EXPRESSION OF G-PROTEIN COUPLED RECEPTORS IN YOUNG AND MATURE THROMBOCYTES AND KNOCKDOWN OF GPR18 IN ZEBRAFISH

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In this study, a novel method based on biotinylated antibodies and streptavidin coated magnetic beads was used to separate the thrombocyte subpopulations from zebrafish whole blood. DiI-C18, a lipophilic dye, labels only young thrombocytes when used at low concentrations. Commercially available biotinylated anti-Cy3 antibody was used to label the chromophore of DiI-C18 on the young thrombocytes and streptavidin coated magnetic beads were added subsequently, to separate young thrombocytes. The remaining blood cells were probed with custom-made biotinylated anti-GPIIb antibody and streptavidin magnetic beads to separate them from other cells. Further, thrombocytes are equivalents of mammalian platelets. Platelets play a crucial role in thrombus formation. The G-protein coupled receptors (GPCRs) present on the platelet surface are involved during platelet activation and aggregation processes. So, thrombocytes were studied for the presence of GPCRs. The GPCR mRNA transcripts expressed in the young and mature thrombocytes were subjected to densitometry analysis and pixel intensities of the bands were compared using one way ANOVA. This analysis did not show significant differences between the young and mature GPCR mRNA transcripts but identified a novel GPCR, GPR18 that was not reported in platelets earlier. To study the function of this GPCR, it was knocked down using GPR18 specific antisense morpholino and vivo morpholino. The immunofluorescence experiment indicated the presence of GPR18 on thrombocytes. The results of the assays, such as, time to occlusion (TTO) and time to aggregation (TTA) in response to N-arachidonyl glycine (NAG) as an agonist, showed prolongation of time in GPR18 larval and adult morphants respectively, suggesting that GPR18 plays a role in thrombus formation in zebrafish. In conclusion, our results indicate that GPR18 may be present in zebrafish thrombocytes, it may be involved in thrombus formation and that NAG may be an agonist at GPR18 on thrombocytes.

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LIST OF ABBREVIATIONS

5HT-2A	5-hydroxytryptamine (serotonin) receptor 2A
AA	Arachidonic acid
AC	Adenylate cyclase
ADP	Adenosine diphosphate
alpha-2A	Alpha-2 (α2) adrenergic receptor
ATP	Adenosine triphosphate
bp	Base pairs
CalDAG-GEF1	Calcium and DAG-regulated guanine nucleotide exchange factor1
cAMP	Cyclic adenosine monophosphate
CLEC-2	C-type lectin receptor
DAG	Diacyl glycerol
DiI-C18	1,1'- dioctadecyl-3,3,3',3'- tetramethyl- indocarbocyanine perchlorate
dpf	Days post fertilization
EP3	Prostaglandin E receptor 3
GPCR	G-protein coupled receptor
GPIb-IX-V	Glycoprotein Ib-IX-V
GPVI	Glycoprotein VI
hpf	Hours post fertilization
ICM	Intermediate cell mass

IP3	Inositol triphosphate
MO	Morpholino
NAG	N-Arachidonyl glycine
PAR1	Protease-activated receptor-1
PAR4	Protease-activated receptor-4
PGE2	Prostaglandin E2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PI3K	Phosphatidylinositol 3' -kinase
PIP2	Phosphatidyl inositol-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Phosphokinase C
PKG	Protein Kinase G
PLC	Phospholipase C
Rap1	Ras-related protein 1
Rho-GEF	Rho-Guanine nucleotide exchange factor
RIAM	Rap1-GTP-interacting adaptor molecule
TP	Thromboxane/prostanoid receptor
TTA	Time to aggregation
TTO	Time to occlusion

TXA2	Thromboxane A2
vMO	vivo Morpholino
VWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

1.1 Hemostasis

Whenever there is an injury to a blood vessel, the blood oozes out and this wounded area seals itself to prevent bleeding as a defense response to the injury. This is a tightly regulated and rapid process called hemostasis. At first, at a moments' notice, platelets from the blood adhere to the vessel wall to form a plug and prevent blood loss. This process is called primary hemostasis. During platelet plug formation, many coagulant enzymes participate and form a fibrin clot, which is called secondary hemostasis. According to Virchow's triad, vascular endothelium (blood vessel wall), shear forces (blood flow) and blood components must be present for the hemostasis to occur (1). In fact, the interplay of both platelets and the coagulation system is required for hemostasis (2). The present study compares the G- protein coupled receptors (GPCRs) transcripts of young and mature thrombocytes in zebrafish. The thrombocytes are homologous to mammalian platelets. Platelet functions such as activation, secretion and aggregation are conserved in thrombocytes. Furthermore, thrombocyte GPCRs could be studied to understand the signaling pathways operating in platelets. In this chapter, the events and players of primary hemostasis are discussed with a special focus on the G-protein coupled receptors (GPCRs).

1.1.1 Primary Hemostasis

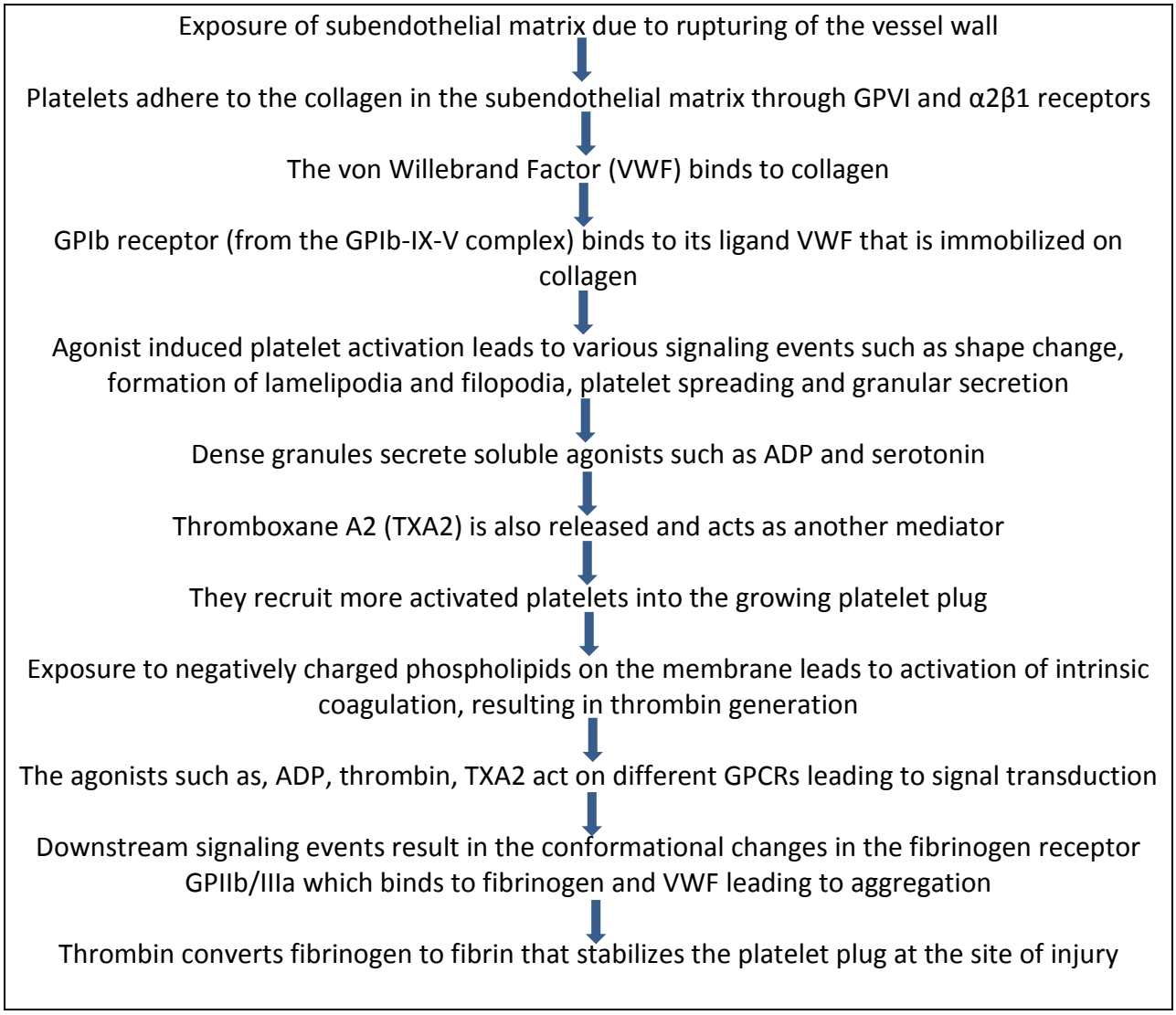

Fig. 1.1. Flow chart for primary hemostasis.

Primary hemostasis is immediately initiated at the site of injury with the exposure of subendothelial matrix, due to the vessel wall rupture. Platelets adhere to the collagen in the subendothelial matrix through GPVI and α2β1 receptors. The von Willebrand factor (VWF), a multimeric adhesive protein, binds to collagen. Platelets roll over the VWF and tethering occurs

at the site of the injury, when the GPIb receptor (from the GPIb-IX-V complex) binds to its ligand VWF that is immobilized on the collagen.

Platelets spread over the subendothelial surface where they are also activated by the coagulant enzyme thrombin that is generated on the platelet surface. Platelet dense granules secrete soluble agonists such as ADP and serotonin which themselves are platelet activators, therefore, they recruit more activated platelets into the growing platelet plug (3). In a similar way, thromboxane A2 (TXA2) is also released and acts as another mediator, responsible for activating other platelets by autocrine or paracrine signaling (4, 5). Membrane phospholipids such as phosphotidylcholine (PC) release arachidonic acid (AA), which produces PGG2 by the action of COX-1 (Cyclooxygenase pathway), which further produces PGH2. PGH2 is converted to TXA2 by the thromboxane synthase (TXS) (6).

Agonist induced platelet activation leads to various signaling events such as shape change, exposure to negatively charged phospholipids on the membrane, leading to activation of intrinsic coagulation (7), further leading to activation of integrin GPIIb/IIIa. Upon activation, GPIIb/IIIa binds to fibrinogen and VWF, facilitating aggregation and platelet plug formation at the site of injury (8).

The agonists act on a wide variety of receptors. For example, ADP activates $P2Y_1$ and $P2Y_{12}$ receptors. Thrombin acts via the PAR1 and PAR4 receptors in humans. These downstream signaling events result in the conformational changes in the fibrinogen receptor GPIIb/IIIa which ultimately interacts with fibrinogen. This is known as “inside-out” signaling. GPIIb/IIIa is a bidirectional receptor. “Outside-in” signaling, after fibrinogen binding, stabilizes the thrombus (9, 10).

1.1.2 Secondary Hemostasis

This involves the coagulation process leading to formation of thrombin which converts fibrinogen to fibrin, culminating ultimately in clot formation (11). The extent of the clot is restricted to the area of injury. Coagulation of blood is a tightly regulated process in which the balance between the pro-coagulant and anti-coagulant reactions is finely maintained (12).

1.1.3 Tertiary Hemostasis

After the formation of a firm platelet plug and fibrin at the site of injury, the necessary steps of hemostasis are attenuation of the activity of the clotting factors and clot retraction. This mechanism of fibrinolysis is termed as tertiary hemostasis. The clot is lysed during the process of fibrinolysis, thus maintaining the vessel integrity (13).

1.2 Thrombus Regulation

The formation of a clot inside the blood vessels is termed as thrombosis (14, 15). The thrombus may be dislodged from its site of formation and enter the circulation (termed embolus). Thrombus formation is regulated by various mechanisms.

Platelet activation is inhibited by the endothelial cell products, nitric oxide (NO) and prostacyclin (16-19). Fibrinolytic factors of the coagulation system such as the TFPI (Tissue factor pathway inhibitor), anti-thrombin III and Protein C and S are involved in thrombus regulation (20).Thrombomodulin modulates the activity of thrombin by converting it from procoagulant to anti-coagulant by changing its substrate (21). PECAM-1 is a cell adhesion

molecule present on platelets as well as endothelial cells. This PECAM-1 negatively regulates the process of thrombus formation (22).

1.3 Origin and Structure of Platelets

Megakaryocytes are the precursors for platelets. They undergo endomitosis, cytoplasmic expansion and granule formation. Platelets are produced in the bone marrow by the megakaryocytic extensions called, 'proplatelets'. Organelles and granules are deposited and trapped into the proplatelet ends. These ends increase in number by a bending and branching process of the proplatelet shaft (23). The nucleus of the megakaryocyte is extruded and undergoes apoptosis. Microtubules from the cytoplasm twist and proplatelet shaft elongates due to sliding of microtubules. The newly formed platelets are released into the blood circulation from the proplatelet ends (24, 25).

Platelets are devoid of nuclei. Platelets are spindle shaped, discoid cells sometimes having teardrop appearance. The ultrastructure of platelets shows the presence of an exterior coat, trilaminar unit membrane, and the area beneath the membrane containing special filaments that form the platelet walls (26, 27). Platelets also have a surface connected open canalicular system (OCS) derived from the demarcation membrane system (DMS) of the megakaryocytes. The matrix of the platelet is known as the 'sol-gel' zone and contains actin filaments, circular band of microtubules, glycogen, mitochondria, alpha and dense granules, and a dense tubular system. They are also involved in several other functions such as, inflammation, tumor angiogenesis and microbial infections (28, 29). There are about 150,000 to 450,000 platelets per microliter of human blood in circulation (26).

1.4 Reticulated Platelets

Thiazole orange (TO) was employed to study the nucleic acid content of platelets by Kienast and Schmitz (30). It was found that a small fraction of platelets were stained by TO and these platelets had RNA fibers which were called reticulum and, therefore, the small fraction of platelets stained by TO was termed reticulated platelets (RP). Reticulated platelets are also the newly synthesized platelets released into circulation and therefore they are called young platelets and have been useful for platelet turnover studies (31). Measurement of RP is an indicator of thrombopoiesis (31-34). TO-positive reticulated platelets from the platelet rich plasma (PRP) are a useful parameter in analyzing thrombocytopenic disorders (32). RP count is important in understanding platelet production, megakaryocyte function, as well as the diagnosis of thrombocytopenic disorders such as ITP (Idiopathic thrombocytopenic purpura) (33). Young platelet rich PRP transfusions were carried out in patients with leukemia and lymphoma. The data from these studies show that a fewer number of transfusions were required when young platelets were used in PRP transfusions, as opposed to other young platelets non-enriched transfusions (34).

1.5 Old Platelets

Platelets vary in size. The smaller platelets showed decreased RNA content and protein synthesis and this finding has been correlated to the their age in development (30). These platelets have been called old or mature platelets. Karpatkin studied young and old platelets in 1969 and found that the old platelets were light and smaller in size due to their reduced metabolic functions (35). Studies conducted by Blajchman *et al.* also showed that older

platelets were smaller in size and also indicated longer bleeding times as compared with the younger platelets (36). Kelton and Denomme's platelet studies demonstrated that the size, density and survival time of the old platelets decrease as they age and binding to IgG increases, which may facilitate their removal (37).

1.6 Major Platelet Receptors, their Ligands and their associated Defects

Platelets possess a number of receptors derived from different receptor families. Platelet activation is achieved through a variety of surface receptors, belonging to different families, such as integrin, immunoglobulin, glycoprotein and G-protein coupled receptors (GPCRs) (38). Fig. 1.2 unveils the major platelet receptors involved in platelet activation and aggregation.

1.6.1 GPIbα

The GPIbα of the platelet binds to the A1 domain of the VWF and mediates the adhesion process in the presence of shear forces (39). Each platelet has about 25,000 copies of this receptor (40, 41).

1.6.2 GPIb-IX-V Complex

GPIb-IX-V complex belongs to the leucine-rich repeat (LRR) family of glycoproteins. In this complex, the GPIbα and GPIbβ subunits are linked via disulfide bonds and are non-covalently associated with GPIX and GPV. This complex interacts with VWF and plays a role in platelet activation (42, 43). Residues from the Ser-rich regions of GPIbα serve as binding sites

for 14-3-3ζ signaling protein. The protein 14-3-3ζ plays a role in GPIIb/IIIa activation (41, 44-47). Bernard-Soulier Syndrome (BSS), or the giant platelet syndrome, is associated with absent (quantitative defect) or abnormal (qualitative defect) GPIb-IX-V receptors. In these individuals, platelet adhesion function is affected. This defect is detected by decreased aggregation in response to ristocetin (48-51).

1.6.3 GPVI

GPVI receptor belongs to the immunoglobulin family, binds to collagen, and also participates in the adhesion reactions (52-54). GPVI binds to fibrous collagen while activating the platelets (55). Absent or abnormal GPVI receptors result in a defect in collagen-induced activation and aggregation of platelets (56).

1.6.4 α2β1 Integrin

The α2β1 (GPIa/IIa) is activated in the presence of agonists and will bind to collagen with high efficiency (12, 57). The integrin enhances GPVI-collagen interactions. Both the receptors work in a synergistic manner (58). Absent or abnormal GPIa/IIa results in a defective adhesion response to collagen (59).

1.6.5 GPIIb/IIIa

Binding of GPIbα and GPVI to VWF and collagen respectively, causes activation of the integrin αIIbβ3 (GPIIb/IIIa) that binds mainly to fibrinogen (or VWF) (60). This marks the beginning of 'aggregation' reaction of platelets. GPIIb/IIIa is activated downstream of GPIb-IX-V

and GPVI as well as the GPCRs such as PARs and ADP receptors (44). There are about 80,000 copies of the GPIIb/IIIa receptor per platelet (61). The GPIIb has binding sites to different substrates such as fibrinogen, fibronectin, vitronectin, and VWF (62). Absent or abnormal GPIIb/IIIa results in the lack of aggregation of platelets in response to the agonists. This defect is known as Glanzmann thrombasthenia. The defect also shows diminished clot retraction (63).

1.6.6 CLEC-2

Recently CLEC-2 (C type lectin-like type II) receptor was found to activate platelets, podoplanin being its physiological ligand. CLEC-2 signaling is via tyrosine phosphorylation of the hemi-ITAM motif, similar to GPVI signaling. CLEC-2 and GPVI pathways may be cross-linked leading to activation of PLCγ2 (4, 64). Very recently shown by Manne *et al.* that a novel agonist, Fucoidan, activates the platelets via SFK-dependent signaling (65).

1.6.7 G-protein coupled receptors (GPCRs) and G-proteins

G-protein coupled receptors (GPCRs) are the membrane receptors that belong to the seven transmembrane (7TM) domains family of receptors. In 2000, the crystal structure of the first GPCR, bovine rhodopsin, was worked out (66, 67), and in 2007, the structure of the β2-adrenergic receptor was solved. In 2012, the Nobel Prize in Chemistry was awarded to Robert Lefkowitz and Brian Kobilka for their studies on GPCRs (68-71). The GPCR superfamily constitutes more than 800 GPCRs, out of which, more than 150 are orphan GPCRs (72).

GPCRs play a vital role in signal transduction and cell to cell communication. Signal transduction via the GPCRs is of utmost importance for the platelets to carry out their role in

hemostasis and thrombosis. GPCRs play a significant role in platelet activation (28). GPCRs are involved in stroke, thromboembolism, atherothrombosis, and deep vein thrombosis. GPCRs are also involved in cancers and metastasis (73-75). Currently, they are studied for their importance in drug development strategies (76). The detailed working of the GPCRs, the G-proteins and GPCRs as targets for drug discovery is discussed in Chapter 4.

The following are the major platelet GPCRs known to play a role in platelet functions.

1.6.7.1 Thrombin Receptors

Thrombin activates the protease-activated receptors (PARs) (77, 78). There are four PARs identified out of which, PAR1, PAR3, and PAR4 are activated by thrombin (77-80). PAR1 and PAR4 are present on human platelets (81). About 1000-2000 copies of PAR1 are expressed per platelet (82). PAR1 is associated with Gq/11 pathway and may also activate Gi/o and G12/13 (83-86). PAR4 signals through Gq and G12/13. (87, 88). PARs are activated by a serine protease. The protease binds to the N terminal of GPCR and cleaves it, thereby forming a new amino terminal. This tethered ligand serves as a signal for activation of these PARs. The termination of PAR1 signaling is mediated by GRKs that phosphorylate the C-terminal (89). The GRKs involve β-arrestins which internalize the receptor by clathrin coated pits. It has been found from the studies conducted in *Par4-/-*mice that platelet activation by PAR4 is not required for the initial and limited accumulation of platelets. However, PAR4 is required for platelet thrombus propagation (90). PARs are also good targets for developing anti-thrombotic drugs. Pepducins targeted at the i3 loop (intracellular loop 3) of the PAR were shown to be potent anti-thrombotic agents (91).

1.6.7.2 ADP Receptors

The purinergic receptors, $P2Y_1$ and $P2Y_{12}$ are activated by ADP (92). About 150 copies of $P2Y_1$ are expressed per platelet (93, 94). The $P2Y_1$ receptor coupled to Gq is involved in ADP induced platelet shape change. $P2Y_1$ is required for calcium mobilization but does not account for full activation. Hence, the presence of another P2Y receptor acting via Gi downstream effectors was predicted (95). $P2Y_{12}$ is coupled to Gi2 pathway leading to inhibition of adenylate cyclase and cAMP production (96). This leads to dense granule secretion, TXA2 formation, aggregation, and also stabilization of thrombus (97, 98). The Gq and Gi pathways initiated by ADP result in releasing the contents of platelet α granules (99). $P2Y_{12}$ signaling occurs via several other downstream pathways such as activation of PI3 kinase (100). Absent or abnormal $P2Y_{12}$ receptors are very rare and result in inhibition of ADP induced platelet aggregation (101).

1.6.7.3 Thromboxane A2 (TXA2) Receptor

TXA2 activates the prostanoid receptor, TPR (Thromboxane/Prostaglandin) (6, 102, 103). TXA2 is produced in platelets by the cyclooxygenase pathway from arachidonic acid (AA). AA is converted to PGG2 via COX-1 catalysis and then to PGH2. TXA2 is synthesized from PGH2 by the enzyme thromboxane synthase (TXS).

Aspirin inhibits COX-1 enzyme by acetylation of serine residue, thereby, decreasing TXA2 production. Platelets lack nuclei and cannot regenerate the COX-1 enzyme. Thus, a low dose of aspirin provides protection from cardiac pathologies to some extent (104). In one study, the TXS inhibitor was shown to reduce the infarct size, myocardial hemorrhage and neutrophil infiltration at the affected area (105-107). The signaling initiated by (Thromboxane/Prostanoid

receptor) TPR causes cytoskeletal modifications, platelet adhesion and vesicle trafficking. The TPR is coupled to Gq protein, which activates PLC-β that further hydrolyses PIP2 to DAG and IP3. IP3 releases Ca^{++} from the intracellular stores. DAG activates PKC (108-112).

1.6.7.4 Epinephrine Receptor

In platelets an epinephrine receptor known as α2A adrenergic receptor is coupled to a Gi subtype variant Gz (113). The Gz pathway decreases cAMP production and increases platelet aggregation. Co-activation of epinephrine and serotonin receptors leads to convergence of pathways at the PLC, initiated by their associated proteins, Gi-βγ and Gqα (114). Thus, release of Ca^{++}, granular secretion and activation of PLA2 and finally platelet aggregation occurs as a result of the signaling events (114).

1.6.7.5 Serotonin Receptor and other GPCRs

Serotonin activates the 5-HT receptor coupled to Gq protein (115, 116). Other platelet GPCRs include LPA (Lysophosphatidic acid) receptors, glutamate receptors, adenosine, prostaglandins, chemokine, vasopressin, succinate, oxytocin and leukotriene receptors (117).

1.7 Diseases Related to Thrombosis or Platelets

The clot if dislodged (embolus) from the artery may reach or block one of the arteries supplying the brain, causing a stroke. The clot may block the artery supplying the heart, leading to myocardial infarction (118). Thrombi in atherosclerotic blood vessels may occlude the

vessels, leading to a heart attack (119). The embolus may result in conditions such as deep vein thrombosis (DVT).

1.8 Adhesive Molecules involved in Hemostasis

1.8.1 VWF (von Willebrand Factor)

It is synthesized in megakaryocytes and endothelial cells. It is stored in the alpha granules of platelets and Weibel-Palade bodies of endothelial cells (120). Large, multimeric VWF supports hemostasis. It mediates the platelet-platelet and platelet-collagen interactions. It is also a carrier of FVIII and thus protects FVIII from proteolysis by APC (activated protein C) (121, 122). Platelets bind to VWF via two receptors, GPIb-α from the GPIb-IX-V complex and the GPIIb-IIIa (123).

VWF defects leads to VWD (von Willebrand disease) (124). Type 1 VWD is characterized by reduction in VWF protein, type 2 VWD is related to abnormal VWF protein, and type 3 VWD is absence of VWF (48, 125). Increased levels of VWF in circulation is a risk factor for atherosclerosis (21).

