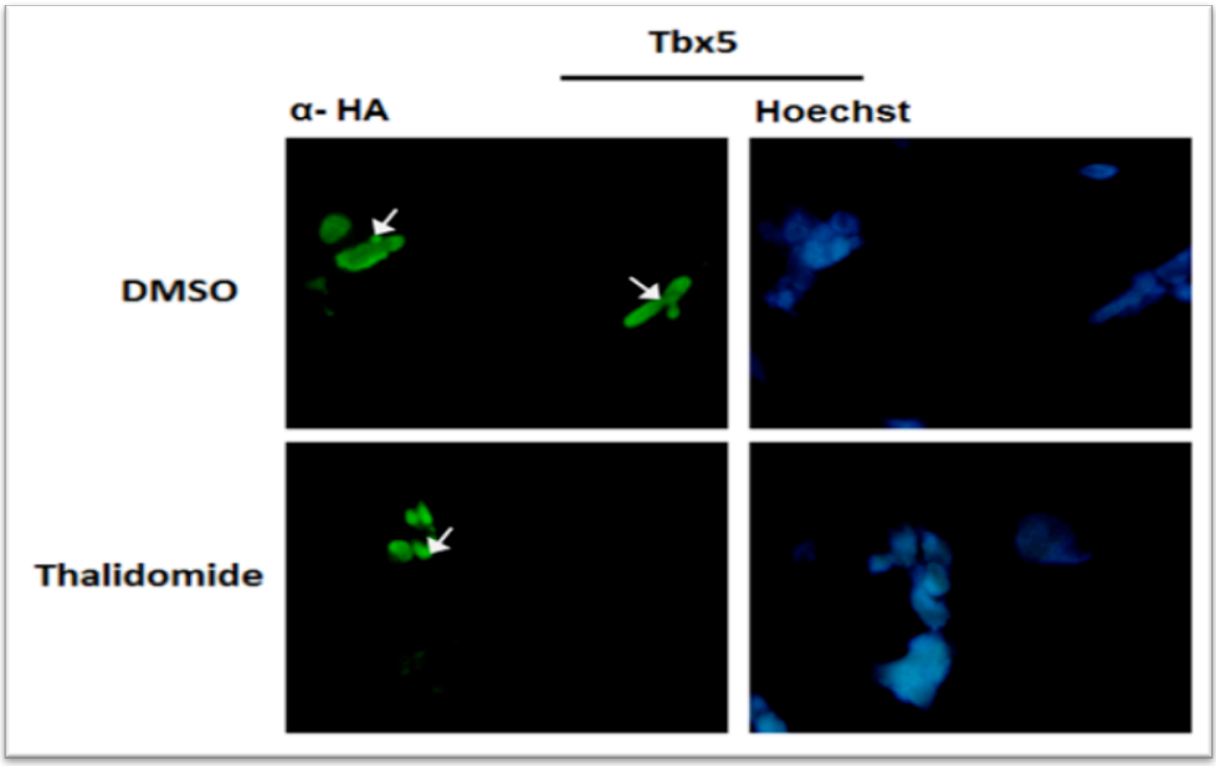


Figure-15: Cellular localization of TBX5 protein in JR1 cells by immunofluorescence. Cells were transfected with TBX5 and treated with 10µL DMSO (as a control) and 10 µL thalidomide (20mg/ml). In the right panel, green indicates the localization of TBX5. In the left panel, blue denotes the staining of the nucleus by Hoechst staining. Images were taken with a fluorescence microscope with a 20x magnification**.**

E. Thalidomide suppressed the functional activity of TBX5

1- Regulation of ANF promoter

The (-700) *ANF* promoter cloned upstream luciferase reporter PXP2 plasmid was used (ANF/Luc). In order to assess the effect of Wt Tbx5 alone on the expression of *ANF* promoter or in the presence of thalidomide, HEK293 cells were transfected with 6 µg of (ANF/Luc) per well and an increasing concentration of the TBX5. After cell lysis, luciferase activity was assayed. Transfections with 200 ng, to 1800 ng of *TBX5* were able to

activate *ANF* promoter by 1 to 17 folds respectively. 10µL of thalidomide (20 mg/mL) suppressed the activity by 8-9 folds compared to 10µL of DMSO at 1800ng of *TBX5.* (Figure-16)

2- Regulation of VEGF promoter

Vascular endothelial growth factor (*VEGF)* is a potent regulatory target for TBX5. VEGF promoter cloned upstream the luciferase reporter plasmid and used (VEGF/Luc). In order to assess the effect of thalidomide on TBX5 gene activation, 10 µL of thalidomide (20mg/mL) and equivalent amount of DMSO was added per well to the different increasing concentration of TBX5. HEK293 cells were transfected with 1.5µg of (VEGF/Luc) per well, and an increasing concentration of TBX5 from 500ng to 1800ng. After cell lysis, luciferase activity was assayed showing that TBX5 was able to activate VEGF promoter gradually reaching up to 9 fold activation. DMSO minimally suppressed this activity, while 10 µL of thalidomide (20mg/mL) suppressed the TBX5 activity by 40% compared with DMSO at the 1800ng concentration of TBX5. (Figure-17)

3- Regulation of VEGF promoter by the combinatorial interaction of TBX5 and GATA4

In order to investigate the effect thalidomide on the functional interaction between TBX5 and GATA4, HEK293 cells were transfected with 2µg of (VEGF/Luc) per well and an increasing concentration of GATA4 (200ng and 400ng) with or without

TBX5 at a concentration of 200ng. After cell lysis was done, Luciferase activity was assayed. 5.5 and 7.5 fold activation was revealed when 200 ng of TBX5 was co-transfected with 200 ng and 400 ng GATA4 respectively. This activation at the same doses decreased to the half when cells were treated with 10µl of DMSO or with 10 µL of thalidomide (20mg/mL). This data suggests that thalidomide will not affect the synergy between TBX5 and GATA4, since the suppression is identical to that of DMSO (Figure-18)

