Transcriptional regulation of human thrombopoietin (TPO)-characterization of a novel gene repressor and PF4-mediated suppression

Author:
Liu, Shengyi

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Transcriptional Regulation of Human Thrombopoietin (TPO)-Characterization of a Novel Gene Repressor and PF4-mediated Suppression

Shengyi Liu

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Department of Medicine,

St. George Clinical School,

Faculty of Medicine,

University of New South Wales

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Abstract

Human thrombopoietin (TPO) is the primary regulator of megakaryopoiesis. It is known that TPO levels in blood are regulated by a mechanism involving its uptake by platelet TPO receptors (c-MPL) and its subsequent intraplatelet destruction. However, this mechanism does not fully explain TPO regulation and there is evidence of an additional regulatory mechanism such as a negative feedback transcriptional mechanism at the level of the bone marrow stroma. This thesis focuses on further investigation of this transcriptional regulation. The aims of the investigation are to: i) characterize the potential repressor(s) of thrombopoietin (TPO) gene transcription; and ii) define the platelet factor 4 (PF4)-mediated pathway that causes suppression of TPO gene expression.

A potential TPO gene repressor, ZEB1, was firstly studied. Promoter analysis showed that there was a small region that greatly reduced TPO promoter activity and this region carries a cluster of four conserved ZEB1 binding motifs. However further analysis revealed that repression of TPO promoter activity did not depend on this ZEB1 cluster, suggesting that ZEB1 is unlikely to be the repressor. This promoter region also contains binding motifs for MZF1. Electrophoretic mobility shift assay (EMSA) showed the binding of MZF1 to the putative binding motif in this region. siRNA knockdown of MZF1 expression reversed the reduced TPO promoter activity. A significant increase of TPO expression was also observed in MZF1 siRNA transfected OP9 cells (surrogate for bone marrow stromal cells). The signaling pathway that mediates PF4-induced repression of TPO expression was then investigated. I found that PF4 is the key factor in the serum that suppresses TPO expression. This suppression is dose-dependent and further experiments revealed that this suppression was through the interaction with LRP1 expressed on OP9 cells. PF4/LRP1 interaction triggered PI3K/Akt signaling pathway with

phosphorylation of Akt. I suggest that PI3K/Akt activation possibly then results in MZF1 activation which then likely migrates into the nucleus and mediates repression of TPO transcription.

In conclusion, the current study identifies a novel intronic transcription repressor (MZF1) of human TPO expression. TPO repression is mediated by PF4 which binds to LRP1, and activates PI3K/Akt signal transduction pathway.

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List of Abbreviations

aa	amino acid
bp, kb	base pairs, kilobase pairs
BSA	bovine serum albumin
°C	degrees celsius
C-/N- terminal	carboxy-/amino- terminal
c-MPL	Myeloproliferative leukemia virus oncogene
Da, kDa	Dalton, kilodalton
DMEM	dulbeco's modified eagle medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid (disodium)
EMSA	electrophoretic mobility shift assay
FBS/FCS	foetal bovine/calf serum
g	acceleration due to gravity (9.8 m/s^2)
h	hour(s)
HSCs	haemotopoietic stem cells
min	minute(s)
LRP1	low density lipoprotein receptor-related protein 1
ng	nanogram
nM	nanomolar
µl, ml, L	microliter, milliliter, litre
µM, mM, M	micromolar, millimolar, molar
mRNA	messenger ribonucleic acid
MK	megakaryocyte
MZF1	myeloid zinc finger binding protein 1
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PF4	platelet factor 4
PI3K/Akt	phosphatidylinositol-3-kinase and protein kinase B
rpm	revolutions per minute
s	second(s)
SDS	sodium dodecyl sulfate
TAE	tris-acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TPO	thrombopoietin
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	unit
ZEB1	zinc finger E-box-binding homeobox 1

Chapter one: Literature Review

Chapter one: Literature Review

1.0 Haematopoiesis

Blood cells such as red cells, leukocytes and platelets play vital biological functions that are important for human survival. Blood cells are produced in the bone marrow by a process, termed haematopoiesis. After their release into the circulation, they survive for a limited period of time, for example platelets survive for an average of 7 days. Blood cells are constantly renewed by their production in the bone marrow, about $1.0 \times 10^{11} \sim 10^{12}$ new cells are produced every day; thus maintaining adequate numbers of different types of blood cells in circulation so that homeostasis is maintained. This is achieved by a tightly controlled system in which a small number of haemotopoietic stem cells (HSCs) give rise fixed numbers of new blood cells by a process of cell differentiation: HSCs differentiate into haematopoietic progenitor cells of different haematopoietic lineages and ultimately into mature blood cells of different types. As shown in **Fig. 1.1**, HSCs give rise to all the blood components such as erythrocytes, megakaryocytes/platelets, lymphocytes, granulocytes and monocytes (Siminovitch et al, 1963). To avoid cell exhaustion, HSCs and haematopoietic progenitor cells undergo proliferation and self-renewal. The self-renewal is achieved by mitosis when HSCs enter the cell cycle, which produces daughter cells. The differentiation allows the maturation of cells, giving rise to different cell lineages (**Fig. 1.1**). Furthermore, the numbers of mature blood cells of different lineages in the circulation are constant within narrow normal ranges, as too few cells or too many cells of any one type could lead to disease or ill health. For example, too few platelets (thrombocytopenia) will lead to bleeding and too many platelets could result in thrombosis. Hence, haematopoiesis is a tightly and precisely regulated process involving growth factors and their receptors (both lineage-specific and multi-lineage), intracellular signaling pathways, transcription factors, post-translation

modifications and other cellular processes. For example, in the case of platelet production, it involves thrombopoietin (TPO), a megakaryocyte-specific growth factor, its receptor (c-MPL), to lesser extent multi-lineage growth factors, signaling pathway involving Jak2 kinase, STAT1 and STAT3, and transcription factors, such as GATA-1, FOG-1, Fli-1 and NFE2.

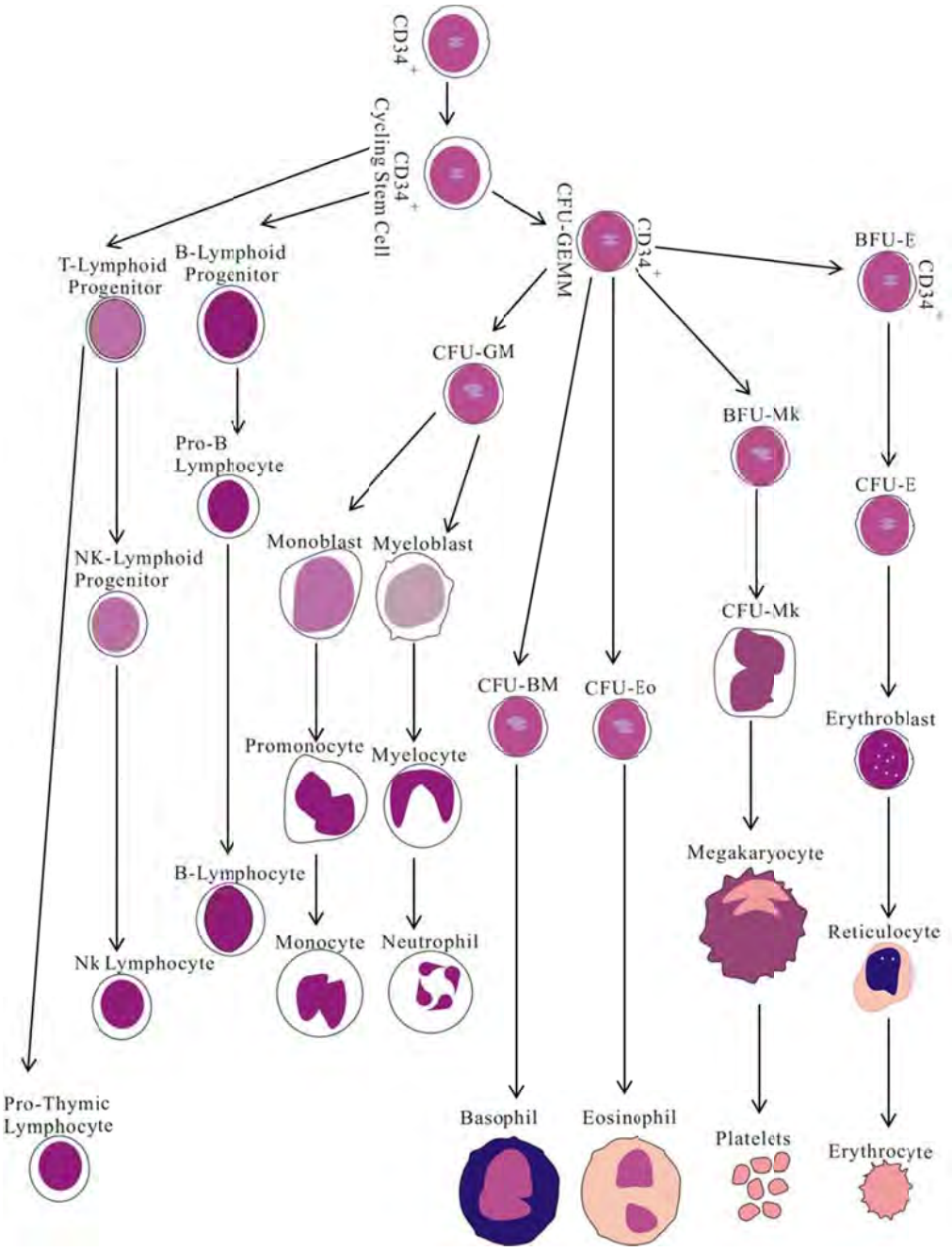


Figure 1.1 The haematopoiesis process. *Figure shows all the blood mature cells developed from haematopoietic stem cells.*

1.1 Megakaryopoiesis and thrombopoiesis

As described above, HSCs either undergo self-renewal or differentiate to a lineage -specific progenitor cells and then finally to mature blood cells of that lineage. In megakaryopoiesis, HSCs go through several stages of maturation before they become mature megakaryocytes (MKs) which are capable of producing proplatelets and platelets. These stages of MK maturation include the pluripotential haematopoietic progenitors, the committed myeloid progenitor cells, CFU-GEMM (colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte), the bipotential erythroid/megakaryocytic progenitors, the megakaryocyte burst-forming cells (BFU-Meg) which have a high proliferative capacity, the colony-forming cells -Meg (CFU-Meg), megakaryoblasts, promegakaryocytes and megakaryocytes (**Fig. 1.2**). Megakaryocyte differentiation is regulated by transcription factors and cytokine signalling. Transcription factors, including Runt-related transcription factor 1 (RUNX1), GATA-1, FOG-1, Fli-1 and NFE2, act both to promote differentiation into certain lineages and to prevent differentiation into other lineages. For instance, GATA-1 works with its co-factor FOG-1 in promoting erythroid and megakaryocyte differentiation (Tsang et al, 1997) while suppressing PU.1 and myeloid differentiation (Chou et al, 2009). Fli-1 up-regulates the GATA-1 activity which promotes maturation of megakaryocytic lineage (Athanasiou et al, 1996), while represses erythroid activity thus inhibiting maturation of the erythroid lineage (Starck et al, 2003). MicroRNAs (miRNAs) miR-150 mediates post-transcriptional modifications and drives megakaryocyte-erythrocyte progenitors (MEPs) differentiation toward megakaryocytes (Lu et al, 2008). The major growth factor that regulates MK differentiation and proliferation is thrombopoietin (TPO). As indicated in the **Fig 1.2**, other growth factors/cytokines (and their receptors) such as stem cell factor (SCF), interleukin(IL)-3, IL-6 and IL-11 are also involved but to a much lesser extent. Mature MKs produce elongated pseudopodia-like structures

called proplatelets, the budding of which gives rise to platelets.

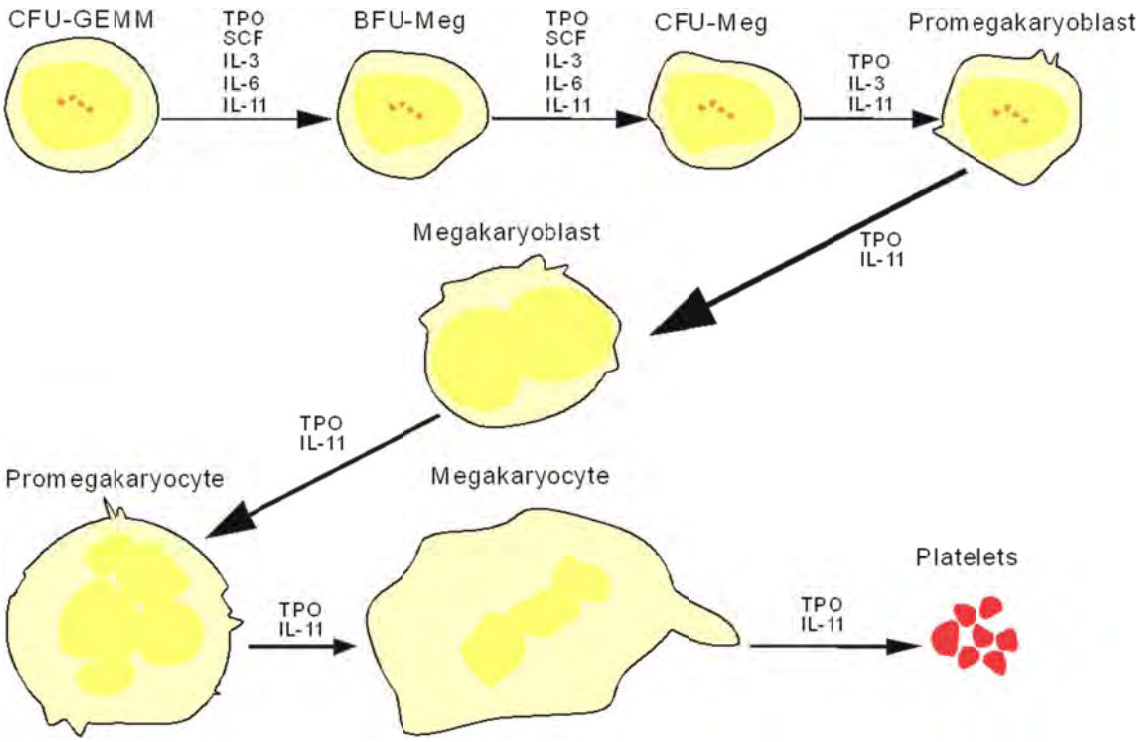


Figure 1.2 The megakaryopoiesis process. *Figure shows the formation of megakaryocytes and platelets. Various growth factors/cytokines mediate every stage of the process among which TPO is the primary regulator.*

1.2 Thrombopoietin (TPO)

1.2.1 Discovery of TPO

Several groups of investigators tried to characterize the c-Mpl (Myeloproliferative leukemia virus oncogene) ligand during the 1990s. de Sauvage (1994) and his colleagues purified Mpl ligand (ML) from aplastic porcine plasma and found that the ML could stimulate *in vitro* megakaryopoiesis. Further *in vivo* investigations showed expression and activation of recombinant ML significantly simulated platelet production. They cloned the ligand after performing N- terminal protein sequencing followed by

PCR from porcine genomic DNA using a pool of primers (de Sauvage et al, 1994). Similarly, Bartley's group purified Mpl ligand, which they called megakaryocyte growth and development factor (MGDF) from aplastic canine plasma and found that the purified MGDF supported the development of megakaryocytes from human CD34+ progenitor cells *in vitro* (Bartley et al, 1994). In another study, Kenneth Kaushansky's group expressed recombinant Mpl ligand and found that it could stimulate *in vitro* megakaryocyte colony formation and increase megakaryocyte size, polyploidization and expression of differentiation makers. Their *in vivo* investigations showed that the injection of Mpl ligand significantly stimulated both development of megakaryocytes and their progenitor cells and platelet production. Based on these observations, Kaushansky and his colleagues suggested that Mpl ligand is the primary regulator of megakaryocyte development, and proposed that the Mpl ligand be termed thrombopoietin or TPO in short (Kaushansky et al, 1994).

1.2.2 Genomic Structure of TPO

In the 1990s, 4 groups reported the human TPO genomic structure independently. Foster et al (1994), Sohma et al (1994), and Gurney et al (1995a) suggested that the human TPO gene spans about 6 kb and consists of 6 exons and 5 introns. On the other hand, Chang and co-workers concluded that the human TPO gene contains 7 exons and 6 introns spanning 8 kb (Chang et al, 1995).

The human TPO gene was mapped to chromosome 3q26-27 and interestingly, a number of chromosomal abnormalities associated with thrombocythemia are found with this region (Pintado et al, 1985; Bernstein et al, 1986; Bouscary et al, 1995). The analysis of TPO cDNA sequence revealed that the encoded product, a polypeptide of 353 amino acids (including a 21-aa signal peptide), consists of a 153-aa N-terminal domain with a high homology to EPO and a 179-aa

C-terminal domain. Studies of the coding region of the TPO gene showed that the intron/exon boundaries are homologous with those of the EPO gene (McDonald et al, 1986; Shoemaker and Mitsock, 1986; Jacobs et al, 1985). Base on this, Fosters et al (1994) proposed that TPO and EPO might have originated from a common ancestral sequence by gene duplication. However, the C-terminal domain of TPO does not bear any similarity to EPO. The possible reason for this, Fosters further proposed, might be due to an insertion into this region of the TPO gene or a deletion in the EPO gene from the ancestral TPO/EPO gene (Foster et al, 1994).

Gurney and his colleagues (1995a) identified a second cDNA variant of TPO, termed TPO-2, which has a deletion of 4 amino acids (LPPQ, residues 112 to 115), however, quantitation of the expression of this variant showed that it was down-regulated 500 times compared to the full-length TPO message. Moreover, this TPO-2 protein did not exhibit biological activity and was unable to support proliferation of Ba/F3-mpl cells (Gurney et al, 1995a). In contrast, a similar splice variant of colony-stimulating factor (G-CSF) containing a 3-aa deletion exhibited full biological activity (Nagata et al, 1986). In a study of erythropoietin, a 5-aa deletion (from residues 122 to 126) was introduced and such deletion was found to alter the conformation of EPO protein and resulted in inhibition of secretion (Boissel et al, 1993). It is likely that the similar deletion observed in the TPO-2 variant might have also induced a conformation change that caused a significant reduced expression of its message and protein, and possibly also loss of its activity. Further investigation in three species (human, pig and mouse) showed a relative abundance of the TPO-2 mRNA compared to TPO mRNA indicating that TPO-2 might play a role in the regulation of TPO expression: TPO expression might be regulated by altering the relative amount of the full length TPO and the TPO-2 (Gurney et al, 1995a). Similarly, in Chang's study, the same variant with 4-aa (LPPQ) deletion was identified, together with another TPO variant produced by an internal splicing

of exon 7 that resulted in an altered reading frame (Chang et al, 1995). These two variants were found to be expressed but exhibited no biological activity.

1.2.3 Characteristics of TPO

Molecular cloning of human TPO showed it consists of 1,774 nucleotides followed by a poly $(A)^+$ tail. The encoded polypeptide is a predicted primary translation product of 353 amino acids. The N-terminal domain is highly hydrophobic which possibly functions as a signal peptide, and a potential cleavage site found at residues 21 indicates a mature product of 332 amino acids (de Sauvage et al, 1994; Lok et al, 1994; von Heijne, 1983). Cloning studies by different groups of investigators revealed various sizes of mature forms of TPO protein in different species, 305 amino acids for rat (Ogami et al, 1995), 332 for humans (de Sauvage et al, 1994; Bartley et al, 1994; Sohma et al, 1994), 335 for murine (Lok et al, 1994; Bartley et al, 1994) and 329 for dogs (Bartley et al, 1994).

The study on the effects of TPO on the M07e cells, a human megakaryoblastic cell line, showed that TPO significantly supported the proliferation of M07e cells (Avanzi et al, 1990). The biological activity was compromised when TPO was chemically reduced by dithiothreitol (DTT) (Kato, 1996). Further investigations revealed the importance of two disulfide bonds between 4 cysteine residues in the N-terminal portion of human TPO for its biological activity. In Kato's study, a cysteine to alamine (Cys→Ala) mutation was introduced at positions 7, 29, 85 and 151 respectively to break the two disulfide bonds between Cys7-Cys151 and Cys29-Cys85. None of the four mutant proteins showed TPO activity (Kato, 1996). The C-terminal domain has no homology with any known proteins and contains potential N-linked glycosylation sites (6 sites in human (Kato, 1996) to 7 in murine (Foster and Lok, 1996)). This domain has the ability to bind to lectin-affinity columns,

which indicates that this portion of TPO is highly glycosylated (de Sauvage et al, 1994). To investigate whether the putative N-glycosylated residues in the C-terminal are essential for the biological activity of TPO, a series of C-terminal deletion mutants of human TPO were generated and assayed. The results showed that these potential glycosylation residues are not directly involved in the biological activity of TPO (Kato, 1996). In another study, a group of truncated forms of TPO was generated by introducing stop codons at different C-terminal positions. Interestingly, all the truncated proteins showed higher biological activity than the full length TPO (Foster and Lok, 1996). These results indicated that like EPO, the full length TPO protein might not be required for its full biological activity but it is essential for its survival in the circulation (Foster and Lok, 1996).

1.2.4 Therapeutic use of TPO

Thrombocytopenia is a common clinical problem due to the decrease in platelet production or an increase in platelet destruction. Cancer patients receiving myelosuppressive treatments often develop severe thrombocytopenia (Kaushansky, 1996; Prow and Vadhan-Raj, 1998). Although platelet transfusion is frequently used to treat for severe thrombocytopenia, it cannot solve the problem in the long term. As TPO is the major regulator of proliferation and differentiation of megakaryocytes and platelet production, for decades, researchers have been trying to develop drugs based on TPO to provide better management of thrombocytopenia.

1.2.4.1 First generation of TPO products

Since the purification and molecular cloning of human TPO, there have been many recombinant TPO products developed and two in particular have undergone intensive clinical evaluation. One is the recombinant human TPO (rhTPO) produced by mammalian cells. It is a full length protein that is

glycosylated and is indistinguishable from endogenous TPO (Vadhan-Raj et al, 2005; Kuter and Begley, 2002). Another TPO product, termed PEG-rHuMGDF, is a truncated form of TPO produced in *Escherichia coli* (*E. coli*). It consists of 163 N-terminal amino acids of TPO and is conjugated to a polyethylene glycol moiety to enhance the stability (Hokom et al, 1995). A single administration of rhTPO effectively stimulated platelet production and increased the number of megakaryocytes (Vadhan-Raj et al, 1997; Vadhan-Raj, 1998). Similar results were obtained with the PEG-rHuMGDF molecule. Administration of PEG-rHuMGDF prior to chemotherapy, led to a dose-dependent increase in the numbers of both platelets and megakaryocytes in advanced cancer patients (Basser et al, 1996; Basser et al, 1997). Unfortunately, further investigations showed that an IgG4 antibody to PEG-rHuMGDF was developed. This antibody inhibited /neutralized endogenous TPO, resulting in severe thrombocytopenia (Li et al, 2001). This adverse effect led to the discontinuation of development of both the recombinant full-length human TPO (rhTPO) and the PEG-rHuMGDF.

1.2.4.2 Second generation of TPO products

The goal of the new generation of TPO products is to develop a therapeutic molecule that bears no structural similarity with wild type TPO to avoid the production of autoimmune antibodies against native TPO. These drugs have been termed TPO-R agonists and are designed to bind and activate the TPO receptor (Kaushansky, 2001). There are three major types of TPO-R agonists: peptide TPO-R agonists, non-peptide TPO-R agonists, and TPO-R agonist antibodies. To-date, phase III clinical trials of two TPO-R agonist compounds, romiplostim and eltrombopag, have been completed. The former is a peptide and the latter is a non-peptide small molecule (Stasi et al, 2010).

The recombinant TPO analog, romiplostim, was developed and produced by Amgen to provide a potential treatment for chronic primary immune

thrombocytopenia (ITP) (Kuter et al, 2008). Romiplostim consists of two disulphide-bonded immunoglobulin IgG1 heavy chain and kappa light chain constant regions. The Fc portion of romiplostim possesses high binding affinity to the FcRn salvage receptor and participates in endothelial recirculation leading to a longer half-life in the circulation. Romiplostim shows a significantly high binding affinity to c-MPL and can efficiently activates it. Moreover, romiplostim bears no sequence and structural similarities to native TPO, hence no production of anti-TPO antibodies was observed upon the administration of this therapeutic agent (Wang et al, 2004; Roopenian and Akilesh, 2007). The *in vitro* investigations showed that romiplostim efficiently competed with the binding of TPO to c-MPL and further studies in various animals (mice, rats and rhesus monkeys) showed that the administration of romiplostim functionally increased platelet counts (Broudy and Lin, 2004; Rice, 2006). The clinical trials of romiplostim demonstrated that it could be safely administered intravenously (IV) and subcutaneously (SC) and the single SC or IV dose of romiplostim successfully led to a significant increase of platelet counts (Wang et al, 2004). In 2008, romiplostim was approved by the U.S. Food and Drug Administration (FDA) for treatment of the chronic ITP in the adult patients who failed multiple prior treatments including corticosteroids, intravenous immunoglobulin, Rho(D) immune globulin and splenectomy.

Eltrombopag is an orally available, hydrazone organic compound designed to be a TPO receptor agonist. This therapeutic molecule was developed and manufactured by GlaxoSmithKline and was approved by the U.S. Food and Drug Administration in 2008. Preclinical investigations revealed that eltrombopag could efficiently promote the differentiation and proliferation of human megakaryocytes and eltrombopag was found to induce a series of signaling transduction pathways (MAPK, JAK-STAT and P38) similar to TPO upon the binding to the TPO receptor (Erickson-Miller et al, 2005). In phase I clinical trials, eltrombopag induced a dose-dependent increase in blood platelet

counts (Jenkins et al, 2007). Additionally, in the phase III clinical trials, patients with chronic ITP who continuously received 50 mg/day of eltrombopag for six weeks showed a significantly higher level of platelets in the circulation (McHutchison et al, 2007).

1.3 TPO receptor (c-MPL)

1.3.1 Discovery of TPO receptor (c-MPL)

The myleoproliferative leukemia virus (MPLV), which causes a severe thrombocytosis in mice, was first identified in 1986 (Wendling et al, 1986). Later in 1989, a further study of the MPLV genome revealed a novel oncogene, v-*Mpl*, which showed a high degree of homology to human c-*MPL* mapped to chromosome 1p34 (Le Coniat et al, 1989). An *in vitro* infection assay of bone marrow cells with MPLV showed that expression of v-Mpl gave rise to immortalized factor-independent hematopoietic cell lines of different lineages (Souyri et al, 1990). The cloning work of v-*Mpl'*s human homolog, c-*MPL*, indicated that c-*MPL* shared high homology with members of the hematopoietic receptor superfamily (Vigon et al, 1992). Inhibition of c-MPL expression negatively regulated *in vitro* megakaryocytic colony formation (CFU-MK), whereas the growth of erythroid or granulomacrophage colonies remained unaltered (Methia et al, 1993). All these studies suggested that c-MPL plays an important role in megakaryopoiesis and that c-MPL may function as the receptor for the cytokine regulating thrombocytosis. In order to further define the role of c-MPL in megakaryopoiesis, the c-Mpl-deficient mice were generated and dramatically reduced platelet and megakaryocyte counts were observed (Gurney et al, 1994). Moreover, c-Mpl- or its ligand TPO- deficient mice showed deficiencies in progenitor cells of multiple hematopoietic lineages (Kimura et al, 1998). The findings of these *in vivo* investigations suggest that TPO and its receptor (c-MPL) are essential for the development of early stages

of hematopoietic stem cell and also the proliferation and maturation of megakaryocytes and platelet production.

1.3.2 Structure of TPO receptor (c-MPL)

The gene of the human TPO receptor, c-*MPL*, located on human chromosome 1q34, consists of 12 exons and spans over 15 kb. Its genomic structure is similar to that of other hematopoietic receptors (Alexander and Dunn, 1995; Bazan, 1990; Mignotte et al, 1994). c-MPL is a member of the class I hematopoietic growth factor super family. These proteins share two main homologous regions: a 5-aa sequence (WSXWS) close to the transmembrane domain, and a cytoplasmic domain that does not exhibit intrinsic kinase activity (Bazan, 1990). For hematopoietic receptors, the most common way of receptor activation is subunit oligomerization. Certain extracellular domains form a dimer interface that stabilizes ligand-induced homodimers. Alexander et al (1995) identified a homologous domain in the c-MPL receptor and showed that TPO-induced c-MPL activation resulted from receptor homodimerization (Alexander et al, 1995).

1.4 TPO/c-MPL signal transduction

1.4.1 Signal transduction upon ligand binding

Activation of various signal transduction pathways occurs upon TPO binding to its receptor. The pathways, as shown in **Fig. 1.3**, are very complex. A detailed account of these pathways is beyond the scope of this study and is not specifically relevant to this thesis. The key players in these pathways are TPO, c-MPL, Shc, Grb2, Jak2, STAT1 and STAT3. Only relevant or important studies related to these signaling molecules will be briefed described below.

Drachman et al (1995) observed tyrosine phosphorylation after TPO

ligand-receptor interaction. This phosphorylation occurred within a very short time period (less than 1 minute) and was capable of supporting the proliferation of engineered c-MPL expressing cells, BaF3 cells. Shc (Src homology 2 domain-containing) (Isoforms: 46 kDa and 52 kDa) phosphorylation occurred within 1 minute on TPO stimulation (Drachman et al, 1995). Shc which contains SH2 and SH3 domains is believed to act as an adaptor protein and tyrosine phosphorylation of Shc mediates its association with other signaling molecules such as Grb2 (Egan et al, 1993). Another signaling molecule, Jak2 which plays an important role in the tyrosine phosphorylation of hematopoietic receptor superfamily, was also investigated by Drachman et al (1995). Jak2 belongs to The Jak family that consists of Jak1, Jak2, Jak3 and Jak4 kinases and their phosphorylation mediates the activation of STAT proteins. An analogous mechanism takes place with signal transduction initiated by another important haematopoietic growth factor, erythropoietin (EPO). In this case, an association of Jak2 with the receptor was found after the binding of EPO to its receptor (Miura et al, 1994; Igarashi et al, 1994). Similarly, a delayed association of Jak2 to c-MPL receptor was found after the binding of TPO to c-MPL. Based on this, Drachman et al. suggested that an earlier kinase might be involved in c-MPL signal transduction process, which caused the phosphorylation of Jak2 followed by a later association with c-MPL (Drachman et al, 1995). Similar results were obtained by Dorsch's study (Dorsch et al, 1995). Moreover, another member of the Jak family, Jak1, was also investigated. Both Jak1 and Jak2 phosphorylation were induced by IL-3 activation (Matsuguchi et al, 1995; Silvennoinen et al, 1993), but only Jak2 but no Jak1 phosphorylation was observed upon TPO stimulation (Dorsch et al, 1995).

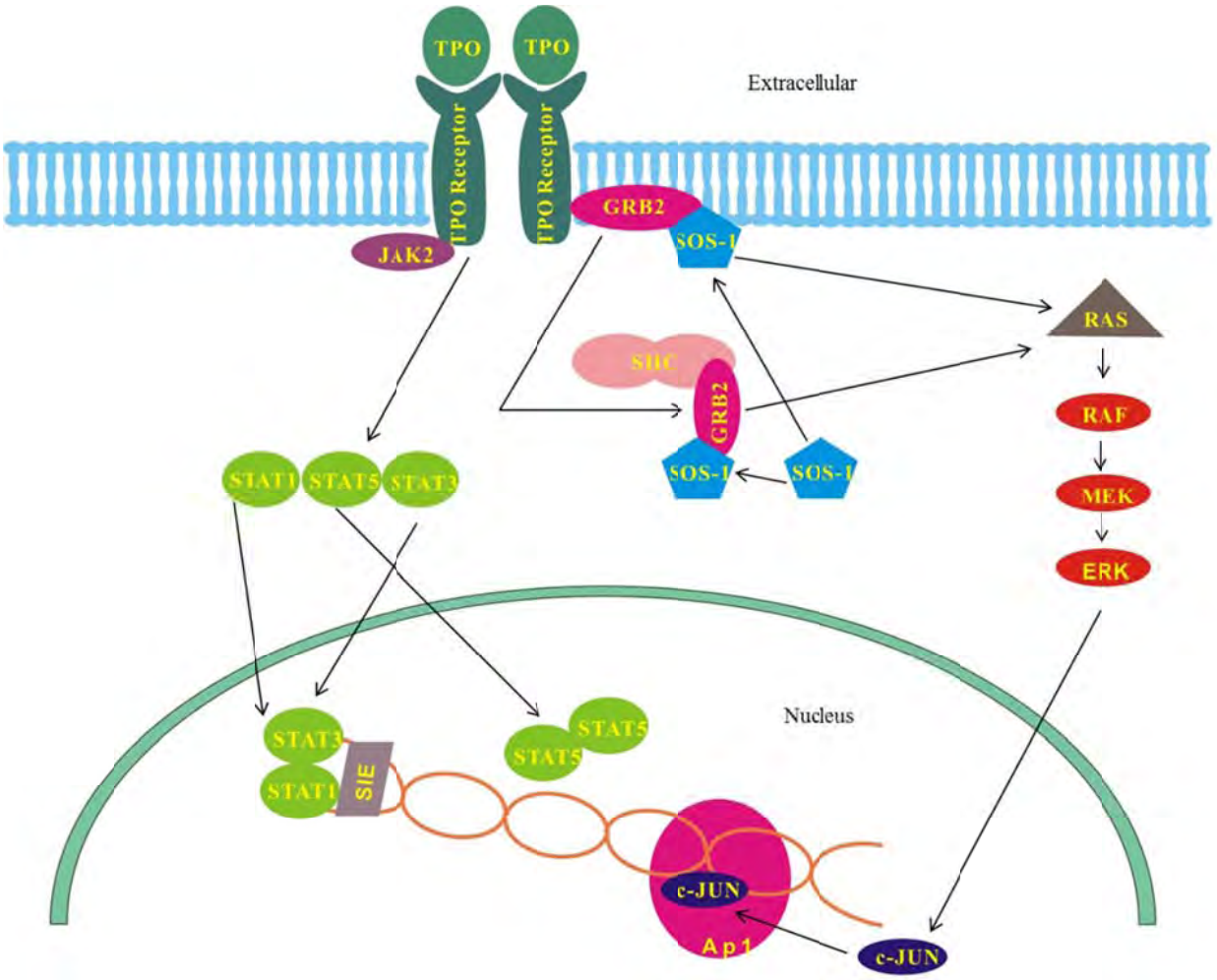


Figure 1.3 TPO/c-MPL-induced signal transduction pathways.

1.4.2 TPO/c-MPL signal transduction mediates TPO biological activity

To investigate the role of signal transduction upon binding of TPO to c-MPL, Porteu et al (1996) identified the existence of two functionally important motifs, termed “box1” and “box2” within the first 69 amino acids of c-MPL’s intracellular domain (Porteu et al, 1996). These two motifs were well conserved in cytoplasmic domains of haematopoietic receptors and were found to contribute to the JAK-STAT signaling pathway (Ihle, 1995). Both Porteu el al (1996) and Gurney et al (1995b) showed that these two motifs were involved in Jak2 phosphorylation and STAT1 and STA3 activation which were responsible

for MK proliferation upon TPO stimulation (Gurney et al, 1995b; Porteu et al, 1996). Alexander et al (1996) identified 33 amino acids within the C-terminal region of c-MPL that were critical for the differentiation of the myelomonocytic leukemia cell lines, WEHI3B-D^+ and M1. Further investigation of this region revealed that mutation of c-MPL residue 599 (Tyr599→Phenylalanine) resulted in loss of c-MPL-induced differentiation activity. In addition, activation of this c-MPL mutant could not induce Shc phosphorylation and the association of Shc with Grb2. Based on these findings, Alexander et al (1996) suggested that the Ras signaling cascade triggered by TPO-c-MPL binding and interaction of Shc with c-MPL at position Tyr599 played a critical role in the TPO- mediated cell differentiation (Alexander et al, 1996)

1.5 Regulation of TPO expression

1.5.1 Receptor-mediated/Physical regulation

Analyses of TPO protein in the circulation showed that the level of TPO is directly related to the platelet count. For many years TPO was thought to be produced at two main sites, liver and kidneys, and at these sites TPO is produced at a constant rate without gene regulation. Consequently blood TPO levels are believed to be regulated by a c-MPL receptor-mediated platelet uptake and subsequent intraplatelet destruction. When the platelet count is high, there is an increase in c-Mpl receptor numbers leading to increased TPO clearance (Kuter and Rosenberg, 1995). In contrast, when there is a low level of platelets in the circulation, TPO accumulates and this results in stimulation of thrombopoiesis (**Fig. 2.1**). However, this receptor-mediated TPO destruction model of regulation does not apply to all situations; only to thrombocytopenia due to decreased platelet production e.g. aplastic anaemia and amegakaryocytic thrombocytopenia (when both platelet and megakaryocyte numbers are reduced). For example, in patients with primary immune thrombocytopenia

(ITP) and other similar conditions when the thrombocytopenia is caused mainly by peripheral platelet destruction (megakaryocyte number is normal or increased), the blood TPO levels are usually not significantly elevated even in patients with markedly low platelet counts. Blood TPO levels in these conditions could not be explained by the c-MPL receptor-mediated mechanism of TPO clearance alone. Furthermore Geddis et al (2006) found that the c-Mpl receptors expressed on endothelial cells did not significantly affect TPO levels (Geddis et al, 2006). Clearly, there is at least another additional mechanism that regulates TPO production. Sungaran et al (1997) showed that TPO was also generated in bone marrow stromal cells. These investigators observed that the levels of TPO transcript in bone marrow stromal cells significantly increased in aplastic anaemia patients with severe thrombocytopenia (Sungaran et al, 1997), suggesting that the elevated blood TPO level in thrombocytopenic patients is not just due to reduced platelet receptor-mediated destruction but also due to increase TPO protein production by bone marrow stromal cells. These authors further showed that there was a feedback regulation of TPO production by bone marrow stromal cells and that the platelet/megakaryocyte alpha-granule proteins PDGF and FGF-2 enhanced, while platelet factor 4 (PF4), thrombospondin (TSP) and transforming growth factor beta (TGF-β) suppressed the TPO expression (Sungaran et al, 1997). As PF4, TSP and TGF-β are substantially more abundant among platelet/megakaryocyte α-granule proteins, the overall effect of α-granule proteins is inhibitory. The inhibitory effect of PF4 predominates; this is well documented. Therefore, these findings strongly suggest that TPO production at least in the bone marrow by the stromal cells involves the transcriptional control. However, the mechanism of the α-granule proteins, particularly PF4, mediated regulation of TPO production is still unknown. **The unanswered questions are:** (1) what is/are the receptor(s) on the stromal cells that bind/interact α-granular proteins (e.g. PF4)? (2) what intracellular signal transduction pathways are activated following ligand/receptor interaction? And (3) what transcription factors, particularly

transcription repressor(s) is/are involved? Preliminary evidence from TPO gene promoter analysis studies suggests the possible involvement of transcription factor ZEB1 or MZF1. The present study aims to address the questions listed above.