1.8.2 Collagen

Collagen binds to GPVI and α2β1 (52, 126). It binds to GPVI specifically at the Gly-Pro-Hyp (hydroxyproline) residues also known as collagen related peptide (123).

1.8.3 Fibrinogen and Fibrin

Fibrinogen bound to the activated platelets, is a substrate for arresting platelets from the flow, mediated by GPIIb/IIIa. Fibrin is the cross-linked insoluble polymer of fibrinogen that holds the platelet plug together at the injury site. Adherent platelets are activated and spread on fibrinogen and fibrin coated surfaces, but only tiny thrombi are formed in absence of other adhesives (8, 127).

1.8.4 Thrombospondin-1

Its function is not clear but it is present in platelet alpha granules and binds to membrane mediating adhesion process (128).

1.8.5 Laminin

Platelets contain laminins 8, 10, 11 and secrete them upon activation. These may be involved in in adhesion, lamelipodia and filopodia formation (129, 130).

1.9 Outside-in Signaling in Platelets

Outside-in signaling in platelets is initiated by binding of fibrinogen to the activated GPIIb/IIIa. Naik *et al.* have showed that CIB (calcium and integrin binding) protein in complex with the GPIIb/IIIa receptor is essential for downstream signaling events such as ADP secretion and platelet spreading reaction (131, 132). Talin and Kindlin proteins bind to the cytoplasmic portion of the β part of the integrin, activate integrin and initiate the clot retraction process (133-136).

1.10 Anti-Platelet Drugs

Aspirin is used for treatment of arterial thrombosis while warfarin is used against venous thrombosis (119). An antithrombotic drug, Dipyridamole, inhibits binding of platelets to collagen. It plays a role in reducing thromboembolic diseases and, along with aspirin, it reduces coronary heart diseases. Dextran, a sucrose derivative, can be used prophylactically to reduce pulmonary embolism and DVT that may occur after surgeries (137).

Most of the above mentioned molecules are used as targets for anti-platelet drugs. For example, Aspirin is used to inhibit the COX-1 enzyme, (138). Since the GPCRs are involved in diseases and pathological conditions, they are crucial targets for many drugs. Overall 50% of the drugs in the global market affect GPCR function, out of which about 30% directly target the GPCRs (139). For example, the ADP receptor, $P2Y_{12}$ is a target of an antithrombotic drug, clopidogrel (44, 138). $P2Y_{12}$ proved to be a good candidate for anti-platelet drugs. The indirect inihibitors of $P2Y_{12}$ include the drugs from the thienopyridine family, namely, clopidogrel, ticlopidine and prasugrel [19, 29]. Drugs such as ticagrelor, cangrelor and elinogrel are used for direct inhibition of $P2Y_{12}$ receptor (38).

1.11 Model Systems for the Study of Mammalian Hemostasis

In 1984, Wu *et al.* studied the role of arachidonic acid metabolic products in thrombus formation in rabbits (140). Mouse models are used to mimic platelet function disorders such as Bernard-Soulier syndrome and Glanzmann thrombasthenia or study platelet biology and receptor studies (141-143). Though extensive work has been done utilizing the mammalian models of hemostasis, the classical genetics approach used in other models systems, such as, *C.*

elegans could not be applied to these systems. Therefore, there was a need for a model addressing basic questions in a robust high throughput method facilitated via classical genetics. Hence the zebrafish, a vertebrate model, was introduced.

1.11.1 Zebrafish [*Danio rerio*] as a Model

The *Danio rerio* fish, commonly known as 'zebrafish' was introduced by George Streisinger as a useful vertebrate model by using haploid and diploid animals for various studies (14). The small size of the zebrafish embryos, their transparency and external fertilization allows one to study development, embryogenesis and organ formation. The female may produce 100-200 embryos per week that may mature in 3-4 months allowing high-throughput screening studies. Haploid and homozygous diploid screens, transgenic mutants, forward genetic screens, overexpression and knockdown technology using anti-sense oligo morpholinos (MOs), are the techniques that can yield immense knowledge about the genes, their expression patterns, in normal as well as diseased conditions(144). Zebrafish proves to be an attractive model for hematopoiesis, thalassemia, deficiencies of the immune system (145), cardiovascular system disorders (146), leukemia, anemia and blood clotting diseases (147). It is an ideal model for identification of novel genes and their functions by using genetic methods (146).

1.11.2 Zebrafish [*Danio rerio*] as a Model to Study Hemostasis and Thrombosis

Biochemical studies have shown the presence of the extrinsic, the intrinsic, and the common coagulation pathways as well as the clotting factors in the teleost fish (148). Thrombin or activated Factor II (F IIa) is an important clotting factor present in the mammalian blood

responsible for formation of fibrin from fibrinogen. Presence of this thrombin was detected by injecting hirudin in zebrafish embryos (149). Prothrombin, Factor X, Antithrombin, Protein C, heparin cofactor II as well as Factor VII have been conserved in zebrafish (148, 150).

The zebrafish thrombocyte is equivalent to the mammalian platelet. This has been shown by immunological and functional assays again stressing the utility of this model hemostasis and thrombosis (151). *In vivo* formation of thrombus in the vessel is a powerful assay that can be used to find new genes related to thrombosis (152, 153). The mammalian blood clotting factors are conserved in the zebrafish and overall 85% of the human genes are conserved (14). Homologs and orthologs of platelet adhesion molecules such as the VWF, fibronectin, vitronectin, thrombospondin, P-selectin, platelet integrins and glycoproteins as well are conserved in zebrafish (20). Also, the presence and role of von Willebrand factor (VWF) in hemostasis analogous to human VWF protein was shown in zebrafish (154). Thus, this model is suitable for identifying novel genes involved in hemostasis and thrombosis (155).

Thrombocytes of gnathostomes (non-mammalian vertebrates) such as birds, amphibians, reptiles and fish are nucleated, diploid cells, differentiated from thrombocyte precursor cells. They share the main components of the hemostatic system (20).

Thrombocytes, unlike the platelets, possess a large nucleus and very sparse cytoplasm. They have granules, membrane receptors and a surface connected open cannalicular system (OCS), which are similar to those found in platelets. In response to agonists, such as platelets they show aggregation, agglutination and secretion reactions.

The electron micrographic picture of thrombocytes shows granules and dense bodies present in the cytoplasm. Formed in the kidney marrow, the zebrafish thrombocytes mature in the circulation. They constitute about 1% of the total blood cells in the zebrafish.

There are two types of thrombocytes, young and mature. Young thrombocytes are the newly formed cells that enter the blood circulation in zebrafish. These cells are the only population labeled by Bromo-deoxyuridine (BrdU) within 24 hours after injecting BrdU that then intercalates with the newly synthesized DNA, thus labeling the cells. These thrombocytes can be differentiated from the mature ones in many categories. The young thrombocytes are labeled by a lipophilic dye, DiI-C18 (1,1'- dioctadecyl-3,3,3',3'- tetramethyl- indocarbocyanine perchlorate), when the dye is used at a very low concentration. In the CD41-GFP adult transgenic zebrafish, the young thrombocytes are less intensely fluorescing cells. The young are more active as they have more adhesive and functional receptors as compared with the mature thrombocytes. They also initiate the clot formation process *in vivo* and *in vitro*. They are approximately 10% of the total thrombocytes. These thrombocytes are not labeled by the DiI dye in zebrafish. These are intense fluorescent cells in the GFP-thrombocyte transgenic line. They showed a lesser number of functional and adhesive receptors and hence are less active than the young thrombocytes. They constitute about 90% of the total thrombocyte population.

1.12 Conclusion

Various methods for separating the young and mature platelets have been utilized for more than two decades. At present, there are no reagents to separate the thrombocyte sub populations. There is a need to develop new technology for separation of the thrombocyte sub

populations. The availability of such separation methods will facilitate the studies on thrombocyte maturation, transcriptional variation between young and mature thrombocytes and the differences in the thrombocyte receptor signaling between these two populations. Since the GPCRs play a significant role in platelet activation, secretion and aggregation, isolation of two populations of thrombocytes would be useful to identify GPCRs on thrombocytes. Such identification of GPCRs in thrombocytes may lead to the discovery of novel GPCRs on thrombocytes that may ultimately lead to characterization and identification of newer molecules or effectors and their contribution to the thrombocyte signaling. This may lead to translational research in platelet field and may further help design novel, anti-platelet drugs, to combat thrombosis.

The study was undertaken in order to address these issues. The following aims and sub-aims were achieved in the quest to understand the differences between the thrombocyte subpopulations and the role of a specific GPCR in thrombosis and aggregation studies.

1.13 Aims and Hypotheses

- Aim 1: To develop a method to separate the young and mature thrombocytes from the zebrafish whole blood

  Hypothesis: DiI-C18,a lipophilic dye, at low concentration, labels only young thrombocytes (156). So we hypothesized that antibody to the chromophore of DiI-C18 could be used to specifically stain the DiI-C18 molecules attached to the young thrombocytes. We further hypothesized that since Cy3 dye is almost structurally similar to DiI-C18 except for the number of carbon atoms in the tail

of the chromophore, biotinylated anti-Cy3 antibody could be used to label young thrombocytes and by using streptavidin-coated magnetic beads, they could be separated since biotin and streptavidin form a strong bond. These magnetic beads along with the attached DiI-C18 and young thrombocytes could be separated from the whole blood, by using a magnetic stand. The beads should attach to the magnetic side of the tube wall and thus could be washed and separated from the other cells (blood cells devoid of young thrombocytes). Using this same technique, mature thrombocytes from the above blood cells devoid of young thrombocytes, could be separated using biotin-anti-GPIIb antibody which will bind to thrombocytes in general. In order to achieve this aim, I propose to use commercially available biotinylated anti Cy3 antibody and GPIIb antibodies which are available in our laboratory and other reagents such as streptavidin-coated magnetic beads to separate young and mature thrombocytes. The above approach should yield a method to separate the young and mature thrombocytes.

- Aim 2: To identify the differences in GPCR mRNA levels between the young and mature thrombocytes and also to identify expression of novel GPCR mRNA/s in thrombocytes

  Hypothesis: Since mammalian platelets have a plethora of GPCRs and the human genome has more GPCRs than those which are discovered on the platelet surface, still more GPCRs remain to be identified in platelets. Since zebrafish genome is 85% homologous to human genome in terms of genes and gene numbers, I believe that analyzing zebrafish genome for GPCRs and their

expression in thrombocytes should be translatable to human platelets. Therefore, gel-electrophoresis of RT-PCR products of the young and mature thrombocyte GPCR RNAs will show differences in expression patterns and identify novel GPCR mRNA/s.

To achieve this aim, I will prepare RNA from young and mature thrombocytes and subject to RT-PCRs using a number of primers from the GPCR sequences obtained from zebrafish genome. These approaches should lead to identify the differences in the young and mature GPCR transcripts as well as identify novel GPCR/s in thrombocytes.

- Aim 3: To knockdown the novel GPCR identified in Aim 2 and establish the role of that GPCR in thrombus formation.

  Hypothesis: Since we would have identified a novel GPCR in Aim 2, its function in thrombocyte activation would be elusive. Therefore, knockdown of this novel GPCR using morpholino (MO) and/or vivo morpholino (vMO) that is specific for this GPCR should identify the role of the novel GPCR, in thrombocyte activation and/or aggregation.

In order to achieve this aim, a novel GPCR selected from Aim 2 will be subjected to knockdown using this selected GPCR specific morpholino and/or vivo morpholino and the resulting embryos and/or adult zebrafish will be analyzed for thrombocyte activation/aggregation. The results of such analyses should delineate role of the novel GPCR and may lead to future therapeutic target in treatment of thrombosis.

1.14 Significance of the Research

This results from the specific aims proposed in this study should prove to be important in several ways. First, it will introduce a novel method for separating the thrombocytes from the whole blood of the zebrafish. This technique can also be used to separate some other cells such as neurons because some neurons have been specifically labeled by DiI-C18. Second, thrombocytes can be separated from very small amounts of blood. Therefore, in cases where the available sample is in miniscule amounts (0.1 to 1.5 ml), as in, the infant heel blood, this technique can be applied using *in vitro* labeling of platelets with DiI-C18. Third, this study will show for the first time, the comparison of the young and mature thrombocyte GPCR mRNA transcripts and hence facilitates the identification of differences in their expression which may provide insight into young and mature platelet expression of these GPCRs although not at protein level, which will also have to be explored. Four, the identification of novel GPCRs could be further exploited to identify the pathways involved in functioning of novel GPCRs. Finally, since GPCRs have been regarded as a promising target for drug discovery, the knowledge about the novel GPCR signaling should be useful for drug screening and anti-thrombotic drug designing to alleviate thrombotic and bleeding disorders.

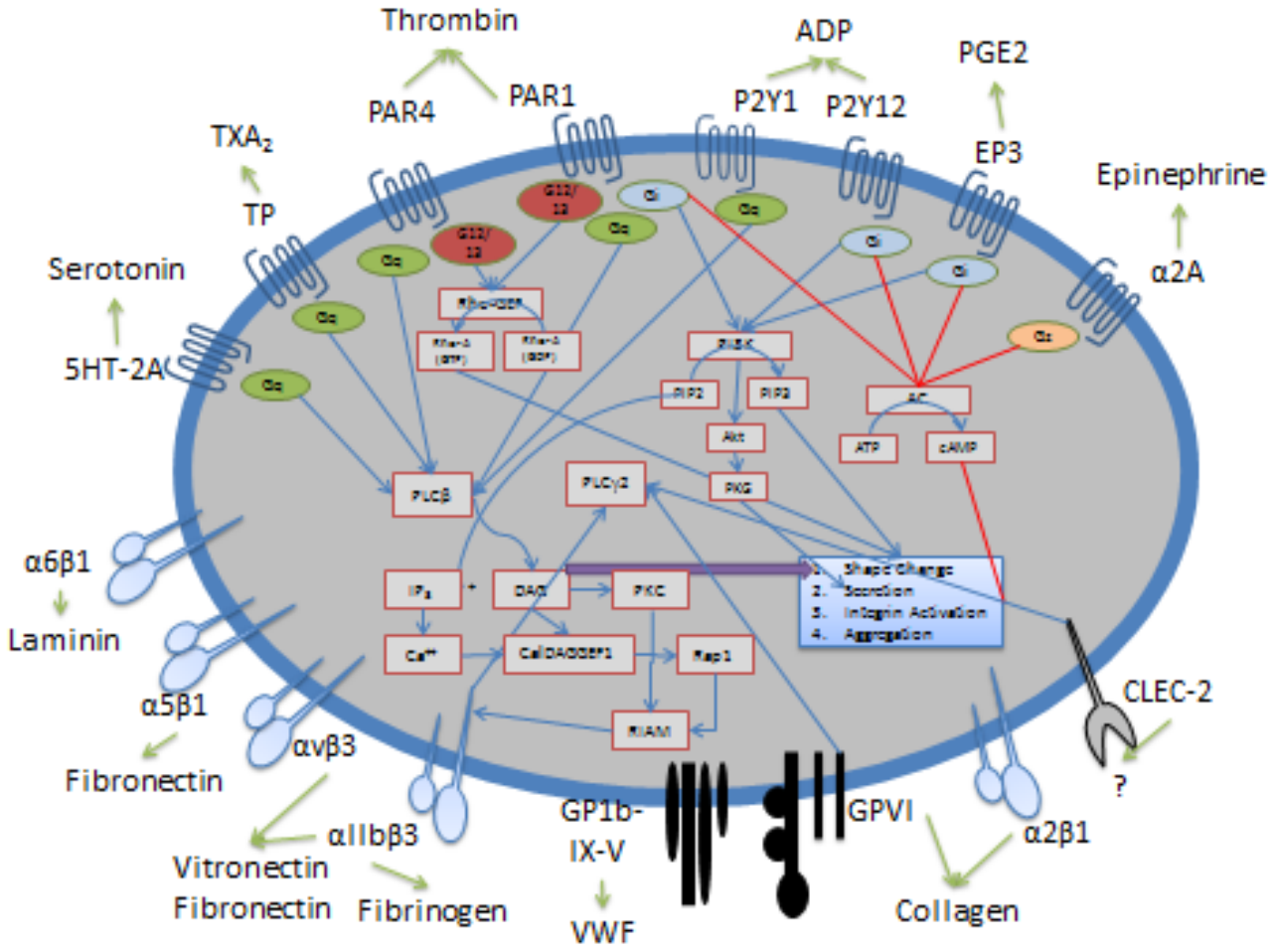

Fig. 1.2. Schematic representation of the major platelet receptors involved in the platelet activation process. Figure is not drawn to scale. In this cartoon, for clarity, GPCRs are shown on one side of the platelet and, on the other side are shown integrins and other receptors. Green arrows indicate the ligands of the receptors. Blue arrows show the stimulated effectors inside the platelet. Red lines indicate inhibition of the enzymes or processes. Full names of abbreviations are mentioned in the list of abbreviations (page viii). This figure has been adapted from Stegner D, Nieswandt B , 2011 and Li Z. *et al.*, Arterioscler Thromb Vasc Biol, 2010 (4, 157).

CHAPTER 2

SEPARATION OF YOUNG AND MATURE THROMBOCYTES IN ZEBRAFISH1

2.1 Introduction

2.1.1 Zebrafish Thrombocytes- Site of Origin

In the teleosts, the kidney is the major the site of thrombocyte production. Thrombopoiesis is initiated in zebrafish embryos within 30 hours post fertilization (hpf) in the 'intermediate cell mass' (ICM) region (158, 159). Initiation of the hematopoietic stem cells (HSCs) production occurs in the ventral side region of the dorsal aorta in zebrafish. Hematopoiesis occurs in the kidney and thymus, and thrombocytes appear in circulation at 36 hpf (158). In adult zebrafish it has been found that the thrombocytes exist as subpopulations, namely young and mature thrombocytes. In this chapter I have accomplished Aim 1 proposed in Chapter 1, which involves separation of young and mature thrombocytes by using DiI-C18 labeling and is described below.

2.1.2 Differential Labeling of Thrombocytes

When DiI-C18 dye was used for *in vivo* labeling of blood cells in zebrafish embryos, our laboratory found earlier that only some of the thrombocytes were labeled with the dye (160). Our laboratory has published these results for the differential labeling of the thrombocyte subpopulations in zebrafish (156) . The thrombocytes released into the circulation were labeled by BrdU after 24 hours of BrdU injections. Since these cells were also found to be labeled by DiI-

1Materials, methods, figures and results are reproduced from the article published in Blood, Cells, Molecules, and Diseases, 48 (3), Kulkarni V., Kim S., Zafreen L. and Jagadeeswaran P., Separation of young and mature thrombocytes by a novel immuno-selection method, 183-187, Copyright Elsevier (2012).

C18 and the half-life of thrombocytes is 4.5 days, our laboratory concluded that DiI-C18 labeled thrombocytes are probably appearing first in circulation and hence termed as 'young' thrombocytes. The other group of thrombocytes, not labeled by DiI-C18, was termed as 'mature' thrombocytes.

2.1.3 Separation of Thrombocyte Subpopulations

In order to study the differences between the two thrombocytes at the nuclear level, it was important to have pure populations of the young and mature thrombocytes. Attempts have been made to separate platelet subpopulations, which are discussed below.

2.1.4 Previously Used Methods for Separating Mammalian Platelet Subpopulations

In 1969, Dr. Karpatkin used specific density oil mixture and a centrifugation technique to separate the 'large-heavy' and the 'light-small' human platelets (35). Blajchman *et al.* obtained the young and old platelets after irradiation and found that the young platelets were more competent and survived longer than the old platelets (36). Thompson *et al.* used 'counterflow' centrifugation to isolate platelets based on their size (161). Gradients of arabinogalactan have been used for separation of high and low density mammalian platelets (162). Traditional methods of FACS sorting cells are also used by most researchers for separating young and mature platelets. But these methods are time consuming, expensive and laborious. In addition they do not yield 100% pure population of thrombocytes. So the following immuno-separation method was designed to obtain specifically antibody-stained selective thrombocyte populations (163).

2.1.5 Novel Method of Separating Young and Mature Thrombocytes

This section focuses on the new method I developed to separate the young and mature thrombocytes from zebrafish whole blood. This method employs specific biotinylated antibodies, for Cy3 and GPIIb antigens, which were used to target the young and the mature thrombocytes, respectively. I hypothesized that the resulting cell populations should be extremely pure due to the use of specific antibodies. This method is quick, inexpensive, easy to follow and more importantly, it is useful for situations where there is a minimal amount of cells available.

2.2 Materials and Methods

2.2.1 Materials

DiI-C18 (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) also called DiI, was purchased from Invitrogen, Carlsbad, CA. Monoclonal Anti-Cy3/5- Biotin, purified mouse immunoglobulin antibody was purchased from Sigma-Aldrich, St. Louis, MO. Custom Antisera GPIIb, biotinylated Anti-GpIIb rabbit antibody and Custom Polyclonal Pre-immune Serum was custom made from Alpha Diagnostic Intl. Inc San Antonio, TX. Streptavidin-FITC was purchased from Sigma-Aldrich, St. Louis, MO. Streptavidin magnetic beads and 6 Tube Magnetic Separation Rack was purchased from New England Biolabs Inc. Ipswich, MA. Oligonucleotide primers were custom made from Biosynthesis, Lewisville, TX. Absolutely RNA miniprep kit was purchased from Stratagene, La Jolla, CA. Superscript One step RT-PCR kit with Platinum Taq was purchased from Invitrogen, Carlsbad, CA. Agarose gel DNA extraction kit was purchased from Roche Diagnostics, Indianapolis, IN.

2.2.2 Methods

2.2.2.1 *In vivo* Labeling of Zebrafish Thrombocytes Using DiI-C18

Zebrafish were injected with 10µl of 20µM DiI-C18 (1,1'- dioctadecyl-3,3,3',3'-tetramethyl- indocarbocyanine perchlorate; DiI-C18) solution in phosphate buffered saline (PBS) (160). The fish were kept for 30-40 minutes at room temperature. Then blood was drawn from each fish with a micropipette tip by making a cut on the lateral side, just above the pelvic fin of the fish (151). 2-4µl blood was collected in a 1.5ml centrifuge tube containing 1µl of 3.8% sodium citrate and stored in ice.

2.2.2.2 Immunofluorescence on Blood Smears

1µl of DiI-C18 labeled blood was collected with micropipette tip and smeared on a glass slide. The blood cells were fixed with methanol for 5 seconds and rinsed with PBS to remove methanol. The cells were incubated under a cover slip with 20µl of 1:20 dilution of the 1mg/ml biotinylated anti-Cy3/Cy5 monoclonal antibody, for 50 minutes at 4°C (151). After removing the cover slip, the blood smear was washed with PBS for several times and incubated with 20µl of 1:100 dilution of 1mg/ml streptavidin-FITC for 50 minutes at 4°C. The smear was washed with PBS, dried and observed under the bright field and the fluorescence at 450-490 nm.

Blood was collected from zebrafish which was not injected with DiI-C18 and the blood smear was processed similarly as described above except that 20µl of 1:100 dilution of 1.7mg/ml anti-GPIIb antibody was used instead of anti-Cy3 antibody to check for labeling of mature thrombocytes.

2.2.2.3 Isolation of Young and Mature Thrombocytes

Zebrafish were injected with 10μl of 20μM DiI-C18, incubated and 2-4μl blood was collected in a 1.5ml tube containing 1μl 3.8% sodium citrate in PBS. To get sufficient number of thrombocytes, 2 or 3 DiI-C18 labeled blood samples from different fish were pooled together to have 10μl total blood volume. 3μl of 1mg/ml biotinylated anti-Cy3/Cy5 antibody was added to 10μl of DiI-C18 labeled blood and the blood sample was incubated for 30 minutes on ice, with intermittent mixing. 100μl of streptavidin-coated magnetic beads were added to this sample with gentle mixing and again incubated for 30 minutes on ice. Then the tube containing this mixture was kept on the magnetic stand for 30 seconds. The beads were pulled towards the magnet along with the antibody and the attached DiI-C18 labeled young thrombocyte while the tube was on the stand. The remaining solution was aspirated and collected in another tube for separating the mature thrombocytes. The beads with young thrombocytes were washed 8 times with 400μl of PBS and resuspended in 100μl of PBS after the final wash in order to proceed with RNA prep. 10μl of 1.7mg/ml biotinylated anti-GPIIb antibody was added to the tube containing the remainder of the blood sample and incubated for 30 minutes on ice. Then 120μl of streptavidin-coated magnetic beads were added to it and incubated for 30 minutes on ice, with intermittent mixing. The tube was then placed on the magnetic stand for 30 seconds to separate the beads along with the mature thrombocytes. These beads were washed 8 times with 400μl of PBS. For washing and separating the beads from PBS, the magnetic stand was used instead of the centrifuge. After the final wash, beads with thrombocytes were re-suspended in 100μl of PBS and then RNA was extracted from the two populations of the thrombocytes.