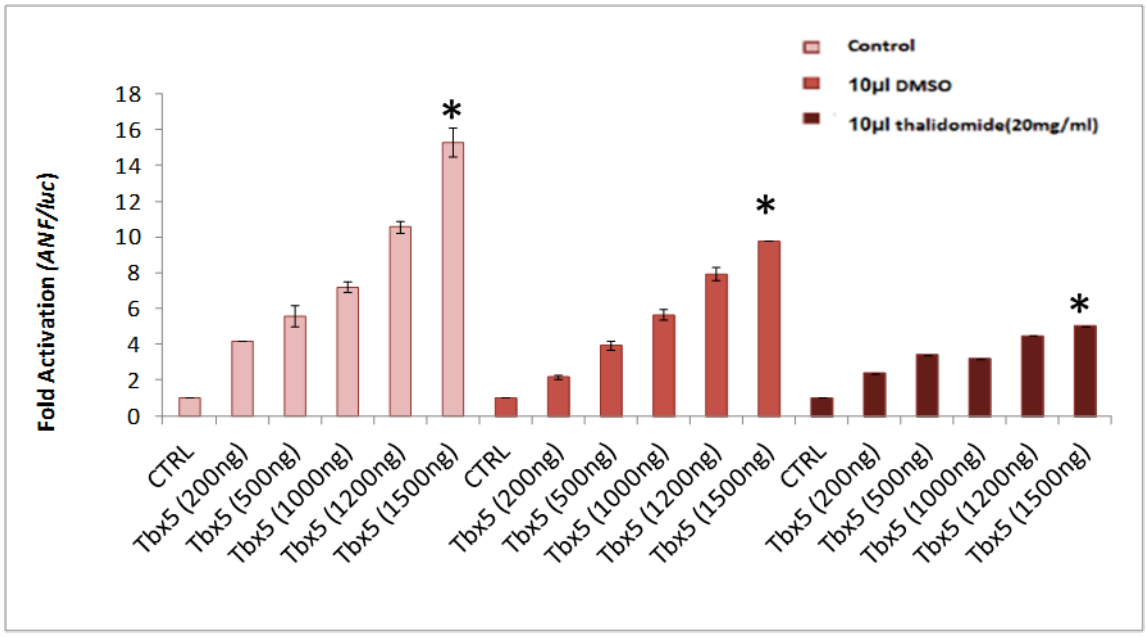


Figure-16: The transcriptional regulation of TBX5 on *ANF* promoter (ANF/Luc) in HEK293 cells in the presence of DMSO or thalidomide: HEK293 cells were transfected with increasing doses of TBX5. At different doses, TBX5 alone activated the *ANF* promoter reaching 17 fold of activation at 1800ng, this activation is supressed by 4 and 12 folds by 10 µL of DMSO or 10 µL of thalidomide (20mg/mL) respectively. The results are presented as fold activation and the values are Mean±SD, $p<0.000$; Paired t-test.

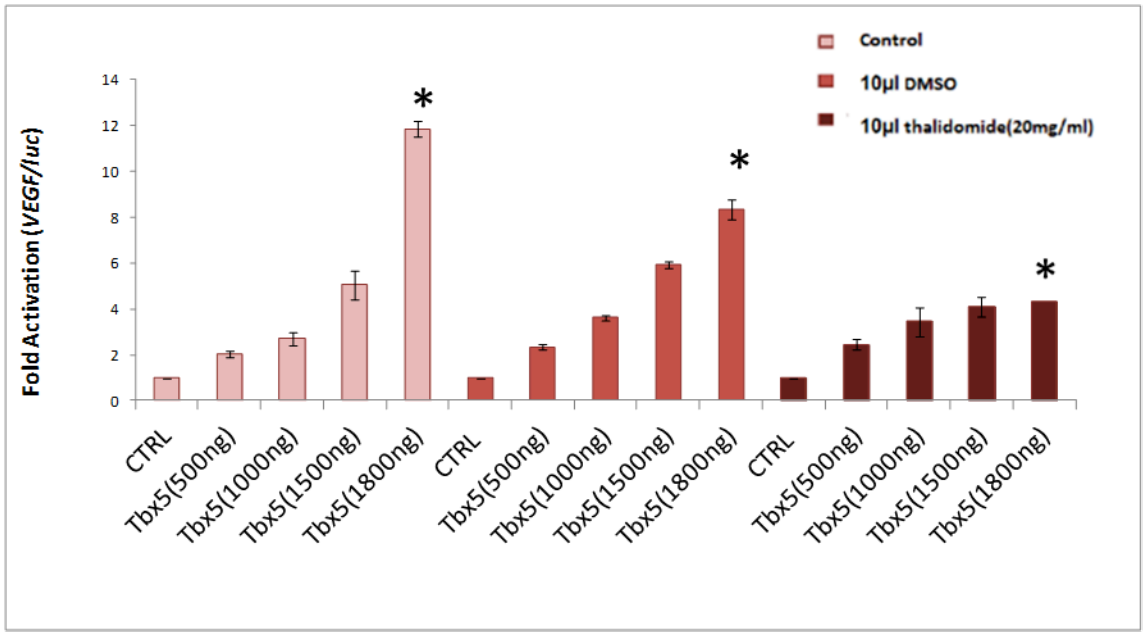


Figure-17: The effect of thalidomide on the transcriptional regulation of TBX5 on *VEGF* promoter in HEK293 cells. HEK293 cells were transfected with increasing doses of TBX5.Three different conditions were conducted, first with no treatment, second with 10µl of DMSO, and third with 10µl of thalidomide(20mg/ml) per well.TBX5 could activate VEGF promoter in a dose dependent mannar reaching 13 folds of activation. This activation is supressed by 3 and 9 folds by 10 µL of DMSO or 10 µL of thalidomide (20mg/mL) respectively.. The results are presented as fold activation and the values are Mean±SD, $p<0.000$; Paired t-test.

To confirm that the results obtained in the luciferase assay were not due to celular death caused by this drug, we performed the MTT assay to check its effect on the HEK293 cells. Thalidomide did not cause significant reduction in HEK293 proliferation; 5µL and 10 µL of thalidomide (20mg/mL) reduced cellular proliferation by 7% and 15% respectively. Similar results were obtained with equvilant amount of

DMSO, by which 5µL and 10µL caused 6% and 11% reduction in the cellular prolefiration (Figure -19).