1.5.2 Gene regulation-TPO gene promoter

Despite recent advances in our understanding of the molecular and functional biology of TPO and c-MPL, the promoter structure and transcriptional control of TPO gene remain largely unknown. The organization of the TPO genomic region is yet to be agreed upon, with some investigators suggesting that it consists of 6 exons and 5 introns (Foster et al, 1994; Sohma et al, 1994; Gurney et al, 1995a), while others suggested 7 exons and 6 introns (Chang et al, 1995; Dordelmann et al, 2008). Thus, it is believed that there are two alternative promoters in the 5' upstream regulatory region, called P0 and P1. It was shown that TPO transcription preferably initiated from P1, with only 10% initiating from the P0 promoter (Ghilardi et al, 1998; Wiestner et al, 1998). Using 5'-RACE assay, Kamura et al (1997) defined a total of six different TPO transcription initiation sites from +1 to +51, with the major sites located at positions +24 and +25 (Kamura et al, 1997). There was no putative TATA box observed in the defined multiple transcription initiation sites. They further showed that a 7-bp sequence -ACTTCCG- from -109 to -103 in the 5'-upstream regulatory region is an active binding site for E4TF1/GABP, a member of the Ets family of transcription factors. Functional analysis showed that a construct bearing one more E4TF1 binding sites further upstream of the wild type binding site in the TPO gene promoter region exhibited increased promoter activity. This further confirmed that the E4TF1/GABP is a functional enhancer for the expression of TPO (Kamura et al, 1997).

1.5.3 Potential transcription factors in TPO regulation

(a) Transcription factor, ZEB1

The zinc finger E-box binding protein 1 (ZEB1, also known as δEF1, Nil-2-a, Tcf8, Bzp, Areb6, Meb1, Zfhx1a and Zfhep) is a transcription factor that consists of a POU-like homeodomain and two zinc finger clusters, and it binds to the consensus site 5'-CACCT-3' with high affinity (Funahashi et al, 1993; Genetta et al, 1994; Sekido et al, 1996). Genetta et al. reported that ZEB1 actively suppressed the transcription of the immunoglobulin heavy chain enhancer (Genetta et al, 1994) and the expression of interleukin-2 (IL-2) was inhibited by the binding of ZEB1 to the IL-2 promoter (Yasui et al, 1998). In addition, it was shown that ZEB1 binds to a subset of E boxes in the promoter of muscle genes and represses gene expression (Postigo and Dean, 1997). Myotube formation and expression of important differentiation makers (myosin heavy chain and myogenin) were suppressed when ZEB1 was overexpressed in C2C12 cells (Postigo and Dean, 1997). Moreover, a ZEB1 cluster containing six ZEB1 binding consensus sites 5'-CACCT-3' was found in intron 1 of human p73 gene, which strongly inhibited the p73 promoter activation (Fontemaggi et al, 2001).

(b) Transcription factor, MZF1

Another important transcription factor which may potentially play a role in TPO regulation is MZF1 (myeloid zinc finger binding protein). MZF1 was found to be primarily expressed in myeloid cells and human bone marrow cells from patients with CML and healthy donors (Hromas et al, 1991). Its role as a transcriptional repressor has been well defined in many studies. A MZF1 binding site localized in the fourth region of human FcεRI β-chain gene, an important molecule in allergic reactions, was found to be a transcription repressor. Inhibition of endogenous MZF1 in KU812 cells by antisense oligonucleotides reversed the suppression and significantly increased the

transcription of FcεRI β-chain gene (Takahashi et al, 2003). MZF1 also serves as a transcriptional repressor of ERCC1, an essential molecule in nucleotide excision repair (Yan et al, 2006). Its role in haematopoiesis and tumorigenesis has also been investigated. It was suggested that MZF1 negatively regulated embryonic stem cells to undergo a hematopoietic commitment and erythromyeloid colony formation (Perrotti et al, 1995). In another study, haematopoietic progenitor cells from the *Mzf*1$^{-/-}$ mouse underwent a significantly increased long-term haemopoiesis, and the *Mzf*1$^{-/-}$ mouse developed solid tumor in the liver that consisted of myeloid cells (Gaboli et al, 2001) suggesting that MZF1 may have a role in repressing haematopoiesis and promoting tumor genesis.

1.5.4 Feedback mechanism of TPO regulation

1.5.4.1 Role of α-granule proteins

There are mainly three different types of platelet granules, namely α-granules, dense granules and lysozymes. The α-granule is the most abundant of platelet granules. α-granules formed in megakaryocytes are transferred to platelets and contain various factors, such as insulin-like growth factor 1, platelet factor 4 (PF4), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and proteins that play important roles in coagulation processes including thrombospondin (TSP), fibronectin, and von Willebrand factor (vWF). Insulin-like growth factor 1 (IGF-1), also named somatomedin C, is a hormone that is structurally similar to insulin. It is an important hormone in human growth in childhood and also shows anabolic effects in adults. Platelet-derived growth factor (PDGF) is a growth factor that regulates cell growth and division, and it participates in blood vessel formation. Transforming growth factor beta (TGF-β) is a cytokine that plays a vital role in cell proliferation, cellular differentiation and many other processes. It has three isoforms, TGF-β1,

TGF-β2 and TGF-β3. TGF-β is widely expressed in most tissues in the human body. Thrombospondin (TSP) family consists of TSP-1 to TSP-5 and they possess antiangiogenic activities. TSP-1 is one of the major constituents of platelet α-granules and plays a role in platelet aggregation and thrombosis. Fibronectin, a high molecular weight glycoprotein is involved in cell adhesion, growth, migration and differentiation. It also regulates wound healing. von Willebrand factor (vWF) is a glycoprotein in blood plasma. It is important in hemostasis and it mediates platelets adhesion to vascular injury sites. Of these proteins, the most relevant to regulation of TPO production and hence most relevant to the present study is PF4, which will be discussed further below.

1.5.4.2 Platelet factor 4 (PF4)

PF4, also known as chemokine C-X-C ligand 4, is a member of CXC chemokine family. It is one of the platelet α-granular proteins that are released by the activated platelets (Rucinski et al, 1990; Holt and Niewiarowski, 1985). The gene of human PF4 has been located to the human chromosome 4 (O'Donovan et al, 1999) and it encodes an 8 kDa protein that consists of 70-aa with a high binding affinity to heparin (Eisman et al, 1990; Petersen et al, 1999; Sachais et al, 2004). The major biological function of human PF4 is believed to neutralize heparin and consequently block the activity of antithrombin III (ATIII). PF4 has a high binding affinity to glycosaminoglycans (GAGs) such as heparin, heparin sulfate (HS), dermatan sulfate(DS) and chondroitin sulfates (CS) (Petersen et al, 1999; Loscalzo et al, 1985; Sachais et al, 2004). PF4 is cleared away from the circulation when it binds to endogenous or exogenous heparin, and thus it has a short plasma half-life of 17 minutes (Rao et al, 1983). A purification method of platelet factor 4 from activated platelets using a heparin sepharose column has been established, due to its high binding affinity to heparin (Handin and Cohen, 1976; Levine and Wohl, 1976).

1.5.4.3 Biological activities of human PF4

The inhibitory effect of PF4 on haematopoiesis has been well studied. In 1980s it was reported that platelet-released constitutes were able to inhibit the growth of megakaryocytes (Vainchenker et al, 1982; Messner et al, 1982; Kimura et al, 1984). It was later confirmed that it was the platelet α-granule protein PF4 released by activated platelets that inhibited megakaryocytic colony formation, proliferation and maturation of progenitor cells (Gewirtz et al, 1989; Han et al, 1990; Gewirtz et al, 1995). PF4 was found to regulate haematopoiesis by binding to the human $CD34^+$ hematopoietic progenitor cells (Dudek et al, 2003). Moreover, it was suggested that PF4 could suppress the proliferation of myeloid progenitor cells through the formation of heteromultimers between tetrameric PF4 and dimeric IL-8 (Dudek et al, 2003; Nesmelova et al, 2005; Daly et al, 1995). PF4 also plays an important role in blood coagulation by blocking the anti-coagulant effect of heparin and anti-thrombin III which is a potent inhibitor of thrombin and factor Xa (Conley et al, 1948; Rosenberg and Damus, 1973).

1.5.4.4 PF4 receptor and signal transduction

1.5.4.4.1 PF4 receptor

The biological functions of PF4 in different physiological processes have been well studied, however, information about the PF4 receptor is very limited. CXCR3-B, one of the variants of CXCR3, derived from an alternative spliced form of CXCR3, has been characterized as the PF4 receptor in endothelial cells (Lasagni et al, 2003). PF4 showed a highly selective binding affinity only to CXCR3-B. Overexpression of CXCR3-B resulted in strongly decreased DNA synthesis and enhanced HMEC-1 cells apoptosis (Lasagni et al, 2003). However, it has not been elucidated whether there is a similar interaction between the murine CXCR3 and PF4 (Kowalska et al, 2010). Moreover, it is noted cells that do not express CXCR3-B are found to respond to the PF4 treatment (Pervushina

et al, 2004). Thus, there is a high possibility that CXCR3-B may not be the only receptor for PF4.

1.5.4.4.2 PF4-induced signal transductions

Evidence obtained from a variety of studies reveals that PF4 is involved in many signal transduction pathways in neutrophils, granulocytes, monocytes and macrophages. PF4 induces the adhesion of neutrophils to endothelial cells in the presence of tumor necrosis factor (TNF). In PF4-dependent adhesion, PF4 was shown to directly activate src-kinases and TNF acted as the co-stimulus activating the P38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (Kasper and Petersen 2011). There is increasing evidence that PF4 can induce both short-term responses, such as formation of reactive oxygen species (ROS), phagocytosis and adherence, and also long-term responses, such as cell survival, differentiation and cytokine production (Scheuerer et al, 2000; Pervushina et al, 2004; Kasper et al, 2007). Unlike the PF4-induced signaling in endothelial cells which needs the co-stimulatory TNF, PF4 directly induces different signaling pathways in monocytes that lead to a variety of physiological processes such as, phagocytosis, respiratory burst, survival and secretion of cytokines. Among these processes, PF4 mediates respiratory burst by inducing rapid activation of PI3K, Syk and P38 MAPK signal transduction. PF4-mediated survival and differentiation of monocytes were found to be regulated by a delayed activation of Erk. Also, the activation of JNK and Erk was believed to control PF4-dependent cytokine expression in monocytes (Kasper et al, 2007).

PF4 was also found to negatively regulate some signaling pathways. Erk signaling transduction induced by basic fibroblast growth factor 2 (FGF2) could be disrupted by PF4, however, FGF2-induced PI3K signaling was not affected by PF4 (Sulpice et al, 2002). Similar regulation was also observed in PF4-mediated signaling transduction in human erythroleukemia cells (HEL)

(Liu et al, 2002).

1.5.4.5 Low density lipoprotein receptor-related protein (LRP1)

Low density lipoprotein receptor-related protein, also known as LRP1, has been shown to be a receptor of PF4 in some biological settings (see section, “1.5.4.5.3 Interaction of PF4 and LRP1” below). LRP1 belongs to the low-density lipoprotein (LDL) receptor superfamily. This family consists of seven structurally related members including LRP1 (Herz et al, 1988), LRP1b (Liu et al, 2001), megalin/LRP2 (Farquhar et al, 1995), LDL receptor (Nykjaer and Willnow, 2002), very low-density lipoprotein receptor (VLDL) (Takahashi et al, 1992), apoE receptor 2 (Kim et al, 1996) and multiple epidermal growth factor-like domains 7 (MEGF7) (Nakayama et al, 1998). The LDL receptor was found to regulate cholesterol homeostasis by mediating endocytosis of cholesterol-rich LDL particles. LRP1 was found to be widely expressed in many tissues and to regulate the cell surface protease activity (Boucher et al, 2003; Herz and Hui, 2004).

1.5.4.5.1 Structural properties of LRP1

The LDL receptor family contains cysteine-rich clusters (CR) that serve as the target for ligand binding. The regions that are rich in CR were defined with clusters I, II, III, IV and V in LRP1 and further investigations showed the cluster II and cluster IV are the binding targets for LRP1 ligands (Neels et al, 1999; Willnow et al, 1994). Another important founding of the LDL receptor family is the existence of the EGF precursor-like regions consisting of two cysteine-rich EGF repeats that might fold as a beta-propeller domain (Springer, 1998). This structure was then found to be essential for release of their ligands in the low pH environment of the endosomal compartments (Rudenko et al, 2002). All the LDL receptor members consist of a transmembrane domain and a cytoplasmic domain. Previous studies showed that the cytoplasmic domain of

LRP1 contained 100 amino acids within which the two leucine and NPxY motif can be tyrosine phosphorylated by the platelet derived growth factor β (PDGF-β), connective tissue growth factor (CTGF) and v-Src (Boucher et al, 2002; Loukinova et al, 2002; Barnes et al, 2002).

1.5.4.5.2 Biological activity of LRP1

Numerous studies investigating the biological activity of LRP1 showed that this receptor is expressed in many cell types and can bind to a wide range of ligands. It is mainly involved in two physiological processes, endocytosis and various signaling pathways. LRP1 interacts with over 40 different ligands and mediate their endocytosis (May and Herz, 2003). The signaling pathways triggered and regulated by LRP1 have been reported in many tissues, including vessel walls, neurons, lung and many other tissues. LRP1 was found to be expressed abundantly in the liver and it mediated the plasma removal of different types of molecules, including proteinase inhibitor complexes such as α_2M (Sottrup-Jensen, 1989; FitzGerald et al, 1995; Poller et al, 1995), serine proteinase inhibitors (serpins) (Silverman et al, 2001), enzymes and cofactors that are essential in the processes of blood coagulation and fibrinolysis such as factor VIII (fVIII) (Lenting et al, 1999; Kowalska et al, 2010), and certain lipoprotein particles (Ulrike, 1998). Studies in different cell types also revealed a protective effect of LRP1 against the formation of atherosclerosis. LRP1 inhibits the proliferation and migration of smooth muscle cells by suppressing the PDGF signaling pathways (Boucher et al, 2003). Studies of LRP1 deficient mice showed a significant two-fold increase in atherosclerotic lesions compared with that of healthy mice (Espirito Santo et al, 2004). Two other *in vivo* studies also indicated a protective capacity against atherosclerosis by the targeted deletion of LRP1 in macrophages; such a deletion strikingly induced atherosclerotic lesions in the aortic root and proximal aorta area (Hu et al, 2006; Overton et al, 2007). LRP1 could also modulate cell migration and it was reported that inhibition of LRP1 using either an antibody against LRP1 or

receptor associated protein (RAP), an antagonist of LRP1, resulted in inhibition of cell migration (Okada et al, 1996).

1.5.4.5.3 Interaction of PF4 and LRP1

Several studies have shown the interaction between PF4 and LRP1. The interaction is involved in different physiological processes such as the development of atherosclerosis and heamatopoiesis. PF4/LRP1 interaction up-regulates expression of E-selectin via activation of the transcription factor NF-kappa B (Yu et al, 2005). E-selectin, a member of the adhesion receptor family, which is expressed in the endothelium of human atherosclerotic lesions (van der Wal et al, 1992; Bensch et al, 1995) and promotes atherosclerosis (Dong et al, 1998; Collins et al. 2000).

PF4 is thought to be the negative regulator of megakaryopoiesis and a study focusing on the mechanism of such regulation showed that LRP1 is the target receptor for PF4. In this investigation, LRP1 was found to be abundantly expressed on large, polyploid megakaryocytes and blocking of LRP1 by using either a specific antibody against LRP1 or receptor-associated protein (RAP), an antagonist of LRP1, reversed the inhibition of megakaryocyte colony formation by PF4. *In vivo* evidence is provided by a study in which transgenic $hPF4^{High}$ mice were found to have thrombocytopenia and fusion of RAP to PF4 of these mice (blocking PF4/LRP1 interaction) significantly restored the platelet counts (Lambert et al, 2009).

1.5.4.5.4 LRP1 signal transduction

(i) LRP1 and phosphatidylinositol 3-kinases (PI3Ks)

The PI3K/Akt signaling pathway is an important signal transduction pathway in many physiological processes such as cancer progression, insulin metabolism and cell growth, differentiation and proliferation. The activation of receptor tyrosine kinases in many receptors such as B and T cell receptors, cytokine

receptors, and G protein coupled receptors leads to the production of 3-phosphorylated phosphoinositides, PIP_3 (phosphatidylinositol (3,4,5)-triphosphate) by PI3K. These lipids provide the docking site for the pleckstrin homology domain (PH) of Akt and its upstream activator PDK1. The harboring of Akt and PDK1 results in the translocation of Akt and PDK1 to the plasma membrane (Franke et al, 1997). The translocation causes the partial phosphorylation of Akt on threonine 308 and a full phosphorylation is completed by the further phosphorylation of Akt on serine 473 induced by the TORC complex of the mTOR protein kinase.

The binding of TSP1 to calreticulin-LRP1 complex negatively regulates cell adhesion and enhances cell motility by focal adhesion disassembly. Further investigation showed that the interaction of TSP1 and calreticulin-LRP1 induced PI3K/Akt signal transduction that led to a suppressed apoptotic signaling in suspended fibroblasts (Pallero et al, 2008). LRP1 deficient fibroblasts were found to fail to induce the PI3K/Akt pathway and the corresponding TSP1-mediated anchorage-independent survival (Pallero et al, 2008). A similar anti-apoptotic signaling induced by the LRP1-mediated PI3K/Akt signal transduction was also reported in neurons. The disruption of LRP1 expression in neurons results in a suppressed activity of PI3K/Akt signal transduction and promotes the activity of caspase-3 that directly causes increased neuronal apoptosis (Fuentealba et al, 2009).

(ii) LRP1 and mitogen-activated protein kinases (MAPK)

MAPK activation is triggered by various types of stimuli including ultraviolet irradiation, heat shock, osmotic shock, cytokines and growth factors. MAPKs are actively involved in gene expression, mitosis, cell survival/apoptosis, differentiation and proliferation (Pearson et al, 2001). There are three major members of the MAPK family including MAPK/ERK, p38 MAPK and JNK (c-Jun). In MAPK signaling pathway, ERK1 and ERK2 are the key components

that are activated by a wide range of receptors. LRP1 could also functionally mediate the PDGF-dependent activation of both PI3K and MAPK/ERK by forming a signaling complex with PDGFR-β in endosomal compartments (Muratoglu et al, 2010). A study on the macrophage-derived cell line, J774, revealed that α2M* could promote the proliferation of J774 by directly binding to LRP1 and activating MAPK/ERK (Bonacci et al, 2007).

Chapter Two: Hypothesis and Aims

Chapter Two: Hypothesis and Aims

2.1 Introduction and Hypothesis

Human TPO is an important protein that regulates thrombopoiesis. It primarily regulates the proliferation and differentiation of megakaryocytes and the production of platelets. A well described mechanism of regulation of TPO in the circulation is c-MPL-mediated TPO uptake by platelets. According to this mechanism, there is an inverse correlation between the TPO level and the platelet count in the circulation: c-MPL-mediated removal of TPO occurs when platelet numbers are high, leading to a decrease in the blood TPO level; in contrast, TPO accumulation occurs when the platelet counts are low, and this results in a rise in blood TPO levels (**Fig. 2.1**). However there is published evidence that the level of TPO in the circulation might also be regulated by a feedback control mechanism at transcriptional level. When platelet counts are high, TPO mRNA and protein levels in bone marrow stromal cells are suppressed, mainly through the action of α-granule-derived proteins, such as platelet factor 4 (PF4), thrombospondin (TSP) and transforming growth factor-beta (TGF-β) (Sungaran et al, 1997; Sungaran et al, 2000) (**Fig. 2.2**). Recent work also suggested a serum dose-dependent suppression of murine TPO expression and this suggests the possibility that the suppression may be mediated by a transcriptional repressor, which is most likely ZEB1. ZEB1 binding sites were identified between 250 bp upstream to approximately 1.9 kb downstream in the middle of intron 2 of murine TPO (McIntosh and Kaushansky, 2008). Moreover, a recent study revealed that one of the human platelet α-granule protein, PF4, can inhibit megakaryopoiesis by binding to the low-density lipoprotein receptor-related protein 1 (LRP1) expressed on megakaryocytes (MKs) (Lambert et al, 2009) suggesting the possibility of an autocrine feedback loop via released TPO on MKs themselves. Similarly there

may also be a paracrine mechanism in which PF4 (released from platelets and megakaryocytes) binds to LRP1 on bone marrow stromal cells and suppresses TPO gene transcription and expression, and consequently TPO protein production. Decreased TPO then leads to suppression of megakaryopoiesis and platelet production.

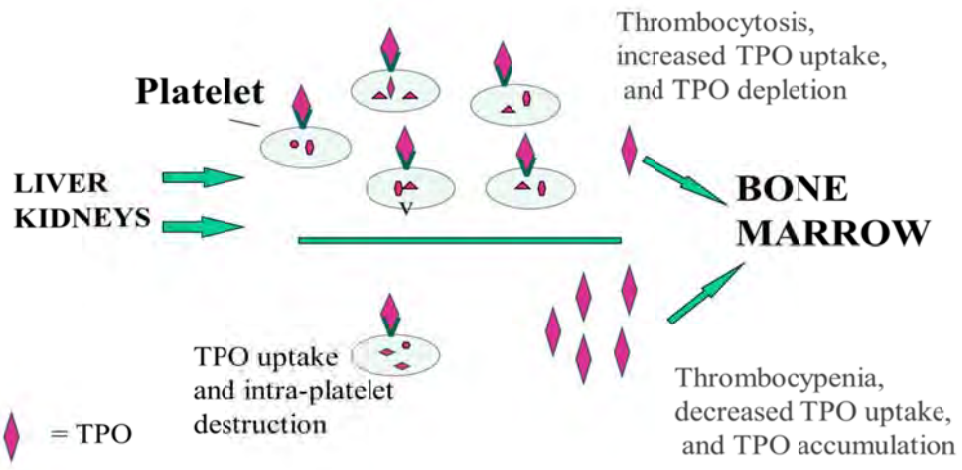


Figure 2.1 Mechanism 1: Receptor-mediated/mechanical regulation of blood TPO levels.

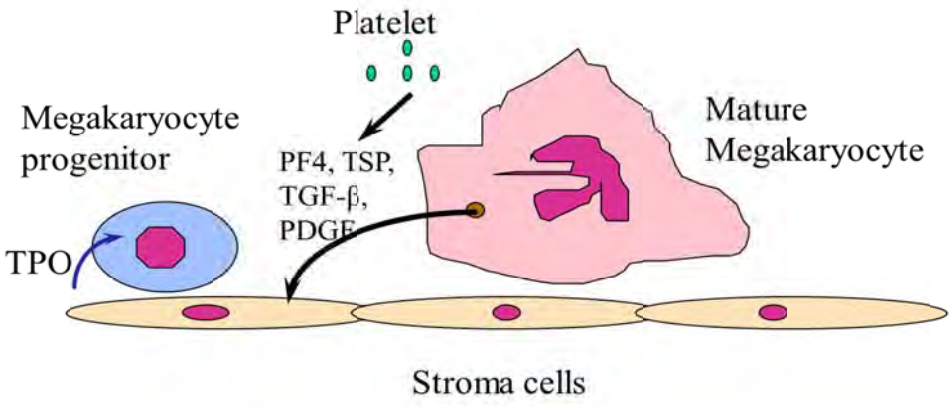


Figure 2.2 Mechanism 2: Negative Feedback mechanism. *Platelet granular proteins inhibit TPO expression in bone marrow stroma cell*

Hypothesis:

The hypothesis of my study is that the human TPO expression is negatively regulated by a transcriptional repressor and that the human TPO gene contains the critical repressor response element. We further hypothesize that PF4 may also suppress the expression of TPO through its association with LRP1 (**Fig. 2.3**).

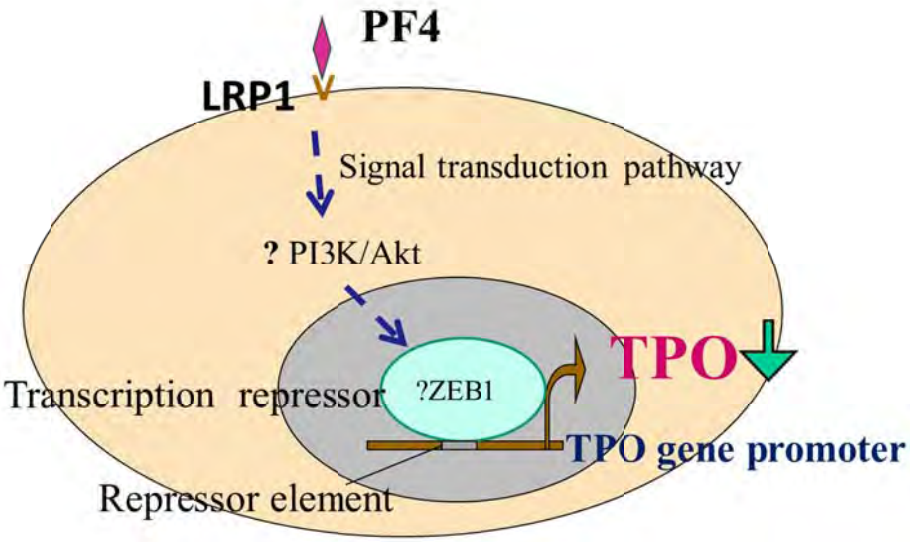


Figure 2.3 Mechanism of PF4 suppression of TPO production by bone marrow stroma cells.

The questions to be answered by this study are:

1. Is there a repression element in the human TPO gene?

2. If there is, where is it located in the human TPO gene? And what is putative repressor?

3. Does the putative repressor bind to the identified repression element of the TPO gene promoter? Does binding of the repressor suppress transcription of the TPO gene?

4. As serum is rich in platelet granule proteins, is PF4 the main factor in serum that suppresses the expression of TPO?

5. Do bone marrow stromal cells express the LRP1 receptor? If so, does PF4 suppress the expression of TPO through LRP1?

6. Is there any correlation between the expression of the transcriptional repressor and PF4 suppression of TPO?

2.2 Aims

The main aim of this study is to identify the transcriptional repressor of human TPO gene and to explore the mechanisms for PF4 suppression of TPO gene expression. Additional aims are (i) to identify the PF4 receptor on OP9 cells (equivalent to bone marrow stromal cells), and (ii) to characterize the signal transduction pathway. Thus, I attempted to define a novel control mechanism of human TPO gene expression.

Specifically, the objectives are:

1. To locate the repression element in the TPO promoter. Series of 5'- and 3'-deletion DNA fragments of human TPO gene were amplified and cloned into the reporter gene vector, to generate TPO promoter luciferase constructs. The constructs were transiently expressed in various cell types and the corresponding promoter activities were determined by luciferase assay.

2. To investigate whether the defined repression element binds the putative transcriptional factor by gel electrophoresis mobility shift assay (EMSA).

3. To investigate whether the putative repressor functionally suppresses the expression of TPO. Specific siRNA that inhibited the transcription of the putative repressor was employed and the corresponding expression of TPO was determined by relative quantitative real-time PCR assay.

4. To investigate suppression of TPO mRNA expression by serum and PF4 in OP9 cells, using relative quantitative real-time PCR assay. PF4 used in the study was purified from platelet rich plasma by using heparin sepharose beads and high performance liquid chromatography (HPLC).

5. To investigate LRP1 expression on OP9 cells by flow cytometry.

6. To investigate whether PF4 suppresses TPO expression through LRP1 (**Fig 2.3**) by using a specific antibody or receptor antagonist against the LRP1 that blocks PF4 binding to the receptor.

7. To investigate whether there is any correlation between the putative transcriptional repressor in OP9 cells and PF4 suppression of TPO mRNA expression.

Chapter Three: General Methods and Materials

Chapter Three: General Methods and Materials

3.1 Cell culture

3.1.1 Bacterial strains

The *E. coli* strain DH5α was purchased from Invitrogen (CA, USA). Another *E. coli* strain ER2925 was acquired from New England Bilolabs® (NEB) (MA, USA).

3.1.2 Culture of HeLa, HepG2 and MCF7 cell lines

The human cervical carcinoma cell line (HeLa cells), the human hepatocarcinoma cell line (HepG2 cells) and the human breast cancer cell line (MCF7 cells) were purchased from American type culture collection (ATCC, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), penicillin (100 units/ml) (Invitrogen), and streptomycin (100 μg/ml) (Invitrogen) at 37°C in a 5% CO_2 incubator.

3.1.3 Culture of OP9 cell line

The mouse stromal cell line, OP9 cells, was purchased from American type culture collection (ATCC) and was maintained in Alpha minimal essential medium (Invitrogen) supplemented with 20% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator.

3.1.4 Culture of P19 cell line

The mouse embryonal carcinoma cell line, P19 cells, was purchased from

American type culture collection (ATCC) and was maintained in Alpha minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator.

3.2 General Methods

3.2.1 Molecular biology and kits

Marker for DNA electrophoresis, DNA ladder, was purchased from Promega Corporation (WI, USA). Marker for Western blot assay, SeeBlue® Pre-Stained Protein Standard, was purchased from Invitrogen. Wizard® Plus SV miniprep kit was purchased from Promega and Wizard® DNA PureLink™ HiPure Plasmid Maxiprep kit was purchased from Invitrogen. The QIAquick® gel extraction kit was obtained from Qiagen (VIC, Australia).

3.2.2 General chemicals

30% acrylamide/bisacrylamide (29:1) was obtained from Bio-Rad Laboratories Inc (CA, USA). Phenol/Chloroform/Isoamyl alcohol (24:24:1) saturated with TE [pH 8.0] was purchased from Progen Industries (QLD, Australia). Chemicals purchased from Sigma-Aldrich (MI, USA) are: bovine serum albumin (BSA), diethyl pyrocarbonate (DEPC), dimethyl sulphoxide (DMSO), dithiothreitol (DTT), ethanol, 2-mercaptoethanol (β-ME), methanol, magnesium chloride, NP-40, isopropyl alcohol, sodium bicarbonate, sodium dodecyl sulphate (SDS), sodium chloride, triton X-100, boric acid, TEMED, ethylene diamine tetra-acetic acid (EDTA), HEPES, guanidine hydrochloride, ethidium bromide (EB), Tris, Glysine, Formaldehyde solution.

3.2.3 Establishment of human TPO promoter-luciferase fusion constructs

3.2.3.1 Extraction of genomic DNA from HepG2 cells

The genomic DNA was extracted by using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Briefly, cells were collected and spun down by the centrifugation at 12, 000 rpm for 10 s and the pellet washed once with PBS. Cells were spun down again and 600 μl of nuclei lysis solution were added and mixed by pipetting. 3 μl of RNase solution was added to cells and mixed by vortexing. The mixture was incubated for 30 min at 37°C and allowed to cool down to room temperature on the bench. 200 μl of protein precipitation solution was added and mixed by vortexing. The mixture was incubated on ice for 5 min and then centrifuged at 12, 000 rpm for 5 min and the upper clear supernatant was transferred to an autoclaved eppendorf tube containing 600 μl of 100% isopropanol. The sample was mixed by inversion and gentle tapping, and then centrifuged at 12, 000 rpm for 1 min at room temperature. The supernatant was discarded and 600 μl of 70% ethanol was added. The sample was centrifuged again at 12, 000 rpm for 1 min. The ethanol was aspirated and the pellet was air-dried for 30 min. 100 μl of rehydration solution was added and the DNA was rehydrated for 1 h at 65°C. The extracted DNA was used directly or stored in -20°C until use.

3.2.3.2 Polymerase chain reaction (PCR)

A series of DNA fragments covering various regions of human TPO gene were amplified by PCR using either Expand high fidelity^{plus} PCR system (Roche Applied Science, NSW, Australia) or KOD DNA polymerase (Merck Millipore, VIC, Australia). The sequence recognized by restriction enzyme was designed into the primers for the DNA amplification (detailed PCR settings for each TPO

fragments are described in relevant sections).

3.2.3.3 Gel extraction

The PCR products were separated by gel electrophoresis and the specific products were separated from the nonspecific using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions:

The gel slice containing the DNA band was excised with a clean, sharp scalpel. The gel slice was weighed. 1 volume of diffusion buffer was added to 1 volume of gel (i.e., 100 µl for each 100 mg of gel) and incubated at 50°C for 30 min. The sample was centrifuged for 1 min. The supernatant was carefully removed using a pipette. The supernatant was passed through a disposable plastic column containing a Whatman GF/C filter to remove any residual polyacrylamide. The volume of the recovered supernatant was determined and 3 volumes of Buffer QG were added to 1 volume of supernatant and mixed. The color of the mixture was checked that it was yellow. A QIAquick Spin Column was placed in a provided 2 ml collection tube. To bind DNA, the sample was applied to the QIAquick Spin Column and centrifuged for 60 s. Flow-through was discarded and QIAquick Spin Column was placed back into the same collection tube. To wash, 0.75 ml Buffer PE was added to the column and centrifuged for 60 s. Flow-through was discarded. QIAquick Spin Column was placed back in the same tube and the column was centrifuged for an additional 1 min at maximum speed. QIAquick Spin Column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick Spin Column and centrifuged for 1 min.

3.2.3.4 Phenol/chloroform/Isoamyoalchohol purification

The amplified fragment was purified by using Phenol/Chloroform/Isoamyl alcohol (Progen Industries). Briefly, PCR product was diluted in 400 µl of

Milli-Q water and then 400 μl of PCI was added to the sample. The sample was vortexed for 5 s and subjected to centrifugation in a benchtop centrifuge at 6,000 rpm/min for 2 min. The top clear phase containing the PCR product was transferred to a new tube and 40μl of sodium acetate and 1ml of cold absolute ethanol were added. The mixture was vortexed for 5 s and then subjected to a centrifugation at 4°C, 13,000 rpm/min for 10 min. After the centrifugation, the pellet on the bottom was washed with 200 μl of 70% ethanol and then subjected to a centrifugation at room temperature, 13,000 rpm/min for 5 min. The purified DNA was left on bench until dry and then dissolved in a certain amount of nuclease free water. The dissolved DNA was subjected to a concentration measurement and then used directly or kept in -20°C until use.

3.2.3.5 Measurement of DNA/RNA concentration

The DNA concentration was measured using the NanoDrop 2000 (Thermo Scientific, DE, USA) and following the manufacturer's instructions. Briefly, 1 μl of nuclease free water was measured as the control for the baseline reading and then 1 μl of DNA/RNA dilution was measured.

3.2.3.6 Restriction enzyme digestion and ligation

As shown in **Fig. 3.1**, the amplified DNA fragments were digested by the specific restriction enzymes at a 37°C for 3 h and then ligated into the luciferase reporter vector PGL3-Basic (Promega). For each ligation, 2 μl of 10 × ligase buffer, 1 μl of T4 DNA ligase (NEB, MA, USA), and ~100 ng (molar ratio of 1:3 vector to insert) of vector and insert were mixed by pipetting. Ligation was incubated at 16°C for 1 h.

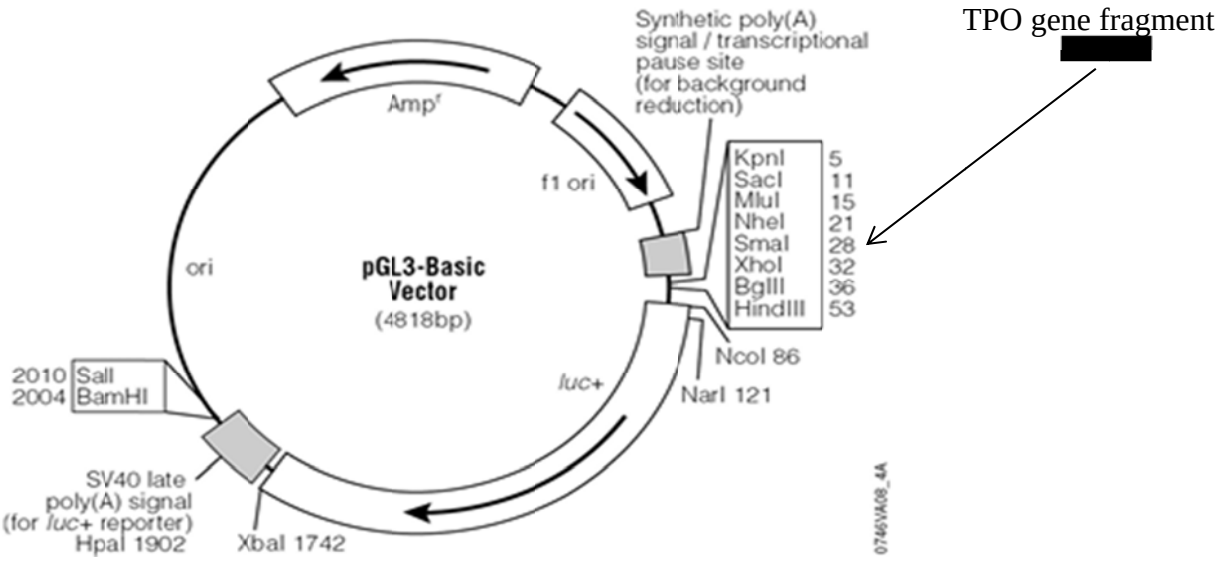


Figure 3.1 Cloning of TPO-luciferase plasmids. *TPO fragments amplified by PCR were ligated into multiple restriction digestion sites of PGL3-Basic vector.*

3.2.3.7 Transformation of *E.coli* (DH5α) with the ligated plasmid

Preparation of Luria Bertani broth (LB) agar plate

2% agar plate with 75 µg/ml of ampicillin was prepared for the growth of *E.coli*. Briefly, 8 LB capsules and 20 g of agar were dissolved in 300 ml of distilled water and autoclaved. After the autoclave, the broth were left at room temperature and allowed to cool down to about 55°C and then 450 µl of 50 ug/ml of ampicillin was added and mixed gently. The broth was poured gently to avoid bubbles. The LB plates were used directly or wrapped with foil and kept at +4°C for up to one month for use later.