2.2.2.4 RNA Preparation from Thrombocytes and Zebrafish Whole Blood

The Stratagene RNA miniprep kit was used to prepare RNA from the isolated young and mature thrombocytes. RNA prep procedure: 200μl Lysis buffer with 1.4μl of β-mercaptoethanol was added to the bead suspension. After 5 minutes of incubation, beads were separated using the magnetic stand, and the remaining cell suspension was filtered through a pre-filter spin cup. Ice cold ethanol (70%) was mixed with the product of centrifugation collected in a tube. Then this mixture was centrifuged using RNA binding spin cup. RNA spin cup was washed with low salt wash buffer and DNase solution was added to it and incubated at 37°C. The spin cup was washed with high and low salt buffers. After washing, 40μl of elution buffer was added to elute the RNA.

The eluted RNA was stored at -20^{o}C or used for RT-PCRs. Purity of the isolated RNA was checked by RT-PCR. Young thrombocyte RNA was amplified using primers for cell specific markers. Zebrafish Pu.1 (forward and reverse) was used as a neutrophil marker, β-globin was used as red cell marker and GPIIb was used as thrombocyte specific marker. Elongation factor-1α (EF1-α) was used as an internal control for RT-PCR (164). Zebrafish whole blood RNA was amplified using the same cell specific gene markers and used as a positive control. The following primers were used for RT-PCRs. For EF1-α, (5' CGGTGACAACATGCTGGAGG 3', 5'-ACCAGTCTCCACACGACCCA-3') [13], Pu.1 , (5’-TCAAATGAAAAGCAGCGTCATATTC-3’, 5’-CCATAGCACATCATGAAAGTTCAC-3’) (165), β-globin (5’ GGCCTGTGGGGAAAGCTC 3’, 5’ GTTGTCGGGATCCACATGCAG 3’) (166) and GPIIb (5’ CAGCTGGACAGAATGAAGCA 3’, 5’ GGGAGTCAGCCAAGCTGTAG 3’) were used. RT-PCR was performed for 40 cycles, at a specific melting temperature for each of the primer pairs. The RT-PCR products were electrophoresed

on 2% agarose gel. The product sizes were 220 base pairs (bp) for EF1-α, 200 bp for Pu.1, 269 bp for β-globin and 199 bp for GPIIb primers. The GPIIb primers were designed using Primer 3 software to amplify zebrafish GPIIb gene. The EF1-α, Pu.1, and β-globin primers were picked as is from published article references.

2.3 Results

2.3.1 Separation of Young and Mature Thrombocytes

DiI-C18, a lipophilic dye, at lower concentration (10μM-20μM), labels only the young thrombocytes. Since the DiI-C18 labels the young thrombocytes and both the young as well as mature thrombocytes can be labeled with antibody raised against GpIIb, we reasoned that if the DiI-C18 binds to the cell membrane lipid bilayer, and if there is an antibody that recognizes the DiI-C18 chromophore we should be able to separate the young thrombocytes from the blood cells. Since remaining mature thrombocytes could be separated by using the antibody against GpIIb from the blood cells, we hypothesized the anti Cy3 antibody should bind to DiI-C18 since Cy3 and DiI-C18 have similar chromophores (Fig. 2.1). Furthermore, since antibodies can be biotinylated, the separation should be amenable to magnetic separation using streptavidin conjugated beads. Such cells should be pure and should be utilizable for biochemical and molecular studies (Fig. 2.2).

2.3.2 Recognition of DiI-C18 by Anti-Cy3 Antibody

Thrombocytes were labeled with DiI-C18 by *in vivo* injection of this dye intravenously into zebrafish and the blood was collected and a small aliquot was checked for the DiI-C18

labeled thrombocytes using a fluorescence microscope. The remaining blood was fixed and probed with biotinylated anti-Cy3 antibody followed by staining with streptavidin FITC. A small aliquot was placed under a cover slip and the fluorescence of the thrombocytes was observed. Green colored labeled thrombocytes were observed under 450-490 nm. No green cells were observed in the control slide, not labeled with DiI-C18 (data not shown). This indicated that the anti-Cy3 antibody reacted with DiI-C18 and was bound to DiI-C18 chromophore on the surface of the thrombocytes, whereas the antibody did not bind to the DiI-C18 negative thrombocytes in the control smear. These thrombocytes can be differentiated from the other blood cells in the bright field image seen in Fig. 2.3.

2.3.3 Principle of Separation of Young and Mature Thrombocytes

The young thrombocytes were separated from the zebrafish whole blood that was labeled with DiI-C18. Remarkable similarity was noticed between the chromophores of DiI-C18 and another dye, Cy3 (Fig. 2.1). Commercially available biotin anti-Cy3/Cy5 antibody was used against the DiI-C18 labeled young thrombocytes, and custom made biotinylated anti-GPIIb antibody was used against the mature thrombocytes. Excess unbound antibody was washed away with PBS (phosphate buffered saline) after incubation. Streptavidin magnetic beads were used to bind to these biotinylated antibodies (streptavidin and biotin form a strong bond). A magnet was used to separate the thrombocytes, drawing them away from the rest of the blood cells, by using biotin-anti-Cy3 antibody and streptavidin-coated magnetic beads. (Fig. 2.2). Immunofluorescence experiments showed that the young thrombocytes labeled by DiI-C18 were labeled by anti-Cy3 and streptavidin FITC (Fig. 2.3). Mature thrombocytes were labeled by

anti-GPIIb antibody and detected by streptavidin FITC (Fig. 2.3). The microscopic observations of the beads showed almost a pure population of thrombocytes (Fig. 2.4). Subsequently, RNA was isolated from the separated populations and young thrombocyte RNA was used to check the purity of the isolated thrombocytes by RT-PCR and also by using cell-specific RT-PCR primers in these amplifications (Fig. 2.5).

2.3.4 Development of Zebrafish Specific Antibody

To separate the mature thrombocytes from the blood cells we developed biotinylated anti-GPIIb antibody. This custom-made antibody was raised in rabbits against the zebrafish αIIb peptide RGGTDIDDNGYPDLIIG that was obtained from Ensembl database.

2.3.5 Labeling of Thrombocytes with Biotinylated Anti-GPIIb Antibody

To test the efficacy, of this antibody we probed zebrafish blood smear with biotinylated anti-GPIIb antibody, again followed by probing with streptavidin FITC. We found all the thrombocytes were labeled by this antibody. The control slide in which pre-immune rabbit antisera was used instead of the anti-GPIIb antibody did not show green thrombocytes under 450-490 nm (data not shown). These results provided the basis for the separation of young and mature thrombocytes by dual labeling.

2.3.6 Isolation of Young and Mature Thrombocytes

To test whether streptavidin-coated magnetic beads will pull the DiI-C18 labeled young thrombocytes attached to biotinylated anti-Cy3 antibody; the magnetic beads that pulled the

young thrombocytes were spread on slides and examined for DiI-C18 labeled cells. As predicted, we found the DiI-C18 labeled cells stained with anti-Cy3 antibodies attached to beads after separating them from the blood. Under the light microscope, the washed beads showed the attached thrombocyte but also, in addition, occasional red cells. After separation of the young thrombocytes from the whole blood, the remaining blood cells were used to separate the mature thrombocytes. Subsequently, the mature thrombocytes were pulled using the biotinylated anti GPIIb antibody and streptavidin magnetic beads. The beads were washed several times to get rid of the contaminating red cells and the white cells.

2.3.7 Purity of Isolated RNA

The young thrombocytes prepared by the above method were used in preparation of RNA. Purity of these thrombocytes was checked by RT-PCR using the cell specific markers such as thrombocyte marker GPIIb, neutrophil marker Pu.1 and red cell marker β-globin. As a control EF1-α was used. Whole blood RNA was used as a positive control. The whole blood RNA control showed positive amplification for all the three cell markers (Pu.1, β-globin and GPIIb) and the control EF1-α, whereas the young thrombocyte RNA showed only the GPIIb amplification (Fig. 2.5). These results suggested that the method developed here provides pure thrombocyte preparation.

2.4 Conclusion

In conclusion, this chapter describes a method for separation of young and mature thrombocytes for the first time and demonstrated its applications. Since the dye labeling is not

only feasible in fish but also in other species, this is a generally applicable method for separation of thrombocytes from any species. Further, it will have applications for separating other cell types such as specific populations of neurons because DiI-C18 labels distinctly certain populations of neurons.

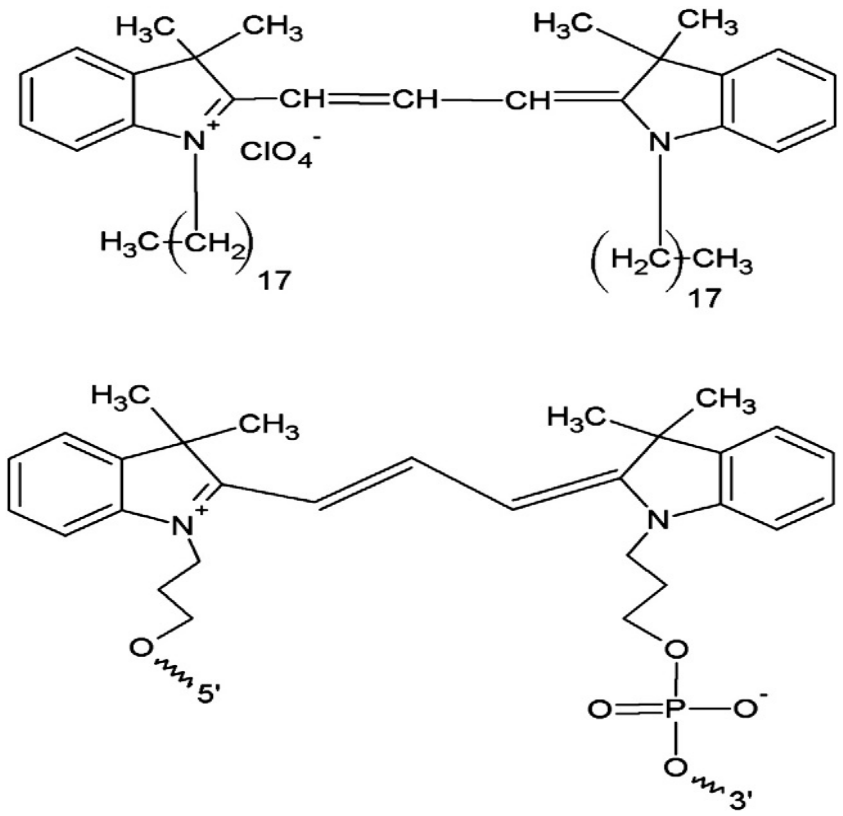

Fig. 2.1. Comparison of the structures of two dyes, DiI-C18 (top) and Cy3 (bottom). Note the similarity between the heterocyclic chromophores of the dyes. Diagram is not drawn to scale.

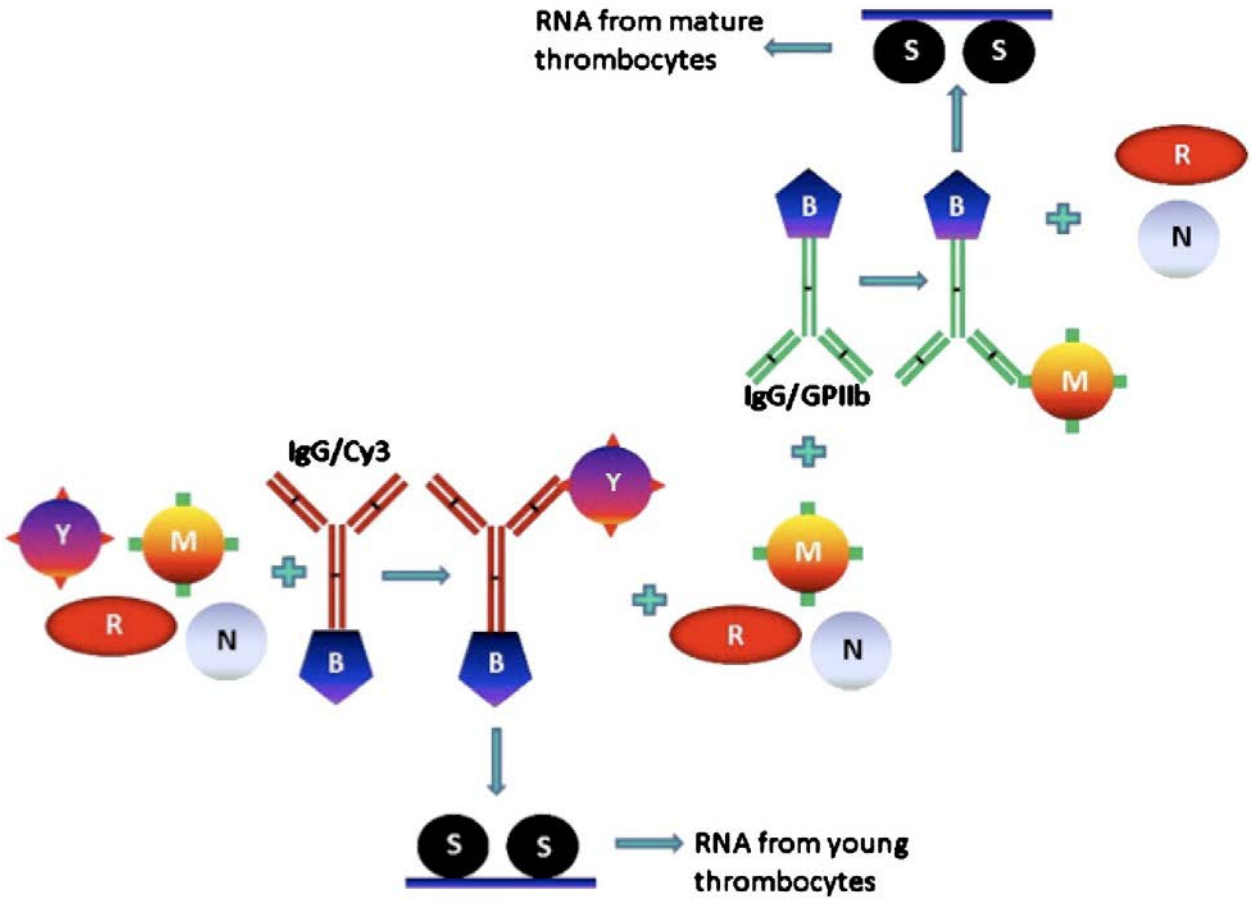

Fig. 2.2. Principle behind separation of young and mature thrombocytes from whole blood using biotin antibodies and streptavidin magnetic beads. Y, young thrombocyte; M, mature thrombocyte; R, red cell; N, neutrophil; B, biotin labeled antibody; S, streptavidin beads attached to magnet. IgG/Cy3 and IgG/GPIIb are represented as Y shaped structures. Thrombocyte and neutrophil are shown in shaded circles. The triangular and square structures on the shaded circles (young and mature thrombocytes) represent Cy3 and GPIIb respectively. Red cells are shown in shaded ovals. Diagram is not drawn to scale.

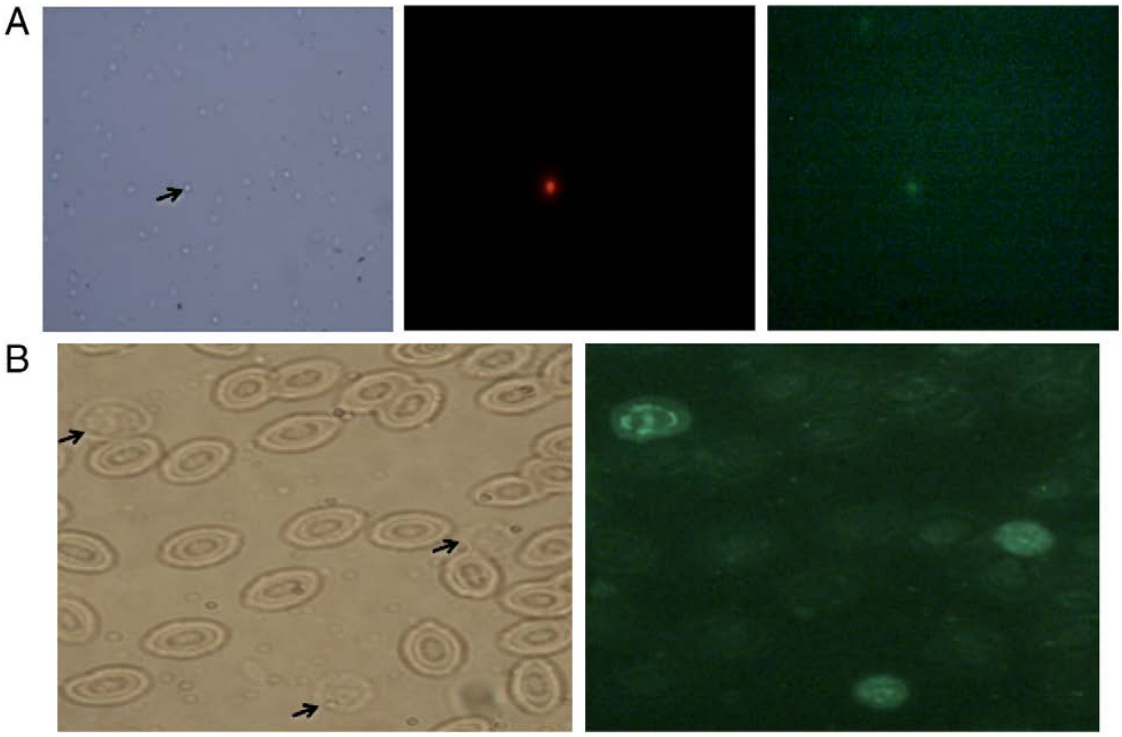

Fig. 2.3. Immunofluorescence images of blood cells under different wavelengths. A, images showing blood cells incubated with anti-Cy3 antibody kept under a cover slip. Left panel, bright field image; middle panel, fluorescence image of the DiI-C18 labeled thrombocyte; right panel, fluorescence image of the streptavidin FITC labeled thrombocyte. B, images showing blood smears probed with biotinylated anti-GPIIb antibody followed by streptavidin FITC. Left panel, bright field image; right panel, fluorescence image of the streptavidin FITC labeled thrombocyte. Arrows indicate the thrombocytes.

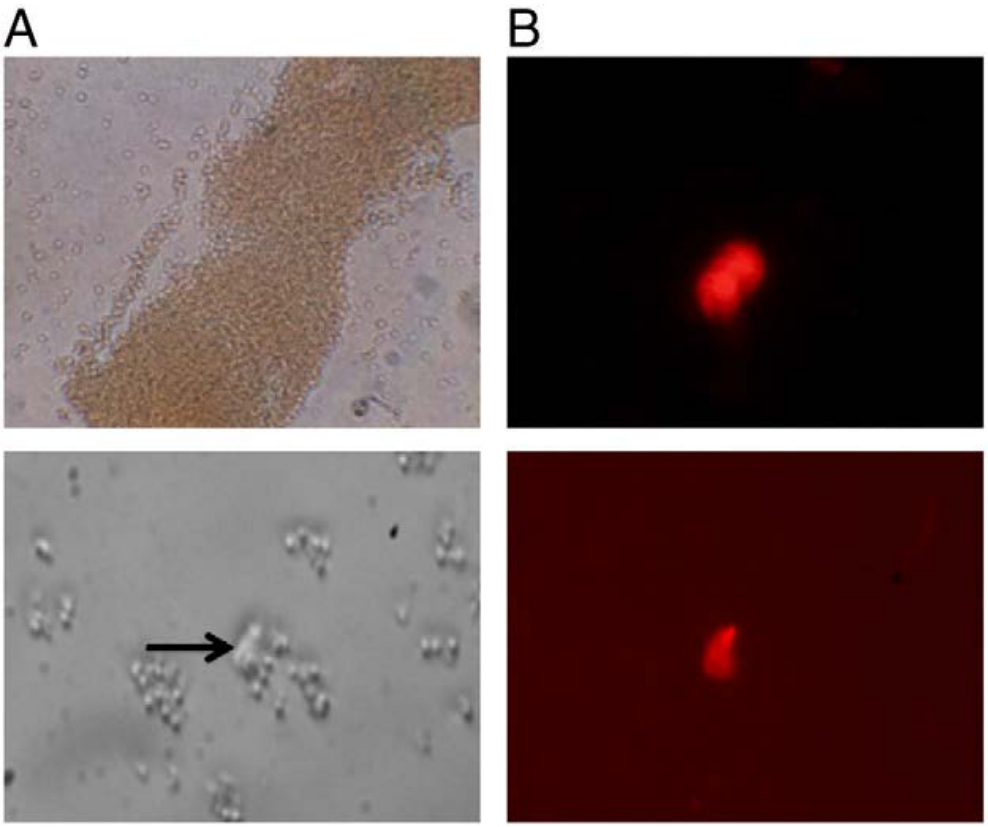

Fig. 2.4. Streptavidin magnetic beads after pulling the DiI-C18 labeled young thrombocytes. The streptavidin beads bind to the biotin anti-Cy3 antibody already bound to DiI-C18 on the surface of young thrombocyte. A, bright field images and B, fluorescence images of streptavidin magnetic beads on a slide.

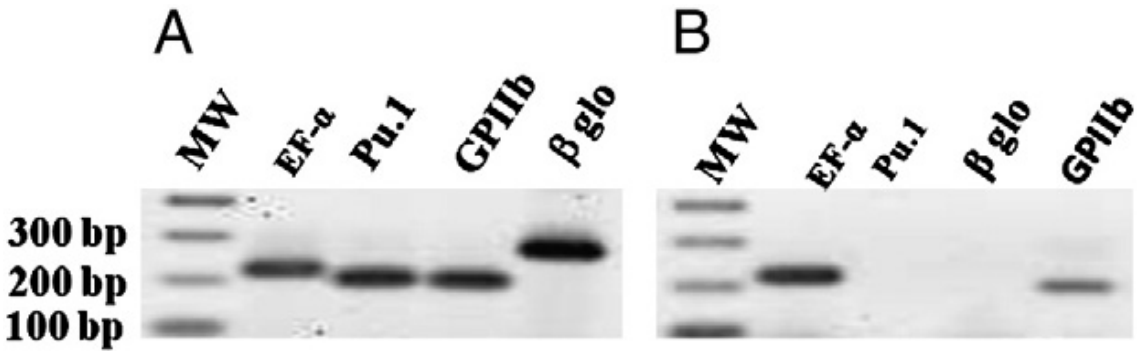

Fig. 2.5. Proof for purity of the isolated young thrombocytes. (A) Whole blood RNA control showing positive amplification for EF1-α (labeled as EF-α), Pu.1, GPIIb, and β-globin (labeled as β glo) genes. (B) Young thrombocyte RNA showing positive amplification for EF1-α and GPIIb genes. The RNA was extracted by using the novel separation method.

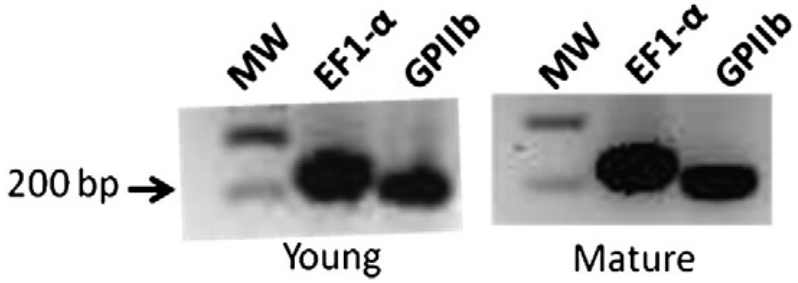

Fig. 2.6. Presence of GPIIb transcripts in mature thrombocytes. Left and right panels show the positive amplification for EF1-α and GPIIb in both young and mature thrombocytes, respectively.

CHAPTER 3

EXPRESSION OF GPCR TRANSCRIPTS IN YOUNG AND MATURE THROMBOCYTES

3.1 Introduction

In this chapter, I describe the various thrombocyte subpopulations and their parallels with the heterogeneity of platelets. Further, the chapter also describes GPCRs found in thrombocytes, and culminates in analysis of expression of GPCR transcripts in the young and mature thrombocytes.