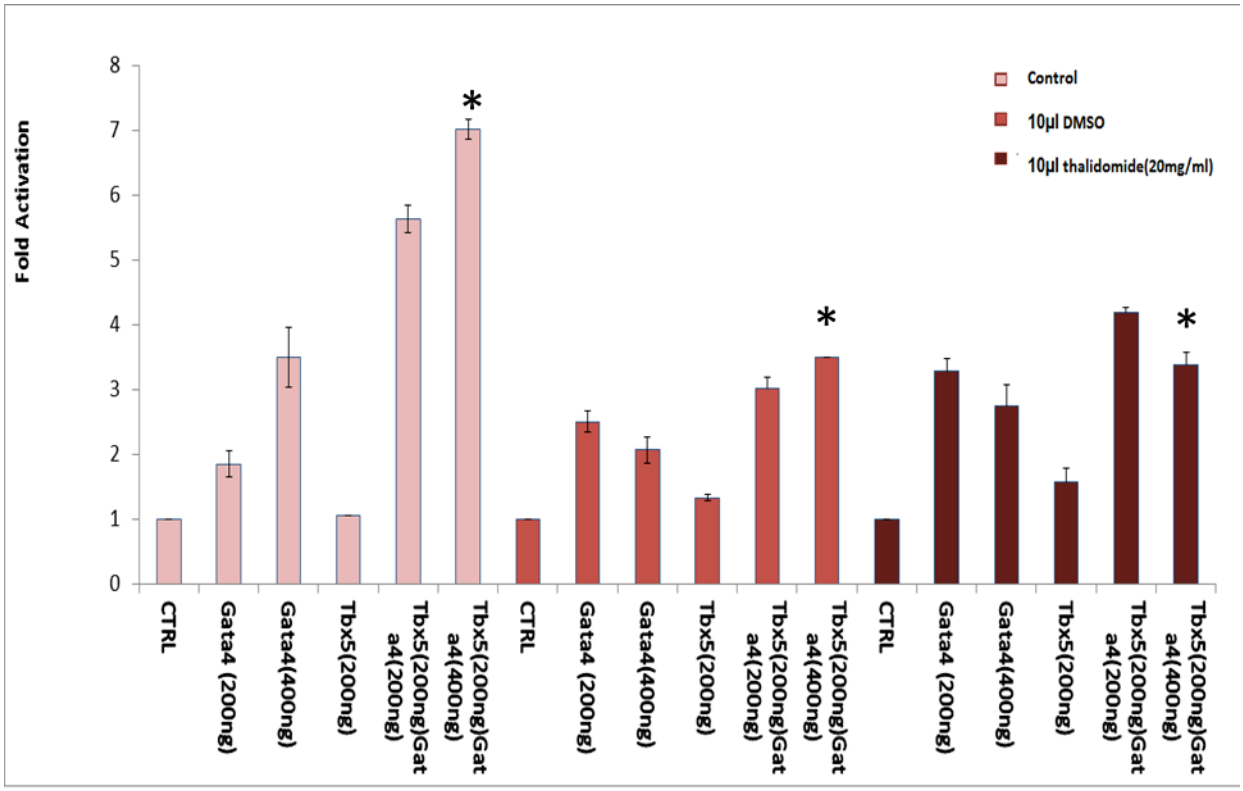


Figure-18: The effect of thalidomide on combinatorial interaction of TBX5 and GATA4. HEK293 cells were transfected with two different doses of GATA4 (200 ng and 400 ng), TBX5 (200ng), and a combination of both. GATA4 could activate transcription in dose dependent manner. Co-transfection of 400 ng GATA4 and 200 ng TBX5 synergistically activated the promoter by 7.5 folds. However, in the presence of DMSO or thalidomide this activation was decreased to its half. The results are presented as fold activation and the values are Mean±SD, $p<0.000$; Paired t-test.

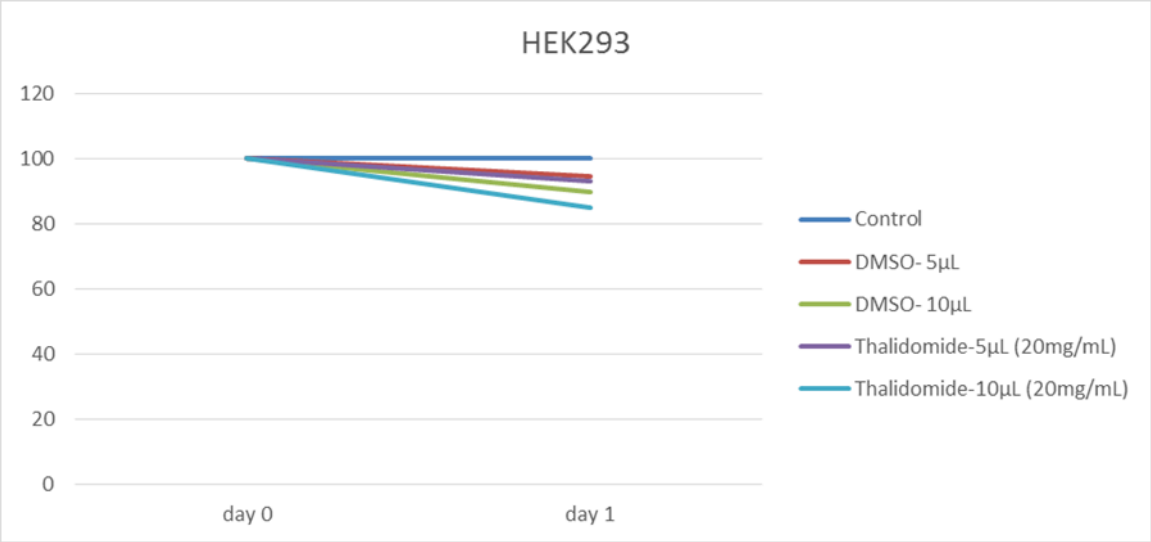


Figure-19: **Effect of thalidomide on cell viability of Hek293 cells determined by MTT assay.** Percentage of cell viability as determined by MTT assay show percentage of absorbance reading from treated cells vs. untreated cells from triplicate wells. Cells were treated for 24 h at 37˚C with various amounts of thalidomide (20mg/mL) or dimethyl sulfoxide (DMSO). After incubating for 24 hours the cells were subjected to the MTT assay. Data are presented as the mean ± S.D. ($p<0.005$)