Transformation of *E.coli*

100 µl of DH5α was thawed on ice and 3 µl of the ligated plasmid was added to the top of the cells. The cells were mixed by gently flicking the tube and then the mixture left on ice for 30 min. The cells were heat shocked at 42°C for 45 s in a water bath and then transferred into 300 µl of SOC medium. The cells were

cultured in a shaking incubator at 37°C, 215 rpm, for 60 min and 100 μl of cells were then plated on a prepared LB agar plate. The cells were incubated in a 37°C incubator overnight to allow the single colonies to grow.

3.2.3.8 Screening of positive clones

Harvest of cloned plasmid

6-12 single colonies were picked up and cultured in 2 ml of LB medium supplemented with 75 μg/ml of ampicillin on a shaking incubator at 37°C, 210 rpm for 16 h. The plasmid amplified by *E. coli* was extracted and purified by using Wizard® Plus SV miniprep DNA Purification System in accordance to the manufacturer's instructions. Briefly, each pellet suspended in 250 μl of Cell resuspension solution and lysed in similar volume of cell lysis solution. The respective mixtures were then incubated for approximately 5 min and 10 μl of alkaline protease solution added. After 5 min, the mixtures were neutralised with 350 μl of neutralization solution. Lysates were centrifuged at room temperature for 10 min. The cleared lysate was transferred to a spin column and centrifuged at room temperature for 1 min. The columns were washed with 1 ml of column wash solution and the DNA eluted in 80 μl of nuclease-free Water

Restriction enzyme digestion

In order to select the correct ligated plasmid, the purified plasmid was digested by specific restriction enzymes purchased from New England Biolabs (MA, USA). Every 0.5 μg of DNA was digested by 1 μl of restriction enzymes (20, 000 units/ml) at 37°C for 3 h.

DNA electrophoresis

The digested plasmid was run on the agarose gel by the DNA electrophoresis. 1% agarose gel was prepared by adding 1.0 g of agarose into 100 ml of 1 × TAE

buffer and dissolving by microwave for 1 min. It was allowed to cool down to approximately 60°C on the bench and 2 µl of ethidium bromide (EB) was added and then mixed well by swirling. The gel was poured slowly into the tank and bubbles were carefully removed. The comb was inserted and it was covered with foil. It was allowed to cool down on bench for 1 h. The digested plasmid was loaded and run at 100 volts for about 45 min.

3.2.3.9 Amplification of TPO promoter/luciferase reporter constructs

E.coli cells with the cloned plasmid were grown in 500 ml of LB medium supplemented with 75 µg/ml of ampicillin. The amplified plasmid DNA was extracted by using DNA PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 500 ml of overnight *E. coli* cultures were harvested by centrifugation at 3,000 rpm for 10 min. 10 ml of lysis buffer was added to cells and mixed by pipetting till the lysate mixture was thoroughly homogenous. The cell debris was spun down and the upper clear supernatant was transferred to the pre-equilibrated DNA binding column. The solution in the column was allowed to drain by gravity. The column was washed with 60 ml of washing buffer and the washing was allowed to drain by gravity. 15 ml of elution buffer was added to the column and the DNA was allowed to be eluted out by gravity. The eluate was collected and added to 10.5 ml of isopropanol. The sample was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was removed and the DNA pellet was resuspended in 5 ml of 70% ethanol. It was centrifuged at 12,000 rpm for 5 min at 4°C again and then the supernatant was carefully removed. The DNA pellet was allowed to dry in a clean fume hood. The DNA pellet was resuspended in 300 µl of TE buffer. The concentration was measured as described before and DNA was aliquoted and stored at -20°C or used directly.

3.2.3.10 DNA Sequence

Sequencing PCR

The concentration of the purified DNA was measured and sent to The DNA Sequencing Service (Ramaciotti Centre, UNSW, ABI 3730 Capillary Sequencer, Applied Biosystems, USA) to confirm the fidelity of the DNA sequence. The sequencing sample was prepared by using BigDye® sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The reaction was set up as below (Table 3.1) and the thermal cycling profile was set as: 96°C for 5 s, followed by 25 cycles consisting of 96°C for 12 s, 50°C for 7 s and 60°C for 4 min.

Table 3.1 Sequencing assay

Reagent	Amount
BigDye®	2.0 μl
5 × Reaction buffer	1.8 μl
Template DNA	0.5 μg
Primer	10 pmol
Water	Top up to 25 μl

Purification and sequencing of PCR product

The PCR product was then purified by ethanol precipitation. 20 μl of distilled water and 80 μl of 95% ethanol were added into the tube containing the PCR product and mixed by inversion and then incubated at room temperature for 15 min. The DNA was spun down at 12,000 rpm for 20 min. The supernatant was removed and the DNA pellet was resuspended in 250 μl of 70% ethanol. The DNA was spun down again at 12,000 rpm for 10 min and the supernatant was removed. The DNA pellet was air-dried for 15 min and the product was then sent for sequencing.

3.2.4 Transient transfection and Luciferase assay

3.2.4.1 Transient transfection

In order to evaluate the transcription activity of the human TPO promoter luciferase reporter constructs, the plasmids were transiently transfected into various cell lines. The details of each transfection methods are described in the subsequent chapters.

3.2.4.2 Luciferase assay

Cell lysis

1× working lysis buffer was prepared by diluting 5 × passive lysis buffer (Promega) in Milli-Q water. The spent culture medium was discarded and cells were washed with PBS twice. 250 µl of 1 × passive lysis buffer was applied to cells and rocked for 10 min at room temperature. Lysates were transferred to eppendorf tubes vortexed for 10 s and then the cell debris was removed by centrifugation at 4°C, 10,000 rpm for 3 min. The supernatant was collected into eppendorf tubes and then used for Luciferase assays or kept at -80°C until use.

Luciferase assay

The Luciferase assay was performed according to the manufacturer's instructions. Briefly, the LARII reaction buffer was prepared by dissolving LARII reagent in 10 ml of LARII buffer. The Stop&Glo reagent was prepared by diluting 50 × Stop&Glo reagent in Stop&Glo buffer to 1 × working solution. For each sample, 50 µl of cell lysate was mixed with 100 µl of LARII by vortexing and placed in the Turner luminometer TD20/20 (Turner Designs) to detect the firefly luciferase activity. 100 µl of Stop&Glo reagent was then added into the sample, mixed by vortexing and placed back into the luminometer to detect the renilla luciferase activity. The normalized luciferase activity was given by the ratio between the reading of firefly luciferase and renilla luciferase.

3.2.5 Electrophoretic mobility shifting assay (EMSA)

3.2.5.1 Oligonucleotide annealing

Single-stranded oligonucleotides with sequences that corresponded to the putative protein binding sites of the TPO promoter were purchased from Sigma-Aldrich and then annealed. The reaction was set up as shown in Table 3.2 and the mixture was heated to 96°C for 10 min and allowed to cool down to room temperature. The annealed oligos were then labelled with ^{32}P.

Table 3.2 Anneling reaction

Reagent	Amount
10 × Annealing buffer	5 µl
5’ Oligo-Sense (100 µM)	10 µl
5’ Oligo-Anti sense (100 µM)	10 µl
Distilled water	25 µl

3.2.5.2 ^{32}P labelling

The annealed oligos were labelled with ^{32}P by using the T4 PNK (NEB) according to the manufacturer’s instructions. The reaction was prepared as follows:

Table 3.3 ^{32}P labelling

Reagent	Amount
10 ×PNK buffer	4 µl
T4 PNK	4 µl
Annealed Oligo (20 pmole/µl)	2 µl
γ.^{32}P.dATP	8 µl
Distilled water	22 µl

The mixture was incubated at 37°C for 1 h and then the reaction was deactivated by incubating the mixture at 68°C for 10 min. The labelled oligos were then purified with G50 spin columns (GE Healthcare, NSW, Australia), according to the manufacturer's instructions. Briefly, the column was centrifuged at 2,000 rpm for 1 min and then the labelled oligos were added into the column and spun for 2 min. The clean oligos were collected and the efficiency of labelling and purification were assessed scintillation counting. Briefly, the sample was prepared by diluting 1 μl of labelled oligonucleotides into 1 ml of high-Scintilation solution (PerkinElmer, MA, USA). The samples were placed into the scintillation counter to read the efficiency of labelling.

3.2.5.3 Extraction of the nuclear protein

The spent medium was discarded and cells were washed by cold PBS twice. The cells were lysed in solution A for 10 min and then vortexed for 10 s. The nuclei were pelleted by spinning at 12,000 rpm for 10 s. The nuclei were then resuspended in solution C. The nuclei were lysed in solution C on ice for 20 min and then vortexed for 10 s. The cell debris was spun down and the nuclear proteins were collected and saved at -80°C.

3.2.5.4 Electrophoresis

A TBE gel (50 ml) was prepared as shown below:

Table 3.4 TBE gel

Reagent	Amount
30% Acrylamide	8.3 ml
10 × TBE	2.5 ml
10% APS	0.5 ml
TEMED	50 μg
Distilled water	38.1 ml

The gel mixture was carefully poured into the cassette (to avoid bubbles) and allowed to set. The binding reaction of nuclear extract, oligo probes and specific antibodies (in super shift assay only) was set up as listed in the following table:

Table 3.5 EMSA binding reaction

Reagent	Amount
2 × Binding buffer	10 µl
Nuclear extract	2 µl
Poly DI·DC (1 mg/ml)	1 µl
Antibody	1 µl
32p- Probe	1 µl
Distilled water	5 µl

The binding reaction was incubated on ice for 30 min before electrophoresis. The gel was pre-run at 200 volts for 30 min and each sample was mixed with 1µl of 20 × loading dye and then the sample was run at 250~260 volts for ~3 h. The gel was transferred and placed on a piece of whatman blotting paper. The gel was then dried in a gel drier at 80°C for ~2 h. The dried gel was exposed to Kodak BioMax X-ray film (Sigma-Aldrich) in a cassette at -80°C for ~18 h and then the film was developed to detect the radiolabeled probes.

3.2.6 Western blot

3.2.6.1 Preparation of cell lysate

Cells were washed with ice-cold PBS twice. The PBS was drained and the lysis buffer was added to the cells (1ml/1.0 × 10^6 cells). The cells were scraped off the culture vessels and then transferred to an autoclaved eppendorf tube. The cells were incubated in the lysis buffer and agitated at 4°C for 30 min. The cell lysate was spun using a benchtop centrifuge at 4°C, 12,000rpm, for 20 min. The

supernatant was collected and used for Western blot.

3.2.6.2 Measurement of protein concentration using the Bradford protein assay

The protein concentration was measured by Bradford protein assay. Briefly, 200 µg/ml of BSA solution in PBS was prepared. 2 ml of the Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories) was diluted in 10 ml of distilled water and the dilution was dispensed into autoclaved eppendorf tubes (1 ml each). The BSA dilutions were prepared for standard curve. Dilutions of BSA solution were prepared as shown below:

Table 3.6 Standard BSA solution

BSA (200 µg/ml)	PBS buffer	Total protein amount
10 µl	90 µl	2 µg
20 µl	80 µl	4 µg
30 µl	70 µl	6 µg
40 µl	60 µl	8 µg
50 µl	50 µl	10 µg
60 µl	40 µl	12 µg
70 µl	30 µl	14 µg

For each dilution, 100 µl of BSA solution was mixed with 1 ml of dye reagent by vortexing. For each measurement, 200 µl of mixture was transferred into a 96-well plate in triplicate. The protein sample was prepared in different dilution: dilute 1 µl, 2 µl and 4 µl of protein sample in 99 µl, 98 µl and 96 µl of PBS respectively (Dilution factors: 100, 50 and 25). Then, 100 µl of protein dilution was mixed with 1 ml of prepared dye reagent by vortexing. For each measurement, 200 µl of mixture was transferred into a 96-well plate in triplicate and analysed using a plate reader.

3.2.6.3 Sample preparation

The sample was prepared by mixing protein from cell lysate (10 µl), 2 × loading dye (12 µl) and 1M DTT (2.2 µl), incubated at room temperature for 10 min, and then heated at 100°C for 3 min. The sample was chilled on ice and spun down. The prepared samples were stored at -20°C until use.

3.2.6.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The concentrated running buffer (10 ×) was prepared by mixing 121 g of Tris, 238 g of HEPES and 10 g of sodium dodecyl sulphate polyacrylamide gel in distilled water (total volume 1L). The samples were loaded and run at 100 volts for 1 to 2 h.

3.2.6.5 Protein transfer

The transfer buffer (1 ×) was freshly prepared every time when use by mixing 14.4 g of Glysine, 3.3g of Tris and 200 ml of Methanol in distilled water (total volume 1L). The transfer membrane (Immobilon-P, filter type PVDF, Merk Millipore, MA, USA) was rinsed with methanol for 1 min and washed in transfer buffer. The gel was equilibrated in transfer buffer for 5 min and the transfer sandwich was assembled. The protein was transferred at 90 volts for 1 h at 4^{o}C.

3.2.6.6 Blocking

The membrane was blocked with 5% skim milk at 4^{o}C overnight in PBS-T buffer. The PBS-T buffer was prepared by mixing 100ml of 1M Tris [pH 7.4], 60 ml of 5 M Sodium chloride and 10ml of 10% T-20 in distilled water (total volume 2 L).

3.2.6.7 Antibody blotting

The blocked membrane washed with PBS-T 3 times (5 min each time). The primary antibody was diluted in 2.5% skim milk in PBS-T to a concentration according to the manufacture's instruction. The membrane was incubated by rocking on a rocker at room temperature for 1 h. The membrane was then washed with PBS-T 3 times (10 min each time) and the membrane was incubated with the secondary antibody, incubated with Western Lightning® Plus-ECL enhanced chemiluminescence substrate (PerkinElmer) and the signal developed according to the manufacturer's instructions.

Chapter Four: The Minimal Promoter of Human TPO Gene

Chapter Four: The Minimal Promoter of Human Thrombopoietin (TPO) Gene

4.1 Introduction

As discussed in Chapter one, for a long time, the classic receptor-mediated regulation mechanism of TPO indicates that the production of TPO mainly in liver and kidney remains constantly without gene regulation. TPO level in the circulation is regulated by a c-MPL-mediated platelet uptake and subsequent intraplatelet destruction. However, this mechanism can only explain the situation in thrombocytopenia due to decreased platelet production such as aplastic anaemia and amegakaryocytic thrombocytopenia. It does not explain the TPO levels in patients with ITP and other similar conditions when the thrombocytopenia is caused mainly by peripheral platelet destruction, the blood TPO levels are not significantly elevated even in patients with markedly low platelet counts. Moreover, another study showed the c-Mpl expressed on endothelial cells does not significantly regulate TPO levels (Geddis et al, 2006). A recent study also indicated that TPO expression is under circadian control by CLOCK, a circadian transcription factor (Tracey et al, 2012). Another important study by Sungaran et al (1997) found that TPO transcript in bone marrow stromal cells significantly increased in aplastic anaemia patients with severe thrombocytopenia, indicating that there is an enhanced TPO production apart from the reduced c-MPL- mediated destruction. Sungaran et al (2000) further demonstrated the presence of a feedback regulation of TPO production by bone marrow stromal cells and showed that the platelet/megakaryocyte α-granule proteins PDGF and FGF-2 enhanced, while platelet factor 4 (PF4), thrombospondin (TSP) and transforming growth factor beta (TGF-β) suppressed TPO expression (Sungaran et al, 2000). Moreover, as PF4, TSP and TGF-β are more abundant among platelet α-granule proteins, the overall effect of α-granule

proteins was inhibitory. However, the mechanism whereby α-granule proteins, particularly PF4, mediate regulation of TPO production remains to be elucidated. These studies clearly indicate TPO production at least in the bone marrow stromal cells is negatively regulated transcriptionally. My study aims to investigate the transcriptional repressor for human TPO. My hypothesis is that human TPO expression is negatively regulated by a transcriptional repressor and that the human TPO gene contains the critical repressor response element.

The study described in this chapter mainly aims to localize the position of potential repressor response element in the promoter region of the human TPO gene. For the human TPO gene promoter, initial analysis revealed that the human TPO gene spans about 6 kb and contains 6 exons and 5 introns (exon 1 to exon 6 and intron 1 to intron 5 in **Fig. 4.1 A**) (Foster et al, 1994; Sohma et al, 1994; Gurney et al, 1995a). Later Chang and colleagues (1995) reported that the gene spans about 8 kb and contains 7 exons and 6 introns (extra exon 0 and intron 0 in **Fig. 4.1 A**) (Chang et al, 1995; Dordelmann et al, 2008). Thus, there are two alternative promoters for the TPO gene, promoter 0 and promoter 1 (**Fig. 4.1 A**). Further investigations found that 90% of the transcription starts from promoter 1 and 10% from promoter 0 (Ghilardi et al, 1998; Wiestner et al, 1998). We propose that promoter 1 contains the elements that drive the main transcript of human TPO and, therefore it will be the focus of our work. In this chapter, a series of TPO promoter luciferase DNA plasmids covering various regions, ranging from -627 to +688 in the human TPO promoter region were constructed by polymerase chain reaction (PCR) and direct molecular cloning. The constructs were transiently expressed in the mouse bone marrow stromal cell line OP9 (used in this thesis as a surrogate of bone marrow stromal cells) and the human cervical carcinoma cell line, HeLa cells. The corresponding promoter activities were determined by the luciferase assay. According to the luciferase activities, the minimal promoter was localized to the -202 to +14 in the TPO promoter region. Other constructs encompassing the minimal promoter

region and additional downstream sequences showed significantly suppressed promoter activities, suggesting the existence of repression element(s) within the +14 to +688 region in intron 1. The following chapters (Chapter five and six) will focus on the accurate localization and identification of the strong repressor in this region. On the other hand, the potential enhancing transcriptional element(s) in the TPO promoter had been also investigated by Kamura et al (1997). They found that the one of the Ets transcriptional factors family, E4TF1/GABPα, was capable of enhancing the expression of human TPO confirming the finding of these authors (Kamura et al, 1997). In the present study, using electrophoretic mobility shifting assay (EMSA), I was able to confirm that the motif, -ACTTCCG- from -109 to -103 in the upstream of regulatory region of human TPO gene, was the functional binding target of E4TF1/GABPα.

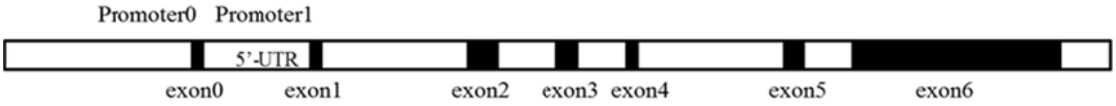
4.2 Materials and Methods

4.2.1 Cell culture

In this chapter, various types of cell lines were used including, HeLa cells, HepG2 cells and OP9 cells. The culturing conditions were stated in section 3.1.

4.2.2 TPO promoter luciferase constructs

A:



B:

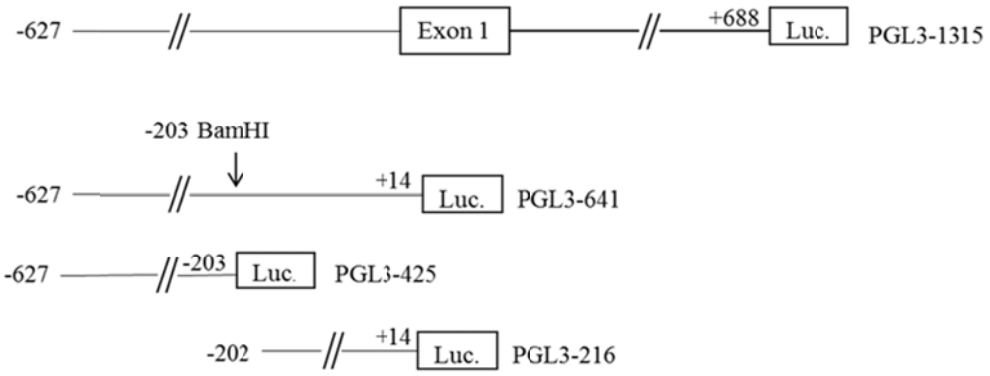


Figure 4.1 Cloning of human TPO promoter constructs (PGL3-1315, PGL3-641, PGL3-425 and PGL3-216). *A: the structure of human TPO gene. Different researchers believed human TPO gene contains 6 exons and 5 introns (Foster et al, 1994; Sohma et al, 1994; Gurney et al, 1995a) or 7 exons and 6 introns (Chang et al, 1995). Thus, there are two alternative TPO promoters (P0, P1) and it was shown that about 90% of transcription starts from P1 and 10% from P0 (Ghilardi et al, 1998; Wiestner et al, 1998). B: TPO promoter luciferase constructs. Four TPO promoter luciferase DNA plasmids*

(PGL3-1315, PGL3-641, PGL3-425 and PGL3-216) covering -627 to +688 in the TPO promoter 1 region were built. PGL3-1315 and PGL3-641 were built by direct cloning. A BamHI restriction enzyme site presents at -203 in the 5'-flanking region of human TPO gene. PGL3-216 and PGL3-425 were collected by digestion of fragment 641 by restriction enzyme BamHI and then subcloning into PGL3-Basic luciferase reporter vector.

4.2.2.1 Construction of PGL3-1315

A 1.3 kb TPO promoter fragment (-627 to +688) was amplified by PCR using genomic DNA from HepG2 cells as template (Primer set-Fp: 5'-GTATAGATCTAGACGAGCACCTAAGCTCAG-3', Rp: 5'-CTGCAGATCTCCACTAACATATCTGTCTC-3'). The PCR condition is shown in the table below:

Table 4.1 PCR of fragment 1315

Reagent	Amount
5 × Reaction buffer	10.0 μl
$MgCl_2$ (25 mM)	2.0 μl
dNTPs (2 mM)	5.0 μl
Primers (Sense/Anti-sense)	2.0 μl each
Template DNA (HepG2 genomic DNA)	5.0 μl (~ 0.5 μg)
DNA polymerase (Expand high fidelity^{plus} PCR system)	0.5 μl

The PCR thermal cycling program was set up as: an initiation denaturation at 94°C for 2 min, followed by 35 cycles consisting of 20 s denaturation at 94°C, 30 s annealing at 62°C and a 1.5 min extension at 72°C, followed by a 7 min extension at 72°C.

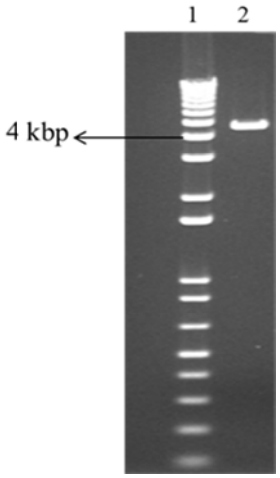


Figure 4.2 DNA electrophoresis of digested PGL3-Basic vector. *The PGL3-Basic vector was digested by BglII/HindIII (lane 1: DNA marker, lane 2: digested PGL3-Basic vector).*

As shown in **Fig. 4.2**, the plasmid PGL3-Basic was digested with BglII and HindIII, the resulting product is seen as a sharp and bright single band in lane 2. The fragment was extracted and used in the DNA ligation directly or stored in the -80°C until use.

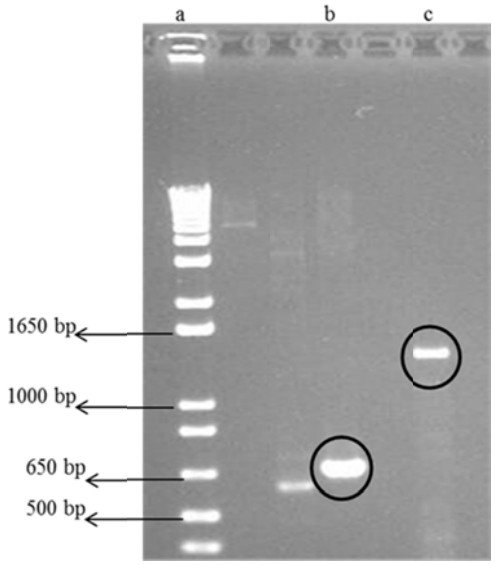


Figure 4.3 PCR amplification of fragments 1315 and 641. *Fragment 1315 and 641 were amplified by PCR and the products separated by DNA electrophoresis (lane a: DNA marker, lane b: fragment 641 and lane c: fragment 1315).*

The amplified product was purified as described in section 3.2.3, and then digested with BglII/HindIII and cloned into BglII/HindIII sites of the PGL3-Basic vector. The digestion and ligation were set up as shown in Table 4.2, Table 4.3 and Table 4.4.

Table 4.2 HindIII digestion of fragment 1315

Reagent	Amount
10 × Digestion buffer 2	2.0 µl
Fragment 1315	2.0 µl (~ 0.5 µg)
HindIII (20 U/µl)	0.5 µl
Distilled water	15.5 µl

The reaction was incubated at 37°C for 3 hours and purified. The product was then redigested with BglII as shown in the table below:

Table 4.3 BglII digestion of fragment 1315

Reagent	Amount
10 × Digestion buffer 2	2.0 μl
Fragment 1315	2.0 μl (~ 0.5 μg)
BglII (10 U/μl)	1.0 μl
Distilled water	15.0 μl

Table 4.4 Ligation of plasmid PGL3-1315

Reagent	Amount
Ligation buffer	1.0 μl
PGL3-Basic (BglII/HindIII digested)	2.0 μl
Fragment 2864 (BglII/HindIII digested)	5.0 μl
T4 ligase	1.0 μl
Distilled water	1.0 μl

The mixture was incubated at 16°C for 1 hour. The ligation was then transformed into *E. coli*. The cells that were successfully transformed were cultured, and the cloned plasmid PGL3-1315 was amplified and the fidelity of the sequence was confirmed as described in section 3.2.3.10.

4.2.2.2 Construction of PGL3-641

A 641-bp fragment containing the region from -627 to +14 of the human TPO gene was amplified by PCR using genomic DNA from HepG2 cells as the template and after digestion with BglII and HindIII, the resulting fragment was ligated into BglII/HindIII sites of PGL3-Basic to obtain the PGL3-641. The PCR condition is shown in Table 4.5:

Table 4.5 PCR of fragment 641

Reagent	Amount
10 × Reaction buffer	5.0 μl
$MgCl_2$ (25 mM)	3.0 μl
dNTPs (2 mM)	5.0 μl
Primers (Sense/Anti-sense)	4.0 μl each
Template DNA (HepG2 genomic DNA)	5.0 μl (~ 0.5 μg)
KOD DNA polymerase	0.5 μl
Distilled water	27.5 μl

The PCR thermal cycling program was set up as: an initiation denaturation at 98°C for 2 s, followed by 30 cycles consisting of 15 s denaturation at 98°C, 30 s annealing and extension at 68°C, followed by a 2 s extension at 68°C. The amplified product (lane c, **Fig. 4.3**) was purified by PCI method and then digested with BglII/HindIII and cloned into BglII/HindIII sites of PGL3-Basic vector.

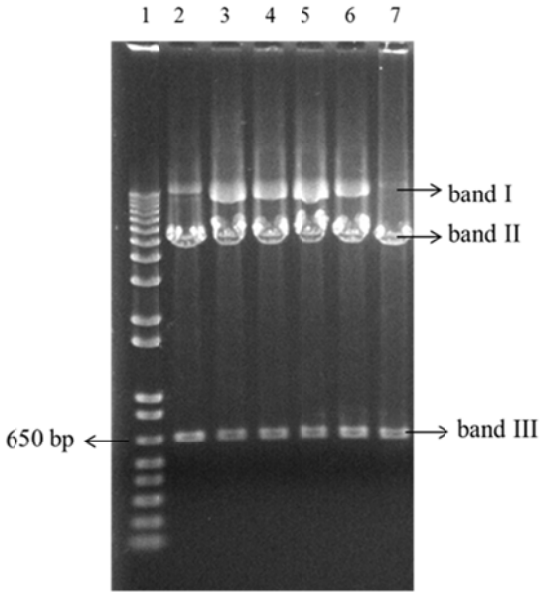


Figure 4.4 Screening of PLG3-641 cloning. *The PGL3-641 colonies were checked by restriction enzyme digestion and DNA electrophoresis (lane 1: DNA marker, lane 2 to lane 7: PGL3-641 colonies).*

Single colonies grown from the transformed cells were picked randomly. The DNA was extracted and digested by BglII/HindIII. All the six colonies contain the expected fragment 641 (**Fig. 4.4**, band III). The top band (band I) is the undigested plasmid and band II is the linearized PGL3-Basic vector. The fidelity of the cloned DNA was confirmed by sequencing. A maxi-prep of plasmid DNA was obtained for use in subsequent experiments.

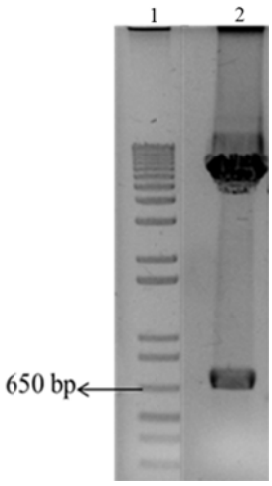


Figure 4.5 DNA electrophoresis of Maxi-prep product (PLG3-641). *The Maxi-prep product was digested by restriction enzymes and checked by DNA electrophoresis (lane 1: DNA marker, lane 2: Maxi-prep DNA of PGL3-641). The expected band was observed in lane 2.*

4.2.2.3 Construction of PGL3-425 and PGL3-216

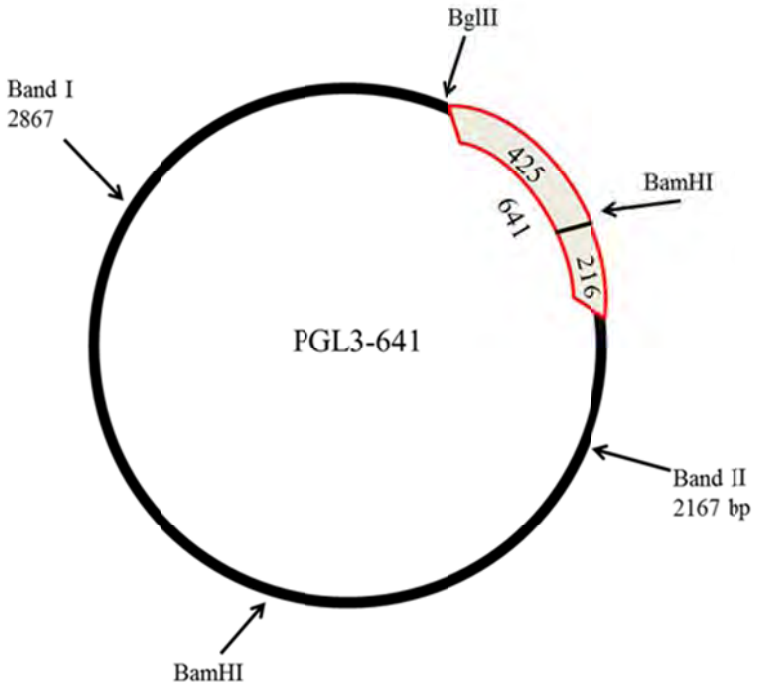
There is a BamHI site at the -203 position in the 5'- flanking region of the TPO gene within the fragment 641, thus fragments 425 (**Fig. 4.6 A**) and 216 (**Fig. 4.6 B**) were collected from the digestion of PGL3-641. Firstly, the fragment 641 was extracted from plasmid PGL3-641 by BglII/HindIII digestion (the digestion was set up similarly as described in Table 4.2 and Table 4.3). The digested plasmid was run by DNA electrophoresis and the separated fragment 641 was extracted and purified as described in section 3.2.3. The fragment 641 was then digested with BamHI as shown below (Table 4.6):

Table 4.6 BamHI digestion of fragment 641

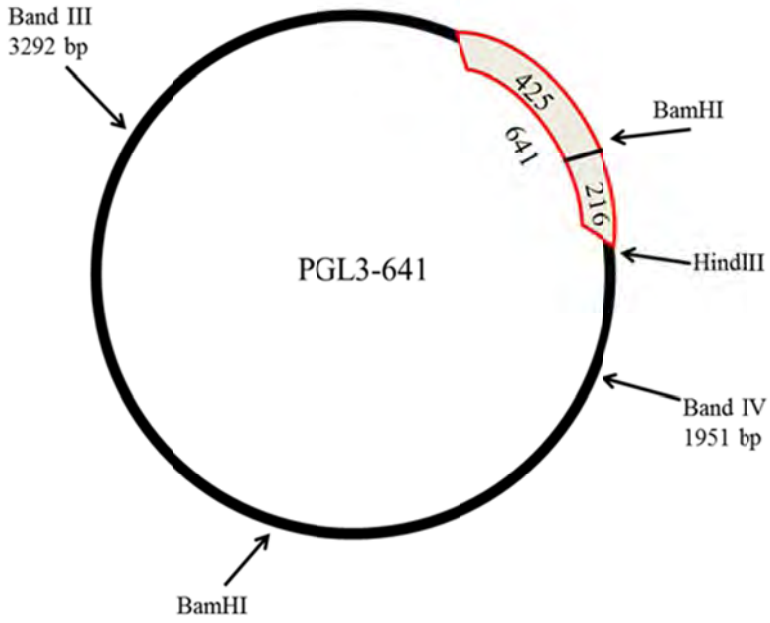
Reagent	Amount
10 × Buffer 2	2.0 µl
Bam HI (20 U/µl)	1.0 µl
HindIII/BglII digested 641	2.0 µl (~ 0.5 µg)
Distilled water	15.0 µl

These two fragments (425, 216) were extracted and purified (section 3.2.3). The fragment 216 was then ligated into the BglII/HindIII sites of PGL3-Basic vector. The fragment 425 with BglII/BamHI ends was ligated into the BglII site of PGL3-Basic vector (The BglII and BamHI sites are compatible for ligation).

A:



B:



C:

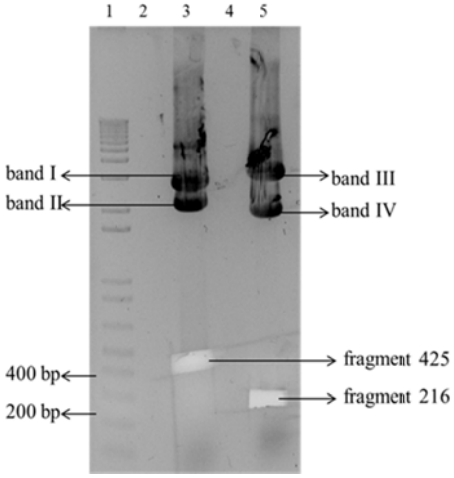


Figure 4.6 Purification of fragments 425 and 216. *A: fragment 425 (-627 to -203) was excised by digestion of plasmid PGL3-641 with BglII/BamHI. B: fragment 216 (-202 to +14) was excised by digestionof plasmid PGL3-641 with HindIII/BamHI C: DNA electrophoresis and gel extraction of plasmid PGL3-641 digestion (lane 1: DNA marker, lane 3: BglII/BamHI digestion of plasmid PGL3-641, lane 5: HindIII/BamHI digestion of plasmid PGL3-641).*

As the digestion map shown in **Fig. 4.6 A**, the BglII/BamHI digestion of plasmid PGL3-641 produced three fragments (lane 3 in **Fig. 4.6 C**): band I (2867 bp), band II (2167 bp) and fragment 425 (425 bp). The Digestion was run by DNA electrophoresis and the fragment 425 was collected by the gel extraction. Similarly, fragment 216 was obtained by digesting the plasmid PGL3-641 with restriction enzymes BamHI and HindIII. As seen in **Fig. 4.6 B**, the BglII/BamHI digestion produced three fragments (lane 5 in **Fig. 4.6 C**): band III (3292 bp), band IV (1951 bp) and fragment 261 (216 bp). Fragment 216 was purified by the gel extraction.

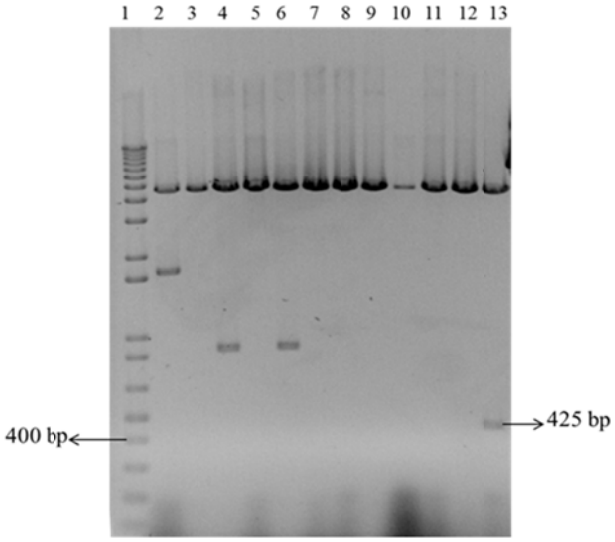


Figure 4.7 Screening of PLG3-425 cloning. *The PGL3-425 colonies were checked by restriction enzyme digestion and DNA electrophoresis (lane 1: DNA marker, lane 2 to lane 13: PGL3-425 colonies).*

The purified fragment was then ligated into PGL3-Basic vector. Twelve single colonies grown from the PGL3-425 ligation were randomly picked and cultured. DNA was extracted, digested with NehI/HindIII and separated on a 1% agarose gel (**Fig. 4.7**). One positive clone containing an insert of the expected size (lane 13) was obtained. Three colonies contained unknown inserts (lane 2, lane 4 and

lane 6) while the rest contained empty PGL3-Basic vector. The identity of the insert was confirmed by DNA sequencing as described before (Maxi-prep DNA is shown in appendices).