3.1.1 Why GPCRs?

GPCRs play a crucial role in the signaling pathways. They also play an important role in the activation of platelets which is required for primary and secondary hemostasis. There are different GPCRs present on the platelets such as the protease activating receptors (PARs), the ADP receptors, thromboxane A2 receptors, and the prostacyclin receptors. The different GPCRs and their functions are listed in table 4.1. The signals are transmitted via the G-proteins to the inside of the cells or platelets. Activated GPCRs transduce stimulatory as well as inhibitory signals, to bring about the platelet activation, secretion, and aggregation (28). The second messengers such as the cyclic AMP (cAMP) and the calcium (Ca^{++}) bring about the platelet responses. When the cAMP levels increase, the platelet adhesion, aggregation and secretion reactions are inhibited; whereas, decrease in cAMP levels causes mobilization of Ca^{++} and phosphorylation of myosin light chain kinase (MLCK). These calcium levels lead to contraction of cytoskeleton and secretion of granular contents from platelets (13).

Another example is activation of GPIIb/IIIa receptors via G-protein signaling. Platelet aggregation is stabilized by the activation of $P2Y_{12}$ receptor through its ligand ADP. $P2Y_{12}$ receptor is coupled with Giα containing G-protein. The activated Giα inhibits enzyme AC (adenylate cyclase) thereby reducing the formation of cAMP. This further results in reduction of PKC activation. This activates the downstream effectors finally resulting in the activation of GPIIb/IIIa receptors. The $\beta\gamma$ subunits also activate their effectors such as PI-3K and Akt, leading to GPIIb/IIIa activation (167).

3.1.2 Heterogenous Platelet Populations

Dr.Karpatkin performed kinetic (Diisopropyl fluorophosphate) DFP^{32} labeling experiments in rabbits and found that as the platelets age in circulation, there is a transition from "large-heavy" to "small-light" platelets. He further described the larger platelets as younger platelets that are newly released from bone marrow and the small platelets as older platelets (35). The large-heavy platelets aggregate faster in response to agonists and also released more ADP, ATP and PF-4 as compared with the small, older platelets (168). Blajchman *et al.* also reinstated that the young platelets are effective in reducing bleeding times and are hemostatically more active than old platelets. When they analyzed platelet subpopulations for different membrane glycoproteins, their studies showed no qualitative differences between the young, old and the normal (heterogeneous) platelets (36). The ^{75}Se-methionine labeling studies done by Thompson *et al.* suggest that platelet function is affected by platelet size and age, and show that no relation exists between platelet size and age (161). Stractan gradient studies on human platelets demonstrated that young platelets were larger and more dense. They became

smaller and less dense due to protein loss and revealed increased IgG level (37). Thompson *et al.* claimed that differences in platelet size were present before the platelets were released into circulation and that the larger platelets were not the younger but those having more lifespan (169). Alberio *et al.* in 1999, stimulated platelets with two agonists, thrombin and convulxin (GPVI activator), and found high expression of factor V from alpha granules in about 31% of platelet population. This population was also labeled with thiazole orange, which suggested that the above activated platelets had enrichment of young platelets. In another study, they found platelets lost reactivity with thrombin and collagen as they aged (170)[(171). Thus, platelet function and platelet size and age have been a controversial issue.

3.1.3 Zebrafish Thrombocytes and Subpopulations

Thattaliyath *et al.* injected Bromo-deoxy-Uridine (BrdU), a chemical that intercalates with the newly synthesized DNA, in zebrafish. Then at 24 hours post injection, they injected DiI-C18 into the same fish and then blood was collected and smeared onto a slide. Most of the BrdU cells that were detected by anti-BrdU antibodies were also found to be labeled by DiI-C18. These new cells were termed as the 'young thrombocytes'(156).

When the blood cells were labeled with a low concentration (20 μM) of lipophilic dye DiI-C18 (DiI) *in vivo* and *in vitro*, only the young thrombocytes were found to be labeled. These looked red under fluorescence 510-560 nm, B-2 filter. Whereas all the thrombocytes were labeled green when observed under G-2A filter, 450-490 nm. These young thrombocytes constitute 10% of the total thrombocyte population (156). The other thrombocytes that circulated in the blood were not labeled with DiI-C18 (DiI negative) and these were older

thrombocytes in circulation termed as the 'mature thrombocytes' (156). The young thrombocytes were found, more so than the mature thrombocytes, to have more functional receptors such as the adhesion, and fibrinogen receptors. The functional ability was shown by antibodies against GPIb and GPIIb receptors (151). The young thrombocytes are the first players to initiate the clot formation process at the site of injury. Hence, these young thrombocytes are more active than the mature thrombocytes (156). Recently, Jagadeeswaran *et al.* demonstrated that the young thrombocytes have more GATA1 promoter activity which is lost with simultaneous gain of the FLI1 expression during the thrombocyte maturation process (172).

3.1.4 Comparison of Young and Mature Platelets

Amisten *et al.* (2008) gave an account of highly expressed GPCRs in human platelets (117). However, not much is known regarding the GPCR expression in thrombocytes and, in fact, no attempt has been made to see the differences in mRNA levels not only between young and mature thrombocytes but also between reticulated platelets and mature platelets. In this study, the young and the mature thrombocyte transcripts were amplified using the GPCR primers and then qualitatively compared in order to identify the differences between them using the densitometry analysis.

3.2 Materials and Methods

3.2.1 *In vivo* Labeling of Zebrafish Thrombocytes Using DiI-C18

Zebrafish were injected with 10µl of 20µM DiI-C18 (1,1'- dioctadecyl-3,3,3',3'-tetramethyl- indocarbocyanine perchlorate; DiI-C18) (Invitrogen, Carlsbad CA) solution in

phosphate buffered saline (PBS) (160). Blood was collected by using the same protocol as discussed in Chapter 2.

3.2.2 Preparation of RNA from Whole Blood and RT-PCR

Whole blood RNA (Stratagene RNA miniprep kit) was prepared from zebrafish blood and amplified using 84 GPCRs from the zebrafish genome mentioned in Table 3.1. The RT-PCR (Invitrogen, Carlsbad CA) products were subjected to gel electrophoresis on 2% gel. Product size of the primers (Biosynthesis, Lewisville TX) was 600 base pairs (bp) for each GPCR.

3.2.3 Isolation of Young and Mature Thrombocytes and RNA Preparation

Young and mature thrombocytes were separated from the whole blood by using the biotin antibody and streptavidin magnetic bead technology, as mentioned in chapter 2. RNA was prepared from the thrombocyte populations using the Stratagene RNA miniprep kit.

3.2.4 RNA Preparation from Total White Cells

Blood was collected from wild type zebrafish in a tube containing 3.8% sodium citrate. The tube was centrifuged for 5 minutes, at 200g. The white cell layer was collected and RNA was prepared using the Stratagene miniprep RNA kit.

3.2.5 RT-PCR and Gel Electrophoresis

The white cell layer RNA was amplified using the 37 GPCR primers that showed positive amplifications with whole blood RNA. The young and the mature thrombocyte RNA showed

positive amplifications for 8 GPCRs with the total white cell RNA. One-step superscript RT-PCR (from Invitrogen, Carlsbad, CA) was used for RT-PCR.

The following primers were used as controls for all the experiments. For EF1-α (as an internal control), (5' CGGTGACAACATGCTGGAGG 3', 5'-ACCAGTCTCCACACGACCCA-3') [13], and GPIIb (thrombocyte marker) (5' CAGCTGGACAGAATGAAGCA 3', 5' GGGAGTCAGCCAAGCTGTAG 3') were used.

RT-PCR was performed for 40 cycles, at a specific melting temperature of 50° C. The RT-PCR products were electrophoresed on 2% agarose gel. The product sizes were 220 base pair (bp) for EF1-α and 199 bp for GPIIb primers. The GPIIb primers were designed using Primer 3 software to amplify the zebrafish GPIIb gene. Other primers for GPCR cDNA were ordered from Biosynthesis, TX. The EF1-α primers were picked as it is from published article references.

For each RT-PCR reaction, 12.5μl of 2X reaction mix and 0.5μl of platinum *Taq* mix (provided by the kit), 0.02μg of (whole blood) RNA, water, 20μM forward and reverse primers were used to make the final reaction volume of 25μl. RT-PCR conditions were programmed for one cycle of cDNA synthesis and pre-denaturation at 50°C for 30 min, 94°C for 4 min and denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and elongation at 72°C for 1:30 min, for 40 cycles.

3.2.6 Densitometry Analysis of the Band Density

Band density for each of the bands was analyzed and average pixel intensity was measured using Quantity One software program. Pixel intensity was measured for each band keeping the area of measurement constant. Fold changes in the expression of young and

mature thrombocytes were calculated using EF1-α as control. The values for young and mature thrombocyte RNA were plotted on a graph. The statistical analysis was performed using one-way ANOVA.

3.3 Results

3.3.1 RT-PCRs using Whole Blood RNA

The biotin-antibody and streptavidin magnetic beads method (described in chapter 2) of cell separation was used for analysis of young and mature thrombocytes. Initially, I used 84 sets of primers derived from GPCR sequences selected from the zebrafish genome and the whole blood RNA was used in amplification reactions. Thirty seven of these reactions displayed positive amplifications on gel electrophoresis at the 600 base pairs (Fig. 3.1). Table 3.2 shows the list of these 37-GPCRs. The GPCRs presenting positive amplifications with whole blood RNA were selected and new primers were constructed having a product size of 200-220 base pairs (Table 3.4).

3.3.2 RT-PCRs Using Total White Cell RNA

The 37 GPCRs which staged positive amplifications with whole blood were used in amplifying the total white cell RNA. Ten of these GPCRs exhibited positive amplifications (Fig. 3.2). These ten GPCRs were then used to amplify young and mature thrombocyte RNA as follows.

3.3.3 RT-PCRs using Young and Mature Thrombocyte RNA

The eight GPCRs that displayed positive amplifications with total white cell RNA were used for amplifying isolated thrombocyte RNA. Young and mature thrombocyte RNAs were amplified using the new GPCR primer sets (forward and reverse) exhibited in Table 3.5. Elongation factor1-α (EF1-α) was used as an internal control for RT-PCRs. Elongation factor 1-α is required for translation in the eukaryotic cells (173, 174). The products of the RT-PCRs were subjected to gel electrophoresis. The resulting bands were subjected to densitometric analysis. The results exposed no significant differences between the expression of the young and the mature GPCR mRNA transcripts (Fig. 3.4). This experiment was repeated three times and statistical analysis was performed by One-way ANOVA, the P values ranged from 0.091 to 0.971 and alpha= 0.05.

3.4 Conclusion

The immuno-separation method I developed was used to isolate the thrombocyte populations. RNA isolated from the thrombocytes was used to compare the GPCR expression in young and mature thrombocytes. When RNA prepared from the young and mature thrombocytes was subjected to RT-PCRs with the GPCR primers, some differential expression patterns of these GPCRs were found in the young and mature thrombocytes by qualitative observations. However, on comparing the bands produced by the young and mature transcripts by densitometry along with statistical methods, there were no significant differences between young and mature thrombocytes. Interestingly, the differences in receptor levels were noted in the earlier work. Thus, our data led to the interpretation that there may be translational control

differences between young and mature thrombocytes since we did not observe notable differences between them. Nevertheless, the research in this aim has led to the identification of a 'novel' GPCR in thrombocytes: GPR18. This is the first finding and, therefore, I pursued by knockdown methods to evaluate the role of GPR18 in the process of thrombocyte activation. This study is presented in chapter 4 of this dissertation.

Table 3.1. List of GPCR primers used to amplify zebrafish whole blood RNA.

Note: The names and the GPCR cDNA sequences used are based on the older version of the database available at the time of study.

Code	Name of the GPCR	Forward Primer (5'-3')	Reverse Primer (5'-3')
9604	Type-1 angiotensin II receptor (AT1)	GTCTGGCCGGAGAGGACT	CGCTGGCGTAGAGGTTGA
10296	Similar to EBV-induced G-protein coupled receptor 2 (EBI2) [*Danio rerio*]	CTAGACCAGGAGGTGCCATT	CATTGACAACAAAGGCAGTG
18953	Similar to bradykinin receptor, beta 2 [*Bos taurus*]	GGCTCGCTTGAGAAAAGGAG	CCCAGATCACCAGGCTGTAG
22016	Similar to GPR34-like GPCR [*Gallus gallus*]	CCCAGCATTTCTCACAGTGTT	ATTATCGCAAGCGTGCTTCT
25413	Similar to Agtr1 protein [*Danio rerio*]	CTTGTATCAGACAGAAGAATCCAAA	CAGCAGGCATGAGAGGAAC
26052	Similar to somatostatin receptor type 1 subtypeB [*Danio rerio*]	TTCAAGAACCTGGAGGATGG	GCCCATCAGAAAGGCGTAT
27120	Similar to cysteinyl leukotriene receptor type 2[*Gallus gallus*]	ATACGGTCCCCACAAACAAC	ACACAGAGATGATCGCGAAA
27382	G protein-coupled receptor 34b [*Danio rerio*]	AAGCTTGCGTGCATTAGACA	AACTCCAGCAATTGACACAGC
28893	Similar to Chemokine receptor-like 1 (G-proteincoupled)	GTGGCTCTGTTGCTTGCTG	CAAGTATGATCAACACCGATGC
32650	Similar to Gpr92 protein [*Gallus gallus*]	TGAAGCCAGGACTCACTCAT	GACGATGCTAAACACCATGC
34893	Similar to opioid receptor-like protein Zf-orl [*Danio rerio*]	TGGAGTTGGTCGAGTTGTCTT	CACGATCCCAGTGCTTTCTT
34987	Similar to Proteinase activated receptor 1 precursor(PAR-1) (Thrombin receptor) [*Danio rerio*]	TGAATTTCATCGACAACTTCAATC	GGCTTAGAGATGGGCGTGT
41935	Protein-coupled receptor 80 [*Danio rerio*]	CGCTCAGATGACAGTGATGC	TCCCAATATGGTGAGAATCCA
43629	Similar to somatostatin receptor type 1 subtypeA [*Danio rerio*]	ACAGTGGCAACGAGAGCTT	GGAACGTATAAACCACGAACG
44732	Similar to G protein-coupled receptor 7 [*Gallus gallus*]	GAACATCACGAGCCCCTTTA	AGCACGCAGCTTTTCCTCT
44742	Similar to G protein-coupled receptor 80 [*Danio rerio*]	CGCTCAGATGACAGTGATGC	TCCCAATATGGTGAGAATCCA
45017	Pyrimidinergic receptor P2Y4 [*Macaca mulatta*]	AACCACTGCTCTCCCTGCT	GGCTACGACCAACCAAACTG
48866	Similar to somatostatin receptor type 3 [*Danio rerio*]	GCAGCACTGGACATATGGAG	CGGCCATATTATGTTGCAGA
51531	Similar to angiotensin II type-2 receptor [*Gallus*	GGGGAGACATGCAGAGAATC	CTCCCCAAAAACCCAGTTGT
51604	Similar to Chemokine receptor-like 1 (G-proteincoupled	CAGTGGACGGGTAAGAGGAA	TGGACGTCACGGTACACAAT
54107	Similar to C-C chemokine receptor type 2 (C-C CKR-2) [*Bos taurus*]	ATGCCAACAACAACGTTTGA	TGGAATGATAAGCCCAAGGA
54108	Similar to C-C chemokine receptor 8 like [*Gallus gallus*]	ATGCCAACAACAACGTTTGA	TGGAATGATAAGCCCAAGGA

Code	Name of the GPCR	Forward Primer (5'-3')	Reverse Primer (5'-3')
55274	G protein-coupled receptor 34a [*Danio rerio*]	TTCAGCTCAGTGTGGGACA	GAACACCAGCCAGAACACG
56245	Similar to CC chemokine receptor 4 [*Gallus gallus*]	ATGGTTGACACTAATTGGAGTGATT	TGTCTGATTGTAACCGGTCATT
56273	G protein-coupled receptor 55 [*Mus musculus*]	ATGAGAGGTGGCAAGAAGGA	TTCAGCAGCAGAAACAGCAG
57319	Similar to G protein-coupled receptor LPSenhR-1 [*Danio rerio*]	TCACCAGTGTCCCAGCTACT	GACCGACAATGAAGCTCAGG
58877	G protein-coupled receptor 80 [*Danio rerio*]	CGCTCAGATGACAGTGATGC	TCCCAATATGGTGAGAATCCA
60098	Similar to EBV-induced G-protein coupled receptor 2(EBI2) [*Danio rerio*]	TCAGACGATGGCAGAGAATG	GGCATGAAAAACCCAATGAC
60136	Similar to Burkitt lymphoma receptor 1 [*Canis familiaris*]	GCAGACAGCACAGAGACAACA	TGCGTCTCCAGCTGATATTG
60521	Novel protein similar to vertebrate G protein-coupled receptor 43 (GPR43) [*Danio rerio*]	TGGACGGTGCATCTCAGTAA	CTTGATGTAGCAGAAGCAGCA
61673	Similar to somatostatin receptor type five subtype C [*Danio rerio*]	TTCCTGATGAAACGAGCTGA	TGTACATGTTGGTGACCGTCT
62041	G protein-coupled receptor 68 isoform 3 [*Pan troglodytes*]	CTCCTCACTGCCTTCCTTTG	CCACACACCTGGCAGGATAG
62129	Platelet-activating factor receptor isoform 6 [*Pan troglodytes*]	TTGGAGGGTAGAGCAGAGTGA	TTACTGCCTGGAAGCGGTTAT
63114	Similar to Chemokine receptor-like 1 (G-proteincoupled receptor DEZ) [*Danio rerio*]	CAAGGAGGGTTTGAAGGTACA	AGCAAAGTTGTTGAAGCAGTTG
63664	Chemokine orphan receptor 1 isoform 3 [*Pan troglodytes*]	CCAAGATGAGTGTGAACGTGA	AAACTGCTTTGCAGACGATG
63752	Similar to Lysophosphatidylcholine receptor G2A(G2 accumulation protein) [*Danio rerio*]	ATCATGCGCTATTCGGAGTC	GGCTTACATAAACGGTCCACA
66804	Similar to P2Y-like G-protein coupled receptor isoform 1 [*Gallus gallus*]	TTGGATCAGCTCCTTGGTTT	CGGAGCTTGATGGACTTCAC
66833	Angiotensin receptor [*Anguilla anguilla*]	GTCTGGCCGGAGAGGACT	CGCTGGCGTAGAGGTTGA
66866	Similar to C-C chemokine receptor-3 [*Danio rerio*]	CCGCTCCACACTACATCAGA	CTTCCACTGTGAAACGGATG
66869	Similar to C-C chemokine receptor-3 [*Danio rerio*]	CCGCTCCACACTACATCAGA	CTTCCACTGTGAAACGGATG
67542	Similar to G protein-coupled receptor 65 [*Gallus gallus*]	GATGATCATGCCCTGGATAAG	TGCTTGGTAGATTTTATGGTAACAA
67835	Similar to pyrimidinergic receptor P2Y, G-protein	ATGATGGAAAACGCCACCT	AACCAAAATGACTACAGAAGGAAGA
68065	Similar to Proteinase activated receptor 1 precursor (PAR-1) (Thrombin receptor) [*Danio rerio*]	AACCAAAGACAGCTGTTTACATCA	CCTGAGGCCTTTCCTAGGTG
68684	To type 2 somatostatin receptor, partial [*Danio rerio*]	CACCGTCATGTATTTCCTGGT	TTGCTGGAGCTCACCTTGAT

Code	Name of the GPCR	Forward Primer (5'-3')	Reverse Primer (5'-3')
68982	EBV-induced G protein-coupled receptor 2 isoform2 [*Pan troglodytes*]	CAGGAGGTGCCATTTCTGAC	CATGGGCATTGACAACAAAG
69578	Similar to Proteinase activated receptor 2 precursor(PAR-2) (Thrombin receptor-like 1) [*Danio rerio*]	CCACTCAGTTTCGCAGGAGT	ATACAGAGGCACGGTTAAAGC
69601	Similar to Leukotriene B4 receptor [*Gallus gallus*]	TGAAGCTGGTAGTTTGCATGA	GCTGCTTCGATTGGTAAGTCA
69931	EBV-induced G protein-coupled receptor 2 [*Homo sapiens*]	CAGGAGGTGCCATTTCTGAC	CATGGGCATTGACAACAAAG
70013	Pyrimidinergic receptor P2Y4 [*Macaca mulatta*]	AACCACTGCTCTCCCTGCT	GGCTACGACCAACCAAACTG
70273	G protein-coupled receptor 68 isoform 3 [*Pan troglodytes*]	CTCCTCACTGCCTTCCTTTG	CCACACACCTGGCAGGATAG
70544	Similar to chemokine (C-C motif) receptor 9 isoform A [*Canis familiaris*]	CCTGCTCAGAACCCACAA	GCCTGGGCAATAGCAATGTA
71727	CXC chemokine receptor-2 [*Cyprinus carpio*]	ACAGCTGTCTCACTTTCTTCCA	TCTCTTCTGGATCAGTGTACTTGTG
72398	Similar to G-protein coupled purinergic receptorP2Y8 [*Danio rerio*]	ATGAATCGGAGGTCATCAAA	GGAGGATGAACATGCCAAAT
73879	Similar to P2Y purinoceptor 2 (P2Y2) (P2U purinoceptor	AACTCCATGAGCCCCATGAC	TGGCAGCCCTAGGATAAAGA
74167	Similar to G protein-coupled receptor 25 [*Macaca mulatta*]	CAGGGGCCTGGGACTACT	ACACGAAGGTCAGCAGCAG
75538	Cysteinyl leukotriene receptor 1 [*Macaca mulatta*]	CCATTCATACGCATTGACCA	GGATGATAAATCCCACTACCAG
75542	Similar to G protein-coupled receptor 23 [*Rattus norvegicus*]	CCAGCCCGAGGAATAACAT	CGGCAGCAGAAAACAAAGAG
76884	Similar to Relaxin 3 receptor 1 (Somatostatin- and angiotensin-like peptide receptor) (G-protein coupled receptor SALPR) (GPCR135) [*Danio rerio*]	GAGCTGTTGCAGGACAGGAT	GAAGCCCACCAGGATCTTCT
77221	Similar to Proteinase activated receptor 2 precursor(PAR-2) (Thrombin receptor-like 1) [*Danio rerio*]	CGGTGTCCGAGAGCTACAG	CTTGCAGTTGAACGGTTTGA
78228	Similar to G protein-coupled receptor 43, partial [*Danio rerio*]	TGGACGGTGCATCTCAGTAA	CTTGATGTAGCAGAAGCAGCA
78547	Coagulation factor II receptor [*Pan troglodytes*]	GTTCTGTTGGTGACGGGTCT	TCGCCAAGTGAGCGAGTC
79155	Similar to coagulation factor II receptor-like 3[*Gallus gallus*]	CTGGGACTTTCCCACTGGT	TCCTGCCTAGGGAGAACATC
79175	Similar to Relaxin 3 receptor 1 (Somatostatin- and angiotensin-like peptide receptor) (G-protein coupled receptor SALPR) (GPCR135), partial [*Bos taurus*]	TTGTGAAGTCGCTGGATATGA	CAGGAAAGCGCAGTAAACAA
79962	G protein-coupled receptor 109B [*Pan troglodytes*]	GAACAACTCCTCATTGTGCTG	GAGCAGTAGATGACGATGAACG

Code	Name of the GPCR	Forward Primer (5'-3')	Reverse Primer (5'-3')
79962	Similar to Nicotinic acid receptor 2 (G-proteincoupled receptor 109B) (G-protein coupled receptor HM74) (G-protein coupled receptor HM74B) [*Homo sapiens*]	CACTTTGCTGGAGCATTCAC	GCTGAAGCTGATGCACACAT
81304	Similar to Probable G-protein coupled receptor GPR4(GPR19) [*Canis familiaris*]	TGGCAAGTCTCAGAGGAAGTG	GTGCTAGGAAGCCAAGGAAA
81413	Purinergic receptor P2Y, G-protein coupled, 4 [*Mus musculus*]	ACCAGTGCAGAATCCTTGCT	CATGACTGCCGAACTGAAGTA
81417	Purinergic receptor P2Y2 isoform 3 [*Pan troglodytes*]	CACCGGGTAGGAGTCTTGTC	GAAAAGAAAGCGGACGAACTT
81420	Purinergic receptor P2Y2 isoform 3 [*Pan troglodytes*]	CACCGGGTAGGAGTCTTGTC	GAAAAGAAAGCGGACGAACTT
83417	Similar to P2Y-like G-protein coupled receptor isoform 1 [*Gallus gallus*]	GACAGAACTTCCCACCTTGC	GCAATGAAGGGTGGTGTGA
83569	Similar to G protein-coupled receptor 18 [*Canis*	TCCTCAAGTGTATCGGATTGC	CCATGAATGAGGTTATCCACGA
83628	Similar to P2Y-like G-protein coupled receptor isoform 1 [*Gallus gallus*]	GACAGAACTTCCCACCTTGC	GCAATGAAGGGTGGTGTGA
83850	Similar to Relaxin 3 receptor 1 (Somatostatin- and angiotensin-like peptide receptor) (G-protein coupled receptor SALPR) (GPCR135) [*Danio rerio*]	TTGTGAAGTCGCTGGATATGA	CAGGAAAGCGCAGTAAACAA
84357	Similar to purinergic receptor P2Y, G-protein coupled, 5 [*Rattus norvegicus*]	CTCACAATACAGCCGGCAAC	GCAGCACAGCCTACAGGAG
84735	Similar to Lysophosphatidylcholine receptor G2A(G2 accumulation protein) (G-protein coupled receptor 132) [*Danio rerio*]	ATCATGCGCTATTCGGAGTC	GGCTTACATAAACGGTCCACA
84847	Similar to G-protein coupled receptor RDC1 homolog (Chemokine orphan receptor 1) isoform 1 [*Danio rerio*]	CAGGTCACTGAAGTGTTGACG	GATTTCTCAGCCTGAAGGAAGTA
85046	Similar to purinergic receptor P2Y, G-protein coupled, 5 [*Rattus norvegicus*]	CTCGGTCTCCCGCCTCTT	CAACAGACAAAGGCACGCTAT
85816	Similar to G protein-coupled receptor 109A [*Bos taurus*]	CTCCTTGCTGGAGCATTCAC	GCTGAAGCTGCTGCACAAAT
86114	Chemokine (C-C motif) receptor 2 isoform 1 [*Pan troglodytes*]	AGGGTTTGCCTGACTCACAC	GAGATGGCCAGATTGAGCA
86331	Novel protein similar to vertebrate G protein-coupled receptor 43 (GPR43) [*Danio rerio*]	TGGACGGTGCATCTCAGTAA	CTTGATGTAGCAGAAGCAGCA

Code	Name of the GPCR	Forward Primer (5'-3')	Reverse Primer (5'-3')
86760	Similar to Proteinase activated receptor 3 precursor(PAR-3) (Thrombin receptor-like 2) (Coagulation factor II receptor-like 2) [*Canis familiaris*]	GCCATCTTTGCAGCCATT	CAGTCCCCGGTAAGTGAAAG
86767	Similar to Proteinase activated receptor 3 precursor(PAR-3) (Thrombin receptor-like 2) (Coagulation factor II receptor-like 2) [*Canis familiaris*]	GCCATCTTTGCAGCCATT	CAGTCCCCGGTAAGTGAAAG
87394	Similar to P2Y purinoceptor 2 (P2Y2) (P2U purinoceptor 1) (P2U1) (ATP receptor) (Purinergic receptor) [*Danio rerio*]	CAATGCCAGCGATCTGTACT	GCGTTGCAGACTAACACGAC
87397	Similar to P2Y purinoceptor 2 (P2Y2) (P2U purinoceptor 1) (P2U1) (ATP receptor) (Purinergic receptor) [*Danio rerio*]	CAATGCCAGCGATCTGTACT	GCGTTGCAGACTAACACGAC
87684	Similar to putative G-protein coupled receptor [*Gallus gallus*]	GCAGCTGAGCTTTCTCATCC	AAACCGTATTGCCATGGTG
87773	Similar to G protein-coupled receptor 1 [*Bos taurus*]	GCATCTGTGCAACATGACATT	TGCCTCAGCAAAATGAGTTC

Table 3.2. List of GPCRs that showed positive amplifications using zebrafish whole blood RNA.