F. Effect of Thalidomide on Protein-Protein Interaction

TBX5 can interact with other cardiac transcription factors such as GATA4 for synergistic activation of target gene. In order to assess the effect of thalidomide on the physical interaction between these two transcriptional factors, co - immunoprecipitation (CoIPs) assay was done. For this aim, the expression vectors encoding GATA4 (flag-tagged), and TBX5 (HA-tagged), were transiently transfected in HEK293 cells. Nuclear extractions and western blots were done to retrieve the target proteins.(Figure-20) Six times the quantity of proteins loaded for western blot were used for immunoprecipitation. TBX5 proteins were immunoprecipitated with HA antibody in the presence of the same amount of GATA4 proteins. Western blot was carried out using

anti-Flag antibody. Membrane stripping and subsequent western blot analysis was performed with HA- antibody in order to detect TBX5 proteins. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD).The results obtained showed a significant physical interaction between these two transcription factors as expected. This interaction was reduced slightly in the presence of either 10 µL DMSO or, 10 µL of thalidomide (20mg/mL) suggesting that this suppression is not due to thalidomide. Thus thalidomide will not affect the interaction between TBX5 and its partner GATA4.

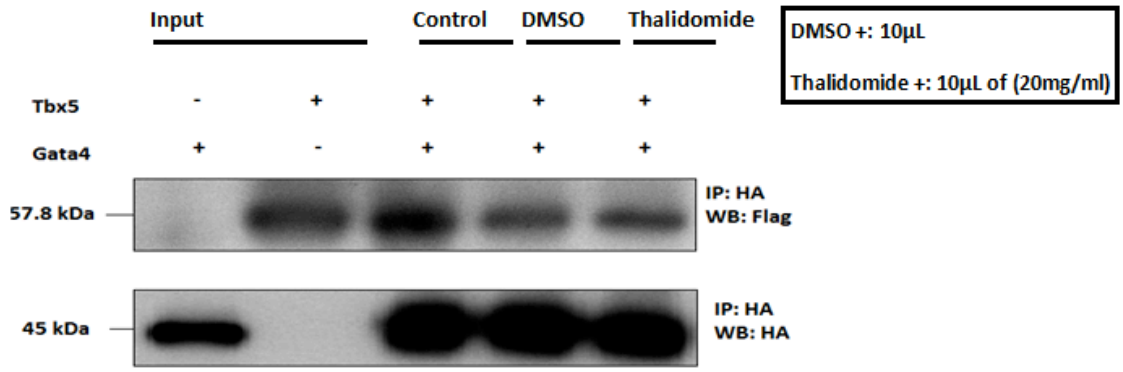


Figure-20: The physical interaction between TBX5 and GATA4 in the presence of thalidomide. Co-immunoprecipitation showed a strong interaction between TBX5 and GATA4 that was not affected significantly by the presence of 10 µL DMSO or thalidomide (20mg/ml).

G. Effect of thalidomide on JR1 cells' proliferation

To assess the effect of thalidomide on JR1 cells we used 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cleavage assay.

Thalidomide reduced JR1 proliferation in a concentration-dependent manner compared with the untreated condition and vehicle control (5-10µL DMSO). 5µL and 10 µL of thalidomide (20mg/mL) reduced JR1 cell proliferation by 24% and 42% after 24hrs of the treatment respectively. While DMSO reduced cell proliferation by 8% and 12% with 5µL and 10 µL respectively.

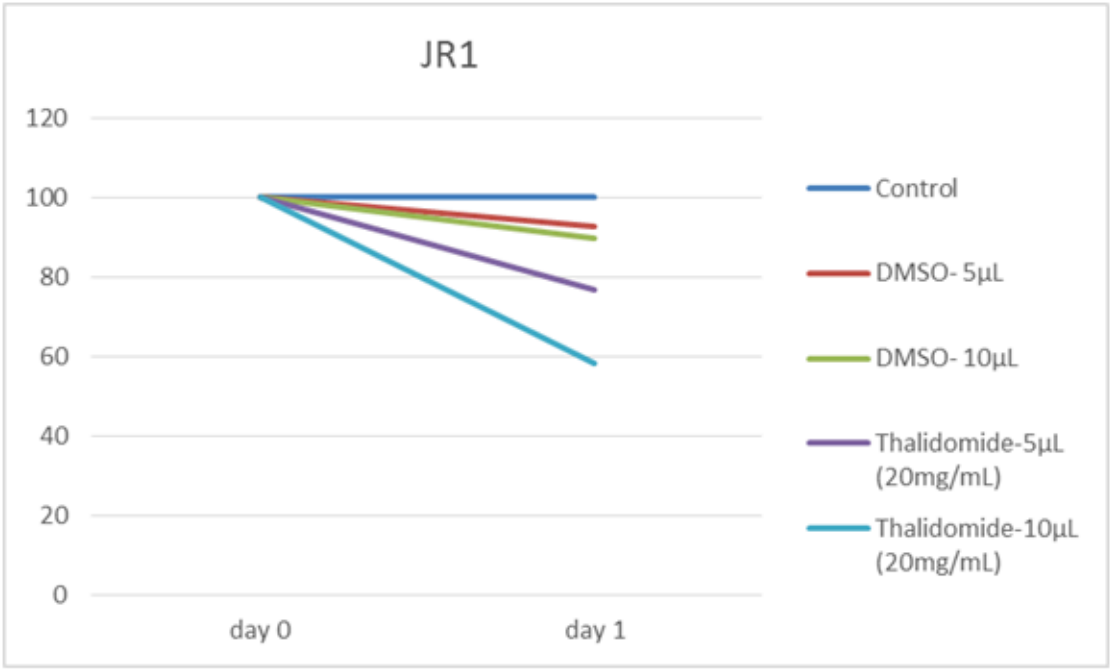


Figure-21: Effect of thalidomide on cell viability of JR1 cells determined by MTT assay. Percentage of cell viability as determined by MTT assay show percentage of absorbance reading from treated cells vs. untreated cells from triplicate wells. Cells were treated with thalidomide for 24 h at 37˚C with various amounts of thalidomide (20mg/mL) or dimethyl sulfoxide (DMSO). After incubating for 24 hours the cells were subjected to the MTT assay. Data are presented as the mean ± S.D. ($p<0.005$)

H. Thalidomide effect on Jr1 cells' migration

The wound-healing model was used to evaluate the migration potential of JR1 cells in the presence of thalidomide. Three different conditions were assesd; wells with no treatment, wells with 10 DMSOµl and wells with 10 µl thalidomde (20mg/ml) . After 2 and 4hrs from the treatment there was no sign of migration in the three different conditions. While after 24hrs we can only detect the wound in the presence of thalidomide, while in the other two wells (control and DMSO) there was approximate 100% wound healing due to cellular migration.