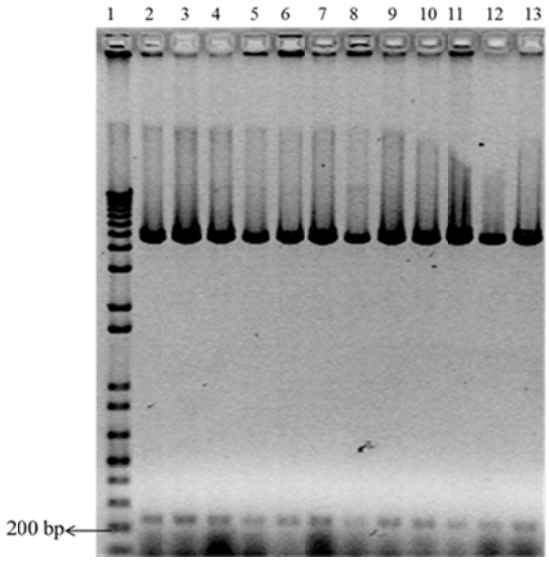


Figure 4.8 Screening of PLG3-216 cloning. *DNA electrophoresis of PGL3-216 colonies. The putative PGL3-216 vectors were checked by restriction enzyme digestion and DNA electrophoresis (lane 1: DNA marker, lane 2 to lane 13: PGL3-216 vectors).*

As shown in **Fig. 4.8**, twelve of the single colonies grown from the PGL3-216 ligation were randomly picked, grown and the DNA extracted and digested with NehI/HindIII. All the twelve colonies contained an insert of the expected size (lanes 2 to 13). The identity of the insert was confirmed by DNA sequencing as described before (Maxi-prep DNA is shown in appendices).

4.2.3 Transient transfection and luciferase assay

4.2.3.1 Transient transfection of HeLa cells

0.5×10^6 HeLa cells were seeded in each well of a 6-well plate 24 h before transfection. Just before transfection the spent medium was replaced with 1.5 ml of fresh growth medium. 2 μg of DNA plasmid, 50 ng of PRL-CMV (Promega) were diluted and mixed in 215 μl of Opti-MEM serum free medium (Invitrogen). 6 μl of Lipofectamine 2000 (Invitrogen) transfection reagent was diluted in 244 μl of Opti-MEM serum and incubated at room temperature for 5 min. The DNA dilution and transfection reagent dilution were mixed and incubated at room temperature for 20 min. The total of transfection mixture (500 μl) was applied to each well containing the HeLa cells in a dropwise manner. The transfected cells were cultured at 37°C, 5% CO_2 for 48 h and then subjected to the Luciferase assay.

4.2.3.2 Transient transfection of OP9 cells

OP9 transfection was done as previously described (Li X, 2010). Briefly, OP9 cells were seeded at a density of 7,000 cells/cm^2 in each well of a 6-well plate a day before transfection. On the day of transfection the cells reached a confluency of ~50%. The spent medium was replaced with fresh growth medium before the transfection. 4 μg of DNA plasmid, 50 ng of PRL-CMV (Promega) were diluted and mixed in 215 μl of Opti-MEM serum free medium (Invitrogen). 6 μl of Optifect (Invitrogen) transfection reagent was diluted in 244 μl of Opti-MEM serum and incubated at room temperature for 5 min. The DNA dilution and transfection reagent dilution were mixed and incubated at room temperature for 20 min. The total 500 μl of transfection mixture was applied to each well containing the OP9 cells in a dropwise manner. The transfected cells were cultured at 37°C, 5% CO_2 for 48 h and then subjected to the Luciferase assay.

4.2.3.3 Luciferase assay

The corresponding promoter activities were measured by the luciferase assay as described in section 3.2.4.2.

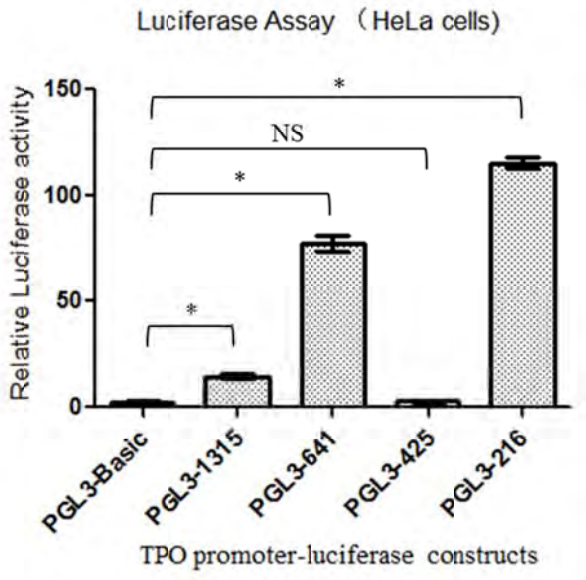
4.2.4 Electrophoretic mobility shifting assay (EMSA)

To investigate the potential transcriptional factors interacting with selected areas in the promoter region of the human TPO gene, the EMSA was performed as described in section 3.2.5.

4.3 Results

4.3.1 Minimal promoter of human TPO gene is narrowed down to -202 to +14

A:



B:

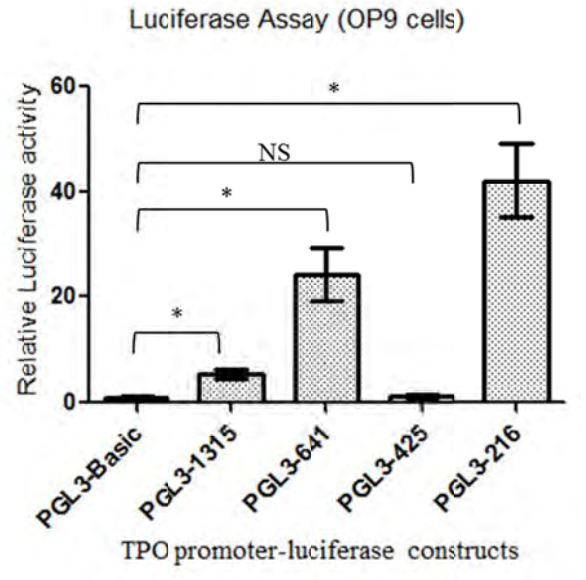


Figure 4.9 Luciferase assays of TPO promoter luciferase plasmids. *2 μg of*

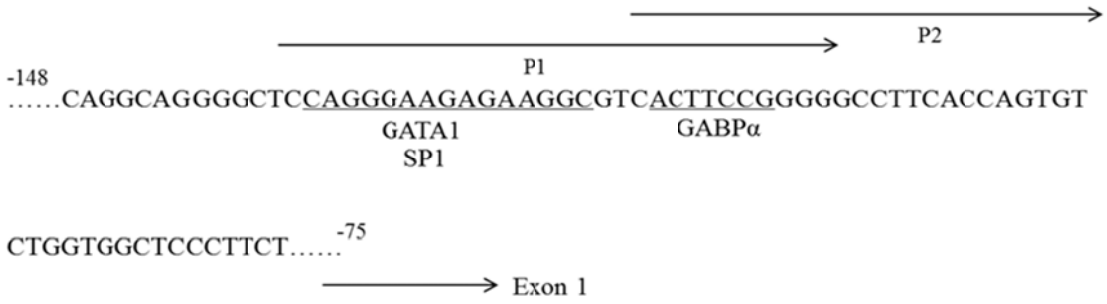
*TPO promoter luciferase constructs and 50 ng of Renilla luciferase expression vectors (PRL-CMV) were transiently expressed in HeLa cells (A), and OP9 cells (B). The data represent the mean of three independent experiments (mean +/- S.D. *P < 0.05, NS: not significantly different).*

In order to localize the minimal TPO promoter the previously generated TPO-promoter constructs: PGL3-1315, PGL3-641, PGL3-425 and PGL3-216 were transiently expressed in HeLa cells and OP9 cells. The corresponding promoter activities were then detected by the luciferase assay. As shown in **Fig. 4.9**, the cloned plasmid PGL3-1315 that covers ~600 bp on both upstream and downstream sides of exon 1 of the TPO gene exhibited a five times higher promoter activity compared with that of PGL3-Basic vector in both HeLa and OP9 cells. The shorter plasmid, PGL3-641 (covers -627 to +14 of human TPO gene, **Fig. 4.1**) that lacks the downstream ~600 bp showed a significantly higher promoter activity (28 and 23 times higher compared to the control-PGL3-Basic in HeLa and OP9 cells, respectively). These results indicated that the region covered by PGL3-1315 contained the human TPO promoter element. However, the much higher promoter activity driven by PGL3-641 suggested the existence of transcriptional repressor(s) binding sites within the +14 to +688 region of TPO. Unexpectedly, PGL3-425 (containing the 5' region of PGL3-641) showed no promoter activity compared with the negative control PGL3-Basic. In contrast, another portion of fragment 641 (-202 to +14) contained in the PGL3-216 vector showed the highest relative promoter activity (42 and 39 times higher compared to the control-PGL3-Basic in HeLa and OP9 cells, respectively). This result indicated that the region covered by this fragment (-202 to +14) is the minimal promoter for the human TPO gene in HeLa and OP9 cells.

4.3.2 Identification of potential transcription factors that bind to motifs within P1 and P2 probes using EMSA

To investigate the potential transcriptional regulators in the defined minimal TPO promoter region, two ^{32}P labeled oligonucleotide probes were designed (P1 and P2 in **Fig. 4.10 A**) and tested in EMSA. P1 contains putative binding motifs for transcription regulators GATA1, SP1 and GABPα. P2 contains GABPα alone. As described in Chapter one, GATA1 and SP1 regulate megakaryopoiesis and thrombopoiesis. GATA-1 works with its co-factor FOG-1 promoting erythroid and megakaryocyte differentiation (Tsang et al, 1997). SP1 regulates the myeloid-specific expression of the human hematopoietic cell kinase gene (Hauses et al, 1998). As mentioned in section 4.1, GABPα binds to motif, -ACTTCCG- from -109 to -103 and enhances human TPO expression (Kamura et al, 1997).

A:



B:

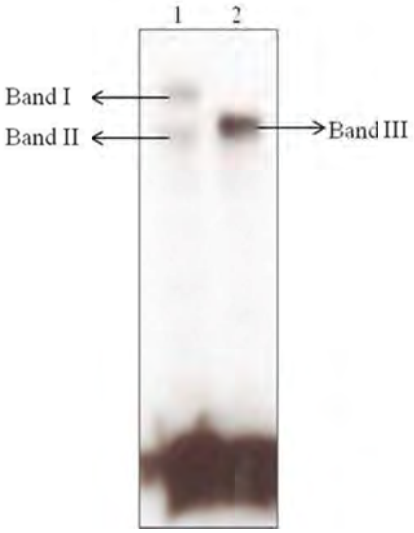


Figure 4.10 Electrophoretic mobility shifting assay (EMSA) showing nucleoproteins that bind to P1 and P2 probes. *A: probes (P1, P2) containing the putative binding motifs for the transcription factors (GATA1, SP1 and GABPα) were designed for the EMSA assay. B: EMSA was done by using ^{32}P labeled probes (P1, P2) and nuclear extracts from HeLa cells.*

As shown in **Fig. 4.10 B**, the binding reaction of P1 and nuclear extract from HeLa cells (lane 1) gave two weak binding signals (bands I and II) while P2 produced a stronger signal (band III). The reason for the weak binding signals from P1 is unclear but may result from nonspecific binding comparing with the much stronger specific binding observed in P2. These results suggested that certain protein(s) in the nuclear extract recognized the labeled probes. To

determine the identity of the bound protein(s), we used antibodies against the likely transcription factors (GATA1, SP1 and GABPα) and analysed the binding in a super-shift assay.

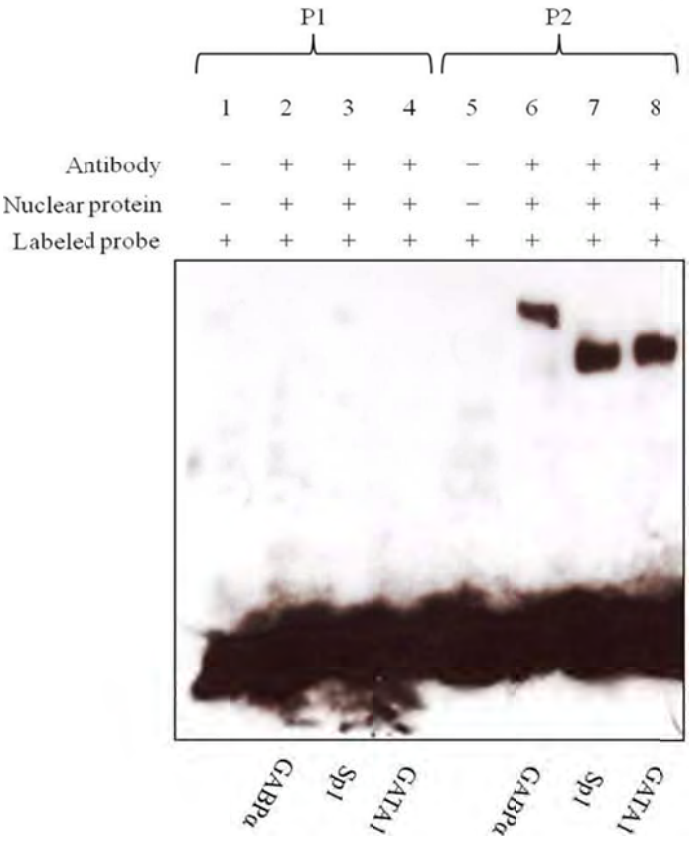


Figure 4.11 Identification of transcription factors using EMSA and specific antibodies. *EMSA was done by using ^{32}P labeled P1 (lane 1 to lane 4), P2 (lane 5 to lane 8) and specific antibodies against the transcription factors shown in the figure. GABPα (lane 2 and lane 6), Sp1 (lane 3 and lane 7) and GATA1 (lane 4 and lane 8).*

As shown in **Fig. 4.11**, with the further modified binding condition (reduced nuclear extract, increased Poly(DI·DC) (deoxyinosinic-deoxycytidylic, a nonspecific protein blocking agent) to reduce the nonspecific binding) the nonspecific binding of P1 disappeared and there is no binding signal detected in reactions containing antibodies (P1: lane 1 to lane 4). P2 showed the strong

specific binding with nuclear protein from HeLa cells and the specific antibody against GABPα successfully super-shifted the retarded band (**Fig. 4.11**, lane 6). This result indicates that the presumed transcriptional regulator motifs in P1 are not functional for the TPO transcription in HeLa cells. The GABPα motif in the region, from -109 to -103, showed binding of this transcriptional regulator. It is interesting to note that both P1 and P2 contain the GABPα recognition motif, however, the P1 did not produce a binding signal. That is probably because P1 may form a secondary structure which inhibits the binding of GABPα or P2 may provide other supporting element(s)/secondary structure(s) that increase protein binding.

To corroborate that the binding of the GABPα motif in P2 to the putative transcription factor in the nuclear extract of HeLa cells, a competition assay was performed using both cold (unlabelled) P2 probe and a mutant P2 probe. The mutant P2 was designed by replacing the thymine (T) at -106 and cytosine (C) at -107 with guanine (G) and adenine (A) respectively (**Fig. 4.12**):

A:

P2: TCACTTCCGGGGGCCTTCACCAGTGTC

Mutant P2: TCACTGACGGGGGCCTTCACCAGTGTC

B:

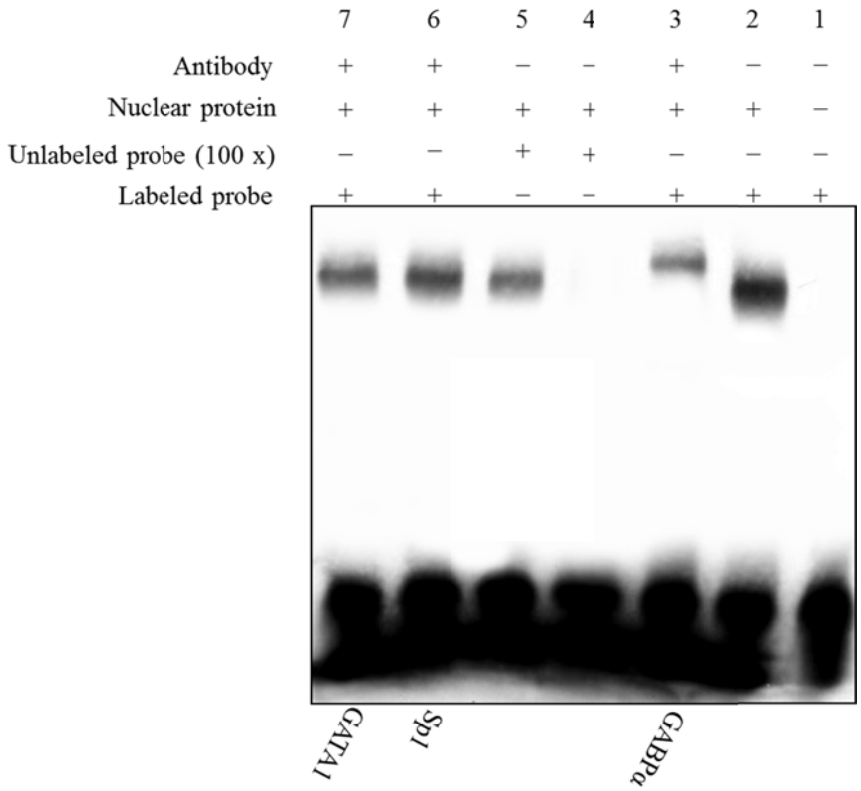


Figure 4.12 EMSA to confirm the binding specificity of radiolabeled P2 probe to the nucleoproteins. *A: the mutant P2 was designed by replacing the thymine (T) at -106 and cytosine (C) at -107 with guanine (G) and adenine (A) respectively. B: EMSA with competition by 100 folds excess of unlabeled probes. Lane 1 contained labeled P2 only. Lane 2 contained the complex of labeled P2 and nuclear extract from HeLa cells. GABPα antibody was added to supershift the complex of P2 and nuclear extract in lane 3. Lane 4 and lane 5 are competition assays performed by using 100 folds excess of unlabeled P2 (lane 4) and mutant unlabeled P2 (lane 5). Lane 6 and lane 7 are supershift assay by using antibody against Sp1 and GATA1 respectively.*

As shown in **Fig. 4.12 B**, a 100 folds excess of unlabeled P2 in the binding reaction caused the disappearance of the signal (lane 4). In contrast, nonspecific competition with the mutant probe did not significantly affect protein binding to the P2 (lane 5). This result further confirmed that the binding of P2 and the putative factor in nuclear extract of HeLa cells was dependent on the presence of an intact GABPα site. Super shift assay with specific antibodies showed that specific antibody against GABPα shifted the complex of P2 and HeLa nuclear extract to a higher position (lane 3) compared to reactions by using antibodies against Sp1 (lane 6) and GATA1 (lane 7). These results indicated that the putative GABPα motif, -ACTTCCG- from -109 to -103 in the upstream of regulation region of human TPO gene is the functional binding motif for GABPα.

4.4 Discussion

In this study, four vectors containing various regions of approximately 600 bp on both sides of exon 1 of human TPO gene were cloned into the reporter plasmid PGL3-Basic vector and their corresponding promoter activities were analyzed by transient transfection in two different cell lines. The results showed that the smallest construct of the TPO gene promoter capable of driving luciferase expression was found in the region -202 to +14 (plasmid PGL3-216). This finding is consistent with a previous study (Kamura et al, 1997), which suggested a minimal promoter region from -102 to +56 of the human TPO gene. Taken together these two findings (from my study and the previous study), the minimal promoter of human TPO gene is located in the-102 to +14 region.

Kamura et al (1997) further revealed that the sequence, -ACTTCCG- from -109 to -103 of TPO gene, is essential for high expression of TPO, and this motif was latter shown to be the binding site for GABPα, a member of Ets family of transcription factors (Kamura et al, 1997). In the present study, this -ACTTCCG- motif in the defined minimal TPO promoter was again shown in the EMSA assay to be the binding site for GABPα. This result further supports GABPα to be functional enhancer of TPO expression. It has been shown that GABPα regulates the megakaryocytic maturation. The heterodimeric GABP complex consisting of GABPα (DNA-binding) and GABPβ (activation and nuclear localization of GABPα) regulates early megakaryocyte-specific gene expression (Rosmarin et al, 2004; Pang et al, 2006). A recent study revealed that myeloid differentiation needs GABP which also partly regulates Gfi-1 expression (Yang et al, 2011). In their study, the number of myeloid cells in GABP knock-out mice dropped significantly, and the residual myeloid cells were dysplastic and immunophenotypically abnormal. As it has been well documented that TPO also regulates differentiation of early myeloid progenitors

(Carver-Moore et al, 1996; Yoshida et al, 1997; Solar et al, 1998), it is possible that GABPα regulation of myeloid differentiation may be in part via enhancing TPO expression.

In conclusion, the present study further localized the minimal promoter of human TPO gene to -202 to +14. The putative transcriptional regulators within the defined TPO promoter region were investigated. Among all the putative transcriptional motifs, the GABPα motif present from -109 to -103 in the upstream regulatory region, was shown to be the a functional binding target for GABPα. Moreover, a further downstream region from +14 to +688 was found to contain a strong transcriptional repressor region for the TPO gene. This finding is consistent with my hypothesis described in the Introduction of this chapter. The studies in the following chapters (Chapters five and six) would attempt to identify the possible novel transcriptional repressor(s) that bind(s) this region of the promoter.

Chapter Five: Characterization of ZEB1, a Potential Repressor for Human TPO Expression

Chapter Five: Characterization of ZEB1, a Potential Repressor for Human TPO Expression

5.1 Introduction

This study aims to explore the transcriptional control of TPO production. As discussed in previous chapters (see section 2.1 and 4.1), plasma TPO level is regulated by a receptor-mediated uptake model. Accumulated evidence indicates TPO production at least in the bone marrow stromal cells is also regulated transcriptionally. It is proposed that human TPO expression is negatively regulated by transcriptional repressor(s) and that the human TPO gene contains critical repressor response element(s). In Chapter four, a region from +14 to +688 was found to contain transcriptional repression element(s) for TPO promoter activity. This region contains binding sites for a number of transcription factors. We noticed the presence of four putative binding motifs for a transcriptional repressor called ZEB1 (**Fig. 5.2**). The zinc finger E-box binding protein 1 (ZEB1), also known as δEF1, Nil-2-a, Tcf8, Bzp, Areb6, Meb1, Zfhx1a and Zfhep, is a well-studied transcription factor that negatively regulates expressions of a number of genes. ZEB1 suppresses the transcription of the immunoglobulin heavy chain enhancer (Genetta et al, 1994) and interleukin-2 (IL-2) (Yasui et al, 1998). Studies of expression of muscle genes revealed that ZEB1 inhibited myotube formation and expression of essential markers for differentiation including myosin heavy chain and myogenin (Postigo and Dean, 1997). ZEB1 protein contains a POU-like homeodomain and two DNA-binding zinc finger clusters (one at its N- and another at its C-terminus) which bind with high affinity to the consensus core sequence, 5’-CACCT-3’ (Funahashi et al, 1993). Two important studies suggested that

ZEB1 is a possible transcription repressor for TPO expression: (1) Fontemaggi and colleagues (2001) identified a cluster of six ZEB1 binding sites within intron 1 of human p73 gene. This ZEB1 cluster was found to strongly inhibit p73 promoter activation (Fontemaggi et al, 2001). A similar ZEB1 cluster consisting of four consensus motifs was localized within a suppression region (+14 to +688) in intron 1 of TPO gene (**Fig 5.2**). (2) A recent study reported a serum-induced suppression on TPO expression in the bone marrow cells, and it suggested that ZEB1 might be the transcriptional suppressor of TPO regulation (McIntosh and Kaushansky, 2008). Based on the above data, I hypothesize in this chapter that the ZEB1 cluster in +14 to +688 region of the TPO promoter binds ZEB1 which mediates inhibition of TPO transcription.

In this chapter, investigations to identify the potential transcriptional repressor of human TPO gene expression are described. In particular, studies were carried out to prove or disprove the hypothesis that the repressor is ZEB1. A series of TPO promoter luciferase constructs that bear binding motifs for ZEB1 were made by PCR and molecular cloning. The plasmid constructs were transiently expressed both in the cell lines that express ZEB1 (HeLa cells and OP9 cells) and also in cell lines that do not express a detectable level of ZEB1 (shown by Western blot, **Fig 5.15**) (MCF7 cells and HepG2 cells). The promoter activities were determined by luciferase assay. EMSA was carried to determine if ZEB1 binds to the ZEB1 motifs in the TPO promoter. To evaluate further the role of ZEB1 in the repression of TPO expression, a ZEB1 mutant, ZEB1-RD (Repression Domain Deficient) which was deficient in repression activity but still retained its ability to bind DNA was generated (Postigo and Dean, 1997; Postigo and Dean, 1999; Postigo and Dean, 2000). ZEB1-RD was to compete with endogenous ZEB1 for binding to TPO promoter.

5.2 Materials and Methods

5.2.1 Cell culture

The HeLa cells, HepG2 cells, OP9 cells and MCF7 cells were cultured as described in section 3.1.

5.2.2 Generation of 3’ -deletion TPO promoter constructs containing the ZEB1 elements

In order to investigate whether the ZEB1 elements in intron 1 of TPO gene were responsible for the suppression of TPO promoter activity, two 3’- deletion TPO promoter constructs bearing two ZEB1 binding motifs (PGL3-886, **Fig. 5.2 A**) and four ZEB1 binding motifs (PGL3-958, **Fig. 5.2 B**) were generated (**Fig. 5.1**) by PCR amplification.

A 958-bp fragment (-199 to +760) of TPO was amplified by PCR using plasmid PGL3-2864 as template (Primer set- Fp: 5’-CCAGGAAAAGATGGATCCC-3’, Rp: 5’-CATGAAGCTTGAGACCAGCTGCCCAGGAAC-3’). The product was digested with BamHI/HindIII and cloned into BamHI/HindIII sites of PGL3-Basic vector. A 886-bp fragment (-199 to +688) was generated by PCR using PGL3-1315 as template (Primer set-Fp: 5’-CCAGGAAAAGATGGATCCC-3’, Rp: 5’-CTGCAGATCTCCACTAACATATCTGTCTC-3’). The product was digested with BamHI/BglII and then cloned into BglII site of PGL3-Basic to obtain the PGL3-886 vector.

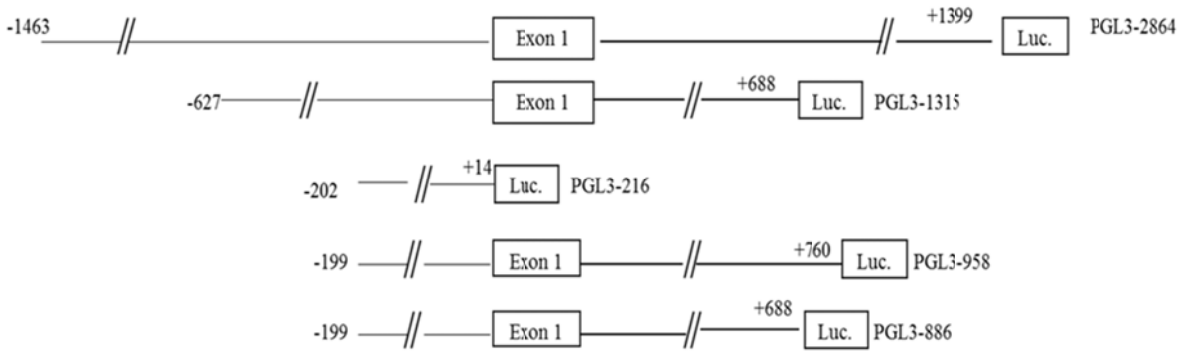


Figure 5.1 TPO promoter luciferase constructs (PGL3-958 and PGL3-886). *The TPO promoter luciferase constructs bearing four and two putative ZEB1 motifs (PGL3-958 and PGL3-886) respectively were designed. The plasmids (PGL3-2864, PGL3-1315, and PGL3-216) are also shown to indicate the regions covered by the new vectors.*

A:

5'…ACTCATGTTGACAGACCTACAGGAAATCCCAATATTGAATC**AGGTG**CAAGCCTC
ZEB1
TTTGCACAACTTGTGAAAGGAGGAGGAAGCCATGTGGGGGGTCCTGTGAAGGAA

CCGGAAGGGGTTCTGCCAAGGGGGCAGGGAGGC**AGGTG**TGAGCTATGAGACAG
ZEB1
ATATGTTAGTG…3'

ZEB1 motifs in PGL3-886

B:

5'…ACTCATGTTGACAGACCTACAGGAAATCCCAATATTGAATC**AGGTG**CAAGCCT
ZEB1
CTTTGCACAACTTGTGAAAGGAGGAGGAAGCCATGTGGGGGGTCCTGTGAAG

GAACCGGAAGGGGTTCTGCCAAGGGGGCAGGGAGGC**AGGTG**TGAGCTATGAG
ZEB1
ACAGATATGTTAGTGGGCGCCTAAGACAAGGTAAGCCCCTA**AGGTG**GGCATCAC
ZEB1
CCAGC**AGGTG**CCCGTTCCTGGGCAGCTGGTCTC…3'
ZEB1

ZEB1 motifs in PGL3-958

Figure 5.2 Putative ZEB1 motifs in the TPO promoter luciferase constructs (PGL3-958 and PGL3-886). *A: two putative ZEB1 binding motifs in PGL3-886. B: four putative ZEB1 binding motifs in PGL3-958.*

5.2.2.1 Cloning of PGL3-958

As shown in **Fig. 5.3 A**, the amplified product, fragment 958 was extracted and purified. The collected fragment 958 was digested by the restriction enzymes and cloned into PGL3-Basic vector to obtain PGL3-958. The single colony from the PGL3-958 ligation was picked and screened by restriction enzyme digestion of mini-prep DNA and DNA electrophoresis. As found in the DNA electrophoresis (**Fig. 5.3 B**), five out of six randomly picked single colonies from the PGL3-958 transformed *E. coli* contained the fragment 958 (lane 1 to lane 5). The plasmid DNA was then amplified by Maxi-prep and the fidelity

sequence was confirmed by sequencing assay. The DNA was concentrated and then stored at -80°C until use.

A: B:

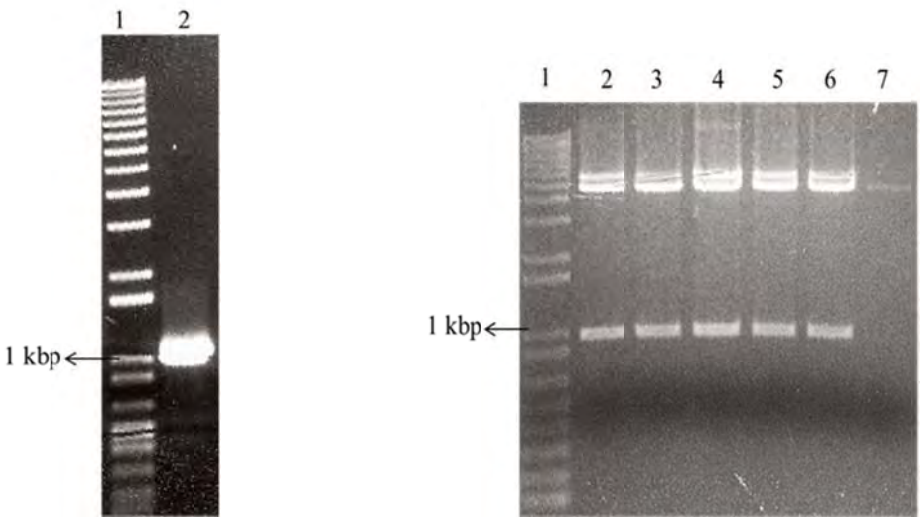


Figure 5.3 Cloning of PGL3-958*. A: DNA electrophoresis of fragment 958. The fragment 958 was obtained by PCR and the product was checked by DNA electrophoresis (lane 1: DNA marker, lane 2: fragment 958 PCR product). B: Screening of PGL3-958 colonies. Colonies containing PGL3-958 were checked by restriction enzyme digestion, and DNA electrophoresis showed inserts of the correct size (lane 1: DNA marker, lane 2 to lane 7: inserts of correct size from PGL3-958 colony).*

5.2.2.2 Cloning of PGL3-886

As shown in **Fig. 5.4**, the PCR successfully amplified fragment 886 (band III), however, there were two additional products (band I and band II) produced probably due to the nonspecific binding of the primers to the DNA template. Band III containing the fragment 886 was extracted, purified and used in a ligation reaction. The transformed cells were screened and plasmid PGL3-886 was purified as described section 5.2.2.1 (**Fig. 5.5**).

A: B:

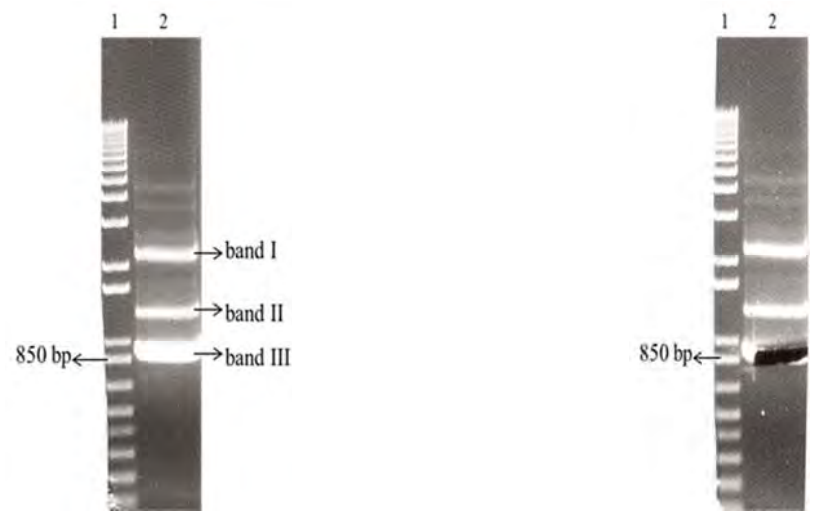


Figure 5.4 DNA electrophoresis of fragment 886. *A: PCR of fragment 886 generated two nonspecific products (band I and band II) and the specific product, fragment 886 (band III). B: gel extraction of fragment 886 (lane 1: DNA marker, lane 2: fragment 886).*

A: B:

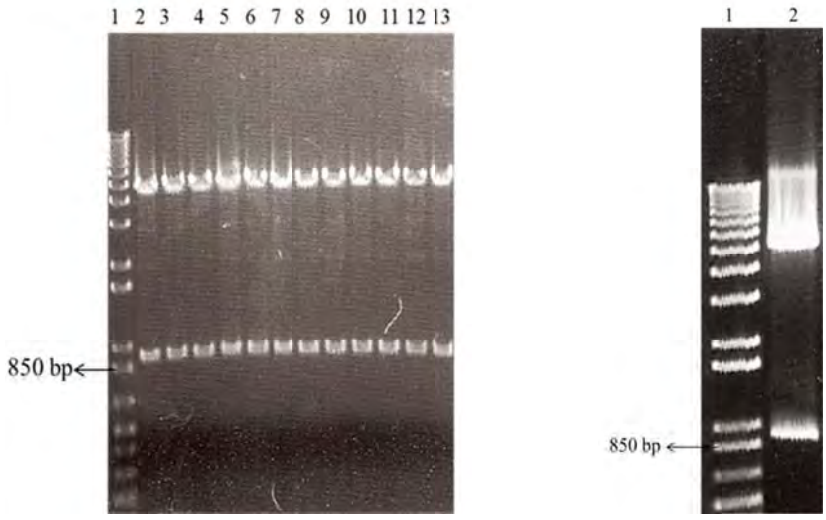


Figure 5.5 Screening of plasmid PGL3-886. *A: DNA electrophoresis of DNA extracted from PGL3-886 colonies. PGL3-886 vectors isolated from several colonies were checked by restriction enzyme digestion (NehI/HindIII) and DNA*

electrophoresis (lane 1: DNA marker, lane 2 to lane 13: PGL3-958 colonies). B: DNA electrophoresis of Maxi-prep of plasmid PGL3-886 (lane 1: DNA marker, lane 2: Maxi-prep of plasmid PGL3-886).

5.2.3 Transient transfection of 3'- deletion TPO promoter luciferase constructs and luciferase assay

TPO promoter luciferase constructs were transiently expressed in HeLa and OP9 cells, and cells were then harvested and subjected to the luciferase assay as described in section 4.2.3.

5.2.4 Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear extracts from HeLa cells and specific antibody against ZEB1 (Santa Cruz Biotechnology, Texas, USA). Probes (P1 to P4 in **Fig. 5.6**) covering the four putative ZEB1 binding sites were labeled with ^{32}P (sequences shown in Table 5a, Appendix C).

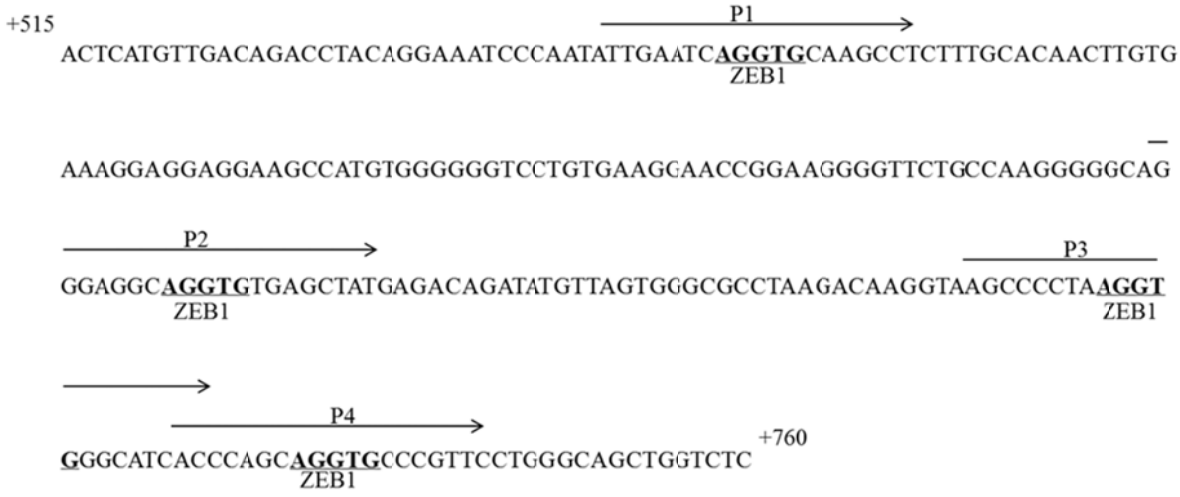


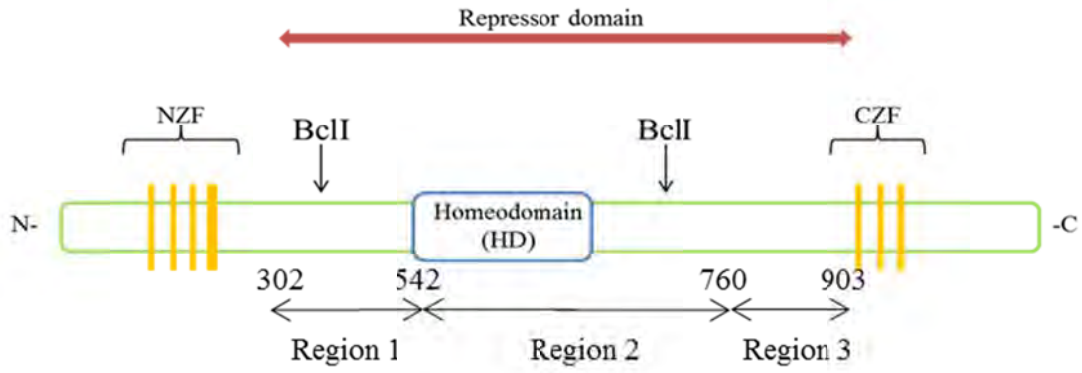
Figure 5.6 Oligonucleotide probes for ZEB1 EMSA. *The oligonucleotide probes containing the four putative ZEB1 binding motifs (termed P1 to P4) as indicated.*

5.2.5 Construction of Zeb1-RD (Repression Domain Deficient) expression vector

Strategy

ZEB1 consists of two zinc finger clusters at both termini, a central homeodomain and binding sites for co-repressors such as CtBP1 and CtBP2 (Furusawa et al, 1999; Wang, 2009) (**Fig. 5.7 A**). The zinc finger clusters function by binding to the specific DNA motif, 5'-CACCT(G)-3', and the region in between (Region 1 to 3 in **Fig. 5.7 A**) is responsible for the repression on various genes (Postigo and Dean, 1999; Postigo and Dean, 2000). According to Postigo and Dean, the most important region is Region 1 (aa 302 to aa 542) as this region exerts transcription repression that is equalled to that produced by the whole region. Region 1 is also the most relevant repression domain as it contained binding sites for haematopoiesis repressors, such as c-myb, ets and TFE-III (Postigo and Dean, 1999). Thus, the strategy was to generate a mutant ZEB1 in which Region 1 was deleted but it still retained its DNA binding ability (N-/C-zinc finger cluster). As shown in **Fig. 5.7 B** this can be achieved by BclI digestion.