Note: Codes and names are based on older version of the database.

Code	Name of the GPCR
10296	Similar to EBV-induced G-protein coupled receptor 2 (EBI2) [*Danio rerio*]
18953	Similar to bradykinin receptor, beta 2 [*Bos taurus*]
27120	Similar to cysteinyl leukotriene receptor type 2[*Gallus gallus*]
27382	G protein-coupled receptor 34b [*Danio rerio*]
32650	Similar to Gpr92 protein [*Gallus gallus*]
34987	Similar to Proteinase activated receptor 1 precursor(PAR-1) (Thrombin receptor) [*Danio rerio*]
41935	Protein-coupled receptor 80 [*Danio rerio*]
44732	Similar to G protein-coupled receptor 7 [*Gallus gallus*]
44742	Similar to G protein-coupled receptor 80 [*Danio rerio*]
48866	Similar to somatostatin receptor type 3 [*Danio rerio*]
51531	Similar to angiotensin II type-2 receptor [*Gallus*
55274	G protein-coupled receptor 34a [*Danio rerio*]

Code	Name of the GPCR
56245	Similar to CC chemokine receptor 4 [*Gallus gallus*]
57319	Similar to G protein-coupled receptor LPSenhR-1 [*Danio rerio*]
60136	Similar to Burkitt lymphoma receptor 1 [*Canis familiaris*]
60521	Novel protein similar to vertebrate G protein-coupled receptor 43 (GPR43) [*Danio rerio*]
63664	Chemokine orphan receptor 1 isoform 3 [*Pan troglodytes*]
66869	Similar to C-C chemokine receptor-3 [*Danio rerio*]
67542	Similar to G protein-coupled receptor 65 [*Gallus gallus*]
67835	Similar to pyrimidinergic receptor P2Y, G-protein
68065	Similar to Proteinase activated receptor 1 precursor (PAR-1) (Thrombin receptor) [*Danio rerio*]
68982	EBV-induced G protein-coupled receptor 2 isoform2 [*Pan troglodytes*]
69601	Similar to Leukotriene B4 receptor [*Gallus gallus*]
69931	EBV-induced G protein-coupled receptor 2 [*Homo sapiens*]
71727	CXC chemokine receptor-2 [*Cyprinus carpio*]
74167	Similar to G protein-coupled receptor 25 [*Macaca mulatta*]
75538	Cysteinyl leukotriene receptor 1 [*Macaca mulatta*]
77221	Similar to Proteinase activated receptor 2 precursor(PAR-2) (Thrombin receptor-like 1) [*Danio rerio*]
78547	Coagulation factor II receptor [*Pan troglodytes*]
81413	Purinergic receptor P2Y, G-protein coupled, 4 [*Mus musculus*]
83569	Similar to G protein-coupled receptor 18 [*Canis*
83628	Similar to P2Y-like G-protein coupled receptor isoform 1 [*Gallus gallus*]
84735	Similar to Lysophosphatidylcholine receptor G2A(G2 accumulation protein) (G-protein coupled receptor 132) [*Danio rerio*]
84847	Similar to G-protein coupled receptor RDC1 homolog (Chemokine orphan receptor 1) isoform 1 [*Danio rerio*]
85046	Similar to purinergic receptor P2Y, G-protein coupled, 5 [*Rattus norvegicus*]
86760	Similar to Proteinase activated receptor 3 precursor(PAR-3) (Thrombin receptor-like 2) (Coagulation factor II receptor-like 2) [*Canis familiaris*]
87397	Similar to P2Y purinoceptor 2 (P2Y2) (P2U purinoceptor 1) (P2U1) (ATP receptor) (Purinergic receptor) [*Danio rerio*]

Table 3.3. List of GPCRs that showed positive amplifications using zebrafish total white cell layer RNA.

Note: The GPCR names and accession numbers are derived from the current NCBI.

Sr. no. For GPCR	Gene Name	Accession number
1	Danio rerio chemokine (C-X-C motif) receptor 7b	XM_690273
2	Danio rerio chemokine (C-X-C motif) receptor 7b	BC154243
3	Danio rerio cysteinyl leukotriene receptor 2-like	XR_117834
4	Danio rerio G protein-coupled receptor 183 (gpr183)	NM_001099241 XM_679670
5	Danio rerio putative protease-activated receptor 2b(PAR2b)	GQ325253
6	Danio rerio coagulation factor II (thrombin) receptor (f2r)	NM_001114846 XM_681694
7	Danio rerio uracil nucleotide/ cysteinyl leukotriene receptor-like	XM_002662449
8	Danio rerio G protein-coupled receptor 18 (gpr18)	XM_679580
9	Danio rerio purinergic receptor P2Y, G-protein coupled 1- like (LOC569401)	XM_692784
10	Danio rerio p2Y purinoceptor 1-like (LOC564730)	XM_688061

Table 3.4. Forward and reverse primers used to amplify total white cell layer mRNA.

Sr. No.	Forward primer	Reverse Primer	Product size in base pairs
1	5'GATGGCATTAATCAGTTCAC 3'	5'GCCATATTATGTTGCAGATT 3'	220
2	5' CATCTTCCTCTTCATCATTG 3'	5' GATCAGGTGTGTAATCTTGC 3'	217
3	5' ACCTGTTCATCTTCTTCCTC 3'	5' AGGTTACATCTCCAAAAACC 3'	210
4	5' GGCCAAATTTAAAGAAAATC 3'	5' GGTCAACACTCAAACAAGTC 3'	216
5	5' AGTATCAGTGGGCTTCTTCT 3'	5' GAACGGTTTGATTGTAGAGA 3'	203
6	5' ATTTATTCGCTCATTTTCG 3'	5' TATATAGTCGGGATGACAGC 3'	219
7	5' GAAAACACAGCAGAGAACAT 3'	5' TTAAATGATAAGTGGCCCTA 3'	202
8	5' TTTACGGCATTATCTTCATC 3'	5' TTAATCCTGCAGAAGATGTC 3'	208
9	5' ATCAATGCAACTGACTGTCT 3'	5' AAACTCAATGCATAAAGCAG 3'	203
10	5' ATGTACATCAGCATCACCTT 3'	5' TCAAAGCACATTTTCTCTTT 3'	200

Table 3.5. Forward and reverse primers used to amplify young and mature thrombocyte GPCR transcripts.

Sr. no. For GPCR	Forward primer	Reverse Primer	Product size in base pairs
1	5' CATCTTCCTCTTCATCATTG 3'	5' GATCAGGTGTGTAATCTTGC 3'	217
2	5' ACCTGTTCATCTTCTTCCTC 3'	5' AGGTTACATCTCCAAAAACC 3'	210
3	5' GGCCAAATTTAAAGAAAATC 3'	5' GGTCAACACTCAAACAAGTC 3'	216
4	5' AGTATCAGTGGGCTTCTTCT 3'	5' GAACGGTTTGATTGTAGAGA 3'	203
5	5' ATTTATTCGCTCATTTTCG 3'	5' TATATAGTCGGGATGACAGC 3'	219
6	5' GAAAACACAGCAGAGAACAT 3'	5' TTAAATGATAAGTGGCCCTA 3'	202
7	5' TTTACGGCATTATCTTCATC 3'	5' TTAATCCTGCAGAAGATGTC 3'	208
8	5' ATGTACATCAGCATCACCTT 3'	5' TCAAAGCACATTTTCTCTTT 3'	200

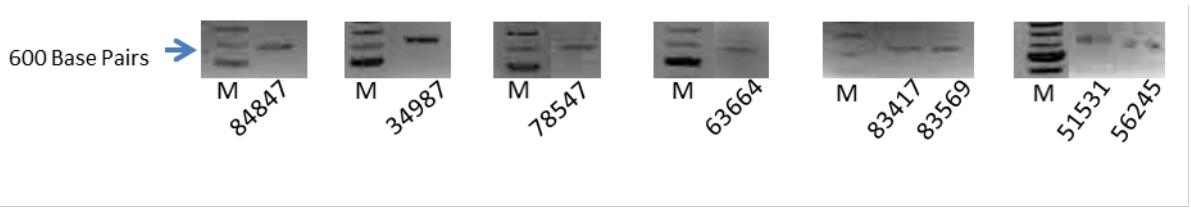

Fig. 3.1. Representative gels showing positive amplifications of the GPCR transcripts using the zebrafish whole blood RNA. The RT-PCR products have a size of 600 base pairs. M stands for molecular weight marker and the numbers below the bands are the GPCR codes based on the older version of the database corresponding to Table 3.1.

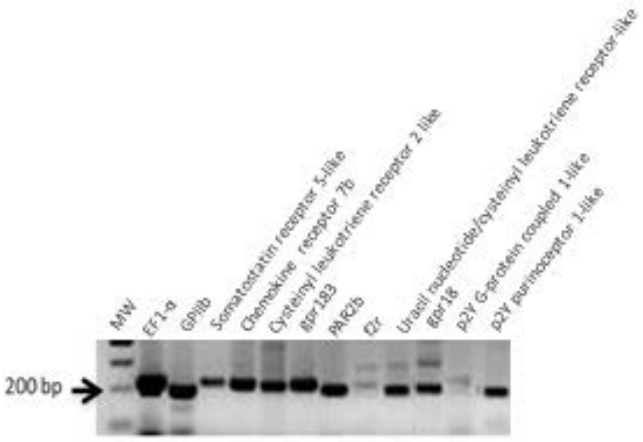

Fig. 3.2. Total white cell layer mRNA amplified by GPCR primers. The total white cell mRNA showed amplification with 10 GPCRs (Table 3.4).

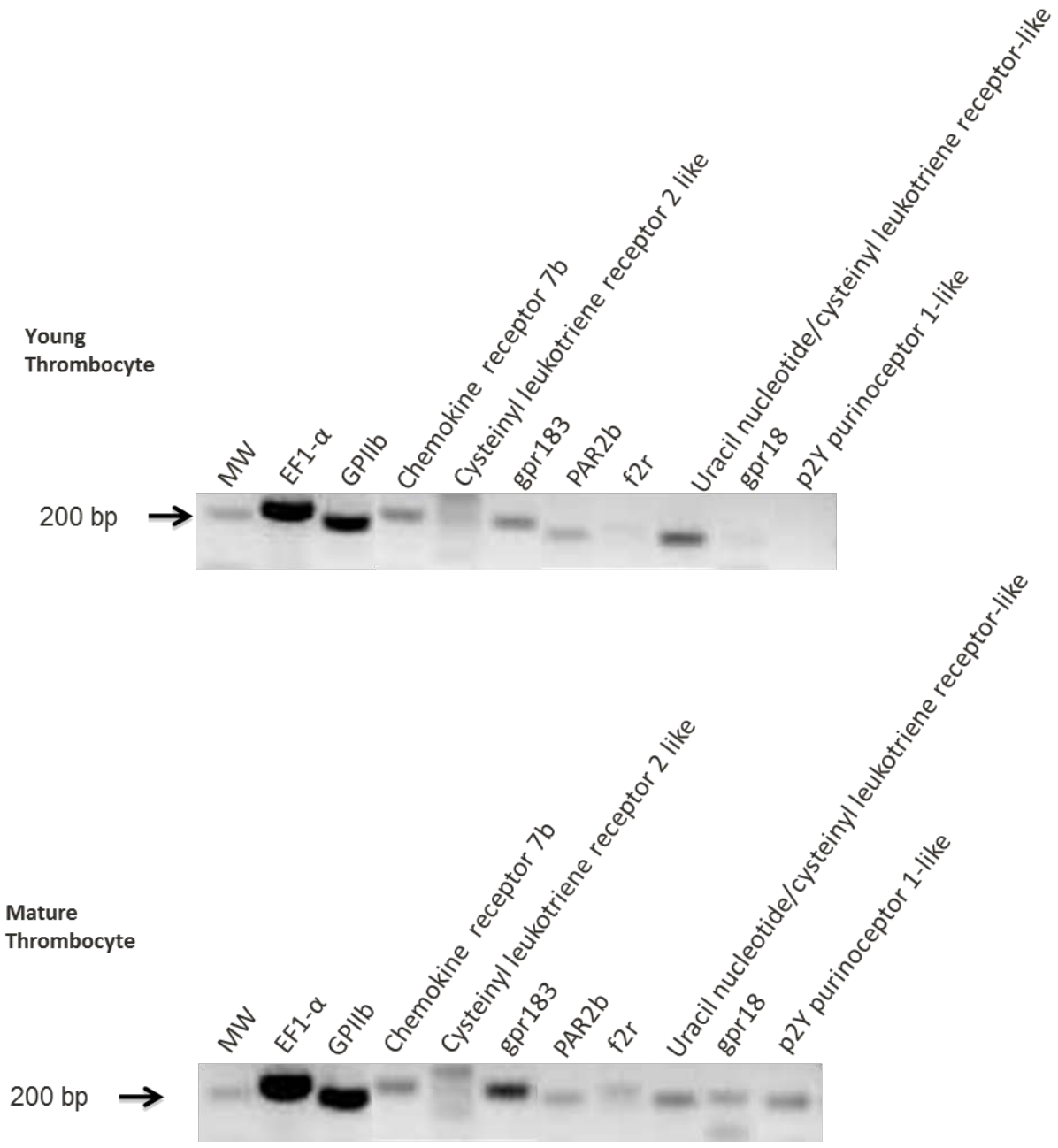

Fig. 3.3. Gel electrophoresis of RT-PCR products of the young and mature thrombocyte RNA. Top image, Young thrombocyte mRNA was amplified using 8 GPCR primer sets. Bottom image, Mature thrombocyte mRNA was amplified using the same 8 GPCR primer sets.

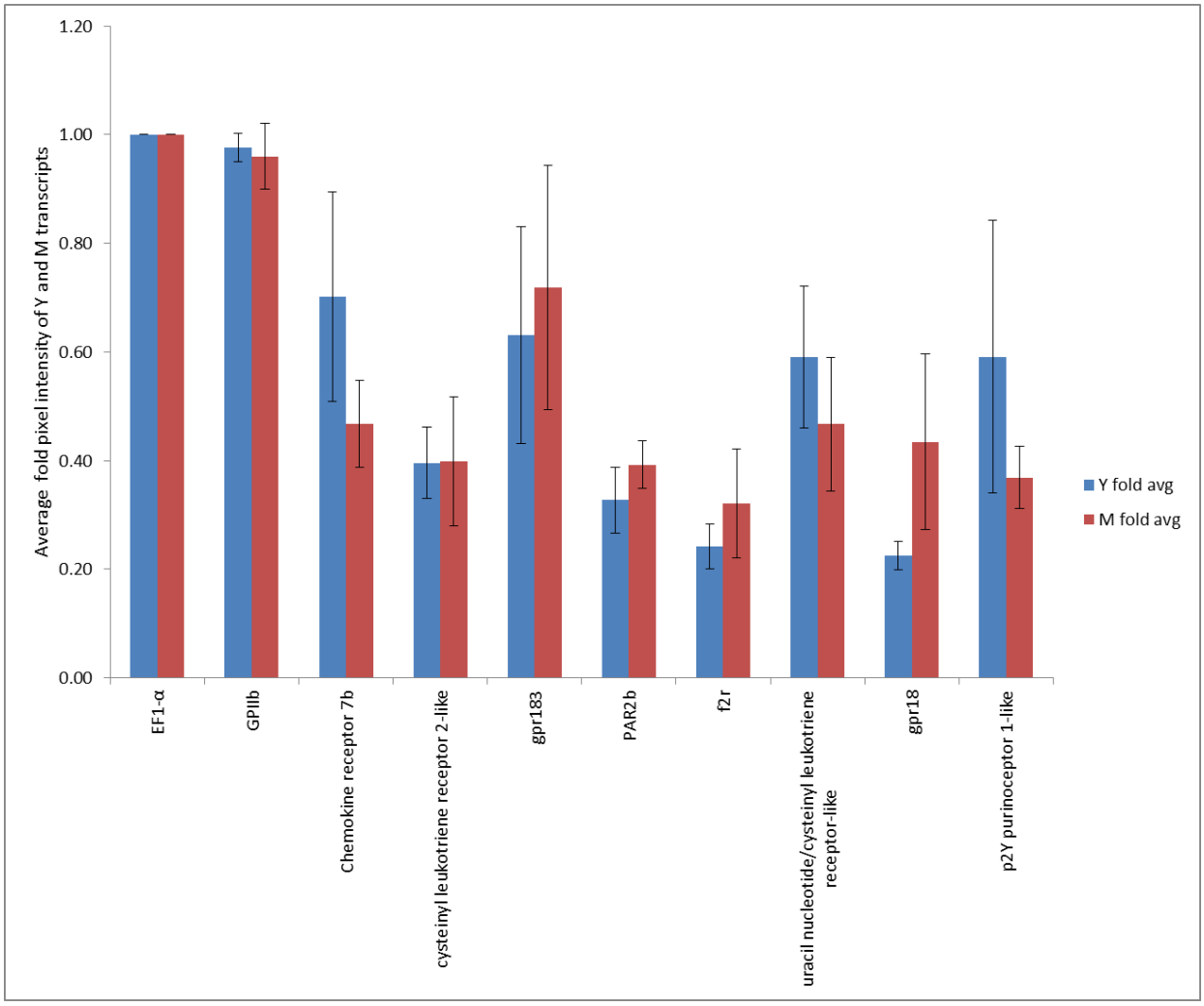

Fig. 3.4. Comparison of young and mature thrombocyte GPCR transcripts. The graph represents the fold changes in young, (Y) and mature, (M) transcripts analyzed by densitometry. Elongation factor 1-α, was used as an internal control (Int). Blue bars represent young transcripts and red bars represent mature transcripts. The X-axis shows fold changes for young and mature transcripts of control, EF1-α, GPIIb and the amplified GPCR genes from Fig. 3.3. The Y-axis represents the fold intensities of pixels measured from the bands produced by gel electrophoresis of young and mature RT-PCR products. The error bars indicate standard deviation. No statistically significant difference was found between the young and the mature transcripts for all the tested genes using one-way ANOVA. The data are an average of three individual experiments. The P-values of the transcripts ranged from 0.091 to 0.971, alpha= 0.05.

CHAPTER 4

KNOCKDOWN OF GPR18*

4.1 Introduction

4.1.1 G-protein Coupled Receptors (GPCRs)

Alfred G. Gilman and Martin Rodbell shared the Nobel Prize for the discovery of G proteins, in 1994 (175). G proteins are associated with the seven transmembrane receptors on the cytosolic side of the cells. The receptors belonging to this family are known as G protein coupled receptors (GPCRs). About 800 GPCRs have been identified in humans (176). GPCRs are involved in various signaling pathways, which are crucial in transmitting different signals from the outside to the inside of the cell. A variety of the first messengers such as the hormones, light, odors, small molecules, pheromones etc. serve as ligands for the receptors (177, 178). Those GPCRs whose physiological ligands are not known are termed as 'orphan GPCRs'. The reverse pharmacology approach utilizes the orphan GPCRs to identify the endogenous ligands (176). When the respective ligands bind to the GPCRs on the extracellular side, they activate the attached G-proteins on the inside of the cells. The activated Gα-GTP subunit and the βγ of the heterotrimeric G-protein may further activate enzymes such as adenylate cyclase or PLCβ which release second messengers, which in turn, activate the downstream effectors that bring about the desired change: for example, transcription of a gene or shape change of a platelet.

Only the major platelet GPCRs known to play a role in thrombosis and hemostasis are discussed below in this chapter.

* Manuscript in preparation. For this study, immunofluorescence and plate-tilt assays were contributed by Dr. Seongcheol Kim and time-to-occlusion assay was performed by Uvaraj Radhakrishnan. Capturing of images and statistical analysis of data was performed by Vrinda Kulkarni.

4.1.2 Structure of GPCRs

GPCRs belong to the family of receptors that have a single polypeptide making seven trans-membrane (7 TM) domains in the plasma membrane of the cell. The 'N terminal' of the receptor lies outside the cell and the 'C terminal' of the receptor lies on the cytosolic side. The receptor polypeptide chain is embedded in the cell membrane having 3 extra-cellular loops, 3 intra-cellular loops, and 7 trans-membrane domains. Ligand binding changes the conformation of the TMs and the ionic bonds between the third and sixth TM domain are broken (179). This is followed by binding of the G-protein to the i2 (intracellular loop 2) or i3 or the C-terminal (178, 180). The peptide hormone ligands bind to the extracellular loops and the N-terminal portion of the receptor, and this triggers the signals inside the cells via the activated G-protein subunits. Larger proteins and glycoproteins bind to the N-terminal tail and then move downwards to interact with the loop and activate the receptor. The biogenic amines, nucleosides and the lipid ligands bind to the hydrophobic pocket of the receptor (180).

4.1.3 GPCR Families

The super family of GPCR is divided in 6 major families as below (181).

Family A, known as Rhodopsin family, is the largest family including odorants and small ligands. Family A is further sub-divided into three groups. Family B is commonly known as secretin receptor family, in which the receptors possess large N terminal extracellular domain. Family C contains the metabo-tropic glutamate receptors, which encompass calcium sensing receptors, taste and olfactory receptors, and pheromone receptors. These also possess large N

terminal extracellular domains. Family D is a fungus pheromone receptor family. Family E is known as cAMP receptor family.

4.1.4 GPCR Dimers/Oligomers

Many studies by BRET and FRET analysis have shown that GPCRs undergo dimerization as well as oligomerization. These dimers/oligomers may affect receptor functions such as the potency of receptors, their affinity for ligands, and their specificity for G proteins. The transmembrane helices I, II, and IV may be involved in dimer formation (182, 183). Some studies show that dimer formation is required for maturation and proper transport of GPCRs from endoplasmic reticulum to the cell membrane (184). Sometimes the GPCR signaling pathway components are concentrated in microdomains such as lipid rafts or caveolae in the cell membrane to ensure signaling (185).