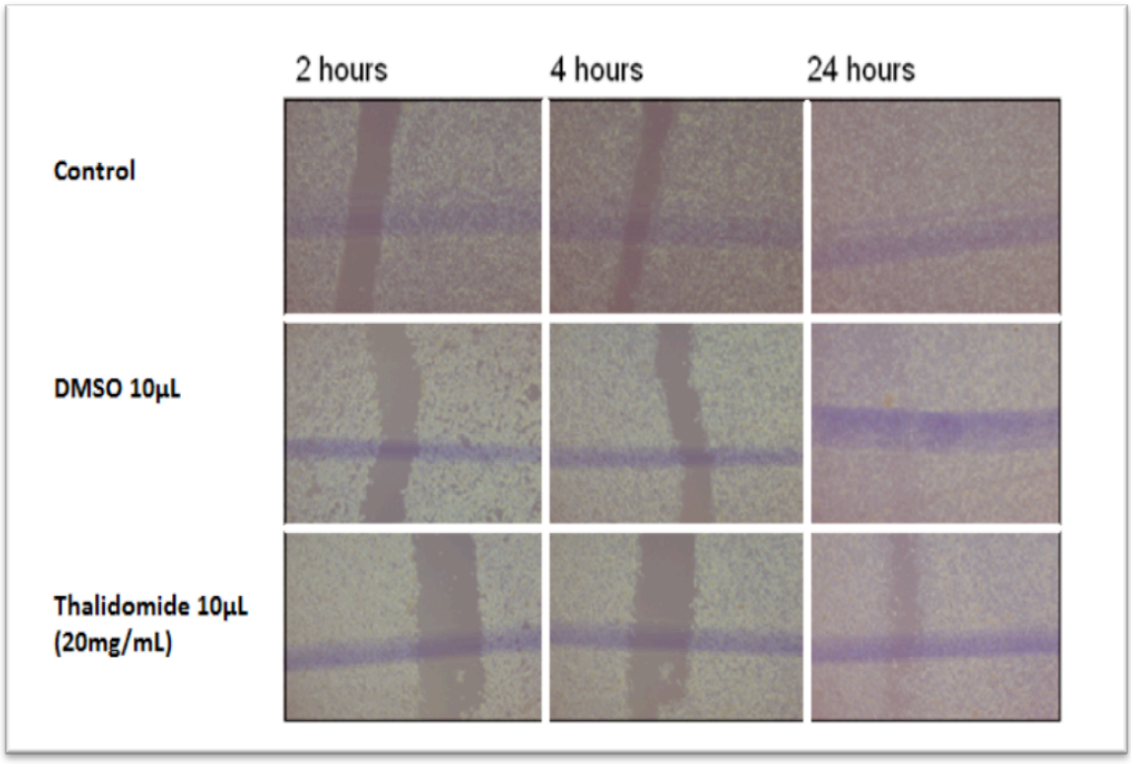


Figure-22: Thalidomide reduces cell migration with respect to the experimental control consisting of only DMSO in a wound-healing assay on JR1 cells. Cells were damaged by mechanical scraping using pipette tip**.** Representative image for the reduction of JR1 cell migration by 10µL thalidomide (20mg/ml) compared with same

amount of DMSO.. Representative monolayer images of cell migration in the wound scrape model at 2, 4 and 24 h are shown. (Magnification, ×10)

I. Effect of thalidomide on the endogenous expression of *TBX5* in JR1 cells

In order to investigate the effect of thalidomide on the gene expression pattern of *TBX5* gene in embryonal rhabdomyosarcoma, JR1 cells were plated in 100 mm culture dishes and when they became 70% confluent they were treated with either 100µl DMSO or 100µl thalidomide (20mg/ml). When cells reach 95% confluency, RNA was collected then reverse transcribed into cDNA. The presence of cDNA was first detected by amplifying the house keeping gene, 18S. All samples showed approximately the same intensity for the amplified band.

TBX5 gene expression was assessed by PCR using *TBX5* cDNA primers. The expected size of *TBX5* cDNA is 328 bp. Upon electrophoresis, *TBX5* cDNA band appeared to be similar in cells treated with thalidomide or its equivalent amount of DMSO. However the bands were slightly upregulated in both conditions if we compared them to the control (untreated cells). (Figure 23).

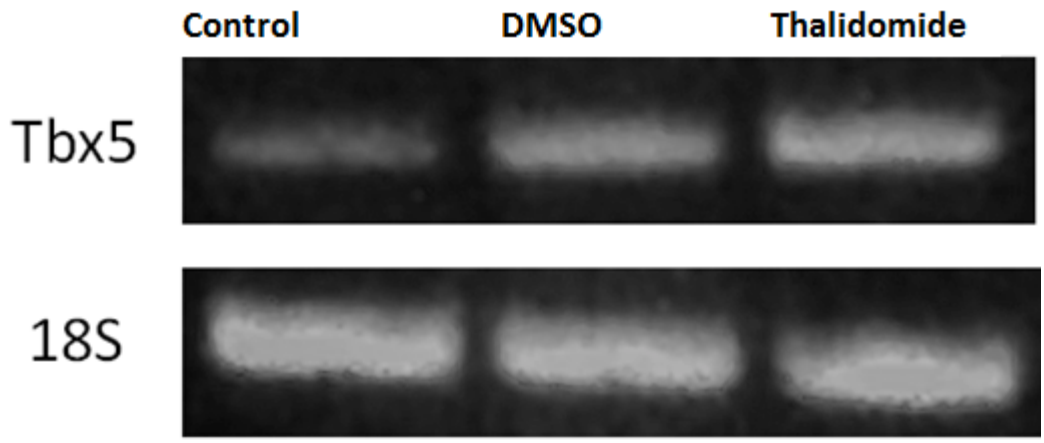


Figure-23: The expression of *TBX5* in JR1 cells: Tbx5 bands (328 bp) after migration on agarose gel, normalized by the expression of 18S. First band represent the expresion of *TBX5* in control wells, second band represent DMSO treated wells and the third for cells treated with thalidomide.