A:



B:

Figure 5.7 Construction of ZEB1-RD (Repression Domain Deficient) expression vector. *A: Diagrammatic representation of ZEB1. The ZEB1 protein contains two zinc finger clusters on both N- terminal end (NZF) and C- terminal end (CZF) that are responsible for the binding to specific DNA motif, notably 5'-CACCT(G)-3'. The region between the two zinc finger clusters functions as the repression domain and contains a homeodomain (HD) and regions for the binding of ZEB1 co-repressors (numbers in the figure are amino acids positions that indicate the regions covers by Region 1 to Region 3). Two BclI restriction*

sites were found within the repressor domain of ZEB1. Digestion with BclI removed most of Region 1 and Region 2 without altering the reading frame. B: Open Reading Frame (ORF) of ZEB1 protein. The zinc finger clusters on N-terminal and C-terminal ends are boxed. The repression domain that was deleted by the BclI digestion is underlined (194 aa/582 bp).

5.2.5.1 BclI digestion and self-ligation

The ZEB1 expression plasmid PCMV-ENTRY-16-ZEB1 (OriGene, MD, USA) was digested by BclI restriction enzyme. The reaction was set up as below:

Table 5.1 BclI digestion of PCMV-ENTRY-16-ZEB1

Reagent	Amount
10 × Buffer 3	4.0 µl
Plasmid DNA	1.0 µg
BclI enzyme	1.0 µl
Milli-Q water	Fill up to a total volume of 40 µl

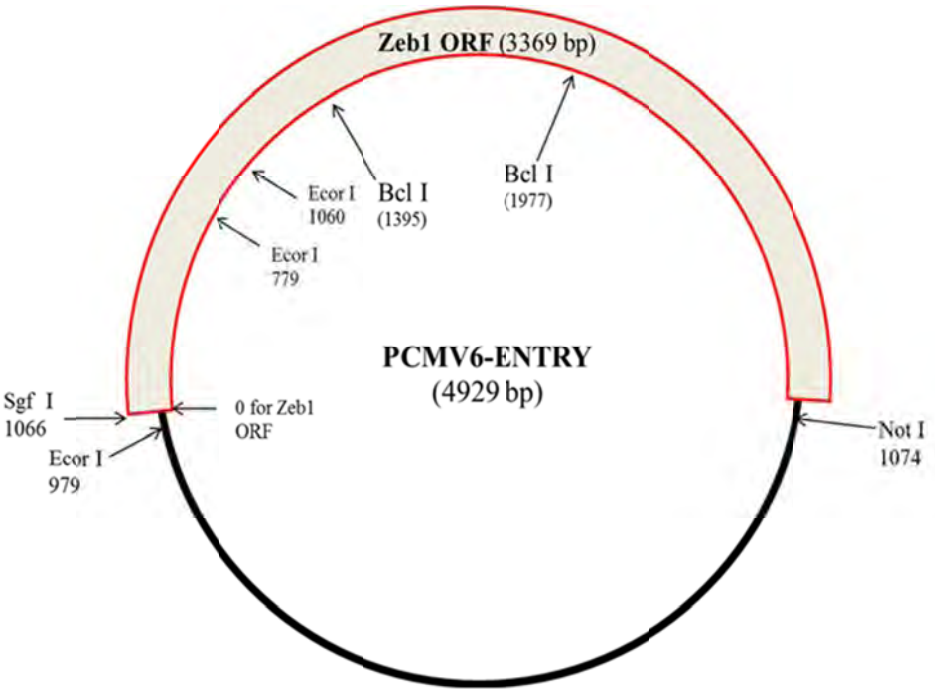
The reaction was incubated at 50 °C for 2 h. The digested plasmid DNA was run by electrophoresis and the correct fragment collected and purified by the gel extraction and ethanol/phenol/chloroform precipitation as described in section 3.2.3.3 and 3.2.3.4. The purified BclI digested DNA was ligated using T4 DNA ligase (NEB). The reaction was set up as below (Table 5.2) and the ligation was incubated overnight at 4°C.

Table 5.2 Self-ligation of PCMV-ENTRY-16-ZEB1

Reagent	Amount
Ligation buffer	1.0 µl
Digested DNA	50 ng
T4 Ligase	1.0 µl
Milli-Q water	Fill up to a total volume of 10 µl

As described in **Fig. 5.7** and **Fig. 5.8 A**, the BclI digestion would remove a fragment of 582 bp from the ZEB1 ORF and as seen in the **Fig. 5.8 B**, band I was collected and then self-ligated and transformed into *E.coli* cells.

A:



B:

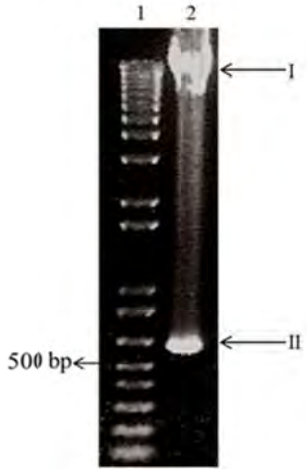


Figure 5.8 DNA electrophoresis of PCMV6-ENTRY-ZEB1 digestion. *A: Map of PCMV6-ENTRY-ZEB1. Some selected restriction sites are indicated. B: the PCMV6-ENTRY-ZEB1 was digested with BclI and the digestion product was run on gel electrophoresis (lane 1: DNA marker, lane 2: PCMV6-ENTRY-ZEB1 digestion product). Vector (band I) lacking the indicated 582 bp (band II) fragment was collected and purified.*

5.2.5.2 Preparation of the competent ER2925 cells

Because BclI is sensitive to Dam and Dcm methylation, an *E. coli* strain, ER2925 (NEB), which is Dam^-/Dcm^- was chosen for the transformation. The ER2925 cells were made chemically competent. Briefly, the cells were suspended in 10 ml of 0.1 M ice cold magnesium chloride ($MgCl_2$) by gentle mixing. Cells were spun (5,000 rpm, 4°C for 5 min) and resuspended in 1.5 ml of 0.1 M ice cold calcium chloride ($CaCl_2$) and incubated on ice for 30 min. The competent cells were dispensed into autoclaved Eppendorf tubes and stored at -80 °C until use.

5.2.5.3 Transformation of competent cells and amplification of the plasmid

The self-ligated plasmid was transformed into the competent ER2925 cells as described in section 3.2.3.7.

5.2.5.4 Screening of positive clones

The transformed ER2925 cells were grown on broth agar plate containing Kanamycin (50 µl/ml, Invitrogen) and Chloramphenicol (20 µg/ml, Sigma-Aldrich) (ZEB1-RD vector provided Kanamycin resistance and ER2925 cells are Chloramphenicol resistant). Single colonies were picked up and grown in 1.5 ml of LB medium containing the antibiotics (Kanamycin and Chloramphenicol). The plasmid was extracted from the cells by using the mini-prep kit as described in section 3.2.3.8 and the mini-prep DNA was used to check if it contained the correct inserts by digestion with EcoRI and NotI. The digestion of wild type PCMV6-ENTRY-ZEB1 produced four fragments of 4850 bp, 2317 bp, 886 bp and 281 bp, while digestion of PCMV6-ENTRY-ZEB1-RD was expected to produce four fragments of 4850 bp, 1735 bp, 886 bp and 281 bp (**Fig. 5.8 A**). As shown in **Fig. 5.9**, 19 out of 20 randomly picked colonies

contained the expected ZEB1-RD construct (lane 2 to lane 7, lane 9 to lane 11 and lane 13 to lane 22): fragments 4850 bp (band I), 1735 bp (band III), 886 bp (band IV) and 281 bp (band V). Lane 12 (wild type PCMV6-ENTRY-ZEB1) was used for comparison. One of the clones was sequenced to confirm that it contained insert with the correct sequence and this clone was used in the subsequent experiments.

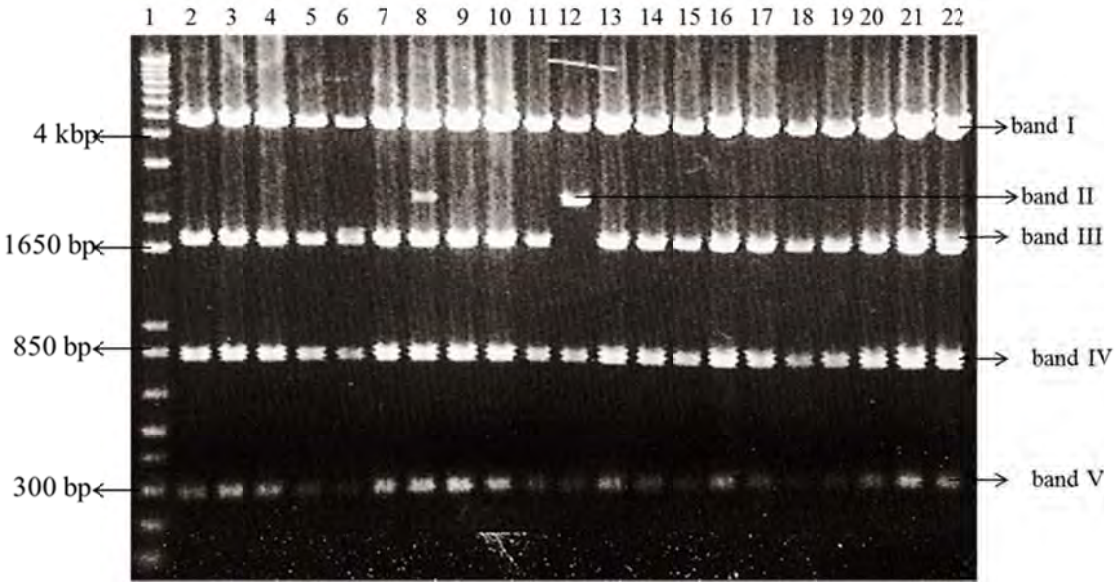


Figure 5.9 DNA electrophoresis of ZEB1-RD colonies. *The ZEB1-RD colonies were subjected to restriction enzyme digestion to check that they contained inserts with the correct size and then DNA electrophoresis was carried out (lane 1: DNA marker, lane 2 to lane 11 and lane 13 to lane 22: ZEB1-RD colonies, lane 12: Wild type ZEB1 expression vector- PCMV6-ENTRY-ZEB1).*

5.2.6 Competitive inhibition of endogenous ZEB1 by ZEB1-RD /Transient transfection and Luciferase assay

In order to investigate whether ZEB1 is responsible for the suppression of promoter activity of the TPO promoter (+14 to +688), ZEB1-RD was co-expressed in HeLa cells together with the plasmids PGL3-886 and

PGL3-958 using lipofectamine 2000 (invitrogen) as described in section 4.2.3.1. Briefly, 0.3×10^6 of HeLa cells were seeded in each well of a 6-well plate 24 h before the transfection. 2 μg of ZEB1-RD was transfected into HeLa cells and cells were cultured for 24 hours. The transfected cells were then transfected with 2 μg of PGL3-886 or PGL3-958 and cultured for another 48 h. The promoter activity was measured using luciferase assay as described in section 3.2.4.2.

5.2.7 Western blot

The efficiency of the ZEB1-RD transfection and protein expression were determined by Western blot as described in section 3.2.6.

5.2.8 Studies on cell lines that do not express endogenous ZEB1 protein

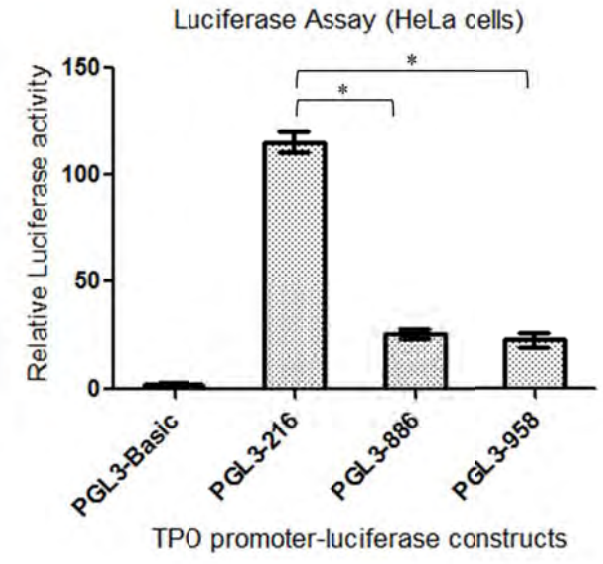
The expression of endogenous ZEB1 in a series of different cell lines was determined by Western blot. These included HeLa, MCF7, HepG2, human bone marrow stromal cells, mouse teratocarcinoma cell line (P19) and OP9. Cell lines that did not express the ZEB1 protein were transfected with PGL3-886 or PGL3-958 and the corresponding promoter activities were measured by luciferase assay.

5.3 Results

5.3.1 Promoter activities of putative ZEB1 elements as determined by transient transfection / luciferase assay

The promoter activities of putative ZEB1 elements containing plasmids (PGL3-886 and PGL3-958) were determined by the luciferase assay. As shown in **Fig. 5.10**, both the TPO promoter vectors showed strongly reduced promoter activities in HeLa and OP9 cells. The repressed promoter activities driven by PGL3-886 and PGL3-958 were similar in HeLa and OP9 cells compared with that of the minimal promoter construct PGL3-216 (about 22% and 27% of that of PGL3-216 in HeLa cells, and 20% and 29% in OP9 cells, respectively). That indicates that the region covered by PGL3-886 (-199 to +668) contains repression element(s) capable of causing reduced promoter activity even without the additional two putative ZEB1 elements in PGL3-958. However, additional experiments are needed to determine whether the observed reduced promoter activity results from the presence of the two ZEB1 elements in PGL3-886.

A:



B:

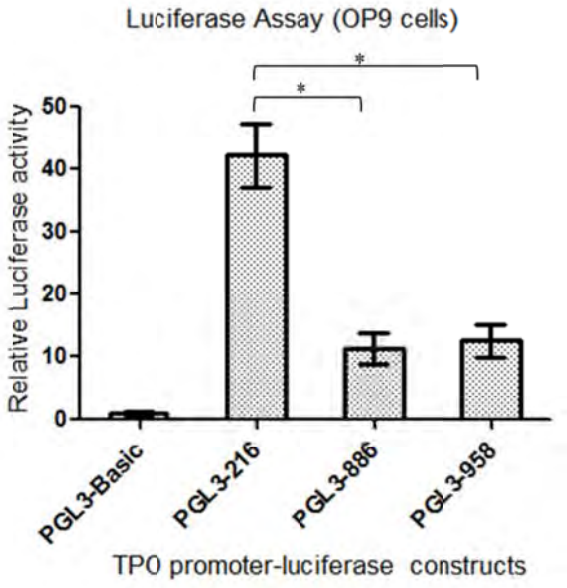
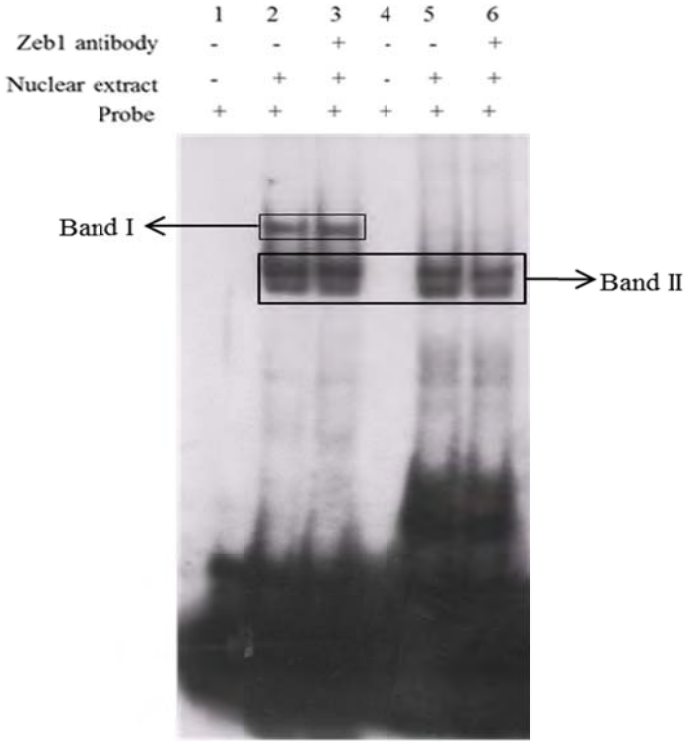


Figure 5.10 Luciferase activities of TPO promoter plasmids (PGL3-216, PGL3-886 and PGL3-958). *2 µg of TPO promoter constructs and 50 ng of Renilla luciferase expression vector (PRL-cmv) were transiently expressed in HeLa (A), and OP9 cells (B). The results show strong repressor activities of pGL3-886 and pGL3-958. The similar repressed promoter activities of PGL3-886 and PGL3-958 indicates that repression element(s) in PGL3-886 is sufficient to cause strong repression even without the additional two ZEB1 motifs in PGL3-958. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

5.3.2 To determine whether ZEB1 binds to the putative ZEB1 motifs in the TPO promoter using EMSA

The study attempted to determine whether the putative ZEB1 elements in the TPO promoter were recognized by nuclear proteins. To this end, nuclear extracts from HeLa cells was collected and EMSA was performed using the probes (P1 to P4) described in section 5.2.4.

A:



B:

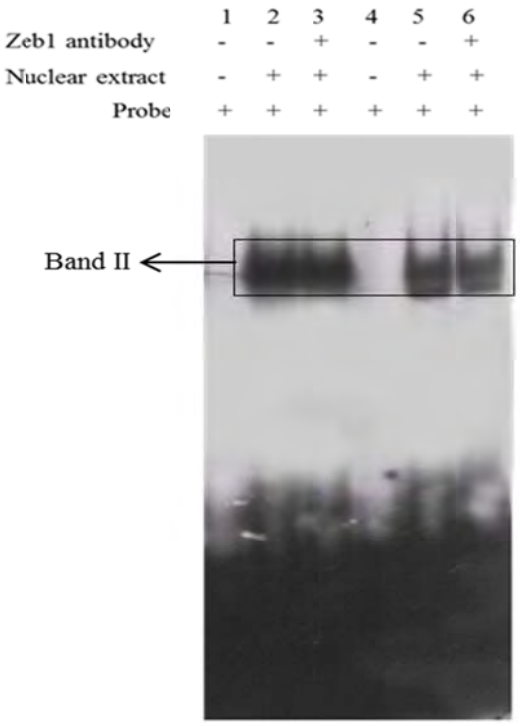


Figure 5.11 EMSA findings showing that the nucleoproteins interacting with Probes P1, P2, P3 and P4 were not ZEB1*. EMSA using* ^{32}*P labeled P1 (lane 1*

to lane 3 in A), P2 (lane 4 to lane 6 in A), P3 (lane 1 to lane 3 in B), P4 (lane 4 to lane 6 in B) and nuclear extract from HeLa cells. Specific anti-ZEB1 antibody was used in lanes 3 and 6 (panels A and B).

As shown in **Fig. 5.11**, all probes showed binding to proteins present in nuclear extract from HeLa cells (band I, II in lane 2 and lane 5). All band IIs were present at the same position suggesting that this might be specific binding. Band I produced by P1 occurred at a higher position may suggest dimerization of P1 or another binding protein(s) with a higher molecule weight. However, addition of an anti-ZEB1 antibody did not supershift the observed bands (lane 3 and 6). This indicates that another protein presented in the nuclear extract, which was not ZEB1, was responsible for the observed binding.

5.3.3 Mutant ZEB1 (ZEB1-RD) could not restore repressed promoter activities

The results from section 5.3.2 suggest that probes P1 to P4, although they contained ZEB1 recognition motifs in the TPO promoter, did not interact with ZEB1 but interacted with another nuclear protein(s) which was not ZEB1. EMSA, is a useful tool to study transcription factors however has its limitation as it is performed *in vitro* with naked DNA probes without taking chromatin structure and accessibility of transcription factors to *in vivo* binding sites into consideration (Pennacchio and Rubin, 2001). Thus, EMSA is most likely reliable when there is a direct binding of transcription factor to its binding sites (**Fig. 5.12 a**), however, there are also other cases *in vivo* that could not be reflected by *in vitro* EMSA (**Fig. 5.12 b**, **c**, **d**) (Wells and Farnham, 2002). Thus, to further and better evaluate the regulation effect of ZEB1 on TPO promoter, a repression domain deficient ZEB1 was designed as described before (section 5.2.5).

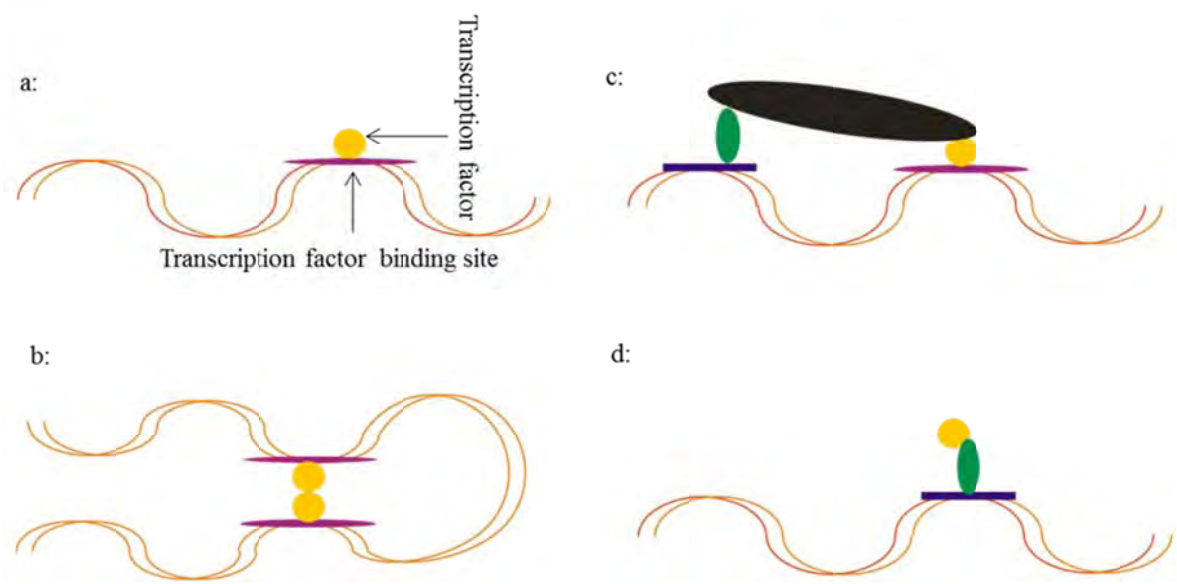


Figure 5.12 Different cases of transcription factor binding. *a) Transcription factor can directly bind to specific DNA motif. b) Looping of DNA brings two binding sites into close proximity that facilitates transcription factor binding. c) Binding of transcription factor with a low affinity can be stabilized by contact of another factor (large dark oval) which is brought to DNA by another transcription factor (small green oval). d) Transcription factor of interest can be recruited to another different transcription factor (small green oval) which binds to a distinct site (modified from (Wells and Farnham, 2002)).*

5.3.3.1 Expression of ZEB1-RD

A mutant ZEB1 protein with compromised repression domain (ZEB1-RD) was transiently expressed in HeLa cells and the expression was evaluated by Western blot.

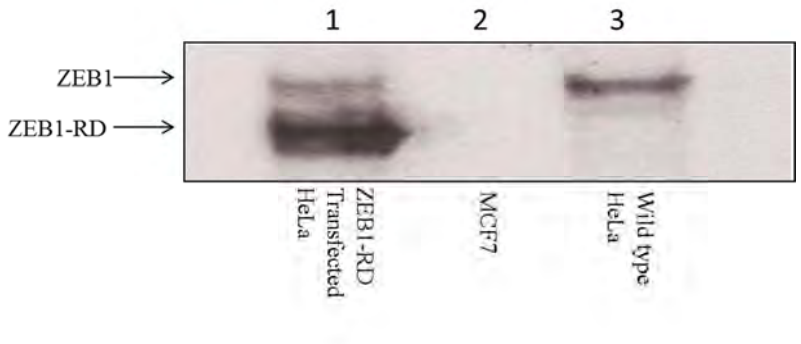


Figure 5.13 ZEB1-RD was successfully expressed in HeLa cells. *ZEB1-RD protein was transiently expressed in HeLa cells (lane 1). MCF7 cells which lack endogenous ZEB1 (also shown in lane 4 in* ***Fig. 5.15****) were used as negative control (lane 2). Endogenous ZEB1 protein expressed in HeLa cells is indicated in lanes 1 and 3.*

Fig. 5.13 shows that ZEB1-RD was expressed when transfected into HeLa cells (lane 1). The upper band (lanes 1 and 3) is endogenous wild type ZEB1, normally expressed in HeLa cells. As ZEB1-RD encodes a product with 194 amino acids deleted from the wild type ZEB1, the ZEB1-RD was observed at a lower position as indicated in lane 1 (the molecular weight difference between ZEB1-RD and wild type ZEB1 is about 21 kDa). In summary, this figure shows that ZEB1-RD was successfully expressed in HeLa cells.

5.3.3.2 Transcriptional activity of ZEB1-RD

We used the repression effect compromised ZEB1-RD construct to investigate the relationship between ZEB1 protein and the repressed promoter activities observed with the vectors PGL3-886 and PGL3-958.

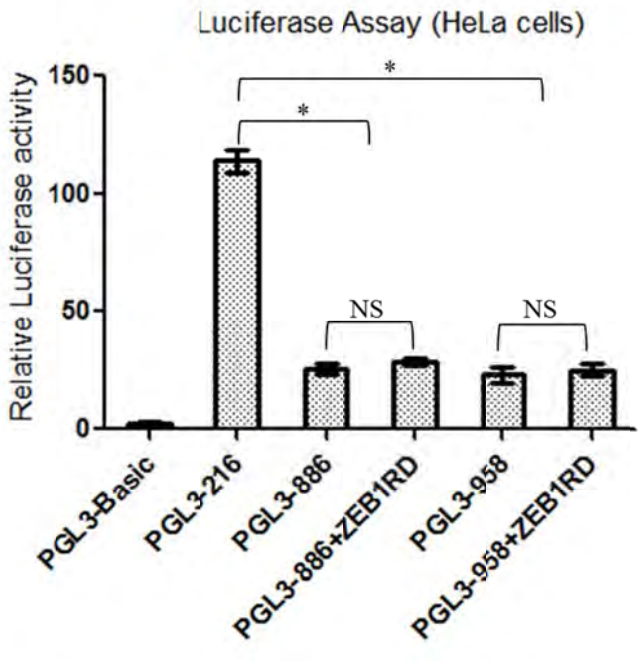


Figure 5.14 Expression of ZEB1-RD did not affect TPO promoter activity. *The repression deficient construct, ZEB1-RD was transiently expressed in HeLa cells together with the TPO promoter constructs. Promoter activities were determined by luciferase assay. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05, NS: not significantly different).*

As shown in **Fig. 5.14**, expression of ZEB1-RD did not restore promoter activity of PGL3-886 and PGL3-958 to the level of PGL3-216. Both PGL3-886 and PGL3-958 showed reduced promoter activities as expected as they contained transcription repressor element(s). Transfecting and expressing of ZEB1-RD in addition to either PGL3-886 or PGL3-958 did not abolish their repressive activities. PGL3-958 and PGL3-886 with ZEB1-RD exhibited 22% and 25% of PGL3-216's promoter activity respectively, compared to 20% and 22% of PGL3-216's promoter activity in the absence of ZEB1-RD, respectively. Over-expression of the repression-deficient ZEB1-RD protein (dominantly negative ZEB1) did not restore the activity of the TPO promoter fragments tested to that of PGL3-216, suggesting that ZEB1 might not be responsible for

the repressed TPO promoter activity observed with PGL3-958 and PGL3-886. Therefore, the putative ZEB1 elements in the intron 1 of the TPO might not be functional.

5.3.4 Investigations on cell lines that do not express endogenous ZEB1 protein

5.3.4.1 Selection of ZEB1-null cell lines

To further confirm that ZEB1 was not involved in transcription repression with PGL3-958 and PGL3-886, I conduct experiments in cells without ZEB1 protein expression, detectable by Western blot.

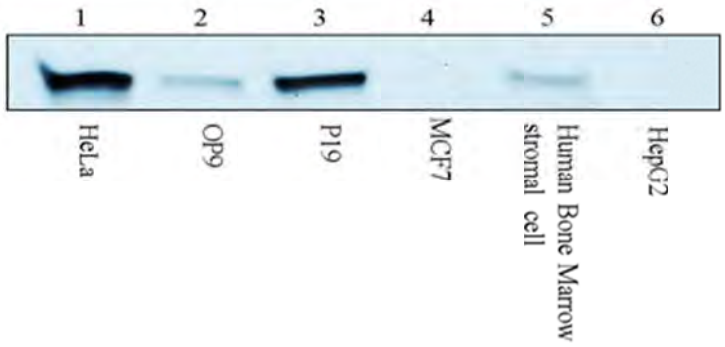


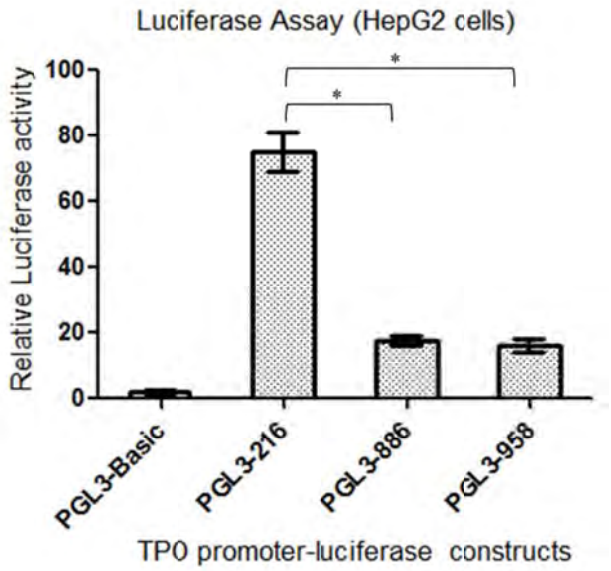
Figure 5.15 Western blot analysis of ZEB1 expression. *A series of different cell lines (indicated in the figure) were cultured and the endogenous ZEB1 expression level was determined by the Western blot.*

As shown in **Fig. 5.15**, HeLa cells (lane 1) and P19 cells (lane 3) showed strong expression of ZEB1. OP9 cells (lane 2) and human bone marrow stromal cells (lane 5) exhibited relatively lower expression of ZEB1, while the human breast cancer cell line MCF7 (lane 4) and human hepatocarcinoma cell line (HepG2) showed no detectable expression of ZEB1 protein.

5.3.4.2 Repressed promoter activities in cells lacking endogenous ZEB1

Western blot showed that MCF7 and HepG2 cells do not express detectable levels of endogenous ZEB1 protein. Putative ZEB1 elements bearing constructs, PGL3-886 and PGL3-958, were transiently transfected into MCF7 and HepG2 cells to determine whether TPO promoter repression would still occur in the absence of ZEB1 protein. Results were shown in following **Fig. 5.16**. PGL3-886 and PGL3-958 still showed significantly reduced promoter activities compared with the minimal promoter construct PGL3-216 in both HepG2 cells and MCF7 cells (**Fig. 5.16**). (PGL3-886 and PGL3-958 exhibited 23% and 21% of PGL3-216's promoter activity in HepG2 cells, and 24% and 26% of PGL3-216's promoter activity in MCF7 cells respectively). These results showed that even in the absence of endogenous ZEB1 protein expression in HepG2 and MCF7 cells, the observed reduced promoter activity still occurred. This further indicated that ZEB1 might not serve as a transcriptional repressor for the TPO promoter.

A:



B:

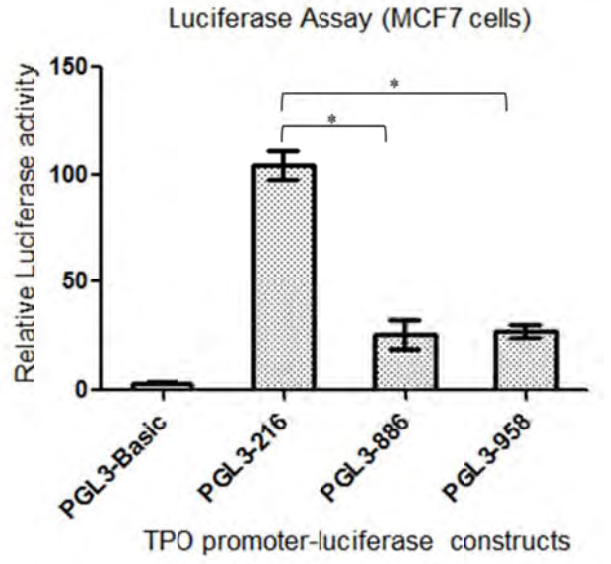


Figure 5.16 Absence of ZEB1 did not prevent repression of the TPO promoter. *Plasmids bearing putative ZEB1 motifs (PGL3-886 and PGL3-958) were transiently transfected into cells that do not express endogenous ZEB1, and the corresponding activities were determined by the luciferase assay (A: HepG2 cells, B: MCF7 cells). The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

5.4 Discussions

The present chapter is concerned with the analysis of the ZEB1 element-containing region of the TPO promoter. ZEB1 is a transcriptional regulator that can both activate and repress genes. It can activate gene in some cases, for example, ZEB1 activates ovalbumin promoter in the absence of steroid hormones (Chamberlain and Sanders, 1999). In an *in vivo* study, ZEB1 mutant (deletion of a zinc finger cluster proximal to COOH terminus) mice were generated, and an impaired development of T cells was observed (Higashi et al, 1997). However, in most studies ZEB1 were found to be a repressor as described before in section 1.5.3. ZEB1 knockout mice had severe T cell deficiency of the thymus and showed skeletal defects (Takagi et al, 1998). For years, the mechanism of ZEB1-mediated gene repression has been of great interest. Generally, ZEB1-mediated repression has two models, either by directly competing with gene activators or cooperating with its co-repressor, notably CtBPs (Furusawa et al, 1999). Postigo and Dean (1999) identified three independent repressor domains (Region 1, Region 2, and Region 3 see **Fig. 5.7**) in ZEB1. Their study further showed that Region 1 was the major repressor domain that was capable of repressing all target genes (except for myogenic factor, MEF2C) repressed by the full length ZEB1. However, Region 3 only inhibited MEF2C among all genes they tested (Postigo and Dean, 1999). On the other hand, a mechanism that involved co-repressors was also studied. A conserved CtBP binding motif identified in ZEB1 (Turner and Crossley, 1998) was found to be essential in the ZEB1-mediated inhibition of MEF2C. And the repression on a MyoD-activated promoter was significantly reduced when CtBP binding motifs in ZEB1 were mutated (Furusawa et al, 1999). A recent study revealed that CtBP2 functioned as co-repressor in ZEB1-induced suppression of IL-2 gene in T cells (Wang et al, 2009).

In my study, both vectors containing fragments of the TPO promoter with ZEB1 elements (PGL3-886 and PGL3-958) showed significantly reduced promoter activities in the luciferase assay. This result agrees with the assumption that the observed reduced promoter activity might have resulted from ZEB1-mediated repression. However, other possibilities cannot be excluded at this stage. For example, despite strong evidence pointing to ZEB1 as the possible repressor, this region interestingly contains many other transcription factor binding motifs, such as binding sites for MZF1, NFκB, AP-1, Elk-1, C/EBP, Sp1, USF and others. Notably, MZF1 is another potential repressor. As I alluded to in section 1.5.3, previous studies showed this transcription repressor had a role in repressing haematopoiesis. In the next chapter I would investigate this transcription factor.

To further explore whether ZEB1 is the transcription repressor, EMSAs were carried out. The strong signal detected in EMSAs (**Fig. 5.11**) indicates that there are nuclear proteins in HeLa cells that specifically bind to the probes tested. However, none of the EMSA bands could be supershifted by specific anti-ZEB1 antibody, indicating that the observed retardation bands may be due to the presence of other DNA-binding protein(s). However, due to the limitation of EMSA itself, it is possible that EMSA did not reflect *in vivo* situation. A study of the transcription factor E2F showed that a great number of E2F promoters bind to the E2F protein *in vivo*, but some of these promoters did not bind the transcription factor *in vitro* in EMSAs (Weinmann et al, 2002). As shown in **Fig. 5.12**, there are mechanisms of transcription factor binding to DNA *in vivo* that will not be detectable by EMSA *in vitro* such as mechanism “b”, “c” and “d” in **Fig 5.12**, particularly mechanisms “c” and “d” which require a co-repressor which may not be present in the nuclear extract used in the EMSA, or mechanism “d” in which the transcription factor binds even the promoter construct does not contain the putative binding site for the transcription factor. For ZEB1 binding, a co-repressor such as CtBP (Turner and Crossley, 1998;

Wang et al, 2009) may be required.