4.1.5 G- proteins

G-proteins couple to the 'Guanine' nucleotide and hence get their name, 'G- protein'. The G-proteins are made up of 3 subunits: α, β and γ. When a ligand binds on the outside of the receptor, the signal is transmitted inside the cell and the GDP bound to the G-protein is replaced with the GTP, thereby activating the internal signaling cascades. This GDP-GTP exchange is catalyzed by GEFs (Guanine nucleotide exchange factors). The GTP binds to the α-subunit of the protein and the Gα-GTP complex dissociates from the G-protein, leaving β and γ subunits together. Both the GTP-α and the β, γ subunits can activate the downstream effectors and can propagate signals causing cellular effect (177, 181, 186). The signal is terminated by the

intrinsic activity of Gα to undergo GTP hydrolysis. The RGS (regulators of G-protein signaling) proteins mediate the signal termination. RGS proteins show GAP (GTPase accelerating proteins) activity for the Gα subunit (187).

In humans, at least eighteen subtypes of Gα proteins, five Gβ proteins and eleven Gγ proteins have been identified (188). Gα anchoring to plasma membrane is via myristoylation and palmitoylation reactions (186). The subtypes of Gα proteins and their role in signaling is summarized in Table 4.2.

4.1.5 Signaling via G- proteins

The G-protein activation region lies in the C-terminal end of the third intracellular loop of the GPCR (178, 179). Upon activation, the GTP-α subunit dissociates from the heterotrimeric G-protein. The Gs-α subunit binds and activates the Adenylate cyclase (AC) enzyme in the plasma membrane. The AC catalyzes the conversion of ATP to cyclic AMP (cAMP). Formation of cAMP in platelets leads to the inhibition of platelet activation.

The Gi-α subunit leads to the signaling cascade that inhibits AC from catalyzing the conversion of ATP to cAMP. This leads to stimulation of platelet activation and aggregation. The Gq-α subunit activates the phospholipase C β (PLC-B) enzyme, which catalyzes the formation of two second messengers, namely, (Diacyl glycerol) DAG and (Inositidine triphosphate) IP3 from (Phosphoinositidine diphosphate) PIP2. DAG activates PKC while the IP3 binds to the IP3 receptor on the endoplasmic reticulum and releases Calcium (Ca^{++}) from the intracellular stores. This further leads to Ca^{++} mobilization into the cell via the CRAC (calcium release activated channel) (157). Calcium and DAG synergistically activate and regulate CalDAG-GEF-I,

which is crucial for integrin signaling (157). Rise in the levels of calcium further activates several molecules such as PKC, calmodulin, nitric oxide synthase (NOS), as well as calcium dependent proteases. CalDAG-GEF-I has been shown to activate Rap 1 and ERK, causes synthesis of TXA2, and also causes granule secretion. Calcium activates the actin-myosin interactions and positively regulates the PI3K/Akt pathway and the Src family kinases (SFKs) (157).

Inhibition of platelet activation is brought about by various signals. For example, the A2 Adenosine receptors (A2A and A2B) are coupled to the Gs protein. On activation of adenylate cyclase, the production of cAMP second messenger level is increased, leading to inhibition of platelet activation (189). Another example is of prostacyclin (PGI2) receptor IP which is also coupled to Gαs pathway. The increased cAMP levels lead to activation of PKA (190). PKA further phosphorylates other proteins which may be involved in regulating platelet functions.

4.1.6 Other Signaling Proteins

RGS (regulators of G-protein signaling) proteins

The RGS proteins bind and modulate the activity of the G-proteins and the GPCRs. They mainly bind to the i3 loop and C-tail end of the receptor. They bind to the Gα subunits and inhibit their activity (191).

4.1.7 Scaffolding Proteins

Various scaffolding proteins interact with the trimeric G-proteins. Their roles are mentioned below (192).

- Proteins belonging to RGS and AGS classes (Regulators and Activators of G-protein signaling)

- AKAPs: A-kinase anchoring proteins help in targeting signaling enzymes such as PKA to specific locations in cells.
- TRP-containing proteins- Tetratricopeptide motif containing proteins react with Gs, Gq and Gi subunits.
- WD40 repeat proteins- these proteins react with activated GPCRs and Gα subunits.

Other proteins include RACK1, Kelch repeat proteins, Radixin, EBP50, KSR-1 etc.

GITs- (GRK interacting proteins) are scaffolding proteins that form homo or heterodimers and are localized throughout the cell, at the periphery and at focal adhesion points. These function in many cellular activities such as GPCR endocytosis, PLCγ activation, Ca^{++} mobilization, activation of MEK1, cytoskeletal regulation via PAK and Rho GTPases and focal complex formation (193).

4.1.8 Termination of the Signal

The signal propagated via the GPCR signaling can be terminated by different mechanisms.

4.1.8.1 Dissociation of GTP-

This occurs due to the intrinsic GTPase activity of the Gα subunit. The GTP is hydrolyzed to GDP and GDP-Gα again associates with the Gβγ subunits to resume the inactive form.

4.1.8.2 Desensitization-

GPCRs that are in a constant stimulation mode are densensitized by the (GRKs). GRKs phosphorylate the stimulated GPCRs and then recruit ß-arrestins that avoid stimulation. This

may be followed by internalization of receptor (194).

GRKs phosphorylate the GPCRs at serine and/or threonine residues. At low agonist concentration, phosphorylation of activated GPCRs is brought about by second messenger dependent protein kinases (PKA, PKC). At high agonist concentration, phosphorylation of activated GPCRs is via GRK activity (178). Homologous desensitization is the attenuation of the response of the particular GPCR subtype. Heterologous desensitization involves attenuation of GPCR subtypes even in the absence of agonist activation (195). This results in temporary inhibition of other GPCRs (196). Agonists also show functional selectivity upon binding to GPCRs (195). The desensitization process may take some hours or even days, rendering GPCRs useful for drug designing strategies (194).

4.1.8.3 Receptor Internalization-

The GRKs phosphorylate GPCRs and subsequently the arrestins bind to the phosphorylated GPCRS, desensitizing them, followed by internalization (195).

Upon internalization, GPCRs are degraded by lysosomes or they are dephosphorylated and recycled to initiate another signaling event (194). Upon internalization, some of the GPCRs have shown to modulate the activity of adenylate cyclase enzyme (197)

4.1.8.4 β Arrestins

β Arrestins negatively regulate the GPCRs. They specifically bind to agonist occupied, and agonist activated GPCRs. These GPCRs are then internalized and desensitized. β Arrestins also act as scaffold proteins in MAPK signaling. They can travel to the nucleus and interact with

transcription cofactors and CREB of genes thereby promoting their transcription. They also regulate transcription indirectly and affect functions such as cell growth, apoptosis and immune responses (198).

The present study as described in Chapter 3 identified 8 GPCRs on thrombocytes. Since it is difficult to study all the GPCRs I identified and, that furthermore, some of the functions of the GPCRs such as, $P2Y_1$, are already known in human platelets, I decided to study one of the GPCRs that is novel and has not been found in platelets so far. I found GPR18 was novel among the positively amplified 8 GPCRs. Therefore, in the present chapter, I have knocked down the function of GPR18 by using antisense morpholinos (MOs) to study the role of GPR18 in thrombocyte function.

4.1.9 GPR18

Studies by Gantz *et al.* on GPR18 show that it is located on chromosome 13q32 in humans and is highly expressed in mammalian spleen and testis. Maximum sequence homology was found with the rat mu opioid receptor (199). Kohno *et al.* found GPR18 expression in hematopoietic cell lines and organs such as brain, skin and small intestine (200). Studies done in metastasis of melanoma showed involvement of GPR18 in the survival of cancer cells, and that it may be constitutively active in cells (73). The Ensembl database shows that the GPR18 receptor protein is 331 amino acids long in humans whereas; it is 314 amino acids long in zebrafish. It clusters with the Epstein-Barr virus (EBI2) receptor on chromosome 13 (201).

4.1.10 N-Arachidonyl Glycine (NAG)

Kohno *et al.* for the first time showed that N-arachidonyl glycine (NAG) is the ligand for the GPR18 receptor. They probed the GPR18 transfected cells with NAG and found that it increases intracellular calcium levels and also inhibits cAMP formation. These data suggested that GPR18 would be coupled to Gαi/o protein (200). Another study on the endogenous lipids and orphan GPCRs, also showed that NAG was able to activate GPR18 via the Gi/o pathway (201). Interestingly, NAG has a high affinity for Gi/o coupled GPR18 and is a partial agonist at Gq/11 coupled GPR92 receptors (202). NAG, a metabolite of N- arachidonyl ethanolamide (AEA also known as anandamide), is synthesized endogenously by two pathways: one is FAAH (fatty acid amide hydrolase) dependent, and another pathway exists is by binding of glycine to arachidonic acid (AA). NAG was found to be involved in cell migration by the activation of GPR18 (203, 204). NAG causes microglial migration through GPR18 activation. GPR18 has similarity with Abn-CBD (Abnormal Cannabinoid receptor) in microglial cells (202). NAG also activates MAPK pathway in GPR18 transfected HEK293 cells (204). NAG induces apoptosis in GPR18 expressing macrophages via the Gi pathway. Thus it acts as an anti-inflammatory lipoamino acid. Burstein *et al.* showed the anti-inflammatory role of NAG and GPR18 signaling (205) (206).

4.1.11 Gene Knockdown Using Morpholino (MO) Injections

Gene knockdown is a method to down regulate the expression of a protein. This method is useful in understanding the function of that particular protein in the signaling pathway. Gene knockdown can be accomplished by different methods. In zebrafish, this is typically achieved by

injecting the morpholino in the embryos (207). Morpholino (MO) is a short antisense nucleotide sequence about 25 nucleotides long in which the sugar in the backbone is modified. The MO carries a six membered morpholine moiety in place of the ribose ring. (208-210). The vivo morpholinos (vMO) are attached to the octa-guanidine dendrimers by covalent linkage which facilitates these vMOs to penetrate effectively into the cells (153). These vMOs have been successfully used in mouse to alter the expression of dystrophin in mouse models of DMD (Duchene muscular dystrophy) (211). This technique has been successfully adopted to knockdown, for the first time, genes in adult zebrafish in our laboratory. Kim *et al.* have displayed the knockdown of GPIIb affects the function of thrombocytes (212). Carrillo *et al.* have created von Willebrand disease-like model by knockdown of VWF with vMOs (154).There are 2 types of MO knockdowns: one is splice-blocking and the other is translational-blocking. This blocking is mostly targeting the splice junctions and because they are anti-sense MOs, they form hybrids with the RNA (208). A gene having more than one exon can be knocked down using the splice blocking morpholino which targets splice junctions, and the spliced products (produced due to the effect of MO) can be checked by RT-PCR and gel electrophoresis (213). The morpholino binds to the RNA thereby, making the RNA inaccessible to the splicing proteins. In this splice blocking, because of the skipping of splicing, the alternatively spliced product will generate a truncated protein product that usually itself results in its lack of functional activity. The product of a gene having single exon can be knocked down using a translation blocking morpholino which targets mRNA around Cozak sequences encompassing the initiator codon. The effect of the gene knockdown can be checked by protein expression studies.

A morpholino can be injected into the single cell stage zebrafish embryos to ensure that each new cell in the mitotically dividing embryo gets a sufficient dose of this morpholino. Since the zebrafish embryos are transparent, they serve as an excellent model to observe the embryonic development and phenotype; in our case, the phenotype that will be generated by knockdown of GPR18, should be prolongation of TTO in laser induced arterial thrombosis model. We asked if GPR18 has a role in thrombocyte function. If we perform vivo MO knockdown of GPR18 in adult zebrafish, it should be possible to study the thrombocyte functions and the downstream pathways of GPR18 *in vitro*, by using thrombocyte functional assays. Thus the function of GPR18 could be easily studied by this approach.

4.2 Materials and Methods

4.2.1 Materials

Control MO and GPR18 vivo morpholinos were purchased from Gene Tools LLC, Philomath, OR. Anti-GPR18 antibody was purchased from Life Span Biosciences, Inc., Seattle, WA. FITC- conjugated anti-Rabbit IgG produced in sheep was purchased from Sigma-Aldrich Co., LLC, St. Louis, MO. Tissue culture mini plates (60 wells) were purchased from Nalge Nunc International, Rochester, NY. Arachidonic acid and N-arachidonyl glycine was purchased from Cayman Chemical Company, Ann Arbor, MI.

4.2.2 Methods

4.2.2.1 Knockdowns Using GPR18 Antisense Oligo Morpholino in Zebrafish Embryos and Adults

GPCR18 sequence was obtained from ENSEMBL database and a GPR18 morpholino (MO), 5' ACACTTGAGGAACTTCAGTCTCCAT 3' was designed and ordered from Gene Tools. Approximately 5-10 nL of MO at a concentration of 1mM was injected in 1-4 cell stage zebrafish embryos with the picospritzer III injector (Parker Precision Fluidics; Hollis, NH). Control MO, 5' CCTCTTACCTCAGTTACAATTTATA 3', was injected in embryos, and larvae were used as controls.

Adult fish were injected with vivo morpholino having the same sequence as above. The procedure followed is the same as discussed in earlier studies on αIIb morpholino from our laboratory by Kim *et al*. (212). Zebrafish were injected with 5 μL of standard morpholino and 5 μL of 400 nM GPR18 vivo morpholino for control and experimental groups, respectively. Blood was collected after 48 hours from both the groups and smeared on slides.

4.2.2.2 Laser Ablations on Larvae

On fifth day post fertilization (5 dpf), the larvae were injured with laser induction under the microscope and the time to occlusion (TTO) in seconds was recorded. For the laser injury, larvae were anesthetized for 2 minutes with 6 μl of 10mM tricaine solution in 0.5 ml of water. Such larvae were resuspended in 0.5 ml of 1.6 % agarose and mounted on their lateral sides on a glass slide before laser injury.

4.2.2.3 Immuno-Staining of Zebrafish Blood Smears

Staining procedure is similar to the one used in the αIIb MO studies (212). Smears were fixed with methanol and rinsed 3 times using 1X PBS. Control and morphant fish blood smears and human blood smears were incubated with 1:20 dilution of 1 μg/μL of anti- GPR18 polyclonal rabbit antibody for 1.5 hours at 25°C. Smears were rinsed using PBS and were incubated with 1:50 dilution of FITC-conjugated anti-rabbit IgG in sheep for 1.5 hours. Smears were rinsed with PBS and then with water. Smears were checked for fluorescence under microscope and images were captured using Nikon Eclipse 80i microscope (212).

4.2.2.4 Plot of Mean Fluorescence Intensities

Mean intensities of fluorescence of the labeled thrombocytes were measured from zebrafish control and the morphant smears using the NIS-elements AR 2.30 software provided by Nikon. The mean intensity of fluorescence for each FITC labeled thrombocyte from control and GPR18 morphant smears was measured. The means for each treatment were plotted using SigmaPlot software.

4.2.2.5 Plate-Tilt Assay

For this assay, vivo MO was injected into the adult zebrafish and blood was collected from the fish after two days as mentioned above. Microtiter plates were used for this assay. Each well had 9.9 μL of PBS, 0.1 μL of 1:5 dilution of agonist in ethanol) , Arachidonic acid (50mg/200μL ethanol) or N-arachidonyl glycine (5mg/100μL ethanol) and 0.5 μL of 3:1 diluted citrated (3.8 % sodium citrate in PBS) blood. Control MO injected fish blood was used as a

control. After every 5 minutes, the plate was tilted at an angle of 45° to check for aggregation or tight button formation at the bottom of the well. The time when the aggregation occurred was noted for both the wells.

4.2.2.6 Statistical Analyses and Graphs

All the data were analyzed using the SigmaPlot 10 Sigma stat integration software. Graphs were plotted using the same software. Data from the studies were analyzed by One-way ANOVA and t-test.

4.3 Results

4.3.1 Laser Ablations on Larvae of Morpholino Injected Zebrafish Embryos

In order to identify the function of the GPR18, the expression of GPR18 was knocked down in zebrafish embryos by injecting GPR18 MO (5' ACACTTGAGGAACTTCAGTCTCCAT 3'). Control MO (5' CCTCTTACCTCAGTTACAATTTATA 3') was injected in 1-4 cell stage of the embryos. The MO was designed at the translational blocking site. I hypothesized that the injected MO would knockdown the function of the GPR18 protein in the larvae for a transient time. Fig. 4.1 shows the graph of the time to occlusion (TTO) required for GPR18 MO injected embryos and laser ablated larvae assayed for arterial thrombosis. On fifth day post fertilization (5 dpf), the larvae were injured in artery and (TTO) in seconds was recorded for 40 larvae (Fig. 4.1). Data from the TTO study was analyzed by Mann-Whitney Rank Sum test, and statistically significant difference in the TTO was seen between the control and GPR18 MO injected

embryos (Fig. 4.1). The GPR18 morphants showed increase in TTO (120 seconds or more) whereas, the control group larvae indicated a TTO of 50-60 seconds.

4.3.2 Immuno-Staining of Zebrafish Blood Smears

To test the presence of GPR18 receptor in zebrafish blood cells, immuno-staining procedure was used. For this experiment, adult zebrafish were injected with GPR18 MO and control group fish were injected with control MO. Blood was collected after 48 hours post injection and smeared on slides. The slides were probed with rabbit anti-GPR18 polyclonal antibody and FITC-conjugated anti-rabbit IgG as secondary antibody. Thrombocytes from the control blood smear and the GPR18 morphant blood are represented in Fig. 4.3. The mean intensity of fluorescence from 9 different thrombocytes from both groups was measured and the average pixel intensity was plotted. The graph showed significantly more fluorescence in the control group when compared with the morphant group, One-way ANOVA and t-test, ($P = <0.001$), (Fig. 4.4).

4.3.3 Thrombocyte Function Studies

Since NAG is a known agonist for GPR18 I wanted to test whether there is a decreased response of GPR18 morphant thrombocytes to NAG. To check the effect of the above agonist on whole blood aggregation I used plate-tilt assay on control and morphant adult fish blood. For this experiment, I injected two separate groups of 4 zebrafish with GPR18 MO and control MO respectively. After 48 hours post injection, blood was collected from the control and experimental groups and added to conical wells in a microtiter plate containing PBS and NAG.

We also used arachidonic acid as an agonist for positive control. Plate was tilted after every 5 minutes to check for delayed migration of cells over the slope or tight button formation at the bottom of the well. The delayed migration or button formation was used as an indicator of aggregation. No significant difference was seen in the control and morphant values for the time to aggregation (TTA) assay in response to arachidonic acid, P= 0.636. However, when N-arachidonyl glycine (NAG) was used as an agonist, the TTA (in minutes) required for aggregation of the GPR18 morphant blood showed statistically significant prolongation, One-way ANOVA and t-test, P= 0.005 (Fig. 4.2).

4.4 Conclusion

The TTO assay for GPR18 MO injected embryos displayed that the morphant larvae required longer time (TTO) to occlude the artery completely than the controls (Fig. 4.1). This experiment was performed *in vivo* using the arterial thrombosis assay and the laser ablation technique (214, 215). Since arterial thrombosis involved mostly thrombocytes, the prolongation of TTO suggests that thrombocytes are affected when GPR18 levels are reduced by knockdown method, which results in reduced thrombus formation *in vivo*.

Overall, around 90% of the GPCRs are devoid of introns (199). GPR18 has only one exon in its coding sequence. Hence, translational blocking morpholino was designed to knockdown the receptor function as MO microinjections are widely used in deciphering gene functions in the developmental program of zebrafish (207). In this study, GPR18 MO was used to knockdown; however, this study can only suggest that knockdown results in defective

thrombocyte indirectly by the above TTO assay. Therefore, we conducted Vivo-MO knockdowns.

The GPR18 vivo MO knockdown resulted in reduced expression of GPR18. This was assessed by measuring mean intensity of fluorescence of thrombocytes (Fig. 4.4). This measurement was required to demonstrate the reduction of protein due to GPR18 knockdown. Others have used western blots to demonstrate the knockdown of protein. However, in our system it is not only important to demonstrate the reduction in protein levels, but also to show the specificity of this protein in thrombocytes. In particular, since thrombocyte population is very limited, we feel that immunofluorescence technique is more sensitive than Western blots, because it requires a higher number of thrombocytes, and so we preferred this method. Even though we developed a method to purify the thrombocytes, the numbers were sufficient to do the RT-PCRs but may not be sufficient to do Western blots.

Because we used Vivo-MO knockdowns we were able to conduct the thrombocyte function studies by the plate-tilt assay in zebrafish which showed that the adult morphant blood takes more time to aggregate in the presence of NAG when compared with the control fish blood. This experiment not only provided evidence that GPR18 is important for thrombocyte aggregation but also provided a clue that NAG may be a ligand for the GPR18 receptor on thrombocytes in zebrafish.

Table 4.1. Major platelet GPCRs, ligands and functions.

GPCR	Ligand	Function	Reference(s)
PAR (protease activated receptor)Family	Thrombin, Cleaved N-terminal of receptor	Coupled to G13: Activates Rho-GEF, phosphorylates myosin light chain, increases contractility, shape change	(28)
P2Y receptors	ADP	$P2Y_1$ coupled to Gq: Regulates Ca^{++} dependent signals, shape change, GPIIb-IIIa dependent aggregation $P2Y_{12}$ coupled to Gi: Activates GPIIb-IIIa by inhibiting cAMP production	(12, 44)
TP receptor	Thromboxane A2	Coupled to PLCβ via Gq: PLCβ forms IP3 and DAG, increases cytosolic Ca^{++} Activates Rho-GEF, phosphorylates myosin light chain, increases contractility, shape change, platelet aggregation	(28, 216)
IP receptor	PGI2	Stimulates Adenylate cyclase (AC), increases cAMP, Inhibits platelet function	(217)
EP3	Prostaglandins PGE2	Inhibits cAMP, stimulates aggregation	(218, 219)
PAF receptor	PAF 4	Platelet aggregation and many other functions such as cell motility, smooth muscle contraction, release of mediators	(220, 221)
Lysophosphatidic acid (LPA) receptors	Lysophosphatidic acid	LPA1, LPA3 coupled to Gi/Gq: Activate plateletGPR 92 coupled to G12/13, Gq: Activates platelet GPR23 coupled to Gs: Inhibits platelet activation	(117)
5-HT2A	Serotonin	Stimulates platelet activation	(117)
α2A- Adrenergic receptor	Epinephrine	Coupled to Gi member, Gz: Acts synergistically with other agonists to inhibit cAMP, Causes platelet activation	(222)
V1A receptor	Vasopressin	Platelet activation	(117)

Table 4.2. Members of Gα protein family and their roles.

Gα Protein Family Members		Role and Effector Molecules	References
Gs		Activates adenylate cyclase	(223)
		Activates Ca^{++} channels	
		Inhibits sodium channels	
	αolf	Activates adenylate cyclase	
Gi	αi1	Activates potassium channels	(223)
	αi2	Inhibits Ca^{++} channels	
	αi3	Inhibits adenylate cyclase	
		Activates PLC	
		Activates PLA_2	
	αz	Inhibits adenylate cyclase	
		Activates cGMP phosphodiesterase	(224)
Gq	αq	Activates PLCβ1, β2, β3	(223)
	α11		
G12		P115RhoGEF	(224)
	α12	Rho activation, cytoskeletal changes in actin	(225)
	α13	Rho activation, cytoskeletal changes in actin	

Table 4.3. Platelet GPCRs, their ligands and associated G-proteins.