CHAPTER IV

DISCUSSION

A. The direct interaction between thalidomide and TBX5

Understanding the exact mechanism of action of thalidomide is essential since this drug and its derivatives are currently being used in several treatments. Nevertheless, even with its diverse valuable properties the usage of thalidomide in human remains limited due to its teratogenic side effects. This drug caused a tragedy between 1958 and 1960 affecting more than 10,000 children worldwide. Wide range of deformities was detected in those babies, but the most striking features were the limb malformations and the congenital heart diseases. These features were very similar to those presented in patients with Holt Oram syndrome, which is a dominant autosomal disease caused by mutation in *TBX5* resulting in upper limb malformations and cardiac septation defects.

Figure-24: Similar features detected in babies with HOS and thalidomide toxicity.(Wikipedia)

These mutations can lead to nonfunctional TBX5 proteins that lack an active DNA binding domain or for the total absence of TBX5 protein. TBX5 belong to a family of transcriptional factors, and it plays an essential role during early development. Its expression in the heart and the limb during embryogenesis explain the features detected in Holt oram syndrome. This led us to question whether thalidomide acts on TBX5 causing the similar features that were observed in HOS patients.

The in silico prediction tool showed an interaction between thalidomide and 3 amino acids (ARG81, ARG82, and LYS226) on the DNA binding site of TBX5, this interaction might inhibit its binding affinity to the DNA consensus. To confirm this prediction, electro mobility gel shift was done showing similar results. Thalidomide could suppress the binding affinity of TBX5 to its DNS consensus by 35% to 45% depending on its concentration. Since this in-vitro experiment can’t reflect what happen

exactly inside the cell, luciferase assay was conducted. TBX5 could activate both *VEGF* and *ANF* promoter in a dose dependent manner, and this activation was suppressed by 9 and 12 folds respectively in the presence of thalidomide. This data suggested that thalidomide suppressed the binding of TBX5 to it DNA consensus and prevented the transcriptional activity, thus blocking the downstream signaling pathway (Figure-24).Though thalidomide didn't affect the nuclear cellular localization or the stability of TBX5 as the immunoflorescence and western blot analysis showed respectively. By this suppression to TBX5 transcriptional activity, thalidomide mimicked the autosomal dominant mutation detected in HOS patients.

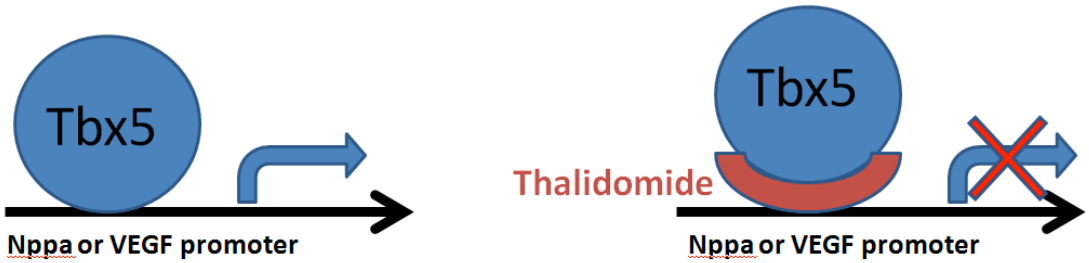


Figure-25: Representation of the mechanism of action of thalidomide. Thalidomide (red structure) interacts directly with specific amino acid in the DNA binding domain of TBX5 (blue structure) and suppressing the activation of both *Nppa* and *VEGF* promoters.

TBX5 interacts with two other transcriptional factors; GATA4 and Nkx2.5 as main partners to regulate the transcription process. To eliminate the possibility that thalidomide interacts with regions responsible for interacting with other transcriptional

factors, co-immunoprecipitation and co-transfection assays were done. The results proved that thalidomide will not inhibit the interaction between TBX5 and GATA4, thus it will not affect the interaction between these cofactors. Further studies should be done to investigate whether thalidomide will affect the interaction of TBX5 with other proteins like Nkx2.5. The luciferase assay showed that thalidomide will not affect the combinatorial interaction between TBX5 and GATA4 on *VEGF* promoter as compared to its equivalent amount of DMSO. One explanation for this is that thalidomide interaction with TBX5 will not prevent the formation of a stable complex between this transcriptional factor and GATA4, which remains suboptimal to promote the transcription of *VEGF* gene.

B. Effect of thalidomide on embryonal rhabdomyosarcoma cells

The necessity to understand thalidomide toxicity is to overcome the limitations in utilizing this drug in patients with tumor-based or inflammatory diseases such as Multiple Myeloma and leprosy. It is also important to use the results obtained to investigate other ways by which thalidomide could be beneficially used. For example; *VEGF* plays an essential role in tumorigenesis which is affected by thalidomide as showed previously. Our results suggest that the effect of thalidomide on cancer cells could be via suppressing the binding affinity of TBX5 to the VEGF promoter. Since our lab has previously showed a novel expression of TBX5 in Jr1 and RH30 (human Alveolar rhabdomyosarcoma) cell lines (Figure-26). Thus we aimed to investigate

whether thalidomide can inhibit proliferation and cellular migration of embryonal rhabdomyosarcoma cell line.