Investigations to assess ZEB1 DNA binding and transcription repression *in vivo* (i.e. intracellular) are necessary to show that ZEB1 is unlikely to be a TPO gene repressor. One of these strategies is to use overexpressed a ZEB1 mutant to repress TPO promoter activity in cells that express ZEB1 endogenously. To generate the ZEB1 mutant, a region of ZEB1 that mediates repressesion of hematopoietic factors (such as c-myb, ets family members, and TFE-III) (Postigo and Dean, 1999), and also contains binding sites for ZEB1 co-repressor (CtBPs), was removed (Furusawa et al, 1999). This ZEB1 mutant is unable to repress TPO transcription but it contains DNA binding domains and should bind DNA. Considering the possibility that only a small amount of ZEB1 is needed to induce repression, two ZEB1 null cell lines were also used. It was found that the absence of ZEB1 did not abolish the repression of transcription activities of the promoter constructs.

Altogether, the studies in this chapter provide strong evidence that ZEB1 is unlikely to be the transcriptional repressor for the human TPO gene, particularly repression mediated by region, +14 to +688, in intron 1 of the TPO gene. Another potential transcriptional regulator alluded to above, MZF1, that may bind this repression region. This will be the subject of the following chapter.

Chapter Six: Characterization of MZF1, a Potential Repressor of Human TPO Expression

Chapter Six: Characterization of MZF1, a Potential Repressor of Human TPO Expression

6.1 Introduction

In Chapter five, I investigated a region of the TPO promoter that carries a DNA cluster containing ZEB1 binding motifs. The results showed that this region contained transcriptional repressor element(s). However, the results also revealed that the ZEB1 motifs in this region were not responsible for the observed suppression on the promoter transcription activity. In this Chapter, to identify the repression element responsible for the suppression of TPO promoter activity, I built a series of TPO gene promoter luciferase constructs that covered all the regions of interest in the first intron of the TPO gene. The region covered (-202 to +688) included the minimal promoter (from -202 to +14) and the region carrying transcription element(s) (+14 to +688). In order to narrow down the location of the repression element, this family of reporter constructs were designed to include consecutive 60 bp deletions from the 3'of the promoter (**Fig 6.1**). The constructs were transiently transfected into HeLa and OP9 cells and the promoter activity of each construct was determined by the luciferase assay. Analysis of the transcriptional profile of these constructs according to the results from luciferase assay suggests that the repressor element is likely to be located between +450 to +509 in the intron 1 of TPO. As described below, the sequence analysis for putative transcriptional regulatory elements within this region (+450 to +509) showed that there is a binding motif for the transcription factor Myeloid Zinc Finger binding protein 1 (MZF1).

MZF1 is a member of the zinc finger transcription factor family and is primarily

expressed in myeloid cells (Hromas et al, 1991). MZF1 has been shown to be a transcriptional repressor in different studies. Transcription of FcεRI β-chain gene, an important molecule in allergic reactions, is negatively regulated by MZF1 and a functional MZF1 binding motif was localized to the fourth intron of FcεRI β-chain gene (Takahashi et al, 2003). A similar intronic MZF1 motif was localized to the first intron of human TPO gene in this chapter. In another study, the expression of ERCC1, a molecule involved in nucleotide excision repair, is transcriptionally suppressed by MZF1 (Yan et al, 2006). Moreover, in a hematopoietic study, MZF1 was found to prevent hematopoietic commitment in embryonic stem cells and to inhibit the formation of erythromyeloid colonies (Perrotti et al, 1995). An *in vivo* study further revealed that the hematopoietic progenitor cells from the *Mzf1*$^{-/-}$ mouse underwent a significantly increased long-term hemopoiesis (Gaboli et al, 2001). It is likely that MZF1 may have a similar suppressive effect on TPO gene promoter activity. Thus, I propose that MZF1 may be a transcriptional repressor for the TPO gene and the MZF1 motif found in the first intron of the human TPO gene may serve as a functional binding target for MZF1.

In this chapter, I carried out experiments to prove or disprove my hypothesis. The major experiments include EMSAs to investigate the putative MZF1 motif in the intron 1 (+457 to +464) of TPO gene is a bona fide binding site for MZF1. I also explored whether knocking-down MZF1 by siRNA would reverse the MZF1 inhibition of TPO gene promoter activity. Further, the regulated expression level of TPO mRNA in OP9 cells was determined when the endogenous MZF1 expression was inhibited by siRNA.

6.2 Materials and Methods

6.2.1 Cell culture

HeLa, OP9, and MCF7 cells were cultured as described in section 3.1.

6.2.2 Establishment of reporter vectors

It is likely that there is a powerful transcriptional repressor element in the downstream region in intron 1 of TPO between +14 to +688 (shown in luciferase assay in section **4.3.1**). In order to further localize the repression element, a series of 3'-deletion TPO promoter constructs were generated (**Fig. 6.1**).

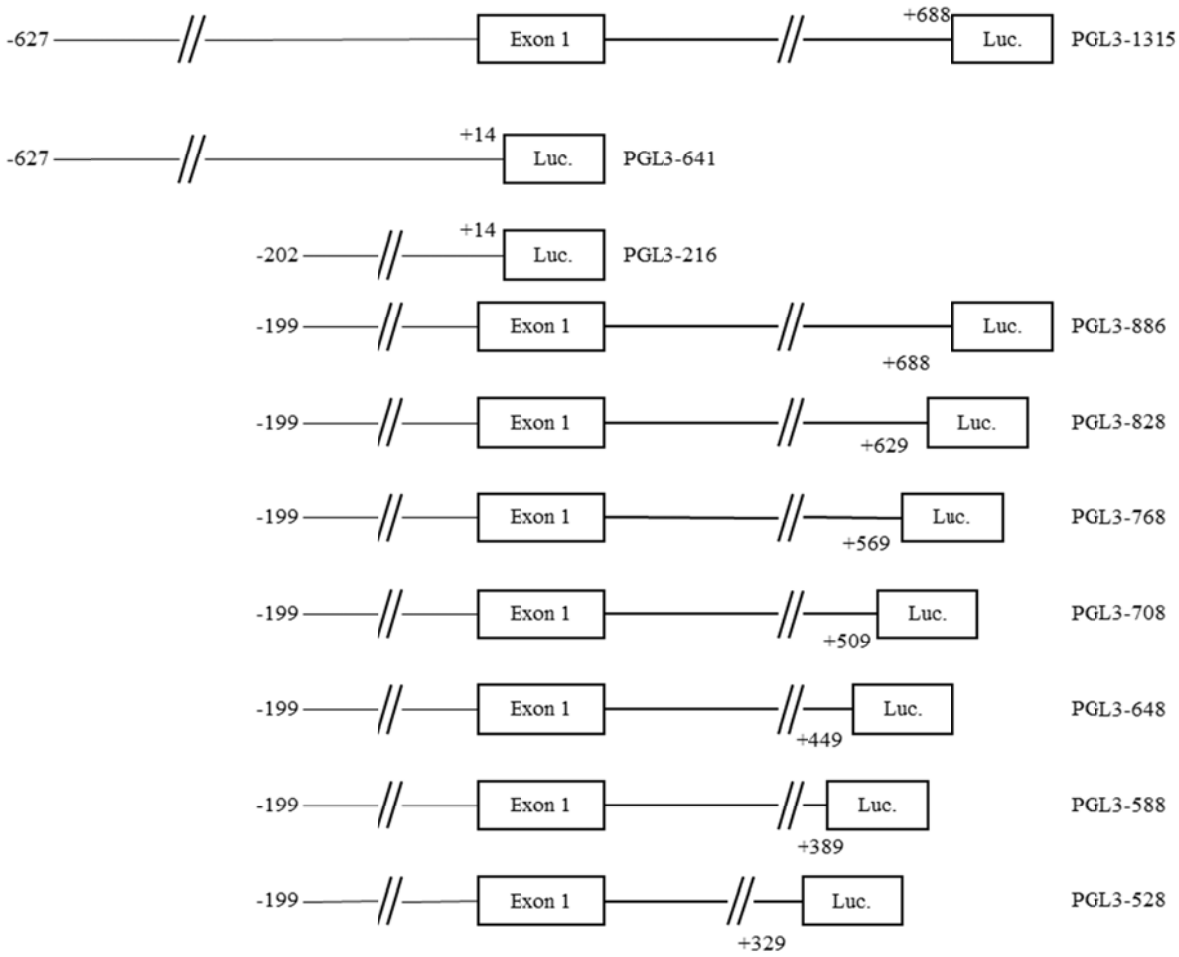


Figure 6.1 Establishment of 3'- deletion promoter constructs. Schematic representation of the vectors used for transient transcription assays. *All vectors contained the previously defined minimal promoter (-202 to +14), and parts of repression region +14 to +688.*

PGL3-886 was used as template and the primers shown in Table 6a (Appendix D) were used for PCR amplification of the following fragments: -199 to +329, -199 to +389, -199 to +449, -199 to +509, -199 to +569 and -199 to +629. The PCR products were cloned into XhoI/HindIII sites of PGL3-Basic vector to get PGL3-528, PGL3-588, PGL3-648, PGL3-708, PGL3-768 and PGL3-828. As shown in **Fig. 6.2**, fragments 528 (lane 2), 588 (lane 3), 648 (lane 4), 708 (lane 5), 768 (lane 6) and 828 (lane 7) were successfully amplified by the PCR. These reactions also generated some nonspecific products (circled bands) thus further

purification was needed. As shown in **Fig. 6.3 A**, the PCR products were well separated by DNA gel electrophoresis and the bands containing fragments 528 (lane 1), 588 (lane 3), 648 (lane 4), and in **Fig. 6.3 B**, fragments 708 (lane 1), 768 (lane 3) and 828 (lane 4) were extracted and further purified. The DNA was then again subjected to gel electrophoresis and the purified DNA was extracted (**Fig. 6.3 C**).

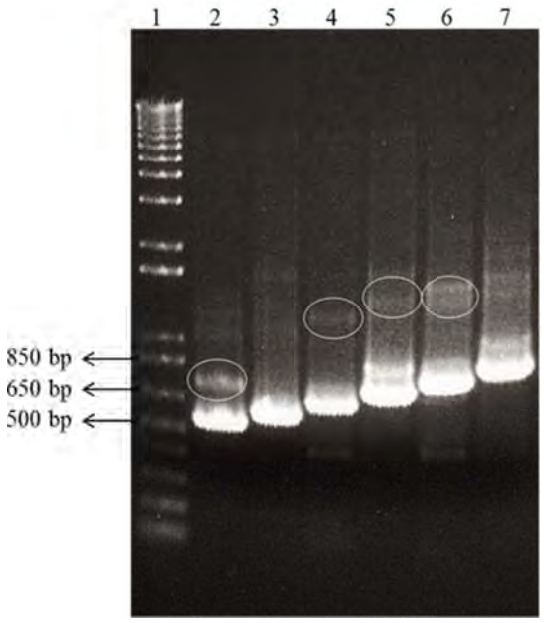


Figure 6.2 DNA electrophoresis of promoter fragments. *The DNA fragments covering various regions of TPO gene as shown in* ***Fig. 6.1*** *were amplified by PCR and the products were visualised by DNA electrophoresis (Lane 1: DNA marker, lane 2 to lane 7: PCR products). Circles indicate nonspecific products.*

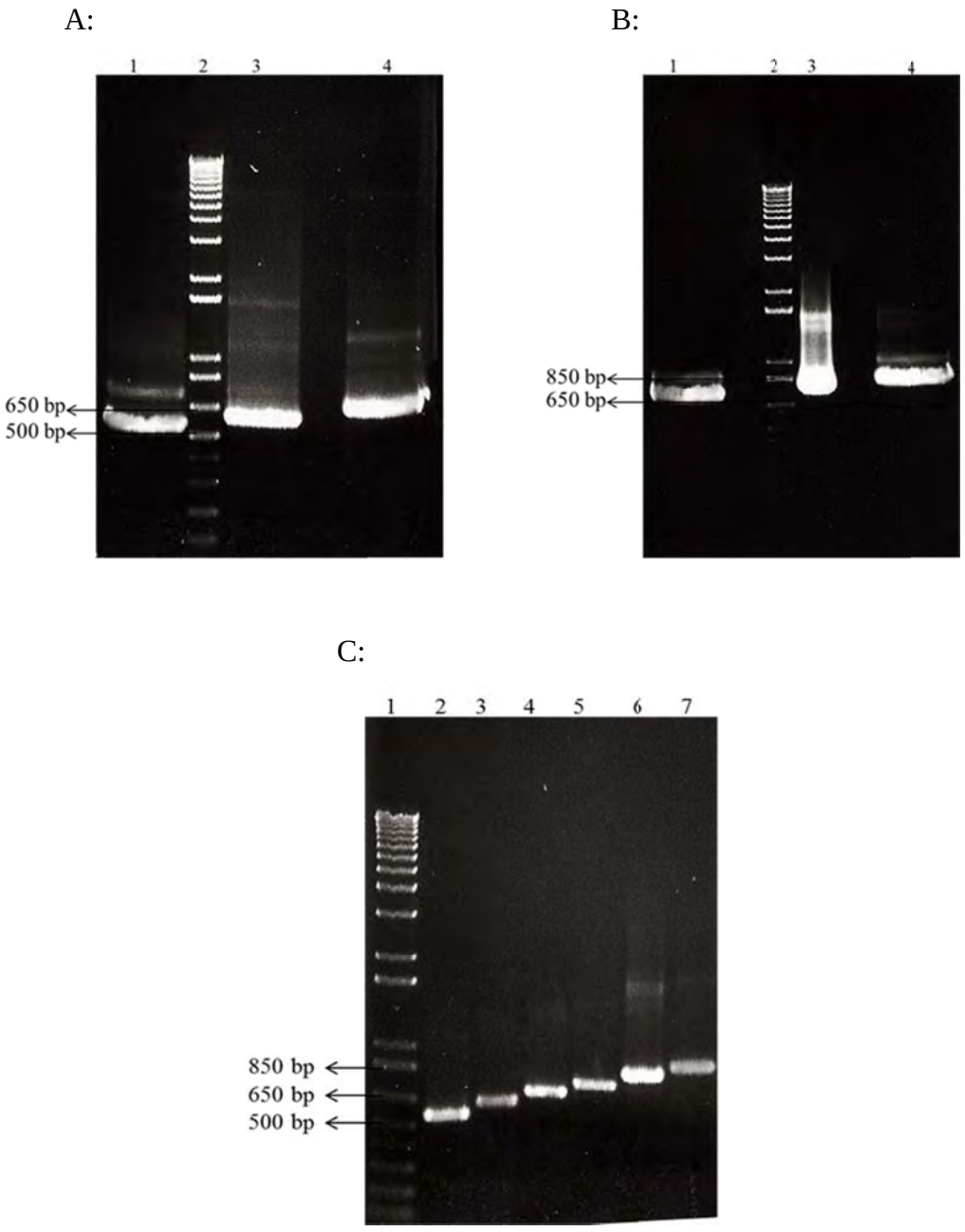


Figure 6.3 Gel extraction of PCR fragments. *A and B: The amplified TPO fragments were separated by DNA electrophoresis. (Lane 2: DNA marker, lane 1, 3 and 4: PCR fragments). C: The extracted PCR products were again subjected to the gel electrophoresis and further purified (lane **1**: DNA marker, lane 2 to 7: PCR products).*

The PGL3-Basic was digested by XhoI/HindIII (see **Fig. 6.4**), purified and used for ligation reactions with the promoter fragments from **Fig. 6.3**.

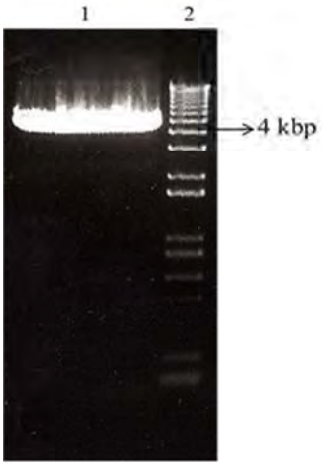
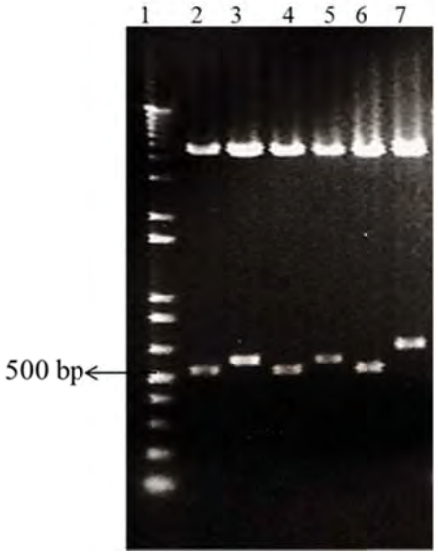


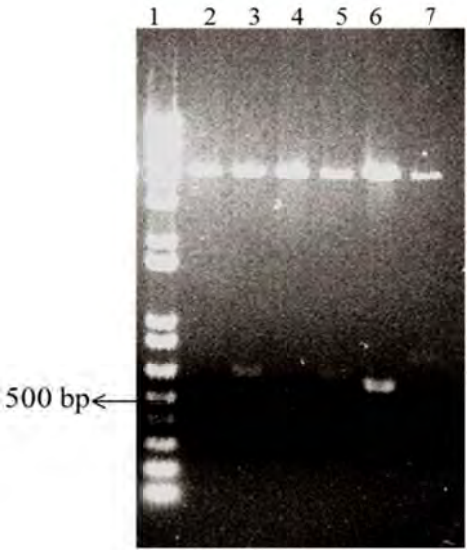
Figure 6.4 DNA electrophoresis of XhoI/HindIII digested PGL3-Basic vector. *The PGL3-Basic was digested by XhoI/HindIII and the digestion was then checked by DNA electrophoresis (lane 1: PGL3-Basic, lane 2: DNA marker).*

The digested TPO fragments were then ligated into PGL3-Basic vector, transformed into *E. coli*, and the single colonies were screened. Six single colonies were randomly selected, and plasmid DNA was extracted and digested with XhoI/HindIII. The digestion was checked by gel electrophoresis. As shown in **Fig. 6.5 A**, three (lane 2, lane 4 and lane 6) out of six colonies contained the plasmid PGL3-528. One (lane 6 in **Fig. 6.5 B**) and five (lane 2, lane 3, lane 5, lane 6 and lane 7 in **Fig. 6.5 C**) out of the six single colonies contained the plasmid PGL3-588 and PGL3-648, respectively. Four (lane 2, lane 3, lane 4 and lane 6 in in **Fig. 6.5 D**) out six picked single colonies contained the plasmid PGL3-708. For the cloning of plasmids PGL3-768 and PGL3-828 all the six single colonies contained the expected plasmids (**Fig. 6.5 E** and **Fig. 6.5 F**). The identity of the clones was confirmed by sequencing.

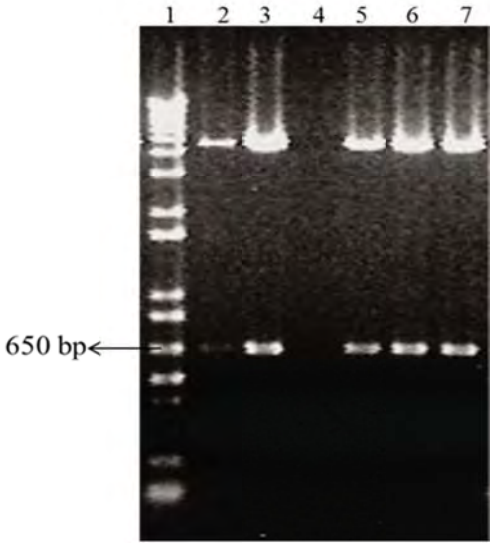
A: PGL3-528



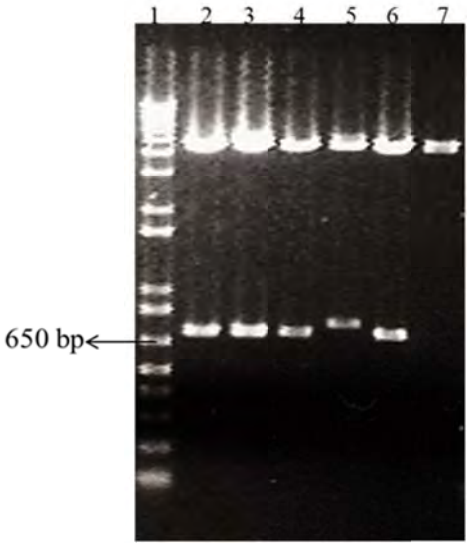
B: PGL3-588



C: PGL3-648



D: PGL3-708



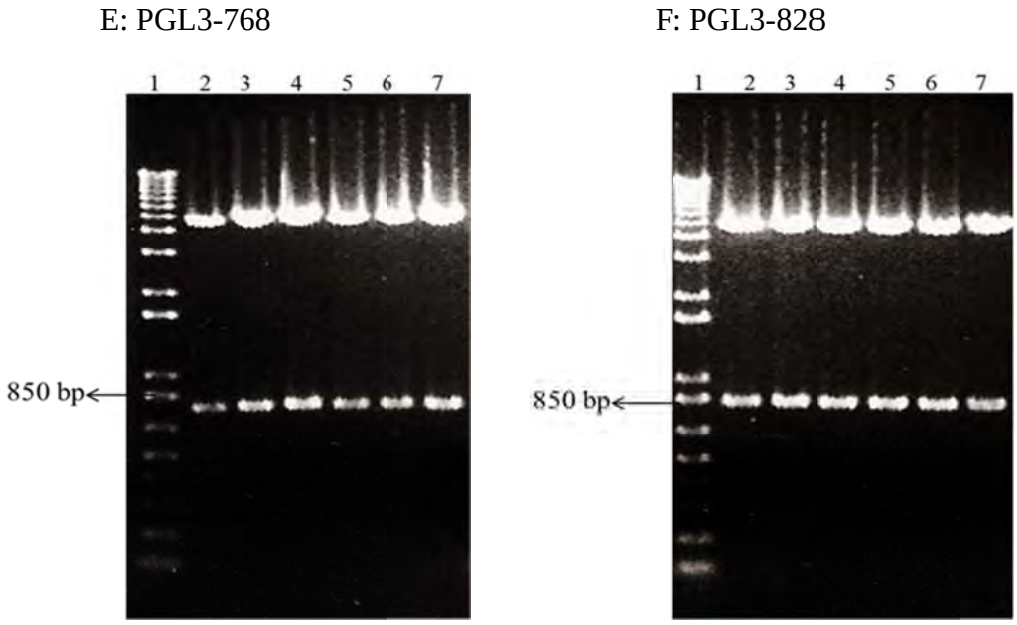


Figure 6.5 Screening of positive colonies. *The TPO promoter colonies were checked by restriction enzyme digestion and DNA electrophoresis (lane 1: DNA marker, lane 2 to lane 7: PGL3-TPO fragments colonies).*

6.2.3 Transient transfection

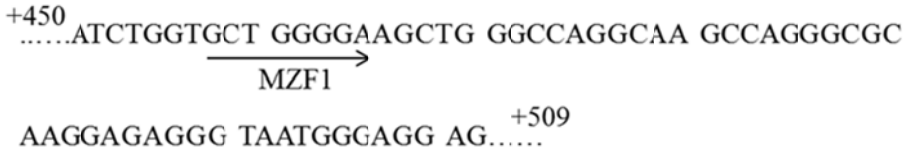
Each of the established TPO promoter constructs was transiently expressed in HeLa cells and OP9 cells as previously described (section 4.2.3). Cells were harvested and the promoter activities were measured by the luciferase assay (section 3.2.4.2).

6.2.4 EMSA investigating MZF1 as the potential repressor of TPO gene expression

In order to investigate whether the putative MZF1 motif present from +457 to +464 (**Fig. 6.6 A**) in the TPO gene was a functional MZF1 binding site, EMSA was performed using nuclear extract from HeLa cells, probe containing sequences corresponding to the putative MZF1 binding region and an

anti-MZF1 antibody (Sapphire Bioscience, NSW, Australia). For competition studies, a 100-fold excess of unlabelled (cold) MZF1 probe (specific competition) or mutant probe (nonspecific competition) was included as shown in **Fig. 6.6 B**.

A:



B:

Specific probe: 5'-ATCTGGTGCTGGGGAAGCTGG-3'

Mutant probe: 5'-ATCTGGTC(G)CTGT(G)GC(G)AAGCTGG-3'

Figure 6.6 Probes used for the MZF1 EMSA. *A: The putative MZF1 recognition site in intron 1 of human TPO gene is shown. B: Specific and mutant probes for the MZF1 EMSA. To generate the mutant probe, three G nucleotides, in the specific probe were replaced with the nucleotides underlined, C, T and C respectively.*

6.2.5 MZF1 Gene Knockdown using siRNA

To investigate whether the endogenous MZF1 expressed in OP9 and HeLa cells is responsible for the observed reduced promoter activity, the endogenous MZF1 was knocked down using mouse/human MZF1 specific siRNA (Santa Cruz Biotech) according to the manufacturer's instructions. Briefly, HeLa and OP9 cells were co-transfected with TPO promoter constructs and with MZF1 siRNA or control siRNA by using transfection reagent (Lipofecatmine 2000 for HeLa cells and Optifect for OP9 cells) (Invitrogen). Approximately 6×10^5 of

HeLa cells and 2 $\times 10^5$ of OP9 cells were suspended in 1.5 ml of complete growth medium and seeded in each well of a 6-well plate. 2 μg of PGL3-Basic, PGL3-216 or PGL3-708 was mixed with 100 pmoles of MZF1 siRNA or control siRNA in 500μl of Opti-MEM I serum-reduced medium (Invitrogen).. 6μl of transfection reagent was added to the mixture and incubated at room temperature for 15 min and then added into the culture. After the transfection, cells were cultured for 48 h without changing medium and subjected to luciferase assay.

6.2.6 Quantitative Real-time PCR

The relative quantitative Real-time PCR was used to evaluate the efficiency of siRNA-mediated knockdown of endogenous MZF1 gene expression.

6.2.6.1 RNA extraction

The total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, the medium was aspirated, and the cells (~ 1×10^6 cells) were washed with PBS. PBS was aspirated, and 0.25% trypsin in PBS was added. After the cells were detached from the dish, medium was added, the cells were transferred to an RNase-free polypropylene centrifuge tube, and centrifuged at 300 × *g* for 5 min. The supernatant was completely aspirated. The cells were disrupted by adding 350 μl of buffer RLT Plus and the cell pellet was loosened by flicking the tube. The lysate was pipetted directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at maximum speed. The lysate was passed through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. The homogenized lysate was transferred to a DNA eliminator spin column placed in a 2 ml collection tube, centrifuged for 30 s at 8,000 *g*, and the flow-through was saved. 350 μl of 70% ethanol was added to the flow-through, and mixed well by pipetting. 700 μl of the sample, including any precipitate,

was transferred to an RNeasy spin column placed in a 2 ml collection tube, centrifuged for 15 s at 8,000 *g* and the flow-through discarded. This was repeated thrice. The RNeasy spin column was then placed in a new 1.5 ml collection tube. 50 µl RNase-free water added directly to the spin column, and centrifuged for 1 min at 8,000 *g* to elute the RNA. The concentration of eluted RNA was measured as described in section 3.2.3.5.

6.2.6.2 cDNA synthesis

The cDNA was synthesized by using the cDNA synthesis kit (SuperScript® III First-Strand Synthesis SuperMix for RT-PCR, Invitrogen). Briefly, the following kit components were combined in a tube on ice. For multiple reactions, a master mix without RNA was prepared:

Table 6.1 Mixture for cDNA synthesis

Reagent	Amount
2 × RT Reaction Mix	10.0 µl
RT Enzyme Mix	2.0 µl
RNA	1.0 µg
DEPC-treated water	fill up to 20.0 µl

Tube contents were gently mixed and incubated at 25ºC for 10 min. Tube was incubated at 50ºC for 30 min, the reaction was terminated at 85ºC for 5 min, and then chilled on ice. 1 µl (2 U) of *E. coli* RNase H was added and incubated at 37ºC for 20 min. cDNA was used in the real time PCR experiment directly.

6.2.6.3 Relative Quantitative Real-time PCR

The reaction was set up according to the manufacturer's instructions by using the Platimum SYBR Green qPCR SuperMix-UDG (Invitrogen). The reaction was set up as below:

Table 6.2 Reaction for relative quantitative real-time PCR

Reagent	Amount
Platinum SYBR Green qPCR SuperMix-UDG	12.5 µl
Primers (forward and reverse primer)	2.5 µl
Template DNA (undiluted/diluted cDNA)	3.0 µl
DEPC-treated water	Fill up to 25.0 µl

The reaction was run in the Rotor gene 6000 (Qiagen), and the program was set as: 50°C for 2 min hold (UDG incubation) and 95°C for 10 min hold. 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s.

6.2.6.4 Data analysis for the RT-PCR

A mathematical model developed by Pfaffl (Pfaffl, 2001) was used to determine the relative quantification of the target gene in comparison to a reference gene. Firstly, relative standard curves for the target gene（MZF1）and reference gene (18s RNA) were obtained by amplification of a serial dilution of cDNA (reverse transcribed 100 ng, 50 ng, 25 ng, 5 ng and 1 ng of total RNA) using the Quantitec assay primer set (Qiagen) for human or mouse MZF1, and human or mouse 18sRNA to generate the reaction efficiency, $E=10^{(-1/slope)}$. Then, the CP (Crossing point at which the fluorescence rises above the background) was obtained for each sample by the Rotor gene software. The relative expression ratio (R) of target gene (MZF1) was calculated using the equation:

$$R= \frac{(E_{Taget})^{\Delta CPTagetControl-Sample)}}{(E_{18sRNA})^{\Delta CP18sRNA(Control-Sample)}}$$

6.2.7 Western blot assay for MZF1 expression

Expression levels of MZF1 protein in siRNA transfected cells were confirmed by Western blot. Briefly, transfected and untransfected cells were harvested and lysed in buffer containing 150 mM sodium chloride, 1% NP-40, 50 mM Tris [pH 8.0] and the proteinase inhibitor cocktail (Sigma-Aldrich). 15 μg of the cell lysates were loaded onto the mini-protean TGX precast gel (Bio-Rad, Gladesvile, Australia) and run by electrophoresis. MZF1 expression was probed with an anti MZF1 antibody (Sapphire Bioscience).The expression of β-actin (Sigma-Aldrich) was used as loading control.

6.2.8 Determination of TPO mRNA expression in OP9 cells in which the MZF1 gene was knocked down

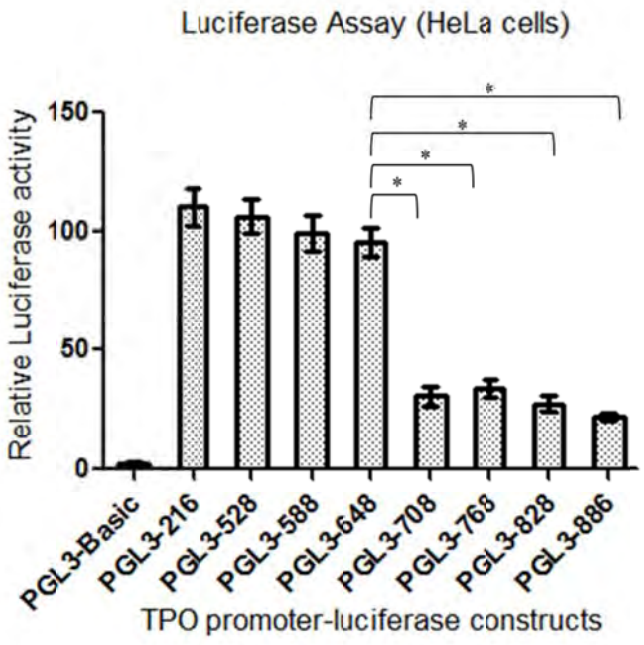
MZF1 siRNA was transfected into OP9 cells to knock down the MZF1 gene and the level of TPO mRNA expression was evaluated by relative quantitative real-time PCR as described in section 6.2.6.

6.3 Results

6.3.1 Repression element in PLG3-708

TPO promoter-luciferase constructs were transiently transfected into HeLa and OP9 cells and the promoter activity was measured by the luciferse assay. As shown in **Fig. 6.7**, clones PGL3-528, PGL3-588 and PGL3-648 showed promoter activity comparable to PGL3-216: approximately 96%, 90%, and 87% of that of PGL3-216 in HeLa cells and approximately 100%, 88% and 86% of that of PGL3-216 in OP9 cells. PGL3-708, PGL3-768, PGL3-828 and PGL3-886 showed significantly reduced promoter activity compared to PGL3-216: 28%, 30%, 25% and 20% in HeLa cells and 31%, 28%, 27% and 27% in OP9 cells, respectively. The strongly reduced promoter activity observed in PGL3-708 indicated the existence of repression element(s) in the region (from +450 to +509) of the TPO gene.

A:



B:

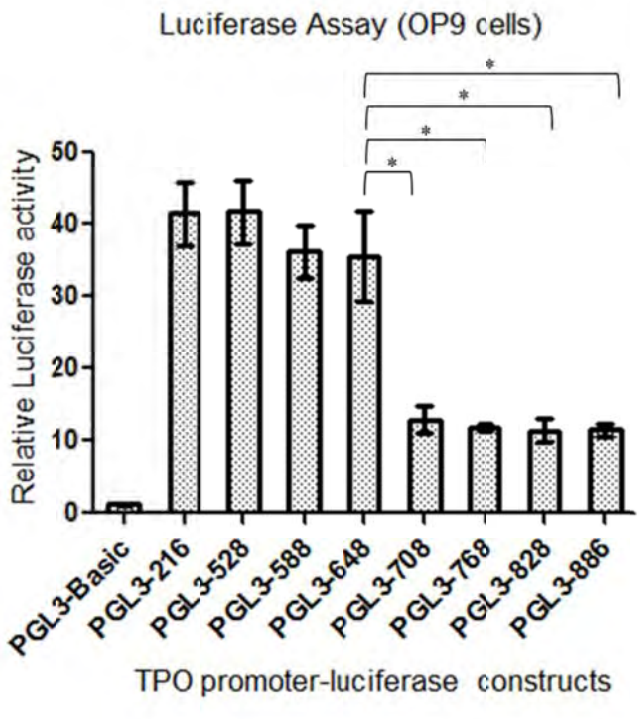


Figure 6.7 Luciferase assay of TPO promoter constructs. *2 µg of TPO promoter DNA and 50 ng of Renilla luciferase expression vector (PRL-cmv) were transiently expressed in HeLa cells (A), and OP9 cells (B). The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

6.3.2 Computational sequence analysis

An internet based computational program (TFSEARCH: http://www.cbrc.jp/research/db/TFSEARCH.html) was used to analyze the putative transcriptional elements in the region between +450 to +509. The analysis showed the existence of a putative binding target for MZF1. This putative MZF1 binding motif (5'-GCTGGGGA-3') from +457 to +464 possesses a guanine rich core (5'-NGNGGGGA-3') as described in a previous study (Takahashi et al, 2003).

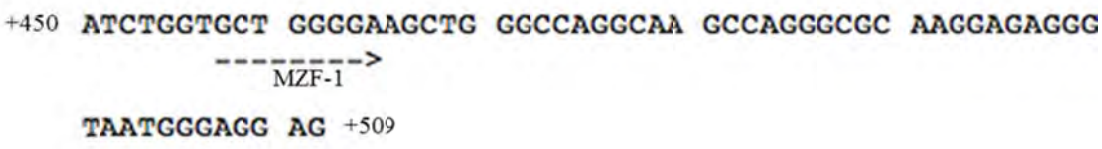


Figure 6.8 The putative MZF1 motif. *The MZF1 motif (underlined arrow) was found from +450 to +509 within the intron 1 of human TPO gene.*

6.3.3 Characterization of the nucleoprotein that binds to putative MZF1 motif

In order to determine whether the putative MZF1 motif is a functional binding target for the MZF1 protein, EMSA was carried out by using the nuclear extract from Hela cells and the ^{32}P labeled/unlabeled (for competition assay) double-stranded oligonucleotide probes (**Fig. 6.6 B**) and as described in section 3.4. Result was showed in following **Fig. 6.9**.