Ligand	GPCR	G-Protein type	References
Epinephrine	α2A adrenergic receptor	G2	(4)
Prostaglandin E2	EP3	Gi	(4)
Serotonin	$5\text{-}HT_{2A}$	Gq	(4)
TXA2 (thomboxane A2)	TPα	Gq	(4), (226)
	TPβ	G12/G13	
ADP	$P2Y_1$	Gq	(226)
	$P2Y_{12}$	Gi2	
Thrombin	PAR1	Gq, G13	(157)
	PAR4	Gq, G13	
Prostacyclin (PGI_2)	IP	Gs	(4)

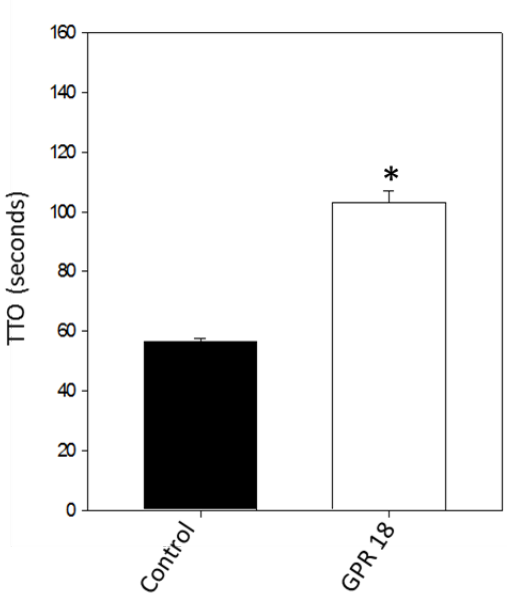

Fig. 4.1. Time to occlusion (TTO) assay for laser ablated GPR18 morphant larvae using *in vivo* arterial thrombosis screen. Control, zebrafish embryos injected with control MO; GPR18, embryos injected with GPR18 morpholino during 1-4 celled stage. On the 5th dpf, larvae from both the groups were laser ablated in the artery between 5th-7th somite region from the anal pore. The time taken to occlude the vessel completely was measured in seconds. The GPR18 morphant larvae showed prolonged TTO as compared with the control group, one-way ANOVA and t-test, *P< 0.0001. The means and SEM are plotted in the graph, n= 42 for each treatment.

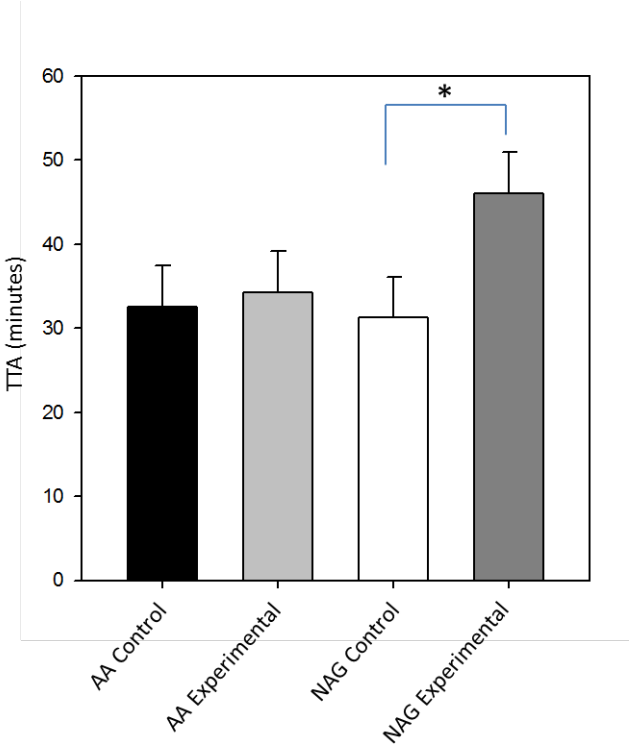

Fig. 4.2. Means of Time to aggregation (TTA) assay for GPR18 morphant blood using AA (Arachidonic acid) and NAG (N-arachidonyl glycine) as agonists. All the bars indicate the time in minutes taken for the control and the experimental blood to aggregate completely. AA Control, the zebrafish injected with control morpholino; AA Experimental, the zebrafish injected with GPR18 vivo morpholino; NAG Control, the zebrafish injected with control morpholino; NAG Experimental, the zebrafish injected with GPR18 vivo morpholino. Statistical comparison against controls was done using one-way ANOVA (AA, P= 0.586 and NAG, P= 0.003) and t-test, *P= 0.0052. GPR18 morphant blood showed significant increase in TTA when compared with control blood TTA in response to NAG. Error bars represent SD, n= 4 for each treatment.

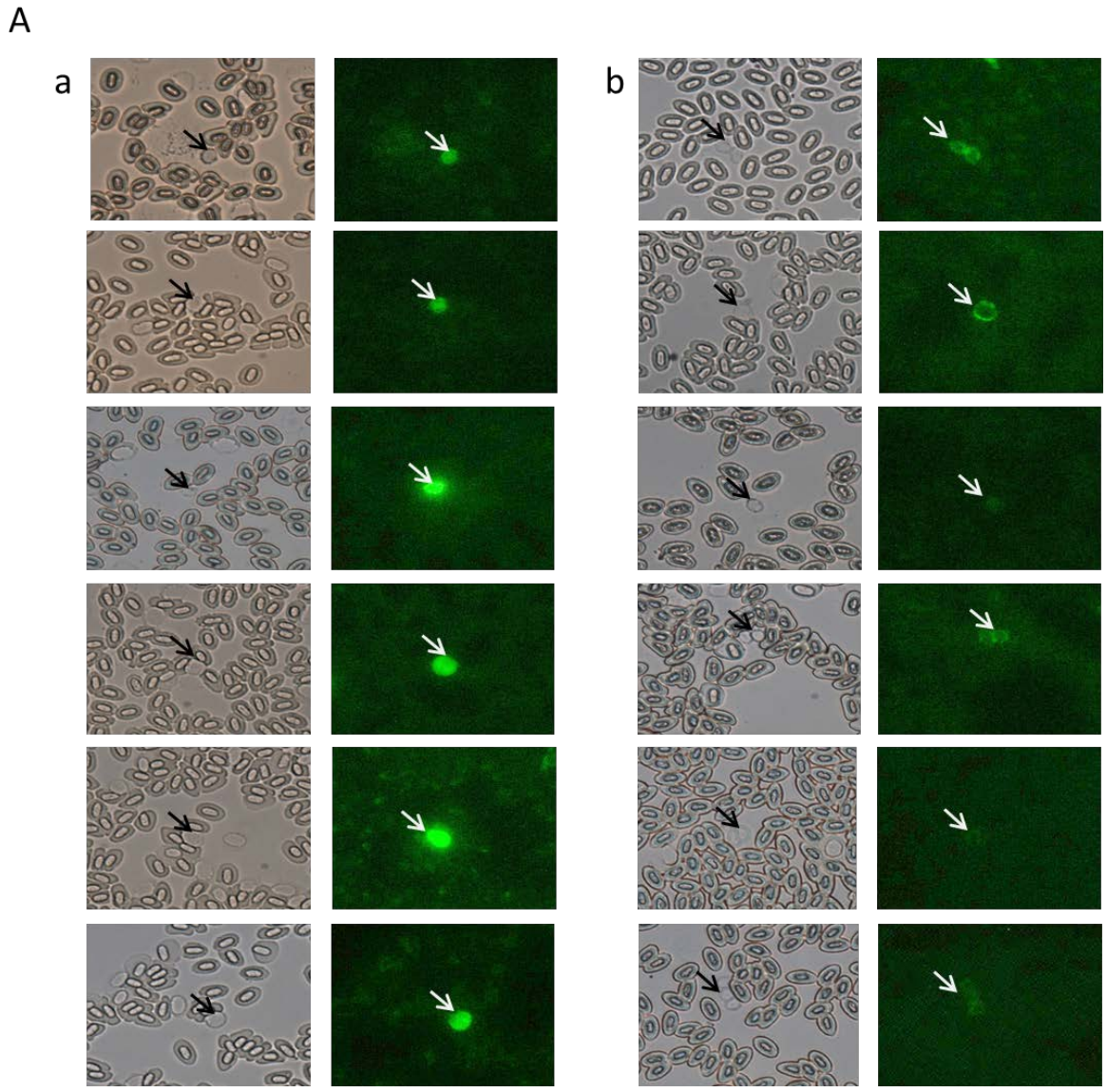

Fig. 4.3. Immuno-staining of the blood smear of the adult zebrafish injected with control morpholino and GPR18 vivo morpholino. Panel (a), representative blood smear images of the control fish injected with control MO; Panel (b), representative blood smear images of the morphant fish injected with GPR18 vivo morpholino. Blood was collected and smeared on slides 48 hours post-injection. Left side of (a) and (b) were images taken under bright light and right side images were taken under fluorescence, respectively. Black and white arrows indicate thrombocytes under bright field and fluorescence thrombocytes labeled with the primary anti-GPR18 rabbit polyclonal antibody and with FITC-conjugated anti-rabbit secondary antibody, respectively. Right side panel (a) images show intense fluorescence when compared with the less intense fluorescence labeling shown by panel (b) images.

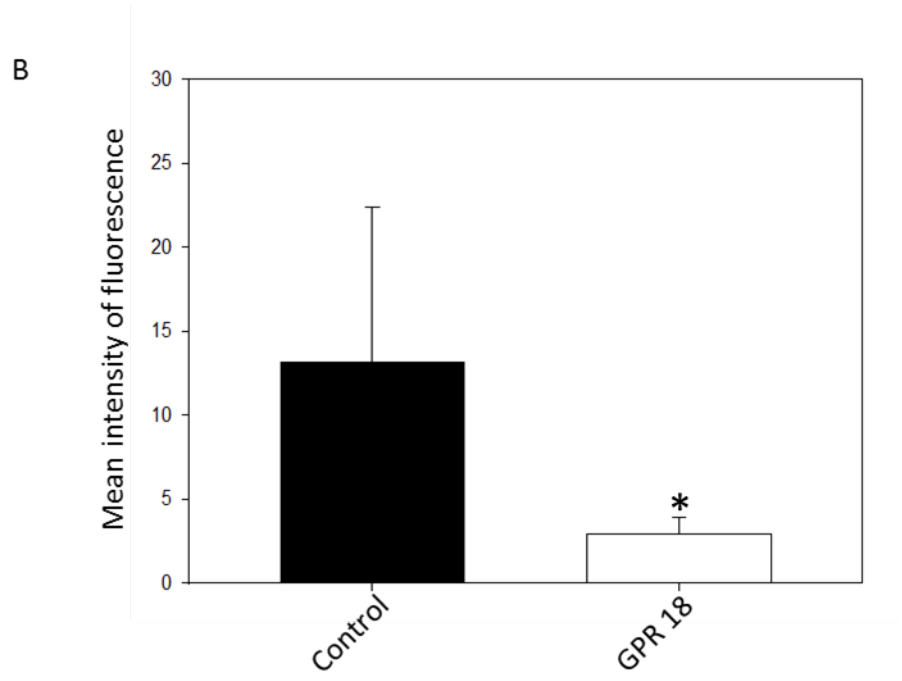

Fig. 4.4. Mean intensities of fluorescence shown by FITC-labeled thrombocytes from control and morphant zebrafish blood from Fig. 4.3. Control, group of fish injected with control MO; GPR18, the morphants injected with GPR18 vivo morpholino. Blood was collected from each group and smeared on slides 48 hours post injections. Slides were stained with anti- GPR18 rabbit antibody and FITC-conjugated anti-rabbit IgG in sheep. Slides were scanned for labeled thrombocytes and images were captured. The mean intensity of fluorescence was measured from these images belonging to each group. The mean intensities were plotted. Labeling intensity was significantly less in the GPR18 group than the control group, one-way ANOVA and t-test, *P= 0.0047. Error bars represent SD, n= 9 per treatment.

CHAPTER 5

DISCUSSION

5.1 Introduction

The focus of this project is the separation of young and mature thrombocytes which further narrows down to knockdown of a G-protein coupled receptor (GPCR), GPR18 in *Danio rerio*. This study has many novel aspects that include a new technique to separate thrombocyte subpopulations. Thrombocyte subpopulations are isolated from zebrafish whole blood for the first time and also the GPCR study on the thrombocytes was conducted for the first time. The study of the GPCRs will not only shed light upon the cell signaling pathways in thrombocytes or platelets and other cells, but also, may lead to the design of novel anti-thrombotic drugs (227-229).

The zebrafish thrombocytes possess all the functional and structural properties of the mammalian platelets except that they are nucleated (20, 151, 160). The thrombocytes also have subpopulations like the platelets (156). In the platelet field, the platelet populations have been separated by centrifugation and flow cytometer. Labeling of the platelets by thiazole orange (TO) dye shows differential labeling between reticulated and old platelets. Young platelets were found to be more active than the older platelets. Whereas, DiI-C18 labels only the young thrombocytes at a low concentration (156). The young thrombocytes have more adhesion and aggregation receptors than the mature thrombocytes (156). Furthermore, *D. rerio* or the zebrafish has been proved to be an ideal model for the study of thrombosis and hemostasis (14, 15). This vertebrate model gives an opportunity to conduct *in vivo* assays with great ease due to the transparent embryos, high fecundity, easy maintenance and forward as

well as reverse genetics (144, 146). With this background, I was interested in understanding the differences between the young and the mature thrombocytes based on their transcriptomes.

5.2 Young and Mature Thrombocytes

5.2.1 Isolation of Young and Mature Thrombocytes

In order to understand the differences between the young and mature thrombocytes, separating them from the blood was necessary. I employed a novel method to separate the thrombocyte populations (Fig. 2.2). In this method I have used anti-Cy3 antibody against the DiI-C18 chromophore on the young thrombocyte. Cy3 and DiI-C18 have very similar structures (Fig. 2.1) and so commercially available biotinylated anti-Cy3 antibody was used. DiI-C18 labeled blood was incubated with the antibody. Then streptavidin-coated magnetic beads that were added to the blood, attached to the biotin antibody and also to young thrombocyte. These beads were separated from the rest of the cells when the tube was kept on a magnetic stand. When the beads were pulled to a side of the tube (due to magnetic attraction), the other blood cells in suspension were aspirated and incubated with the anti-GPIIb antibody to separate the mature thrombocytes in a similar way (163). The thrombocytes isolated by this method were extremely pure. I showed this by extracting RNA from each population and amplifying it using the blood cells specific markers (Fig. 2.5, Fig. 2.6).

This technique is quick, easy to follow, and does not require fancy instruments. Moreover, it is specific and yields almost pure cell populations. This method can be utilized to analyze the reticulated platelets in cases where a lesser amount of blood is available (0.1 to 1.5 ml), such as is the case with infants (230). Another application would be to study thrombocyte

maturation and the changes that occur in the nucleus or the membrane when the thrombocytes mature in the circulation. Interestingly, young and mature thrombocytes form independent clusters while forming a hemostatic plug at the site of injury (156). This opens another area wherein one could study the homotypic and heterotypic interactions between thrombocytes.

5.2.2 Comparison of Receptors of Young and Mature Thrombocytes

For the comparison of properties of young and mature thrombocytes, there are many parameters that one could compare. Because of the extensive scope I limited my studies to compare the levels of GPCR transcripts between young and mature thrombocytes. The reason for selecting the thrombocyte GPCRs is that they play a crucial role in signaling. Platelet activation, downstream signaling inside the cells for secretion events is majorly dependent upon several GPCRs. These events constitute the basis of thrombus formation, and aggregation and also the dissolution of the fibrin clots.

RNA samples prepared from the young and mature thrombocyte populations that were procured from the little amounts of blood collected from the zebrafish were used to perform RT-PCRs. Since the RNA extracted by this method was precious, I used whole blood RNA first to check for the presence of GPCRs in whole blood. All the GPCRs from the zebrafish genome were selected and primers were designed to amplify products of 600 base pairs for each of them (Table 3.1). The GPCRs showing positive results were chosen (Fig. 3.1) and new primers were designed to give products from 200 to 220 base pairs. To further narrow down my study, I centrifuged the zebrafish blood at low speeds to obtain the total white cell layer and prepared

RNA from the same. I found that the total white cell RNA was amplified by some of the GPCR primers (Fig. 3.2). Then, only the GPCRs showing amplifications with the total white cells were used for amplifying the young and the mature thrombocyte RNA. These bands showed differential amplifications in the young and the mature transcripts (Fig. 3.3). My findings suggest that there are no significant differences in the RNA expression patterns of these thrombocytes during maturations (Fig. 3.4). These results suggested that differential expression of receptors between young and mature thrombocytes observed earlier may be due to the post translational regulation. Further detailed studies would be required to support this finding. Nevertheless, our data certainly ruled out the regulation of receptors at the mRNA level.

5.3 Knockdown of GPR18

Despite the lack of differences in levels of GPCR transcripts between young and mature thrombocytes our results revealed at least one novel GPCR in thrombocytes. Since one of these GPCRs also has a novel ligand, NAG, I chose this GPR18 to knock it down in the zebrafish to understand its role in thrombocyte function.

The knockdown of GPR18 was accomplished by injecting morpholino (MO) designed specifically against the GPR18 into 1-4 cell stage embryos. The GPR18 MO injected and control MO injected larvae were screened for TTO on the 5 dpf by laser ablations of the artery. Time required to block the arterial vessel by the thrombus (TTO) was measured in seconds. The results of these studies showed that the morphant larvae showed an increase (average 120 seconds or more) in the TTO, suggesting its role in thrombocyte aggregation indirectly because arterial thrombus is almost all thrombocytes.

The GPR18 cDNA sequence was obtained from the Ensembl online database (Zv9) and translational blocking morpholino oligos were constructed by the Gene Tools LLC. The GPR18 coding region consists of a single exon like most other GPCRs. So splice blocking morpholinos could not be used against the GPR18 gene to achieve the knockdown of the protein.

Since we cannot study any biochemistry of thrombocytes using arterial thrombosis assay, the knockdown of the GPR18 receptor was studied in the adult zebrafish utilizing the immuno-fluorescence studies. The GPR18 vivo morpholino was injected into the adults and blood was collected from the zebrafish after 48 hours to make smears. The smears were incubated with the anti- GPR18 polyclonal antibody in rabbits and then probed with the FITC-conjugated anti-rabbit IgG in sheep as secondary antibody. The thrombocytes from morphant blood smears showed less intensity as compared with the control group smears (Fig. 4.3, Fig. 4.4). The diminished mean fluorescence intensity observed in the morphant thrombocytes suggests that the GPR18 protein was effectively knocked down in the adult zebrafish.

5.4 Ligand Studies for GPR18

In zebrafish, the ligand studies for GPR18 were conducted by utilizing the plate-tilt method of measuring *in vitro* aggregation, that was developed in our laboratory (212). The results of these studies with arachidonic acid (AA) agonist, showed no statistically significant difference between the control and the experimental, whereas the N-arachidonyl glycine (NAG) did show a statistically significant difference (Fig. 4.2). This suggests that the AA (820 µM) did not affect the aggregation of thrombocytes from the zebrafish that were injected with the GPR18 vivo MO. The GPR18 vivo MO injected zebrafish thrombocytes, when compared with the

control, showed prolongation of aggregation time (TTA) in response to the NAG (552 µM) (Fig. 4.2) suggesting that NAG acts as a ligand for the GPR18 receptor. These data also confirm the presence of GPR18 receptor on the thrombocytes and that GPR18 contributes to the aggregation reaction.

5.5 Summary

I have developed a new technique to separate the young and mature thrombocytes from the zebrafish whole blood. Then I prepared RNA from the two populations of thrombocytes and compared them qualitatively using RT-PCR, gel electrophoresis and densitometry analysis. On comparing the transcripts from the two populations, amplified by GPCR primers, I found no differential expression of them. I further knocked down one of the GPCRs, GPR18, to characterize its role in thrombosis and hemostasis. The GPR18 studies show that the receptor is activated in response to N-arachidonyl glycine (NAG) agonist and brings about the activation and aggregation processes in zebrafish thrombocytes.

5.6 Future Perspectives

5.7 Translational Studies

From the GPR18 knockdown studies, GPR18 was shown to be present in zebrafish thrombocytes, and that, it has a role in thrombocyte aggregation. To investigate whether GPR18 is present in platelets or if it plays a role in human platelet aggregation, and to translate our findings to humans, this work can be extended to platelets. In a similar fashion our finding that, NAG is an agonist for GPR18 in thrombocytes can also be used to test whether NAG is a

human platelet activator and the pathway involved. Altogether, the results of the thrombocyte experiments could be translated to human platelets and therefore, should have clinical applications.

Though we understand the mechanisms of platelet activation and aggregation, secretion and shape change in some details, many of the gaps in our understanding of the blue prints of signaling cascades still need to be filled. Our finding of NAG induced GPR18 mediated pathway is one of these gaps and studying this signaling pathway in platelets will not only help in understanding the pathways in other cell types but will also render useful information for designing novel anti-thrombotic drugs.

The separation of young and mature thrombocytes in the present work will initiate new studies on signaling pathways operating in the young and mature thrombocytes. Knockdown approaches can be used to understand the signaling in the thrombocytes. Inhibitors to agonists, receptors or some of the downstream effectors or enzymes that regulate the signaling cascades can be used to understand more about the receptors and their ligands. Furthermore, studies on GPR18, as well as other thrombocyte GPCRs, could be translated to mammalian platelets. Using the human genome sequence, single nucleotide polymorphisms (SNPs) or mutations could be identified using the human genome sequences and could contribute to the knowledge of platelet disorders, for which, molecular basis is not known. Finally, new anti-thrombotic drugs could be designed from the knowledge gleaned from this information.

BIBLIOGRAPHY

1. Virchow R (1856) in Gesammelte Abhandlungen zur wissenschaftlichen Medicin. *Medinger Sohn & Co. Frankfurt aM,* :219-732.

2. Mackman N, Tilley RE, & Key NS (2007) Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arteriosclerosis, thrombosis, and vascular biology* 27(8):1687-1693.

3. Dutting S, Bender M, & Nieswandt B (2012) Platelet GPVI: a target for antithrombotic therapy?! *Trends in pharmacological sciences* 33(11):583-590.

4. Stegner D & Nieswandt B (2011) Platelet receptor signaling in thrombus formation. *J Mol Med (Berl)* 89(2):109-121.

5. Ruggeri ZM (2002) Platelets in atherothrombosis. *Nature medicine* 8(11):1227-1234.

6. Halushka PV, Mais DE, Mayeux PR, & Morinelli TA (1989) Thromboxane, prostaglandin and leukotriene receptors. *Annual review of pharmacology and toxicology* 29:213-239.

7. Norris LA (2003) Blood coagulation. *Best practice & research. Clinical obstetrics & gynaecology* 17(3):369-383.

8. Coller BS, Peerschke EI, Scudder LE, & Sullivan CA (1983) A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. *The Journal of clinical investigation* 72(1):325-338.

9. Coller BS & Shattil SJ (2008) The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood* 112(8):3011-3025.

10. Peerschke EI (1985) The platelet fibrinogen receptor. *Seminars in hematology* 22(4):241-259.

11. Macfarlane RG (1964) An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier. *Nature* 202:498-499.

12. Jung SM & Moroi M (2000) Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets. *Trends in cardiovascular medicine* 10(7):285-292.

13. Austin SK (2009) Haemostasis. *Medicine* 37(3):133-136.

14. Jagadeeswaran P (2005) Zebrafish: a tool to study hemostasis and thrombosis. *Curr Opin Hematol* 12(2):149-152.

15. Jagadeeswaran P, Gregory M, Day K, Cykowski M, & Thattaliyath B (2005) Zebrafish: a genetic model for hemostasis and thrombosis. *J Thromb Haemost* 3(1):46-53.

16. Clutton P, Folts JD, & Freedman JE (2001) Pharmacological control of platelet function. *Pharmacological research : the official journal of the Italian Pharmacological Society* 44(4):255-264.

17. de Graaf JC*, et al.* (1992) Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* 85(6):2284-2290.

18. Radomski MW, Palmer RM, & Moncada S (1987) The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *British journal of pharmacology* 92(3):639-646.

19. Moncada S, Herman AG, Higgs EA, & Vane JR (1977) Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. *Thrombosis research* 11(3):323-344.

20. Lang MR, Gihr G, Gawaz MP, & Muller, II (2010) Hemostasis in Danio rerio: is the zebrafish a useful model for platelet research? *J Thromb Haemost* 8(6):1159-1169.

21. Blann AD & Lip GY (2001) Virchow's triad revisited: the importance of soluble coagulation factors, the endothelium, and platelets. *Thrombosis research* 101(4):321-327.

22. Furie B & Furie BC (2007) In vivo thrombus formation. *J Thromb Haemost* 5 Suppl 1:12-17.

23. Italiano JE, Jr., Lecine P, Shivdasani RA, & Hartwig JH (1999) Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* 147(6):1299-1312.

24. Italiano JE, Jr., Patel-Hett S, & Hartwig JH (2007) Mechanics of proplatelet elaboration. *J Thromb Haemost* 5 Suppl 1:18-23.

25. Thon JN & Italiano JE (2010) Platelet formation. *Seminars in hematology* 47(3):220-226.

26. Shapiro AD (2000) Platelet function disorders. *Haemophilia : the official journal of the World Federation of Hemophilia* 6 Suppl 1:120-127.

27. Brewer DB (2006) Max Schultze (1865), G. Bizzozero (1882) and the discovery of the platelet. *Br J Haematol* 133(3):251-258.

28. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, & Deckmyn H (2011) Platelets at work in primary hemostasis. *Blood reviews* 25(4):155-167.

29. Weyrich AS, Lindemann S, & Zimmerman GA (2003) The evolving role of platelets in inflammation. *J Thromb Haemost* 1(9):1897-1905.