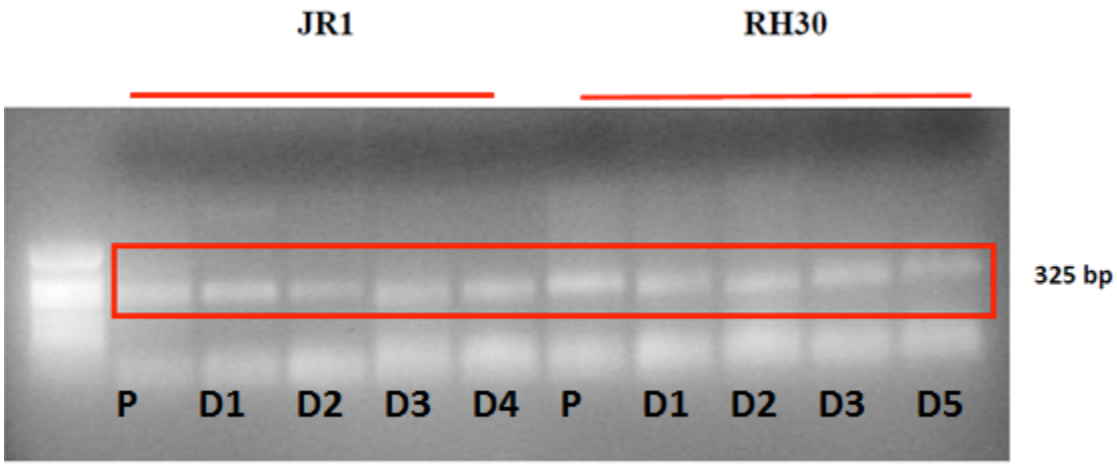


Figure-26: The expression of TBX5: Tbx5 bands (328 bp) after migration on agarose gel. The first five bands are for JR1 from P to d4 and the other five for RH30.

C. Rhabdomyosarcoma (RMS)

Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin thought to arise from cells committed to a skeletal muscle lineage [41]. RMS is the most common soft tissue sarcoma in children and adolescents, accounting for approximately 5% of all pediatric cancers and about one-half of all soft tissue sarcomas[94]. According to the histological classification the two most common RMS are: embryonal (accounts for 75% of RMS cases) and alveolar [41]. Embryonal rhabdomyosarcoma (ERMS) is associated mostly to the head, neck and genitourinary regions. ERMS demonstrates a bimodal age of distribution, frequently diagnosed between 0–5 years while it is less

common in adolescence. On the other hand, Alveolar rhabdomyosarcoma (ARMS) is more likely to be associated with adolescent's tumors [94]. RMS is thought to be mainly sporadic in nature, but some association with genetic syndromes was observed. Examples of familial syndromes that are associated with RMS are neurofibromatosis and the Li-Fraumeni syndrome. Pathologically RMS is classified as small, round, blue-cell tumors of childhood using light microscopy or immunohistochemistry or electron microscopy. Clinically the signs and symptoms are inconstant and they depend on the site of origin of the primary tumor, the patient's age, and if there is metastasis or not. The available treatment for RMS patients is restricted to the classical cancer treatments that include: surgery, radiation therapy, and chemotherapy. The outcome of this combined therapy depends on the stage of the tumor. 70% of non-metastatic tumors can be cured while only 20% of metastatic RMS is currently cured of the disease. These facts encourage researchers to find an antiangiogenic drug that will target the metastatic behavior of RMS.

Although we have numerous ongoing clinical trials, outcomes for high risk RMS have not improved expressively in the last 30 years. Since the diagnosis of such disease is still considered minimal if compared to other tumors. Thus the number and timing of new clinical trials that aim in testing new potential treatment agents is limited. On the in-vitro level there are 18 embryonal and 12 distinct alveolar human RMS cell lines that are being used currently for research purposes. These cell lines differ in their origins, histology, karyotype, and methods of validation. One of the ERMS cell lines that are being used in our lab is JR-1 cells. These cells were derived from a lung

metastasis of a 7-year-old female with a primary uterine mass and they showed poorly differentiated embryonal histology of the biopsy specimen [95].

Since we already proved that thalidomide directly binds to TBX5 and suppresses its binding affinity we chose to assess the effect of this drug on rhabdomyosarcoma cells that endogenously express TBX5. The preliminary results showed significant suppression in cellular proliferation by the MTT assay, as well as suppression in cellular migration revealed by the wound healing assay. The expression of *TBX5* in JR1 cells was not affected by thalidomide as compared with its equivalent of DMSO. More studies should be done to confirm the potential of thalidomide in treating embryonal rhabdomyosarcoma specifically by acting on TBX5 transcriptional properties and not on the mRNA level.

D. Future prospect

As mentioned previously the T-box is the minimal region within the T-box protein that is both necessary and sufficient for sequence specific DNA binding [38].Although there is sequence variations within the T-box between family members, specific residues within this region are completely conserved in all orthologues of a single family member [42]. In addition, the examination of downstream targets and binding-site selection experiments for a number of T-box proteins demonstrated similar DNA consensus sequence TCACACCT by which all members of the family so far examined bind to [38]. Since studies showed that specific residues within the T-box are highly conserved in all members of the family, and we proved that thalidomide is

capable to bind to specific amino acids in the DNA binding region of TBX5; further studies should be done to detect if thalidomide can bind to other members in this family and inhibit their activities.

Analysis showed that TBX5 exit in two main isoforms in mice; TBX5a and TBX5b. TBX5a is the longer isoform that is made up of 518 aa and more prominent in embryonic hearts, while TBX5b is made up of 255 aa and more prominent in adult hearts [96]. The cellular localization also differs between these two isoforms, in which TBX5a is restricted to the nucleus while TBX5b is localized in both the nucleus and the cytoplasm [96].TBX5a can bind to *Nppa* promoter and activate transcription, also it interacts with GATA4 and Nkx2.5 as mentioned above to activate transcription, on the contrary TBX5b cannot activate the *Nppa* promoter or interact with Nkx2.5. Furthermore the luciferase reporter assays showed stronger binding affinity between TBX5a and GATA4 compared with that of TBX5b. Though both isoforms play critical role in heart development, by which TBX5a is more prominent in proliferative developing cells and in regulating cardiomyocyte growth while TBX5b is more prominent in terminally differentiated cells and regulating cardiomyocyte growth arrest. Noted that our work was done by using TBX5a isoform, it might be interesting also to investigate whether TBX5b could activate *VEGF* promotor, or if thalidomide can interact with this isoform affecting its transcriptional properties.