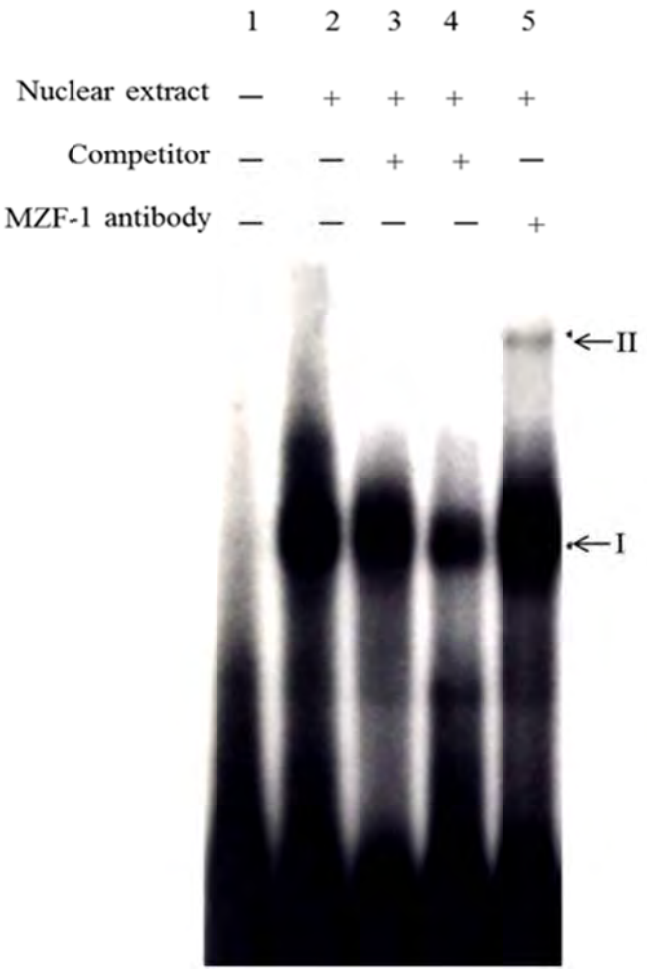


Figure 6.9 Characterization of the nucleoprotein that interacted with MZF1 containing probes using EMSA. *Lane 1 contained unlabeled cold probe only, and 15 µg of HeLa nuclear protein were incubated with the labeled probe (from lane 2 to 5). The binding was competed nonspecifically by excess amount of mutant cold probe (Lane 3, 100:1) and competed specifically by specific cold probe (Lane 4). The specific competition yielded a band (band I in lane 4) of much lesser intensity compared with the nonspecific competition (band I in lane 3) and that without competition (band I in lane 2). Antibody against MZF1 supershifted the specific band to a higher location (band II in lane 5).*

As shown in **Fig. 6.9**, nuclear extract of HeLa cells formed complexes (band I in lanes 2 to 5) with the ^{32}P labeled probe bearing the putative MZF1 motif from +457 to +464 in intron 1 of TPO gene. Specific competition by excess

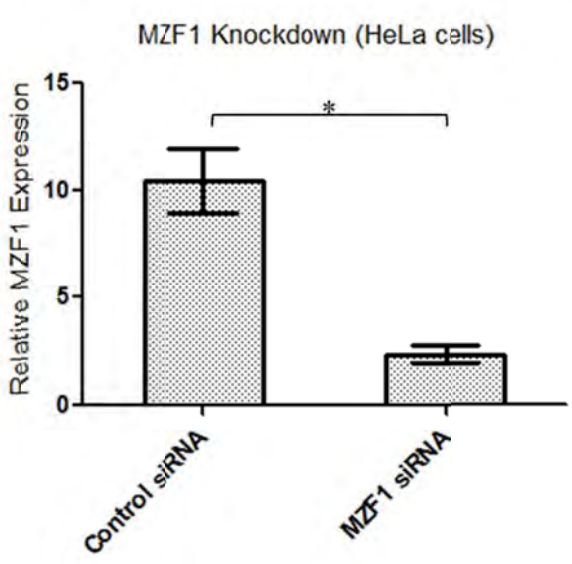
unlabeled (cold) probe (100 times more than labeled probe) gave a band at position I with a much reduced intensity (lane 4) than the one given by nonspecific “competition” with the unlabeled mutant probe (lane 3) and the one without competition (lane 2). The data indicate that there was a complex formed by specific binding of a certain nucleoprotein in the nuclear extract of HeLa cells to the probe. Incubation of an anti-MZF1 antibody with the reaction mixture yielded a supershifted complex, band II (lane 5). These results show that the MZF1 motif from +457 to +464 of TPO gene is probably a functional binding target of MZF1. These results also suggest that MZF1 protein in the HeLa cell nuclear extract bound to the MZF1 motif (+457 to + 464) in intron 1 of TPO gene and repressed gene transcription.

6.3.4 MZF1 Gene knockdown with siRNA

6.3.4.1 MZF1 gene knockdown

To further investigate whether the observed repressed promoter activity of PGL3-708 can be attributed to the putative MZF1 motif in the region, +457 to +464 in the intron 1 of TPO gene, PGL3-708 was co-transfected with MZF1 siRNA into HeLa or OP9 cells to suppress the endogenous MZF1 gene expression. The efficiency of the MZF1 gene knockdown in HeLa and OP9 cells was evaluated by measuring mRNA levels of MZF1 using the relative quantitative real-time PCR and by estimating the MZF1 protein levels using Western blot.

A:



B:

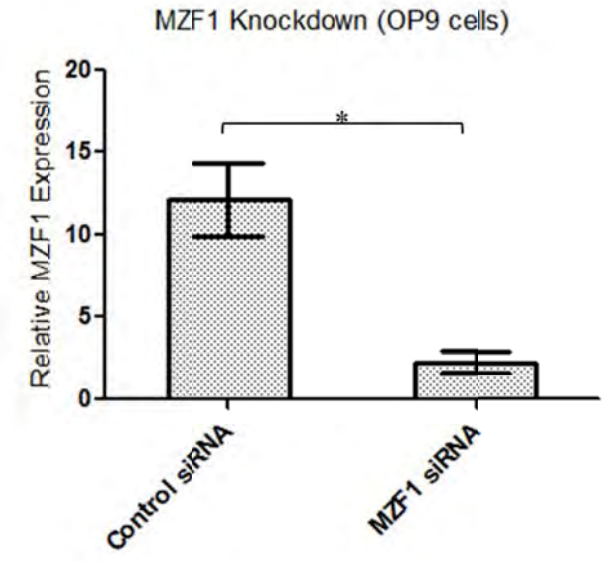


Figure 6.10 Knockdown of MZF1 mRNA expression by MZF1 or control siRNAs. *MZF1 mRNA levels of HeLa (A) or OP9 (B) cells were measured by relative quantitative real-time PCR, after MZF1 specific siRNA or control siRNA transfected into these cells. The relative expression ratio (R) of MZF1 was generated using method introduced in section 6.2.6.4. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

As shown in **Fig. 6.10**, transfection of MZF1 siRNA into HeLa or OP9 cells successfully suppressed the expression of endogenous MZF1. The MZF1 siRNA inhibited the endogenous MZF1 expression by 85% (in HeLa cells) and 80% (in OP9 cells) respectively.

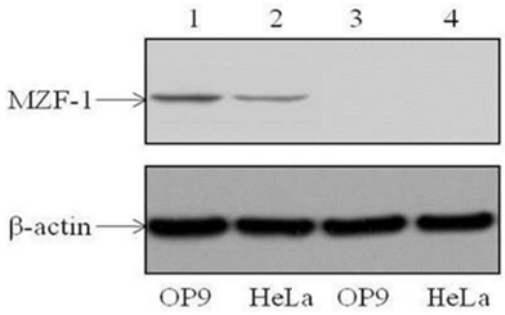
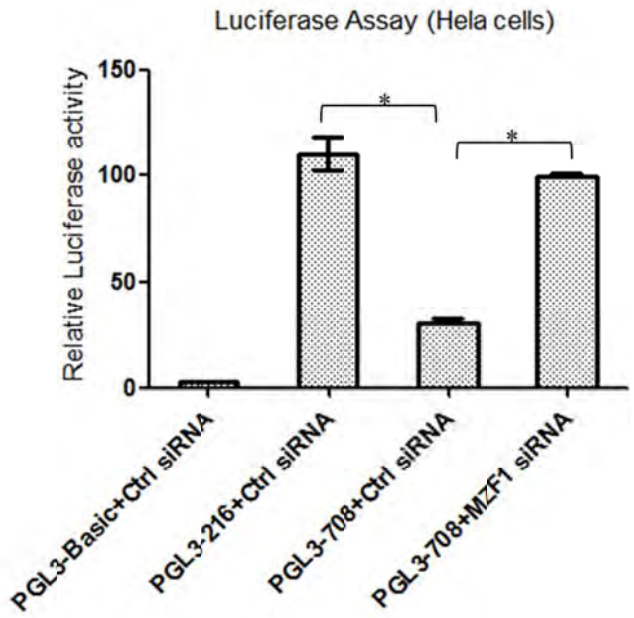


Figure 6.11 Western blot showing suppression of MZF1 protein levels by MZF1-specific siRNA and control siRNA. *The protein expression of MZF1 was evaluated by Western blot, in OP9 (lane 1: transfected with control siRNA and lane 3: transfected with MZF1-specific siRNA) and HeLa cells (lane 2: transfected with control siRNA and lane 4: transfected with MZF1-specific siRNA).*

As shown in **Fig. 6.11**, MZF1 protein expression in cells transfected with control siRNA cells was shown in lane 1 (OP9 cells), lane 2 (HeLa cells) and MZF1 protein expression in cells transfected with MZF1-specific siRNA was shown in lane 3 (OP9 cells), and lane 4 (HeLa cells). The results clearly showed that MZF1 siRNA successfully suppressed endogenous MZF1 protein expression in both OP9 and HeLa cells, reducing MZF1 protein expression to undetectable levels. There was no difference in protein loading in the four samples (β-actin (Sigma-Aldrich), lane 1 to 4)

6.3.4.2 Luciferase assay showing reversal of repression of TPO reporter activity by MZF1 siRNA

A:



B:

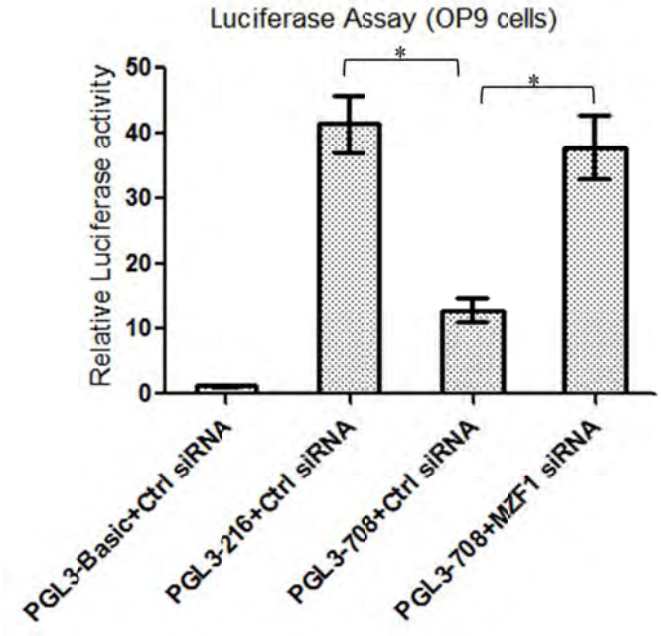


Figure 6.12 Luciferase assay/MZF1 siRNA co-transfection. *The MZF1 motif bearing plasmid PGL3-708 and MZF1 specific siRNA (4th bar) or control siRNA*

*(3rd bar) were co-transfected into HeLa or OP9 cells, and the promoter activity was determined by luciferase assay (A: HeLa cells, B: OP9 cells). In the control experiments, PGL3-Basic (1st bar) or PGL3-216 plasmid (2nd bar) and control siRNA were co-transfected into HeLa or OP9 cells. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

The promoter activity driven by PGL3-708 in HeLa or OP9 cells co-transfected with MZF1 siRNA or control siRNA was determined by luciferase assay. The luciferase assay for siRNA co-transfection revealed that inhibition of MZF1 expression reversed the transcription suppression and brought the promoter activity of PGL3-708 back to approximately 86% of that of PGL3-216 in HeLa cells (PGL3-708 showed 28% of PGL3-216's promoter activity in HeLa cells with a normal endogenous MZF1 expression), 90% in OP9 cells (PGL3-708 showed 31% of PGL3-216's promoter activity in OP9 cells with a normal endogenous MZF1 expression) (see results in **Fig.6.7** for promoter activities of PGL3-708 in HeLa and OP9 cells with normal MZF1 expression). This result suggests that endogenous MZF1 is responsible for the reduced promoter activity observed with PGL3-708 and it also further suggests that MZF1 is a novel intronic transcriptional repressor for the human TPO gene.

6.3.5 Enhanced TPO expression in the OP9 cells after MZF1 gene knockdown

To further investigate how the MZF1 gene knockdown would affect TPO expression, the mRNA level of TPO in the MZF1 siRNA transfected OP9 cells was determined by the relative quantitative real-time PCR assay.

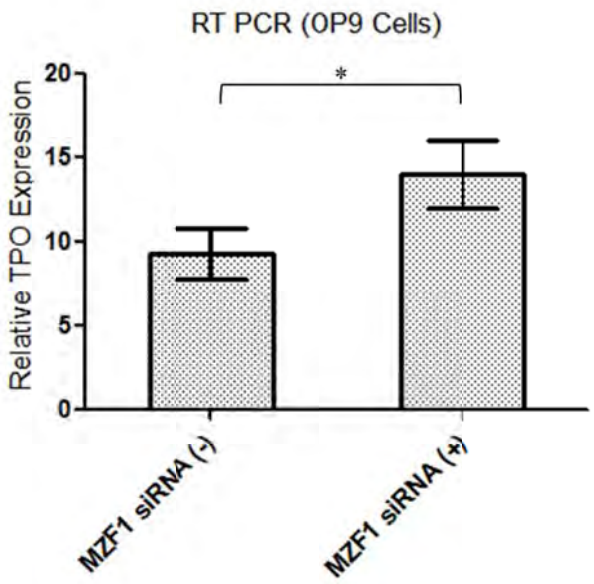


Figure 6.13 Knockdown of MZF1 gene yielded an increased TPO expression. *The endogenous expression of MZF1 in OP9 cells was knocked down by MZF1 siRNA and TPO expression was determined by real-time PCR. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P=0.02 < 0.05).*

As shown in **Fig. 6.13**, knockdown of MZF1 (2^{ed} bar, MZF1 siRNA (+)) in the OP9 cells significantly increased expression of TPO up to over **1**.7 times higher than that of the control in which the cells were transfected by a control (irrelevant) siRNA (1st bar, MZF1 siRNA(-)). This result further suggests that MZF1 is probably the novel intronic transcriptional repressor of the human TPO gene.

6.4 Discussion

In Chapter five, I presented evidence that ZEB1 is probably not a repressor of TPO promoter activity. In this chapter, I attempted to identify the repressor using a series of 3'-deletion TPO promoter-luciferase constructs I generated. Promoter activities of these deletion constructs were measured using the luciferase assay. The results clearly showed that the repressor activity was localized to the region of TPO promoter, +450 to +509. Subsequent sequence analysis indicated the presence of a MZF1 motif 5'-GCTGGGGA-3' from +457 to +464 of TPO gene. The MZF1 motif-bearing TPO promoter construct, PGL3-708, gave a significant reduction of promoter activity, i.e. 28% of that of the control construct, PGL3-216 in HeLa cells and 31% in OP9 cells respectively. EMSA assay further showed that the 5'-GCTGGGGA-3' motif is the functional binding target of MZF1 transcription factor. These results suggest MZF1 is probably the transcriptional repressor of the TPO promoter.

Furthermore, MZF1 siRNA co-transfection successfully blocked the inhibition and restored the promoter activity of the MZF1 motif-bearing construct, PGL3-708, back to 86% in HeLa cells and 90% in OP9 cells of that of the control construct, PGL3-216. It was also shown in this chapter by relative quantitative real-time PCR that when endogenous MZF1 expression was knocked down, the mRNA of TPO in OP9 cells increased significantly. These results suggest that MZF1 is the transcription repressor which binds to guanine-rich motif, 5'-GCTGGGGA-3' at the intronic site, (+457 to +464) and represses TPO expression. The identification of MZF1 as the repressor of TPO gene is not unexpected as MZF1 has previously been reported to be a transcriptional repressor in haematopoiesis. For example, Takahashi et al (2003) found in their study that MZF1 was a transcriptional repressor of the human FcεRI β-chain gene and a guanine-rich motif in its fourth intron of FcεRI β was

identified as the binding site of MZF1 in this context (Takahashi et al, 2003). MZF1 was further explored in another study, there was another factor, FHL3 (four and a half LIM domain protein 3), involved in such MZF1-mediated suppression on FcεRI β-chain gene. FHL3 bound MZF1 *in vitro* and *in vivo* as a cofactor of MZF1, forming a complex whereby repressed FcεRI β-chain gene (Takahashi et al. 2005). It remains a question that whether the co-factor, FHL3, exists in current defined MZF1 repression on TPO. However, the chance is slim as FHL3 has been found to primarily express in skeletal muscle (Li et al, 2001). Very low level of FHL3 expression in HeLa cells (Turner et al, 2003) and no clue for an expression in OP9 cells also indicates that it might be a FHL3-independent mechanism in this study. However, this needs further experiments to confirm in future investigations.

In addition, other studies have shown that MZF1 negatively regulates differentiation of embryonic stem cells and in particular their commitment to the hematopoietic lineages (Perrotti et al, 1995). Furthermore (Gaboli et al, 2001), hematopoietic progenitor cells of the *Mzf*$^{-/-}$ mice were found to have a significantly increased long-term hematopoiesis probably because of deletion of MZF1, a transcription repressor in haematopoietic development, enhances haematopoiesis.

In conclusion, the present study identified MZF1 as a novel intronic transcriptional repressor of human TPO gene expression. I further showed that MZF1 specifically binds to the guanine-rich motif (GCTGGGGA) in intron 1 of TPO gene promoter, from +457 to +464. This work helps to understand further the regulation of human TPO gene expression and provides the scientific basis for further investigations of the role of MZF1 in the regulation of hematopoiesis.

Chapter Seven: PF4 Suppresses TPO Expression through LRP1

Chapter Seven: PF4 Suppresses TPO Expression through LRP1

7.1 Introduction

As discussed in previous chapters (section 1.5 and 4.1), there are two mechanisms that regulate plasma TPO levels. The first is a mechanism in which TPO is taken up by its receptor (c-MPL) and degraded intra-cellularly. When blood platelet levels are high, more TPO is taken up and degraded, resulting in a fall in plasma TPO levels which in turn cause a decrease in plasma TPO level. The reverse occurs when blood platelet levels are low (Fielder et al, 1996). The second is a mechanism in which TPO expression in bone marrow stromal cells is regulated at the transcriptional level. Previous studies showed that transcriptional regulation of TPO expression is mediated by platelet and MK α-granular proteins: PDGF and FGF-2 enhance, while platelet factor 4 (PF4), thrombospondin (TSP) and transforming growth factor (TGF-β) suppress the expression of TPO in human bone marrow stromal cells (Sungaran et al, 2000). The key factor in the α-granular protein releasate is PF4 which suppresses TPO expression and the overall effect is also a suppressive one. Serum contains released α-granular proteins and overall serum effect is also suppressive as shown by McIntosh and Kaushansky (2008). These authors proposed that ZEB1 was the most likely transcription factor responsible for the serum-mediated down-regulation of human TPO expression (McIntosh and Kaushasky, 2008). This transcription factor is known to response to serum treatment and translocate into the nucleus upon serum stimulation (Franklin et al, 1994). ZEB1 is an important member of zinc finger protein family that is expressed widely and functions mainly as a repressor for gene expression (Ponticos et al, 2004; Jethanandani and Kramer 2005; Shirakihara et al, 2007). However, as discussed in Chapter five, I found that ZEB1 was unlikely to cause suppression

of TPO expression. On the other hand, I characterized another zinc finger protein, MZF1 which functions as a transcriptional repressor of TPO expression (Chapter six). However, the mechanism of platelet/MK α-granular protein-mediated transcriptional regulation of the TPO gene has not been fully investigated. In this chapter, the serum-, PF4- and MZF1- mediated suppression of TPO expression were investigated.

PF4, one of the platelet α-granular proteins released by activated or apoptotic platelets and megakaryocytes, belongs to the CXC chemokine family. Previous studies showed that PF4 inhibits colony formation of MKs, proliferation and maturation of MK progenitor cells (Vainchenker et al, 1982; Han et al, 1990; Daly et al, 1995). A recent study revealed that PF4 interacted with the low density lipoprotein receptor-related protein (LRP1) expressed on MKs and inhibited megakaryopoiesis (Lambert et al, 2009). This prompts me to investigate whether stromal cells also express LRP1 using OP9 cells (murine stromal cells) as a surrogate for bone marrow stromal cells. I found high level of LRP1 expression on OP9 cells. This finding is consistent with my hypothesis that PF4 might bind to LRP1 on bone marrow stromal cells, induces intracellular signaling and activates MZF1 which then represses TPO expression (see Chapter six).

I also hypothesize that when PF4 binds LRP1, it activates PI3K/Akt signal transduction pathway. My hypothesis is based on previous studies which showed when a ligand interacted with LRP1 it activated the PI3K/Akt signal transduction pathway. For example, binding of thrombospondin 1 to calreticulin-LRP1 complex activated PI3K/Akt resulting in suppression of apoptosis in fibroblasts (Pallero et al, 2008) and in neurons (Fuentealba et al, 2009). It was also found in nerve regeneration after nerve injury that LRP1-activated PI3K signal transduction significantly suppressed the apoptosis of Schwann cells (Mantuano et al, 2011). Work on the protection of LRP1

against atherosclerosis revealed that LRP1 suppressed the formation of vascular atherosclerotic lesions by regulating the platelet derived growth factor receptor-β (PDGFR-β)-dependent PI3K signal transduction (Zhou et al, 2009). These findings indicate that LRP1-mediated PI3/Akt signal transduction generally leads to a suppressive transcription effect. In summary, I propose that interaction of PF4 and LRP1 may lead to activation of PI3K/Akt signal transduction pathway which in turn activates MZF1, and this ultimately leads to repression of TPO transcription. In this chapter I present data to show involvement of PI3K/Akt signaling in PF4-mediated suppression of TPO transcription.

7.2 Methods and Materials

7.2.1 Cell culture

OP9 cells were cultured as described in section 3.1.3.

7.2.2 Serum treatment on OP9 cells

To investigate the effect of serum on the expression of TPO, OP9 cells were incubated with various doses of serum and TPO mRNA levels determined by relative quantitative real-time PCR. Briefly, 0.5×10^6 of OP9 cells were seeded into each well of a 6-well plate 24 h before addition of serum. The spent medium was removed and cells were washed twice with sterile PBS and seeded in medium supplemented with 20%, 15%, 10%, 5%, 2.5% or 0% of serum. The cells were incubated for 2, 4 or 8 h and then harvested. The total RNA was extracted and cDNA was synthesized as described previously. The mRNA levels were evaluated by relative quantitative real-time PCR. Specific primer pairs used in the amplification were purchased from Qiagen (VIC, Australia).

7.2.3 Purification of PF4

7.2.3.1 Preparation of heparin sepharose beads

PF4 was purified from plasma supernatant of outdated human platelet concentrates using heparin sepharose 6 fast flows (GE Healthcare, NSW, Australia) and according to the manufacturer's instructions. Briefly, 10 ml of heparin sepharose beads were loaded into the chromatography column (GE Healthcare) that was then placed on the AKTA HPLC instrument (GE Healthcare). The heparin sepharose beads were washed with 100 ml of column washing buffer (20 mM HEPES (Sigma-Aldrich), 05 M NaCl, 5 mM EDTA [pH 7.5] with a flow rate of 1 ml/min. The beads were washed with 20 ml of 6 M

guanidine hydrochloride (Sigma-Aldrich) with a flow rate of 1 ml/min, with 40 ml of 70% ethanol and then with 100 ml of the column washing buffer again with a flow rate of 1 ml/min.

7.2.3.2 Protein binding and elution

1.5 L of outdated platelet rich plasma was dispensed into autoclaved 350 ml centrifuge bottles. The plasma was spun at room temperature at 6,000 rpm/min for 20 min to pellet the cell debris and the clear supernatant was transferred to a clean container containing 10 ml of heparin sepharose beads and the mixture was rocked at 4 ºC, and at 150 rpm for 12 h. The mixture was placed in the 4 ºC cold room for 1 hour to allow the beads to settle down and then the upper supernatant was carefully removed with a transfer pipette. The beads were loaded to the column and then placed on the HPLC instrument. Proteins binding to the heparin sepharose beads in the column were eluted using elution buffer (20 mM HEPES, 2 M NaCl, 5 mM EDTA, pH 7.5).

7.2.3.3 PF4 gel filtration

The eluate (which mainly contains PF4 and anti-thrombin III) was then concentrated (which contains using the 3 kDa molecular cut-off concentrator (Merck Millipore) to a final volume of less than 250 µl. The concentrated eluate was loaded onto the gel filtration column (superdex 75 prep grade) (GE Healthcare) and gel filtration buffer (20 mM HEPES, 2 M NaCl, 5 mM EDTA, pH 7.5) was applied. The eluate showed 3 peaks and PF4 was located mainly in the 3rd peak. Fractions in the 3rd peak were pooled, dialysed against PBS and then concentrated. The protein content of the purified PF4 was measured as described before (see section 3.2.6.2) and the purified PF4 was stored at -80 ºC until use.

7.2.4 PF4 treatment of OP9 cells

To investigate the effect of PF4 on the expression of TPO, OP9 cells were incubated with various concentrations of purified PF4 and the corresponding TPO mRNA levels were determined by the relative quantitative real-time PCR. Briefly, 0.5×10^6 of OP9 cells were seeded into each well of a 6-well plate 24 h before the addition of PF4. The spent medium was removed and cells were washed twice with sterile PBS. Serum-free medium supplemented with 0 μg/ml, 5 μg/ml, 15 μg/ml, 30 μg/ml, 60 μg/ml or 120 μg/ml of PF4 was added to the cells and incubated for 8 h. The total RNA was extracted and cDNA was synthesized as described previously and mRNA levels were evaluated by relative quantitative real-time PCR as previously described (see section 6.2.6). The specific primer pairs used in the TPO gene amplification were purchased from Qiagen.

7.2.5 Removal of PF4 from serum

To evaluate the suppressive effect of PF4 in the serum on expression of TPO, the PF4 in serum was removed using heparin sepharose beads which incidentally also removed other heparin-binding proteins (e.g. ATIII). The PF4-reduced serum was then used to treat OP9 cells. Briefly, 1 ml of heparin sepharose beads were added into 10 ml of serum in a sterilized tube and rocked at 4 ºC for 12 h. The beads were pelleted by centrifugation and the supernatant was collected. OP9 cells were cultured with medium supplemented with 20% PF4-reduced serum for 8 h and the corresponding mRNA level of TPO was determined by the relative quantitative real-time PCR.

7.2.6 Detection of LRP1 expression by cell flow cytometry

The expression of LRP1 on OP9 cells were detected by cell flow cytometry. OP9 cells were collected by centrifugation (1,000 rpm for 10 min) and suspended in 1 ml of PBS. Cells were fixed in 4% of formaldehyde for 10 min at 37°C and then chilled on ice for 1 min. 1×10^6 cells were dispensed into each sample tube which contained 2 ml of incubation buffer (0.5% BSA in PBS). Cells were resuspended and blocked in 100 µl of incubation buffer for 10 min at room temperature. Cells were then incubated with anti-LRP1 antibody (mouse monoclonal and reacts with mouse and human LRP1, 1:50 dilution, Abcam, MA, USA) for 1 h at 22°C. Cells were washed in incubation buffer and pelleted by centrifugation. Cells were resuspended in 50 µl of incubation buffer which contained secondary antibody purchased from Invitrogen (Goat Anti-Mouse IgG (H+L) conjugated with FITC, 1 µg per 1×10^6 cells). Cells were incubated with secondary antibody on ice for 30 min. Cells were washed with incubation buffer, pelleted by centrifugation, resuspended in 0.5 ml PBS and analyzed on FACSCalibur (BD Biosciences, CA, USA). An isotype antibody (mouse IgG2a conjugated with FITC, Invitrogen) was used as a control. Data were analyzed using BD CellQuest Pro software.

7.2.7 Blocking of LRP1

LRP1 was blocked using either a specific anti LRP1 antibody or the receptor associated protein (RAP) (Molecular Innovations, MI, USA), which is an antagonist of LRP1. Briefly, 0.5×10^6 of OP9 cells were seeded into each well of a 6-well plate 24 h before treatment. The spent culture medium was discarded and cells were washed twice with sterile PBS. Cells were cultured for 30 min in serum free media supplemented with 30 µg/ml or 40 µg/ml of anti-LRP1 antibody (Abcam). Then 30 µg/ml or 60 µg/ml of PF4 were added to the cells

treated by 30 μg/ml or 40 μg/ml of antibody respectively. Cells were then incubated for 8 h. Cells were also treated in the same way with RAP instead of anti-LRP1 antibody. Total RNA of treated cells was extracted and corresponding TPO mRNA levels were determined by the relative quantitative real-time PCR analysis.

7.2.8 PF4 treatment on OP9 cells and determination of MZF1 expression

To determine whether there was a correlation between the expression of MZF1 and PF4-induced suppression of TPO expression, OP9 cells were treated with various concentrations of PF4 and the corresponding mRNA levels of MZF1 were determined. OP9 cells were treated by PF4 as described in section 7.2.4 and mRNA levels of MZF1 were measured by the relative quantitative real-time PCR.

7.2.9 Detection of PF4 and LRP1-induced signal transduction pathway

7.2.9.1 Western blot/Detection of phosphorylated Akt

1×10^6 of OP9 cells were seeded into 100 mm Petri dishes 24 h prior to PF4 treatment. The spent medium was discarded and cells were washed twice with sterile PBS. Serum free medium supplemented with 200 nM wortmannin (a specific inhibitor of PI3K/Akt obtained from Sigma-Aldrich) was added to the cells and incubated at 37°C in a 5% CO_2 incubator for 30 min. 60 μg/ml of PF4 was then applied to both wortmannin treated and untreated cells. Cells were incubated at 37 °C in a 5% CO_2 incubator for 1 h and the nuclear protein was harvested. 10 μg of nuclear extract was used to estimate Akt phosphorylation level by Western blot. Total Akt was detected with anti-Akt antibody (Santa

Cruz Biotech). The level of phosphorylated Akt was estimated using an anti-phosphorylated Akt antibody (Santa Cruz Biotech). β-actin (Sigma-Aldrich) was used as loading control.

7.2.9.2 Determination of TPO expression in PI3K/Akt inhibited OP9 cells

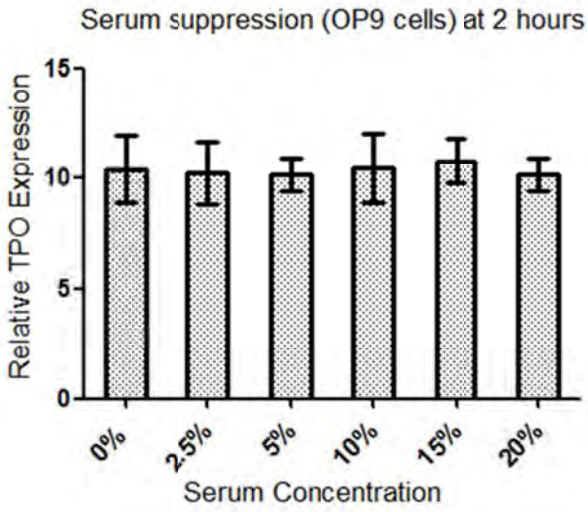
OP9 cells were treated as described in section 7.2.9.1 (cells were incubated with PF4 for 8 h instead) and TPO expression in treated and untreated cells was determined the relative quantitative real-time PCR as described in Chapter six (section 6.2.6**).**

7.3 Results

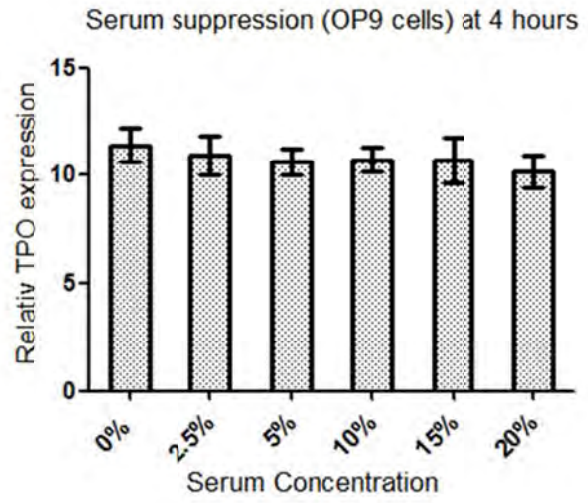
7.3.1 Serum represses TPO expression

To investigate the effect of serum on expression of TPO, OP9 cells were treated with various dilutions of serum and the corresponding TPO mRNA levels were determined by the relative quantitative PCR.

A:



B:



C:

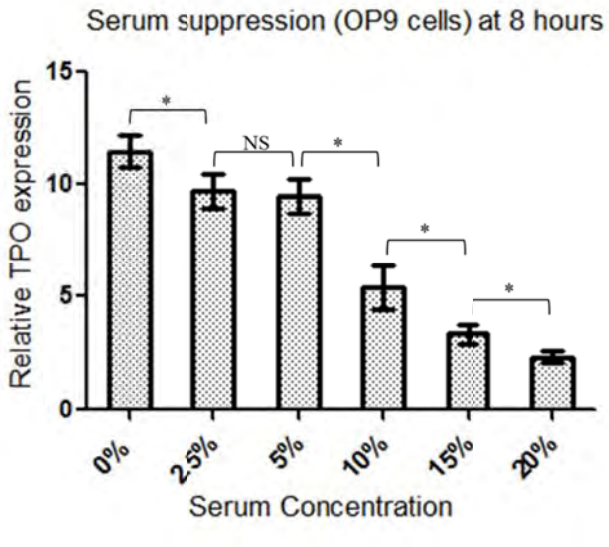


Figure 7.1 Serum represses dose-dependently TPO expression in OP9 cells. *Various dilutions of serum (5% to 20%) were applied to OP9 cells and TPO mRNA levels were determined at different time points (2, 4 and 8 h). The relative expression ratio (R) of TPO was generated using method introduced in section 6.2.6.4. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05, NS: not significantly different).*

Expression of TPO was not significantly affected in the first 4 h of PF4 treatment (**Fig. 7.1 A and B**). However, the 8-hour serum treatment induced a dose-dependent decrease in TPO expression. As shown in the **Fig. 7.1 C**, 2.5% and 5% of serum induced a similar decrease in TPO mRNA and the remaining TPO expression was approximately 85% relative to serum-free condition. 10%, 15% and 20% of serum caused significant decrease of TPO expression and the remaining TPO expression levels were: 49%, 30% and 21% relative to serum free conditions, respectively. This result clearly showed that in OP9 cells, serum could effectively suppress the mRNA levels of TPO and a concentration of 10% of serum in the medium was capable of inducing a substantial decrease in TPO expression. This serum-induced suppression was dose-dependent and

time-dependent as the 8 h but not 2 and 4 h serum treatment could significantly decrease of TPO mRNA level. Alternatively, the TPO mRNA already present in the cells was stable for the first 4 h and reduction was only observed after a longer incubation time. Overall, these results showed that normal serum effectively suppresses TPO expression.

7.3.2 PF4 suppresses the expression of TPO gene

OP9 cells were treated by various concentrations of PF4 and the corresponding TPO mRNA levels were determined by relative quantitative real-time PCR.

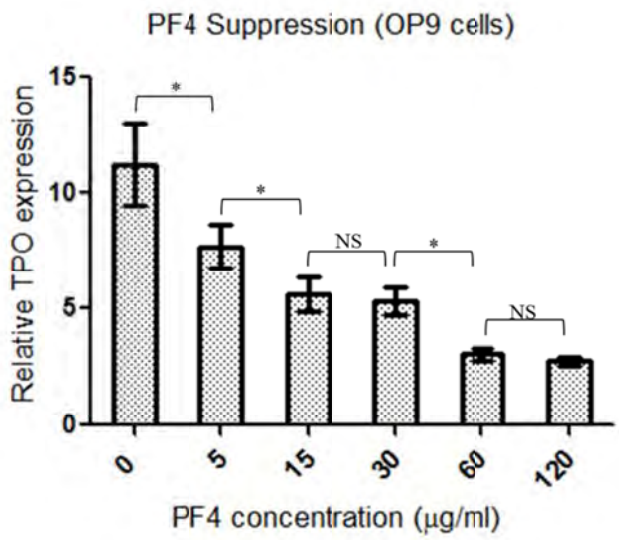


Figure 7.2 PF4 suppression of TPO expression. *Various concentrations of PF4 (5 µg/ml to 120 µg/ml) were applied to OP9 cells. The expression level of TPO gene was determined by relative quantitative real-time PCR. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05, NS: not significantly different).*

As shown in **Fig. 7.2** treatment of OP9 cells with PF4 caused a dose-dependent decrease of TPO expression. 5 µg/ml of PF4 suppressed the mRNA level of TPO to 70% relative to untreated cells. 15 µg/ml and 30 µg/ml reduced TPO expression by approximately 50% relative to untreated cells, while 60 µg/ml

and 120 μg/ml of PF4 reduced TPO expression by more than 75%. This result showed that PF4 was capable of inducing a dose-dependent suppression of TPO expression similar to that observed with serum. As serum is rich in PF4, I sought to determine whether the PF4 in the serum is the key factor in the serum-mediated TPO repression.

7.3.3 PF4-reduced serum

To evaluate the contribution of PF4 in the serum-induced repression of TPO in OP9 cells, PF4 in the serum was removed using heparin sepharose beads. The PF4-reduced serum was then used to treat OP9 cells as described in section 7.2.5.

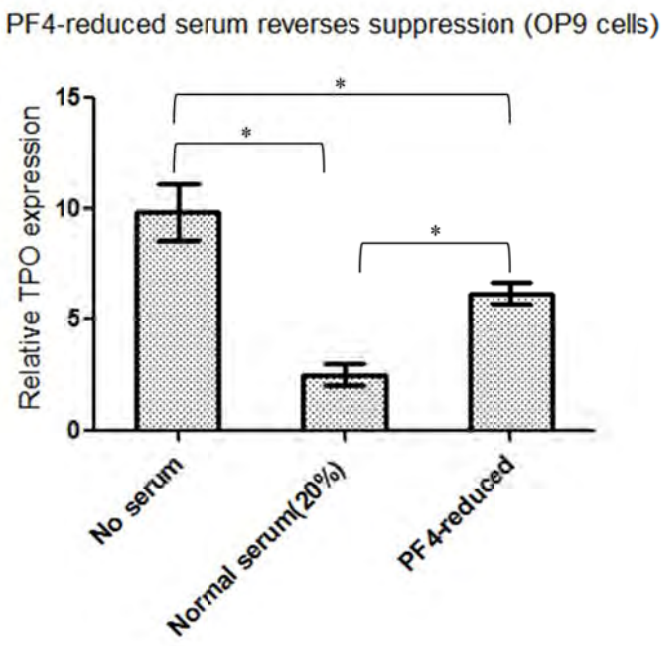


Figure 7.3 PF4-reduced serum reverses suppression of TPO expression. *PF4-reduced serum and control serum were applied to OP9 cells and the mRNA levels of TPO were determined by the relative quantitative real-time PCR. Cells in serum free or 20% normal serum condition were used as controls. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P <*

0.05).

As shown in **Fig. 7.3**, and in agreement with **Fig. 7.1**, the presence of 20% serum down-regulated TPO expression by more than 75% relative to serum free conditions. In contrast, culture with PF4-reduced serum resulted in a significant 2.5-fold increase in TPO expression relative to the expression observed in cells treated with 20% normal serum. The results clearly showed that PF4 was a major factor in serum-induced TPO repression in OP9 cells. However, the possibility that there were other factors in the serum which could also contribute to the suppression of TPO expression could not be entirely excluded, as heparin-sepharose beads also removed other heparin-binding proteins, such as anti-thrombin III (ATIII), in addition to PF4. Also, removal of these proteins from the serum did not completely reverse the TPO suppression; the remaining minor suppression may be attributed to other serum factor(s) or could result from the remaining PF4 that was not removed completely from normal serum.