Many mutations were detected in DNA binding region of TBX5, by which they caused suppression of binding affinity to the T-box binding element (TBE). Among the mutations that caused HOS are G80R and R237Q mutants located within the T-box

domain by which TBX5 failed to bind DNA containing a palindromic T-box binding site. It would be of great importance to assess the effect of these mutations on the binding affinity of TBX5 and on its transcriptional activity as well, comparing the results with those obtained due to thalidomide interaction on TBX5 binding site. Especially that these mutations are very close to the amino acids implicated in the interaction with thalidomide. Thus a similar effect of thalidomide on TBX5 could be another sold evidence of its mechanism of action. Furthermore, generating TBX5 with mutations on one or the three amino acids implicated in thalidomide interaction by side directed mutagenesis should be done. If thalidomide interact exclusively with the amino acids revealed by the in-silico prediction tool it should not be able to bind to the mutated TBX5, thus not affecting its transcriptional activity. Also truncation of the C-terminal and/or N-terminal of TBX5, and detecting whether thalidomide will still show the same results will confirm its ability to bind exclusively on the T-box region.

Using a drug that suppresses embryonal rhabdomyosarcoma proliferation and angiogenesis by acting on TBX5 would be of great importance. Since TBX5del/+ mice exhibit forelimb and congenital heart malformations thus the main role of TBX5 is during embryogenesis and its expression in other tissues (lungs, eyes ….) is minimal. Therefore having a drug that targets TBX5 specifically in embryonal rhabdomyosarcoma to suppress proliferation will be considered as a directed targeted therapy. More studies should be done on other tissues that express TBX5 with no known altered function in HOS patients, to rule out any toxic effect of thalidomide on them.

Another study on zebrafish revealed that deficiency of TBX5 activates multiple signal pathways that increased the rate of apoptosis and ceased the cell growth in the head, heart, fins, and trunk [97]. This conclusion was based on studying several apoptosis related genes (bcl-2, bad, bax, p27 and p57) and apoptosis related proteins (CASPASE-3, CASPASE-8, and BAD). Furthermore, TBX5 knockdown embryos showed a high ADP/ATP which is an indication of apoptosis. Thus thalidomide might cause similar features in embryonal rhabdomyosarcoma cells by binding to TBX5 and down-regulating its transcriptional activity

Further studies should be done to determine the exact effect of thalidomide on embryonal rhabdomyosarcoma cells (ERMS). Since our results showed a possible effect of thalidomide on ERMS cell viability and cellular migration, understanding the mechanism of action is of a great importance. Increased survival of the normal cells depends on VEGF expression. Rise of VEGF will cause an increase in the expression of cell cycle related proteins and thus promote transition from G1 phase to the S phase [98]. In tumor cells VEGF is mainly produced to induce the formation of new vessels toward the tumor site to avoid hypoxia. Therefore VEGF production by tumor is a specific and critical regulator of the angiogenic signaling cascade .Our study revealed that TBX5 regulate *VEGF* promoter, and this activation is suppressed by the presence of thalidomide. Inhibition or suppression of VEGF protein production from tumor cells will result in the regression of the tumor growth. This fact causes the introduction of many drugs that target VEGF specifically. Neutralizing antibodies or introducing dominant negative VEGF receptors into endothelial cells of tumor-associated blood

vessels are being investigated as targeted therapies in treating cancer [99]. So it is important for us to assess whether thalidomide can suppress VEGF transcription by binding to TBX5 in JR1 cells. This could be done by investigating the VEGF expression in JR1 cells pre-treated with thalidomide. On the other hand, if TBX5 is the major player in promoting tumorigenesis in JR1 cells, then overexpressing TBX5 in these cells might be a protective mechanism against thalidomide. Measuring the effect of thalidomide on cellular proliferation and death in JR1 cells that overexpress TBX5, and comparing the results with normal JR1 cells would help in understanding how thalidomide is affecting these cells.

Pomalidomide is a thalidomide derivative that gained the FDA approval on february 8, 2013, for the treatment of specific MM patients. Pomalidomide is constructed by adding an amino group to the fourth carbon of the phthaloyl ring of thalidomide, this extra amino group increased potency of both anti-inflammatory and antiangiogenic properties with reduced toxicities [33]. Yet and the precise mechanism of each corresponding effect to its anti-MM activity remains uncertain. Thus it is important to study whether the addition of this amino group will affect the binding affinity of TBX5.

D. Conclusion

Our study revealed two faces of thalidomide; one related to its teratogenic mechanism of action and a second one related to its benefits in cancer treatment. These results were the first to show that thalidomide bind specifically to TBX5 on its DNA

binding domain suppressing its binding affinity and affecting it transcriptional properties. By this mechanism thalidomide mimicked the autosomal dominant mutation detected in HOS patients. Also no previous studies were done to assess the effect of thalidomide on embryonal rhabdomyosarcoma cells, so we were the first to show its antiproliferative and antiangiogenic effect on JR1 cells and eventually in treating this type of cancer.

It is important to develop strategies to specifically intervene with the toxic activity of thalidomide, while keeping it beneficial properties. This information would also be useful to study whether the newest thalidomide derivative "pomathalidomide" is still holding the positive and beneficial effect of this drug while taking away its side effects.

E. Limitations and drawbacks

Scientific research is always affected by limitations and problems, by which we encountered some of these drawbacks throughout our project. The transfections carried out in this project were done in HEK293 and JR1 cells, to study the transcriptional regulation and functional interaction of TBX5 and GATA4. But the efficiency of transfection varied between the experiments and it didn't exceed 60% in HEK293 cells and 40% in JR1 cells.

Another limitation is that we have assessed the effect of thalidomide on TBX5 physical and functional properties in an in vitro model. The interaction between this molecule and TBX5 was represented outside the cells using the in silico prediction tool

and the EMSA assay, and its suppression to the transcriptional activity was conducted in HEK293 cells. Thus we do not know yet the effect of this interaction in vivo especially that the machinery presented in HEK cells is absolutely different from what is actually present in that of cardiomyocytes.

In vivo models are also essential to validate our hypothetical model. But the main problem is that thalidomide does not disturb the embryonic development of mice and rats, that made research conducted on thalidomide be held in chick's embryos. Moreover, the fact that thalidomide does not induce phocomelia in chicks (but in humans) establishes the limitation of this model organism. Thus, this significant species specificity marks thalidomide research as a complicated field.

Due to the shortage in the radioactive γ-ATP, we didn’t detect the effect of higher doses of thalidomide on TBX5 binding affinity. Though higher doses of thalidomide should be used. Higher doses might cause more suppression or might inhibit the binding affinity to this protein.

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