7.3.4 LRP1 expression on OP9 cells

It's likely that PF4-induced repression of TPO could be mediated through LRP1 as discussed before (section 7.1). Firstly, I used flow cytometry to determine whether LRP1 was expressed on OP9 cells.

A:

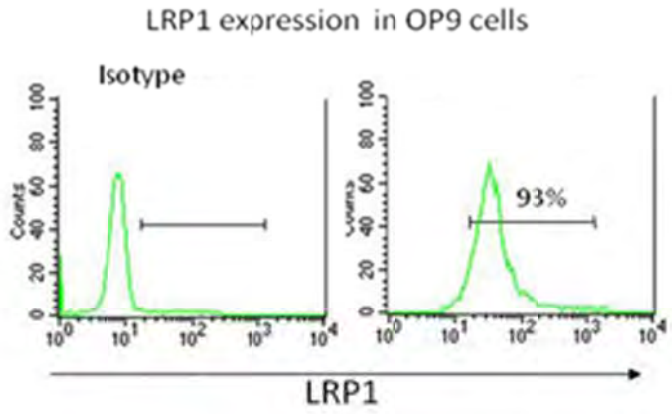


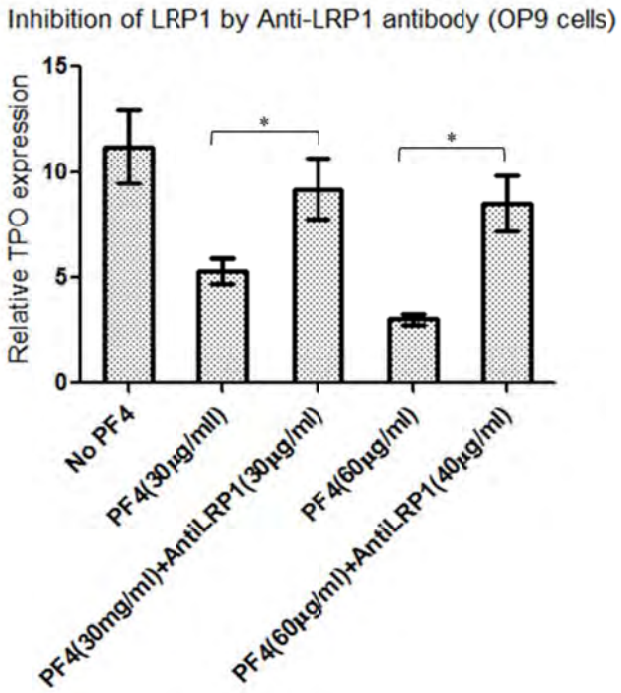
Figure 7.4 Expression of LRP1 on OP9 cells. *The expression of LRP1 on OP9 cells was determined by flow cytometry.*

As shown in **Fig. 7.4**, most of OP9 cells (93%) expressed LRP1. Based on the presence on this molecule on the cells, we proposed that it may serve as a receptor for PF4 and might, therefore, be a mediator of PF4-induced TPO repression.

7.3.5 Inhibition of LRP1 reverses suppression of TPO messages

To test whether PF4 suppressed the expression of TPO through the LRP1 receptor detected on OP9 cells, LRP1 was blocked by either a specific anti-LRP1 antibody or an LRP1 antagonist (RAP). The blocked cells were then treated with PF4 and corresponding expression levels of TPO mRNA were determined by the relative quantitative real-time PCR analysis.

A:



B:

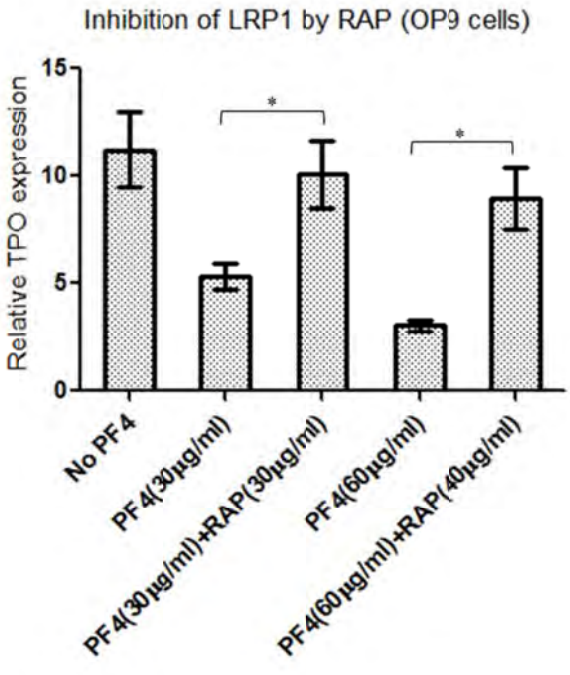


Figure 7.5 Inhibition of LRP1 restores TPO expression. *A: LRP1 was blocked with 30 or 40 µg/ml of specific antibody (Anti-LRP1) or B: 30 or 40 µg/ml of*

*LRP1 antagonist, RAP. 30 and 60 μg/ml of PF4 were applied to both untreated and LRP1 blocked cells and the corresponding expression levels of TPO were evaluated. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

As shown in **Fig. 7.5 A**, blocking by the specific antibody against LRP1 on OP9 cells significantly reversed the inhibition of TPO expression in the presence of PF4. 30 μg/ml of anti-LRP1 antibody restored the suppression of TPO caused by 30 μg/ml of PF4 from 50% back to 82% relative to untreated cells, and 40 μg/ml of anti-LRP1 antibody also efficiently restored the suppression of TPO expression induced by 60 μg/ml of PF4 from 25% back to 76% relative to untreated cells. The significant recovery of suppressed TPO expression strongly suggests that PF4-induced suppression of TPO mRNA was through an interaction with LRP1.

To further confirm the role of LRP1 in PF4-induced suppression of TPO mRNA, LRP1 was blocked using an LRP1 antagonist, RAP. As shown in **Fig. 7.5 B**, blocking of LRP1 by the RAP also efficiently counteracted the effect of PF4 on TPO expression. 30 μg/ml of RAP restored the suppression of TPO caused by 30 μg/ml of PF4 from approximately 50% back to 91% and 40 μg/ml of RPA also significantly restored TPO expression caused by 60 μg/ml of PF4 from 25% to 81% relative to untreated cells, respectively.

The blocking of LRP1 by either a specific antibody against LRP1 or the LRP1 antagonist RAP significantly reversed the PF4-induced suppression of TPO in OP9 cells. These results indicate that PF4-induced suppression was through the interaction between PF4 and LRP1 expressed on OP9 cells.

7.3.6 PF4 does not alter the expression of MZF1

To explore the correlation between the PF4-mediated suppression of TPO expression and the expression levels of MZF1, OP9 cells were treated with various concentrations of PF4 and corresponding mRNA levels of MZF1 determined.

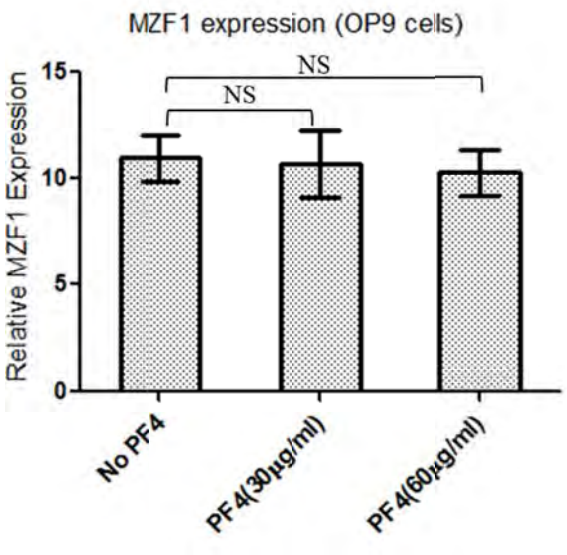


Figure 7.6 PF4 does not alter the expression of MZF1. *Various concentrations (30 and 60 μg/ml) of PF4 were applied to OP9 cells. The corresponding expression levels of MZF1 in untreated (control, no PF4) and treated cells were determined by relative quantitative real-time PCR. The data represent the mean of three independent experiments (mean +/- S.D. bar. NS: not significantly different).*

As shown in **Fig. 7.6**, the treatment with PF4 (30 μg/ml and 60 μg/ml) did not significantly alter the expression of MZF1 in OP9 cells. This result indicates that the suppressed expression of TPO gene may not be through a direct alteration of MZF1 expression. However, it is possible that PF4 might still regulate MZF1 activity in an indirect manner that facilitated MZF1-mediated suppression of TPO. PF4 could trigger intracellular signal transduction, activate MZF1 by phosphorylation or other modification, and subsequently induce

translocation of MZF1 into the nucleus allowing MZF1 to bind to the promoter region of TPO and to repress its transcriptional activity.

7.3.7 Signal transduction

As discussed in section 7.3.6, the intracellular signal transduction pathway whereby PF4 induces TPO down-regulation by MZF1, remains unidentified. I suggested that it is the PI3K/Akt signaling pathway (section 7.1). I provide below the findings of my investigations that support this hypothesis.

7.3.7.1 PI3K/Akt signal transduction

OP9 cells were treated with PF4 and phosphorylation of Akt was assessed by Western blot.

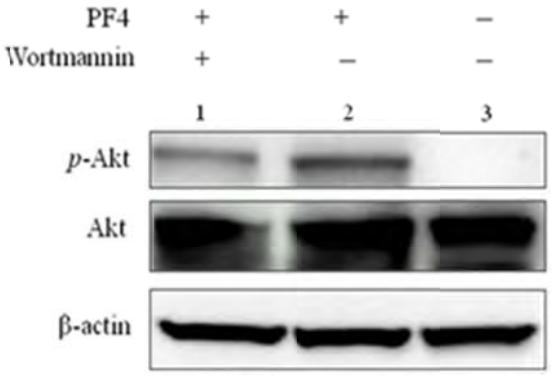


Figure 7.7 PF4 induces Akt phosphorylation. *OP9 cells were pretreated by the drug wortmannin that specifically blocks the activation of PI3K/Akt. PF4 was applied to the drug treated (lane 1) and untreated (lane 2) OP9 cells. The corresponding phosphorylated form of Akt in treated (lane 1, lane 2) and control (lane 3) cells was detected by the Western blot.*

As shown in **Fig. 7.7**, OP9 cells treated with PF4 showed induced Akt

phosporylation (lane 2: *p*-Akt) while no phosphorylated Akt was detected in cells without PF4 treatment **(**lane 3: *p*-Akt). The cells pretreated with the specific inhibitor (wortmannin) showed as expected, a reduced level of phosphorylation of Akt (lane **1**: *p*-Akt) compared with cells without treatment (lane 2: *p*-Akt). This result suggests that the interaction of PF4 and LRP1 on OP9 cells induced PI3K/Akt signal transduction.

7.3.7.2 PI3K/Akt inhibition reversed TPO mRNA down-regulation induced by PF4

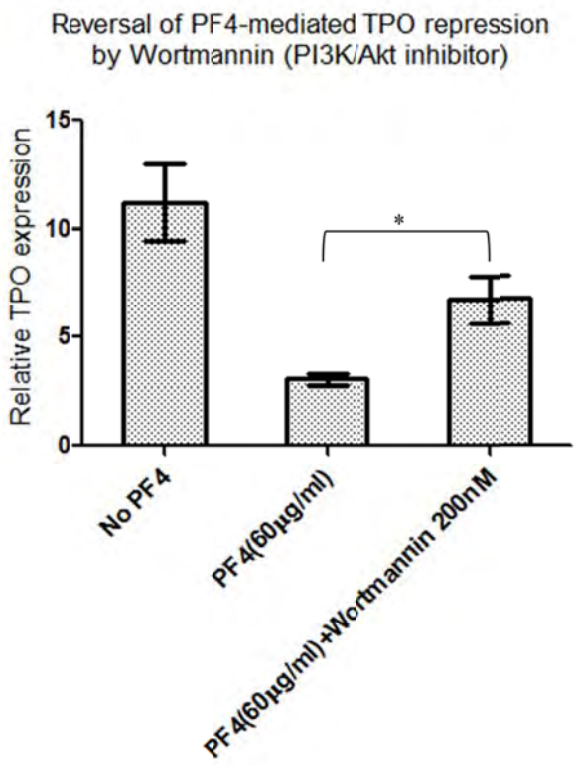


Figure 7.8 Akt inhibition reverses repression of TPO mRNA expression. *PI3K/Akt phosphorylation activated by PF4 was inhibited with wortmannin and the resulting mRNA levels of TPO gene in untreated (buffer/no PF4/no wortmannin) cells, cells treated with 60 µg/ml of PF4, and cells treated with 200 nM of wortmannin plus 60 µg/ml of PF4, were determined by relative*

*quantitative real-time PCR. The data represent the mean of three independent experiments (mean +/- S.D. bar. *P < 0.05).*

To interrogate if PF4-induced phosphorylation of Akt is functionally involved in the PF4-mediated down-regulation of TPO expression in OP9 cells, the activation of PI3K/Akt was inhibited by wortmannin and the resultant TPO mRNA expression in PF4-treated OP9 cells was determined by the relative quantitative real-time PCR. As expected, PF4 caused significantly reduction of TPO expression (**Fig. 7.8)**. The inhibition of PI3K/Akt by wortmannin successfully reversed the suppression of TPO expression and restored it from approximately 25% to 60% relative to untreated OP9 cells.

Altogether, the results clearly indicate that repression of TPO expression induced by PF4/LRP1 interaction is mediated by PI3K/Akt signal transduction pathway. However, the inhibition of PI3K/Akt did not reverse the suppression of TPO mRNA completely, i.e. to the levels observed in untreated OP9 cells. There are two possible explanations for this: 1) the activation of PI3K/Akt was significantly reduced by wortmannin, however there was still a low level of Akt activation remaining compared to the untreated OP9 cells (as showed in lane 1, **Fig. 7.7**, a *p*-Akt band of less intensity was still visible), which might be enough to induce TPO suppression. 2) besides PI3K/Akt pathway, interaction of PF4 and LRP1 might activate other signal transduction pathway(s) that would also induce suppression of TPO expression. The former explanation (1) appears more likely as suggested by Fig 7.7.

7.4 Discussion

The mRNA level of TPO in the mouse bone marrow stromal cells could be effectively suppressed by normal serum. This serum suppressive effect was observed only after a time lag of 8 h after serum treatment. The suppressive effect was dose-dependent; there was a progressive decrease in the levels of TPO mRNA with increasing serum concentrations. It is known that serum is rich in growth factors and cytokines, including TPO. The simplest explanation for this negative regulation of TPO expression is a feedback inhibition: i.e. high blood levels of TPO inhibit the expression of the TPO gene. However, treatment of cells with recombinant TPO did not suppress TPO expression in the bone marrow stromal cells (McIntosh and Kaushansky, 2008). On the other hand, TPO expression in bone marrow stromal cells was efficiently suppressed by the thrombin-activated platelet releasate. It was also known that serum derived from thrombin activated of platelet-rich plasma induced a much greater repression of TPO expression compared with serum derived from platelet-poor plasma. All these observations indicated that the reduced TPO expression did not result from the feedback inhibition by TPO protein itself but from increased levels of repressive factor(s) released from activated platelets.

Previous studies pointed out that the platelet α-granule protein PF4 (present in high concentrations in platelet releasate) suppressed the expression of TPO in bone marrow stromal cells (Sungaran et al, 2000). To investigate the contribution of PF4 in the serum, bone marrow stromal cells were incubated with purified PF4, serum depleted or serum not depleted of PF4, and TPO mRNA expression was then measured by real-time PCR. PF4 efficiently suppressed TPO expression in the bone marrow stromal cells in a dose-dependent manner and removal of PF4 from serum successfully reversed the inhibition. These observations are consistent with the findings of a previous

study (Sungaran et al, 2000), and further confirm that PF4 is capable of inhibiting TPO expression at the transcriptional level. My studies used relative quantitative real-time PCR, which is a more robust technique than that used in previous work. My studies also provide evidence that PF4 is the major serum component that was responsible for the observed serum suppression of TPO expression. Since removal of PF4 did not completely reverse the repression of TPO, the possibility that there might be other factor(s) in serum that also suppressed TPO expression could not be excluded. In fact, previous research found that two other components of platelet α-granule proteins, thrombospondin and transforming growth factor β (TGF-β), also negatively regulated TPO gene expression in bone marrow stromal cells (Sungaran et al, 2000). Further studies are needed to investigate the contribution of thrombospondin and TGF-β in suppression of TPO expression. My findings also support the hypothesis of an alternative paracrine regulatory mechanism of TPO expression. When there is a high level of platelets, more PF4 is released from MKs and platelet α-granules, and this leads to increased inhibition of TPO production by the bone marrow stroma. Conversely, when the platelet count is low, the concentration of PF4 decreases and TPO is expressed at a relatively higher level. This pathway regulates TPO expression at a transcriptional level and it probably occurs hand-in-hand with the c-MPL-mediated TPO regulation.

PF4 has been reported to bind to LRP1 in cells such as megakaryocytes (Lambert et al, 2009). In this study, I observed for the first time high expression of LRP1 on OP9 cells. This finding prompted me to consider the possibility of interaction between PF4 and LRP1 on stromal cells (OP9).

My subsequent observation that LPR1 inhibition reversed, to a great extent, PF4-mediated TPO suppression, clearly pointed to a functional interaction between PF4 and LRP1. The interaction of PF4 and LRP1 may also lead to signal transduction activation (see below for further discussion) and the

subsequent inhibition of TPO gene transcription. Blocking of LRP1 by a specific antibody against LRP1 or by the antagonist, RAP, reversed PF4-induced repression of TPO expression would be consistent of this concept.

Studies in Chapter six revealed that transcriptional factor MZF1 negatively regulated the expression of TPO. In order to investigate the correlation between the expression level of MZF1 and PF4-mediated suppression of TPO, OP9 cells were treated with various doses of PF4 and the resulting mRNA levels of MZF1 were determined. The results showed that the interaction of PF4 and LRP1 did not alter the expression of MZF1 in OP9 cells suggesting PF4/LRP1 interaction did not repress TPO expression by modulating MZF1 expression. However, it remains possible that PF4/LRP1 interaction activates a signal transduction pathway which in turn activates MZF1 (without changes in MZF1 levels) and activated MZF1 then migrates into the nucleus and mediates transcription repression of TPO. Due to my PhD study was close to the end and I was not able to find time to carry out experiments to investigate MZF1 activation and MZF1 nuclear import induced by PF4/LRP1 interaction.

My results also showed that PF4/LRP1 interaction induced PI3K/Akt signal transduction and blocking of PI3K/Akt efficiently restored the suppressed TPO expression. This observation indicates that PF4/LRP1 interaction induces repression of TPO expression via activation of PI3K/Akt signal transduction. As discussed in the preceding section, it is likely that activation of PI3K/Akt signaling pathway leads to activation of MZF1, which probably then translocates into the nucleus and induces its repression of TPO gene transcription.

In conclusion, the present study investigated the serum- and PF4-induced suppression of TPO gene expression. PF4 was found to be a major factor in the serum that repressed TPO expression. PF4 was observed to interact with LRP1

receptors which were expressed abundantly in OP9 cells. PF4/LRP1 interaction did not modulate MZF1 expression but induces phosphorylation of Akt instead, implying that it activates PI3K/Akt signal transduction. If so, it is likely that induction of PI3K/Akt signal transduction activates MZF1, triggers its translocation into the nucleus and facilitates its binding to the specific MZF1 motif in the promoter region (intron 1) of TPO gene. DNA binding of MZF1 causes transcriptional repression of TPO gene. Data provided in this thesis have significantly extended our current knowledge of the mechanism of TPO regulation in the blood, in particular the negative feedback mechanism via MK/platelet alpha-granular proteins, proposed previously by Sungaran et al (2000) and McIntoch and Kaushansky (2008). As TPO is a major regulator of thrombopoiesis, my findings also provide helpful insights into regulation of platelet production.

Final Summary

TPO is the key regulator of megakaryopoiesis. Mechanisms that regulate blood TPO also indirectly regulate platelet production. When blood TPO level is high, MK proliferation and differentiation are enhanced. This leads to an increase in platelet production and a rise in blood platelet level. To keep blood platelets within a normal range, regulatory mechanisms (described below) then decrease blood TPO level and consequently blood platelet number. If not, too low or too high circulating platelet numbers will occur and this will result in disease.

Current evidence suggests that there are two mechanisms of TPO regulation. The first is a mechanism which regulates blood TPO level by a TPO receptor (c-MPL) mediated uptake and intracellular degradation of TPO. This leads to a decrease in blood TPO level when platelet number is high, and vice versa, and this mechanism thus helps to maintain blood platelets within a tight normal range. The second mechanism regulates blood TPO at the gene transcription level. TPO gene expression and TPO production in bone marrow stromal cells are controlled by a negative feedback loop via MK and platelet α-granule proteins, in particular PF4 (Sungaran et al, 2000; McIntosh and Kaushansky, 2008). TPO generated by bone marrow stromal cells probably has a greater influence on megakaryopoiesis than TPO generated elsewhere because this TPO is released at the site of platelet production. However, this transcriptional regulation of TPO has not been fully characterized. The PF4 receptor on the stromal cells, the intracellular signal transduction pathway(s) and the transcription factor involved in this regulatory mechanism have not yet been identified. The studies in this thesis focus on the identification of the receptor for PF4 on stromal cells, the signal transduction pathway and the potential transcription factor that participate in the negative feedback control of TPO production in the bone marrow.

My study started with localization of the repression element in the promoter region of TPO gene. In Chapter four, a minimal promoter of human TPO gene was localized to a region from -202 to +14. Combining this result with a previous finding (Kamura et al, 1997), a minimal promoter of human TPO gene could be fine mapped to -102 to +14. My studies firstly confirmed that a previously defined TPO transcription enhancer, GABPα, binds to -ACTTCCG- from -109 to -103. More importantly, further studies using promoter deletion analysis revealed a profound reduction of promoter activity when the TPO promoter fragment included a further downstream region, from +14 to +688, indicating the presence of a strong repression element(s) in this region.

Having identified the repression region in Chapter four, the potential repression element was then characterized in Chapter five. I found that a cluster of four conserved ZEB1 binding motifs are present in this repression region of the TPO gene. It has been previously proposed by McIntosh and Kaushansky (2008) that the transcription factor, ZEB1, is likely to be responsible for the repression of TPO gene. To prove or disprove this proposal, I used TPO promoter constructs containing two or four of these ZEB1 motifs (PGL3-886, PGL3-958) and showed that both constructs greatly reduced TPO promoter activity (**Fig. 5.10**). However, endogenous ZEB1 in HeLa cells failed to bind to any of the four ZEB1 motifs in EMSAs. To further evaluate role of ZEB1 in TPO gene repression, a non-functional mutant ZEB1 expression plasmid (ZEB1-RD) devoid of repression effect was generated and expressed in HeLa cells. The expected competitive inhibition or reversal of the transcriptional repression by endogenous ZEB1 was surprisingly not observed. Furthermore, cells that do not express detectable ZEB1 still showed repression of TPO promoter activity. Altogether these results strongly indicate that the ZEB1 cluster in this region of the TPO gene and the ZEB1 transcription factor are not responsible for the observed TPO repression.

In Chapter six, to identify the real repression element, a series of TPO promoter constructs with consecutive 60 bp deletions in the repression region were built and then expressed in HeLa and OP9 cells. A substantial reduction of promoter activity was observed within +450 to +509. Further analysis revealed that this region contained a putative binding motif of MZF1, a known transcription repressor. Using EMSA, I clearly showed that MZF1 protein bound to this putative MZF1 motif: (5'-GCTGGGGA-3') located in the TPO promoter region from +457 to +464. Specific siRNA was applied to knock down endogenous expression of MZF1 in HeLa and OP9 cells and the repressed promoter activity was significantly restored when over 80% of endogenous MZF1 expression was knocked down. MZF1 gene knockdown also resulted in significant increase in TPO mRNA as determined by relative quantitative real-time PCR. In conclusion, studies in this chapter successfully identified MZF1 as the novel intronic transcription repressor of TPO. My studies also showed that this transcription factor bound to the MZF1 motif in intron 1 of the TPO gene and mediated transcription repression of the gene.

In Chapter seven, the studies focused on regulatory events upstream to MZF1-mediated transcriptional repression of TPO. My data confirmed the findings of previous reports that serum and MK/platelet α-granular proteins repressed TPO gene expression and that PF4 is the key inhibitory factor in serum (Sungaran et al, 2000) as removal of PF4 from the serum significantly reversed the repression. Prompted by a previously reported finding that PF4-LRP1 interaction inhibited megakaryopoiesis, experiments were carried out to investigate if PF4-LRP1 (as ligand /receptor) interaction similarly will lead to repression of the TPO gene. Firstly, I found high expression levels of LRP1 on OP9 cells (which I used as a surrogate for bone marrow stromal cells). Secondly, the experiments showed that in the presence of PF4, blocking LRP1 by an anti-LRP1 antibody or a LRP1 antagonist (RAP) reversed the repression of TPO gene expression. These findings altogether suggest that PF4 interacting

with its receptor (LRP1) on OP9 cells, led to down-stream events that ultimately induced repression of TPO gene.

Evidence is also provided in this chapter that PF4 activated PI3K/Akt intracellular signal transduction. Treatment of OP9 cells resulted in phosphorylation of Akt. Inhibition of Akt phosphorylation by wortmannin prevented PF4-mediated repression of TPO gene. Altogether these findings indicate that PF4-induced repression of TPO gene in stromal cells (OP9) is mediated by PF4-LRP1 interaction which in turn triggered the PI3K/Akt signal transduction pathway. PI3K/Akt signaling then activated MZF1 which ultimately became bound to its specific binding motif in intron 1 of TPO gene and caused repression of TPO transcription.

Future directions

The studies in this thesis investigate transcriptional regulation of human TPO. MZF1 was identified as the novel transcription repressor of TPO, PF4 was found to be the key factor in serum that mediated suppression of TPO expression in stromal cells, and LRP1 was observed to be the PF4 receptor with which PF4 interacted and consequently activated PI3k/Akt signal transduction pathway. PI3K/Akt signalling resulted in TPO gene repression possibly by MZF1. Research often leads to more unanswered questions. I listed below further studies that will further extend the studies in my thesis and will provide more insights into transcriptional regulation of human TPO.

In Chapter four, a minimal TPO promoter was mapped to the region, -202 to +14 of the TPO gene. A previous study localized it to -102 to +56 (Kamura et al, 1997). Combining the two findings, the gene regulatory domain could be more precisely mapped to the region, -102 to +14 of the gene. In addition, I confirmed a binding motif of transcriptional enhancer, GABPα in this region of the TPO gene. It would be interesting to further extend this finding to explore how GABPα up-regulates TPO expression, for example to identify (1) the growth factor or the ligand involved (as Sungaran et al (2000) reported PDGF and FGF up-regulated TPO expression; are these factors involved?), (2) the receptor for the ligand, and (3) the intracellular signaling transduction pathway that finally activates GABPα which then enhances TPO transcription.

In Chapter six, a novel intronic transcription repressor (MZF1) was characterized. As discussed in section 6.4, MZF1 mediated suppression requires its co-factor FHL3 in another context. It would be helpful to investigate in future studies if FHL3 or another co-repressor is required for MZF1 to repress TPO transcription. It would be interesting in future study to investigate how

PI3K/Akt signal transduction activates MZF1 (Is it by phosphorylation?) and induces its translocation into the nucleus so that it can perform its function as a gene repressor. Hence identifying MZF1's nuclear localizing signal(s) and its nuclear import mechanism would be of interest.

In Chapter seven, PF4 was proved to be a key factor in normal serum that is capable of inducing a dose-dependent suppression of TPO expression as removal of serum PF4 significantly reversed the suppression. However, it is possible that other factors in serum may also contribute to the suppression, considering that removal of PF4 by heparin sepharose beads would also remove other serum heparin-binding proteins such as antithrombin III. Furthermore detailed studies are required to investigate the role of those proteins relative to that of PF4.

Also, in Chapter seven, I showed that PF4 induced PI3K/Akt signal transduction which resulted in suppression of TPO gene expression. This finding could be strengthened further by showing in future studies that other signaling proteins in the PI3K/Akt are also involved in PF4 induced signal transduction. For example, it can be shown that other proteins in this pathway are phosphorylated as Akt did upon OP9 treatment with PF4. It remains possible that other signal transduction pathway(s) may also be triggered by PF4 as it is known that biological processes can be mediated by multiple signal transduction pathways.

Further investigations to address the unanswered questions listed above will certainly help to better understand transcriptional regulation of TPO and regulation of platelet production in the bone marrow.

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Appendices

Appendix A: Buffers

Buffers for DNA electrophoresis:

50 × TAE buffer (1 L)

Reagent	Amount
Tris	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA Ph 8.0	100 ml
Milli-Q water	Top up to 1 L

10 × loading buffer (20 ml)

Reagent	Amount
Xylene cyanol	25 mg
Bromophenol blue	25 mg
10% SDS	1.25 ml
Glycerol	12.5 ml
Milli-Q water	6.25 ml

Buffers for electrophoretic mobility shifting assay (EMSA):

10 × annealing buffer (50 ml)

Reagent	Amount	Final Concentration
1M Tris-HCl, [pH] 7.5	5 ml	100 mM
5 M $MgCl_2$	10 ml	1 M
5 M $NaCl_2$	10 ml	1M
Nuclease free water	25 ml	

Solution A (500 ml)

Reagent	Amount	Final concentration
1M Hepes, [pH] 7.8	5 ml	10 mM
1M $MgCl_2$	750 µl	1.5 mM
1M KCl	5 ml	10 mM
Distilled water	489 ml	

Solution C (200 ml)

Reagent	Amount	Final concentration
1 M Hepes, [pH] 7.8	4 ml	20 mM
100% Glycerol	50 ml	25%
1 M NaCl	84 ml	420 mM
1 M $MgCl_2$	300 µl	1.5 mM
0.5 M EDTA	80 µl	0.2 mM
Distilled water	61.62 ml	

Protease inhibitors and 1mM DTT were added to solution A and C, freshly prepared every time before use.

2 × binding buffer (5ml)

Reagent	Amount	Final Concentration
1 M Tris-HCl, [pH] 7.8	250 µl	50 mM
100% Glycerol	1 ml	20%
1 M $MgCl_2$	60 µl	12 mM
0.5 M EDTA	10 µl	1 mM
1 M KCl	600 µl	120 mM
1 M DTT	5 µl	1 mM
10 mg/ml BSA	200 µl	400 µg/ml
Distillled water	2.78 ml	

10 × TBE buffer (1 L)

Reagent	Amount
Tris Base	108 g
Boric Acid	55 g
EDTA	9.3 g
Distilled water	Top up to 1 L

20× loading dye for EMSA

Reagent	Amount
1 M Tris-HCl, [pH] 7.5	150 µl
Sucrose	2 g
BPB (Bromophenol blue)	0.01 g

Buffers for Western Blot:

Lysis buffer for western blot assay

Reagent	Concentration
sodium chloride	150 mM
NP-40 (Trion X-100)	1%
Tris (pH 8.0)	50 mM

Appendix B: Maxi-prep DNA of TPO luciferase plasmids:

Maxi-prep of PGL3-425 and PGL3-216:

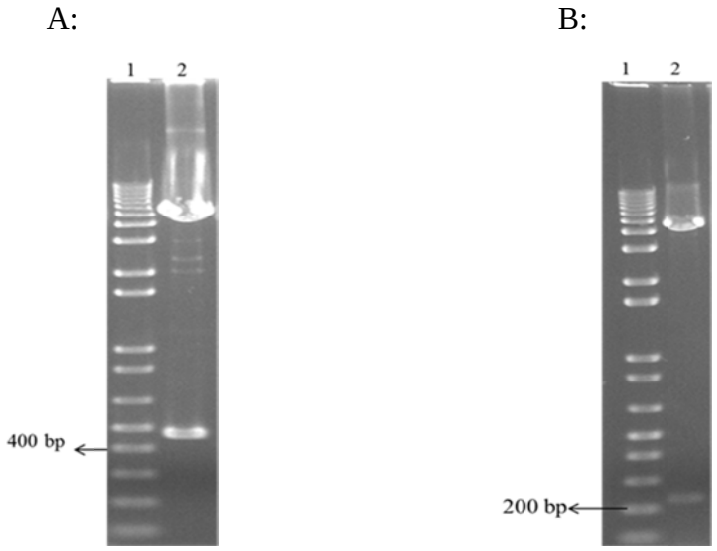

Figure DNA electrophoresis of Maxi-prep product-PGL3-425 (A) and PGL3-216 (B). *The Maxi-prep product was digested with restriction enzymes and checked by DNA electrophoresis (lane 1: DNA marker, lane 2: Maxi-p DNA of PGL3-425/216).*

Maxi-prep of PCMV6-ENTRY-ZEB1-RD:

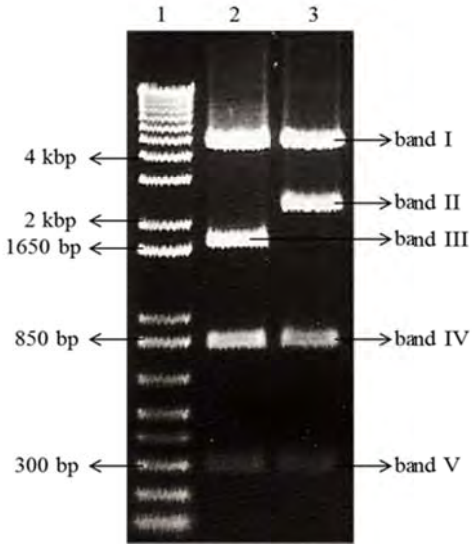

Figure 5a DNA electrophoresis of ZEB1-RD. *The Maxi-p of ZEB1-RD was digested by EcorI/NotI and the digestion was run by electrophoresis (lane 1: DNA marker, lane 2: ZEB1-RD, lane 3: Wild type ZEB1 expression vector-PCMV6-ENTRY-ZEB1). The digestion of ZEB1-RD and wild type ZEB1 expression vector gave different digestion patterns and the corresponding DNA fragments from the digestion were shown as band I to band V in the figure.*

Maxi-Prep of plasmid clones in Chapter six:

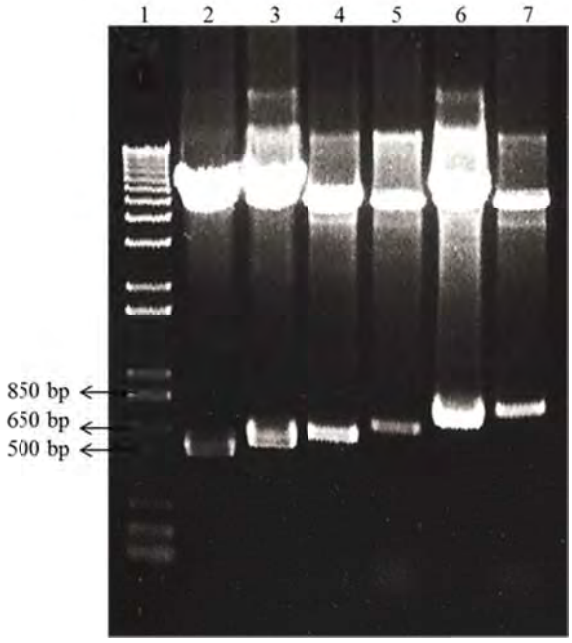

Figure 6a DNA electrophoresis of Maxi-prep products. *The Maxi-prep products were digested by restriction enzymes and checked by DNA electrophoresis (lane 1: DNA marker, lane 2 to 7: Maxi-prep product).*

The plasmid clones amplified and purified by the Maxi-prep were digested with the XhoI/HindIII as shown in **Fig. 6a**, the digestion gave the expected insertion of fragment 528 (lane 1), 588 (lane 3), 648 (lane 4), 708 (lane 5), 768 (lane 6) and 828 (lane 7). The plasmid clones were then subjected to a concentration measurement and stored at -80°C until use.

Appendix C: ^{32}P labelled oligonucleotide probes (ZEB1) used in EMSA

Table 5a Probes for ZEB1 EMSA

Probes	Nucleotide sequences
P1	Forward probe: 5'-ATTGAATCAGGTGCAAGCCTC-3' Reverse probe: 5'-GAGGCTTGCACCTGATTCAAT-3'
P2	Forward probe: 5'-AGGGAGGCAGGTGTGAGCTAT-3' Reverse probe: 5'-ATAGCTCACACCTGCCTCCCT-5'
P3	Forward probe: 5'-AAGCCCCTAAGGTGGGCATCAC-3' Reverse probe: 5'-GTGATGCCCACCTTAGGGGCTT-3'
P4	Forward probe: 5'-CACCCAGCAGGTGCCCGTT-3' Reverse probe: 5'-AACGGGCACCTGCTGGGTG-3'

Appendix D: Primers for PCR amplification in Chapter six

Table 6a Primers used to create the constructs described in section 6.2.2

TPO fragments	Primer pairs	Region covered	Length
528	Fp:5’-CCGCTCGAGTCCCCCTATCCAAA TCTTCTC-3’ Rp:5’-CCCAAGCTTGCTCATGTCCCCTT TCCTTA-3’	-199 to +329	528 bp
588	Fp:5’-CCGCTCGAGGATCCCCCTATCCA AATCTTC-3’ Rp:5’-CCCAAGCTTCTGTGTTTGCTAAG CCCTAAGTG-3’	-199 to +389	588 bp
648	Fp:5’-CCGCTCGAGTCCCCCTATCCAAA TCTTCTC-3’ Rp:5’-CCCAAGCTTTCAGAACCTTAAG CTAACGAGGA-3’	-199 to +449	648 bp
708	Fp:5’-CCGCTCGAGGATCCCCCTATCCA AATCTTC-3’ Rp:5’-CCCAAGCTTCTCCTCCCATTACC CTCTCC-3’	-199 to +509	708 bp
768	Fp:5’-CCGCTCGAGTCCCCCTATCCAAA TCTTCTC-3’ Rp:5’-CCCAAGCTTAAGAGGCTTGCAC CTGATTC-3’	-199 to +569	768 bp
828	Fp:5’-CCGCTCGAGTCCCCCTATCCAAA TCTTCTC-3’	-199 to +629	828 bp

	Rp:5’-CCCAAGCTTCCTTCCGGTTCCTT CACAG-3’		