30. Ault KA (1993) Flow cytometric measurement of platelet function and reticulated platelets. *Ann N Y Acad Sci* 677:293-308.

31. Robinson MS*, et al.* (1998) Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. *Br J Haematol* 100(2):351-357.

32. Fujii T, Shimomura T, Fujimoto TT, Kimura A, & Fujimura K (2000) A new approach to detect reticulated platelets stained with thiazole orange in thrombocytopenic patients. *Thrombosis research* 97(6):431-440.

33. Maruyama H*, et al.* (2009) Reticulated platelet levels in whole blood and platelet-rich plasma of dogs with various platelet counts measured by flow cytometry. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 71(2):195-197.

34. Parco S & Vascotto F (2012) Application of reticulated platelets to transfusion management during autologous stem cell transplantation. *OncoTargets and therapy* 5:1-5.

35. Karpatkin S (1969) Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. *The Journal of clinical investigation* 48(6):1073-1082.

36. Blajchman MA, Senyi AF, Hirsh J, Genton E, & George JN (1981) Hemostatic function, survival, and membrane glycoprotein changes in young versus old rabbit platelets. *The Journal of clinical investigation* 68(5):1289-1294.

37. Kelton JG & Denomme G (1982) The quantitation of platelet-associated IgG on cohorts of platelets separated from healthy individuals by buoyant density centrifugation. *Blood* 60(1):136-139.

38. Moheimani F & Jackson DE (2012) P2Y12 receptor: platelet thrombus formation and medical interventions. *International journal of hematology* 96(5):572-587.

39. Chow TW, Hellums JD, Moake JL, & Kroll MH (1992) Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* 80(1):113-120.

40. Andrews RK, Gardiner EE, Shen Y, Whisstock JC, & Berndt MC (2003) Glycoprotein Ib-IX-V. *The international journal of biochemistry & cell biology* 35(8):1170-1174.

41. Du X, Beutler L, Ruan C, Castaldi PA, & Berndt MC (1987) Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. *Blood* 69(5):1524-1527.

42. Modderman PW, Admiraal LG, Sonnenberg A, & von dem Borne AE (1992) Glycoproteins V and Ib-IX form a noncovalent complex in the platelet membrane. *J Biol Chem* 267(1):364-369.

43. Lopez JA*, et al.* (1994) Glycoprotein (GP) Ib beta is the critical subunit linking GP Ib alpha and GP IX in the GP Ib-IX complex. Analysis of partial complexes. *J Biol Chem* 269(38):23716-23721.

44. Andrews RK & Berndt MC (2004) Platelet physiology and thrombosis. *Thrombosis research* 114(5-6):447-453.

45. Du X, Harris SJ, Tetaz TJ, Ginsberg MH, & Berndt MC (1994) Association of a phospholipase A2 (14-3-3 protein) with the platelet glycoprotein Ib-IX complex. *J Biol Chem* 269(28):18287-18290.

46. Du X, Fox JE, & Pei S (1996) Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *J Biol Chem* 271(13):7362-7367.

47. Gu M, Xi X, Englund GD, Berndt MC, & Du X (1999) Analysis of the roles of 14-3-3 in the platelet glycoprotein Ib-IX-mediated activation of integrin alpha(IIb)beta(3) using a reconstituted mammalian cell expression model. *J Cell Biol* 147(5):1085-1096.

48. Rand ML, Leung R, & Packham MA (2003) Platelet function assays. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 28(3):307-317.

49. Simon D, Kunicki T, & Nugent D (2008) Platelet function defects. *Haemophilia : the official journal of the World Federation of Hemophilia* 14(6):1240-1249.

50. Howard MA, Hutton RA, & Hardisty RM (1973) Hereditary giant platelet syndrome: a disorder of a new aspect of platelet function. *British medical journal* 2(5866):586-588.

51. Caen JP & Levy-Toledano S (1973) Interaction between platelets and von Willebrand factor provides a new scheme for primary haemostasis. *Nature: New biology* 244(135):159-160.

52. Moroi M, Jung SM, Okuma M, & Shinmyozu K (1989) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *The Journal of clinical investigation* 84(5):1440-1445.

53. Tsuji M, Ezumi Y, Arai M, & Takayama H (1997) A novel association of Fc receptor gamma-chain with glycoprotein VI and their co-expression as a collagen receptor in human platelets. *J Biol Chem* 272(38):23528-23531.

54. Jandrot-Perrus M*, et al.* (2000) Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. *Blood* 96(5):1798-1807.

55. Moroi M & Jung SM (2004) Platelet glycoprotein VI: its structure and function. *Thrombosis research* 114(4):221-233.

56. Sugiyama T*, et al.* (1987) A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood* 69(6):1712-1720.

57. Jung SM & Moroi M (1998) Platelets interact with soluble and insoluble collagens through characteristically different reactions. *J Biol Chem* 273(24):14827-14837.

58. Chen H & Kahn ML (2003) Reciprocal signaling by integrin and nonintegrin receptors during collagen activation of platelets. *Molecular and cellular biology* 23(14):4764-4777.

59. Kehrel B*, et al.* (1988) Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood* 71(4):1074-1078.

60. Savage B, Shattil SJ, & Ruggeri ZM (1992) Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *J Biol Chem* 267(16):11300-11306.

61. Wagner CL*, et al.* (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood* 88(3):907-914.

62. Bennett JS (2005) Structure and function of the platelet integrin alphaIIbbeta3. *The Journal of clinical investigation* 115(12):3363-3369.

63. George JN, Caen JP, & Nurden AT (1990) Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood* 75(7):1383-1395.

64. Uhrin P*, et al.* (2010) Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. *Blood* 115(19):3997-4005.

65. Manne BK*, et al.* (2013) Fucoidan is a novel platelet agonist for the CLEC-2 receptor. *J Biol Chem*.

66. Stenkamp RE, Teller DC, & Palczewski K (2002) Crystal structure of rhodopsin: a G-protein-coupled receptor. *Chembiochem : a European journal of chemical biology* 3(10):963-967.

67. Palczewski K*, et al.* (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289(5480):739-745.

68. Latek D, Modzelewska A, Trzaskowski B, Palczewski K, & Filipek S (2012) G protein-coupled receptors - recent advances. *Acta biochimica Polonica* 59(4):515-529.

69. Kobilka BK*, et al.* (1987) An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. *Nature* 329(6134):75-79.

70. Shorr RG, Lefkowitz RJ, & Caron MG (1981) Purification of the beta-adrenergic receptor. Identification of the hormone binding subunit. *J Biol Chem* 256(11):5820-5826.

71. Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* 273(30):18677-18680.

72. Tang XL, Wang Y, Li DL, Luo J, & Liu MY (2012) Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta pharmacologica Sinica* 33(3):363-371.

73. Qin Y*, et al.* (2011) Quantitative expression profiling of G-protein-coupled receptors (GPCRs) in metastatic melanoma: the constitutively active orphan GPCR GPR18 as novel drug target. *Pigment cell & melanoma research* 24(1):207-218.

74. Muller A*, et al.* (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410(6824):50-56.

75. Robledo MM*, et al.* (2001) Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. *J Biol Chem* 276(48):45098-45105.

76. Schlyer S & Horuk R (2006) I want a new drug: G-protein-coupled receptors in drug development. *Drug discovery today* 11(11-12):481-493.

77. Vu TK, Hung DT, Wheaton VI, & Coughlin SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64(6):1057-1068.

78. Ishihara H*, et al.* (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 386(6624):502-506.

79. Coughlin SR (2000) Thrombin signalling and protease-activated receptors. *Nature* 407(6801):258-264.

80. Xu WF*, et al.* (1998) Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A* 95(12):6642-6646.

81. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, & Coughlin SR (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation* 103(6):879-887.

82. Gachet C (2012) P2Y(12) receptors in platelets and other hematopoietic and non-hematopoietic cells. *Purinergic signalling* 8(3):609-619.

83. Babich M, King KL, & Nissenson RA (1990) Thrombin stimulates inositol phosphate production and intracellular free calcium by a pertussis toxin-insensitive mechanism in osteosarcoma cells. *Endocrinology* 126(2):948-954.

84. Brass LF, Manning DR, Williams AG, Woolkalis MJ, & Poncz M (1991) Receptor and G protein-mediated responses to thrombin in HEL cells. *J Biol Chem* 266(2):958-965.

85. Baffy G, Yang L, Raj S, Manning DR, & Williamson JR (1994) G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *J Biol Chem* 269(11):8483-8487.

86. Offermanns S, Laugwitz KL, Spicher K, & Schultz G (1994) G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. *Proc Natl Acad Sci U S A* 91(2):504-508.

87. Xu H, Echemendia N, Chen S, & Lin F (2011) Identification and expression patterns of members of the protease-activated receptor (PAR) gene family during zebrafish development. *Developmental dynamics : an official publication of the American Association of Anatomists* 240(1):278-287.

88. Connolly AJ, Ishihara H, Kahn ML, Farese RV, Jr., & Coughlin SR (1996) Role of the thrombin receptor in development and evidence for a second receptor. *Nature* 381(6582):516-519.

89. Trejo J (2003) Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *The Journal of pharmacology and experimental therapeutics* 307(2):437-442.

90. Vandendries ER, Hamilton JR, Coughlin SR, Furie B, & Furie BC (2007) Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. *Proc Natl Acad Sci U S A* 104(1):288-292.

91. Kuliopulos A & Covic L (2003) Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. *Life sciences* 74(2-3):255-262.

92. Krotz F, Sohn HY, & Klauss V (2008) Antiplatelet drugs in cardiological practice: established strategies and new developments. *Vascular health and risk management* 4(3):637-645.

93. Baurand A*, et al.* (2001) Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. *European journal of pharmacology* 412(3):213-221.

94. Ohlmann P*, et al.* (2010) Quantification of recombinant and platelet P2Y(1) receptors utilizing a [(125)I]-labeled high-affinity antagonist 2-iodo-N(6)-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([(125)I]MRS2500). *Pharmacological research : the official journal of the Italian Pharmacological Society* 62(4):344-351.

95. Cattaneo M & Gachet C (1999) ADP receptors and clinical bleeding disorders. *Arteriosclerosis, thrombosis, and vascular biology* 19(10):2281-2285.

96. Hollopeter G*, et al.* (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 409(6817):202-207.

97. Dorsam RT & Kunapuli SP (2004) Central role of the P2Y12 receptor in platelet activation. *The Journal of clinical investigation* 113(3):340-345.

98. Kahner BN, Shankar H, Murugappan S, Prasad GL, & Kunapuli SP (2006) Nucleotide receptor signaling in platelets. *J Thromb Haemost* 4(11):2317-2326.

99. Quinton TM, Murugappan S, Kim S, Jin J, & Kunapuli SP (2004) Different G protein-coupled signaling pathways are involved in alpha granule release from human platelets. *J Thromb Haemost* 2(6):978-984.

100. Cosemans JM*, et al.* (2006) Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood* 108(9):3045-3052.

101. Gachet C (2001) ADP receptors of platelets and their inhibition. *Thrombosis and haemostasis* 86(1):222-232.

102. Hirata M*, et al.* (1991) Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature* 349(6310):617-620.

103. Kim SO*, et al.* (1992) Purification of the human blood platelet thromboxane A2/prostaglandin H2 receptor protein. *Biochemical pharmacology* 43(2):313-322.

104. Anonymous (1998) Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. The Medical Research Council's General Practice Research Framework. *Lancet* 351(9098):233-241.

105. Belhassen L, Pelle G, Dubois-Rande JL, & Adnot S (2003) Improved endothelial function by the thromboxane A2 receptor antagonist S 18886 in patients with coronary artery disease treated with aspirin. *Journal of the American College of Cardiology* 41(7):1198-1204.

106. Toki Y*, et al.* (1988) Myocardial salvage by a novel thromboxane A2 synthetase inhibitor in a canine coronary occlusion-reperfusion model. *Arzneimittel-Forschung* 38(2):224-227.

107. Patrono C (1994) Aspirin as an antiplatelet drug. *The New England journal of medicine* 330(18):1287-1294.

108. Ogletree ML (1987) Overview of physiological and pathophysiological effects of thromboxane A2. *Federation proceedings* 46(1):133-138.

109. Brass LF, Manning DR, Cichowski K, & Abrams CS (1997) Signaling through G proteins in platelets: to the integrins and beyond. *Thrombosis and haemostasis* 78(1):581-589.

110. Kinsella BT, O'Mahony DJ, & Fitzgerald GA (1997) The human thromboxane A2 receptor alpha isoform (TP alpha) functionally couples to the G proteins Gq and G11 in vivo and is activated by the isoprostane 8-epi prostaglandin F2 alpha. *The Journal of pharmacology and experimental therapeutics* 281(2):957-964.

111. Berridge MJ & Irvine RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312(5992):315-321.

112. Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* 308(5961):693-698.

113. Yang J*, et al.* (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci U S A* 97(18):9984-9989.

114. Shah BH*, et al.* (1999) Co-activation of Gi and Gq proteins exerts synergistic effect on human platelet aggregation through activation of phospholipase C and Ca2+ signalling pathways. *Experimental & molecular medicine* 31(1):42-46.

115. Baumgartner HR & Born GV (1968) Effects of 5-hydroxytryptamine on platelet aggregation. *Nature* 218(5137):137-141.

116. Roevens P & de Chaffoy de Courcelles D (1995) Desensitization and resensitization of human platelets to 5-hydroxytryptamine at the level of signal transduction. *The Biochemical journal* 307 (Pt 3):775-782.

117. Amisten S, Braun OO, Bengtsson A, & Erlinge D (2008) Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thrombosis research* 122(1):47-57.

118. Clemetson KJ (1999) Primary haemostasis: sticky fingers cement the relationship. *Current biology : CB* 9(3):R110-112.

119. Roth GJ (1992) Platelets and blood vessels: the adhesion event. *Immunology today* 13(3):100-105.

120. Sadler JE (1998) Biochemistry and genetics of von Willebrand factor. *Annual review of biochemistry* 67:395-424.

121. Franchini M & Lippi G (2006) Von Willebrand factor and thrombosis. *Annals of hematology* 85(7):415-423.

122. Luo GP, Ni B, Yang X, & Wu YZ (2012) von Willebrand factor: more than a regulator of hemostasis and thrombosis. *Acta haematologica* 128(3):158-169.

123. Ruggeri ZM & Mendolicchio GL (2007) Adhesion mechanisms in platelet function. *Circulation research* 100(12):1673-1685.

124. Ruggeri ZM & Zimmerman TS (1987) von Willebrand factor and von Willebrand disease. *Blood* 70(4):895-904.

125. J. Lilleyman IH, V. Blanchette (Eds) (1999) *Pediatric Hematology (2nd ed.)* (Churchill Livingstone).

126. Nieuwenhuis HK, Akkerman JW, Houdijk WP, & Sixma JJ (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* 318(6045):470-472.

127. Savage B, Saldivar E, & Ruggeri ZM (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* 84(2):289-297.

128. Jurk K*, et al.* (2003) Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 17(11):1490-1492.

129. Nigatu A*, et al.* (2006) Megakaryocytic cells synthesize and platelets secrete alpha5-laminins, and the endothelial laminin isoform laminin 10 (alpha5beta1gamma1) strongly promotes adhesion but not activation of platelets. *Thrombosis and haemostasis* 95(1):85-93.

130. Inoue O*, et al.* (2006) Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI. *Blood* 107(4):1405-1412.

131. Naik UP, Patel PM, & Parise LV (1997) Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J Biol Chem* 272(8):4651-4654.

132. Naik UP & Naik MU (2003) Association of CIB with GPIIb/IIIa during outside-in signaling is required for platelet spreading on fibrinogen. *Blood* 102(4):1355-1362.

133. Calderwood DA*, et al.* (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J Biol Chem* 274(40):28071-28074.

134. Haling JR, Monkley SJ, Critchley DR, & Petrich BG (2011) Talin-dependent integrin activation is required for fibrin clot retraction by platelets. *Blood* 117(5):1719-1722.

135. Montanez E*, et al.* (2008) Kindlin-2 controls bidirectional signaling of integrins. *Genes & development* 22(10):1325-1330.

136. Moser M, Nieswandt B, Ussar S, Pozgajova M, & Fassler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nature medicine* 14(3):325-330.

137. Huebsch LB & Harker LA (1981) Disorders of platelet function: mechanisms, diagnosis and management. *The Western journal of medicine* 134(2):109-127.

138. Michelson AD (2011) Advances in antiplatelet therapy. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* 2011:62-69.

139. Fredholm BB, Hokfelt T, & Milligan G (2007) G-protein-coupled receptors: an update. *Acta Physiol (Oxf)* 190(1):3-7.

140. Wu KK, Hall ER, & Papp A (1984) Modulation of vascular thrombosis by products of arachidonic acid metabolism. *Clinical physiology and biochemistry* 2(2-3):84-90.

141. Ware J, Russell S, & Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. *Proc Natl Acad Sci U S A* 97(6):2803-2808.

142. Hodivala-Dilke KM*, et al.* (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *The Journal of clinical investigation* 103(2):229-238.

143. Graham GJ*, et al.* (2009) Endobrevin/VAMP-8-dependent dense granule release mediates thrombus formation in vivo. *Blood* 114(5):1083-1090.

144. Patton EE & Zon LI (2001) The art and design of genetic screens: zebrafish. *Nature reviews. Genetics* 2(12):956-966.

145. Amatruda JF & Zon LI (1999) Dissecting hematopoiesis and disease using the zebrafish. *Dev Biol* 216(1):1-15.

146. Dooley K & Zon LI (2000) Zebrafish: a model system for the study of human disease. *Current opinion in genetics & development* 10(3):252-256.

147. Berman J, Hsu K, & Look AT (2003) Zebrafish as a model organism for blood diseases. *Br J Haematol* 123(4):568-576.

148. Jagadeeswaran P & Sheehan JP (1999) Analysis of blood coagulation in the zebrafish. *Blood Cells Mol Dis* 25(3-4):239-249.

149. Jagadeeswaran P, Liu YC, & Eddy CA (1997) Effects of hirudin (thrombin specific inhibitor) in zebrafish embryos: a developmental role for thrombin. *Blood Cells Mol Dis* 23(3):410-414.

150. Sheehan J*, et al.* (2001) Demonstration of the extrinsic coagulation pathway in teleostei: identification of zebrafish coagulation factor VII. *Proc Natl Acad Sci U S A* 98(15):8768-8773.

151. Jagadeeswaran P, Sheehan JP, Craig FE, & Troyer D (1999) Identification and characterization of zebrafish thrombocytes. *Br J Haematol* 107(4):731-738.

152. Gregory M, Hanumanthaiah R, & Jagadeeswaran P (2002) Genetic analysis of hemostasis and thrombosis using vascular occlusion. *Blood Cells Mol Dis* 29(3):286-295.

153. Williams CM & Poole AW (2012) Using zebrafish (Danio rerio) to assess gene function in thrombus formation. *Methods Mol Biol* 788:305-319.

154. Carrillo M, Kim S, Rajpurohit SK, Kulkarni V, & Jagadeeswaran P (2010) Zebrafish von Willebrand factor. *Blood Cells Mol Dis* 45(4):326-333.

155. Jagadeeswaran P, Kulkarni V, Carrillo M, & Kim S (2007) Zebrafish: from hematology to hydrology. *J Thromb Haemost* 5 Suppl 1:300-304.

156. Thattaliyath B, Cykowski M, & Jagadeeswaran P (2005) Young thrombocytes initiate the formation of arterial thrombi in zebrafish. *Blood* 106(1):118-124.

157. Li Z, Delaney MK, O'Brien KA, & Du X (2010) Signaling during platelet adhesion and activation. *Arteriosclerosis, thrombosis, and vascular biology* 30(12):2341-2349.

158. Grabher C*, et al.* (2011) Zebrafish microRNA-126 determines hematopoietic cell fate through c-Myb. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K* 25(3):506-514.

159. Khandekar G, Kim S, & Jagadeeswaran P (2012) Zebrafish thrombocytes: functions and origins. *Adv Hematol* 2012:857058.

160. Gregory M & Jagadeeswaran P (2002) Selective labeling of zebrafish thrombocytes: quantitation of thrombocyte function and detection during development. *Blood Cells Mol Dis* 28(3):418-427.

161. Thompson CB, Jakubowski JA, Quinn PG, Deykin D, & Valeri CR (1984) Platelet size and age determine platelet function independently. *Blood* 63(6):1372-1375.

162. Mezzano D, Aranda E, & Foradori A (1986) Comparative study of size, total protein, fibrinogen and 5-HT content of human and canine platelet density subpopulations. *Thrombosis and haemostasis* 56(3):288-292.

163. Kulkarni V, Kim S, Zafreen L, & Jagadeeswaran P (2012) Separation of young and mature thrombocytes by a novel immuno-selection method. *Blood Cells Mol Dis* 48(3):183-187.

164. Lin HF*, et al.* (2005) Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood* 106(12):3803-3810.

165. Lieschke GJ*, et al.* (2002) Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. *Dev Biol* 246(2):274-295.

166. Chan FY*, et al.* (1997) Characterization of adult alpha- and beta-globin genes in the zebrafish. *Blood* 89(2):688-700.

167. Joo SJ (2012) Mechanisms of Platelet Activation and Integrin alphaIIbeta3. *Korean circulation journal* 42(5):295-301.

168. Karpatkin S (1969) Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. *The Journal of clinical investigation* 48(6):1083-1087.

169. Thompson CB, Love DG, Quinn PG, & Valeri CR (1983) Platelet size does not correlate with platelet age. *Blood* 62(2):487-494.

170. Kempton CL, Hoffman M, Roberts HR, & Monroe DM (2005) Platelet heterogeneity: variation in coagulation complexes on platelet subpopulations. *Arteriosclerosis, thrombosis, and vascular biology* 25(4):861-866.

171. Alberio L, Safa O, Clemetson KJ, Esmon CT, & Dale GL (2000) Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood* 95(5):1694-1702.

172. Jagadeeswaran P, Lin S, Weinstein B, Hutson A, & Kim S (2010) Loss of GATA1 and gain of FLI1 expression during thrombocyte maturation. *Blood Cells Mol Dis* 44(3):175-180.

173. Nordnes S, Krauss S, & Johansen T (1994) cDNA sequence of zebrafish (Brachydanio rerio) translation elongation factor-1 alpha: molecular phylogeny of eukaryotes based on elongation factor-1 alpha protein sequences. *Biochimica et biophysica acta* 1219(2):529-532.

174. Negrutskii BS & El'skaya AV (1998) Eukaryotic translation elongation factor 1 alpha: structure, expression, functions, and possible role in aminoacyl-tRNA channeling. *Progress in nucleic acid research and molecular biology* 60:47-78.

175. Milligan G & McGrath JC (2009) GPCR theme editorial. *British journal of pharmacology* 158(1):1-4.

176. Chung S, Funakoshi T, & Civelli O (2008) Orphan GPCR research. *British journal of pharmacology* 153 Suppl 1:S339-346.

177. Jacoby E, Bouhelal R, Gerspacher M, & Seuwen K (2006) The 7 TM G-protein-coupled receptor target family. *ChemMedChem* 1(8):761-782.

178. Hendriks-Balk MC, Peters SL, Michel MC, & Alewijnse AE (2008) Regulation of G protein-coupled receptor signalling: focus on the cardiovascular system and regulator of G protein signalling proteins. *European journal of pharmacology* 585(2-3):278-291.

179. Covic L, Gresser AL, Talavera J, Swift S, & Kuliopulos A (2002) Activation and inhibition of G protein-coupled receptors by cell-penetrating membrane-tethered peptides. *Proc Natl Acad Sci U S A* 99(2):643-648.

180. Bridges TM & Lindsley CW (2008) G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS chemical biology* 3(9):530-541.

181. Tuteja N (2009) Signaling through G protein coupled receptors. *Plant signaling & behavior* 4(10):942-947.

182. Bai M (2004) Dimerization of G-protein-coupled receptors: roles in signal transduction. *Cellular signalling* 16(2):175-186.

183. Milligan G, Ramsay D, Pascal G, & Carrillo JJ (2003) GPCR dimerisation. *Life sciences* 74(2-3):181-188.

184. Terrillon S & Bouvier M (2004) Roles of G-protein-coupled receptor dimerization. *EMBO reports* 5(1):30-34.

185. Ostrom RS & Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *British journal of pharmacology* 143(2):235-245.

186. Oldham WM & Hamm HE (2006) Structural basis of function in heterotrimeric G proteins. *Quarterly reviews of biophysics* 39(2):117-166.

187. Siderovski DP & Willard FS (2005) The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *International journal of biological sciences* 1(2):51-66.

188. Kroeze WK, Sheffler DJ, & Roth BL (2003) G-protein-coupled receptors at a glance. *Journal of cell science* 116(Pt 24):4867-4869.

189. Johnston-Cox HA & Ravid K (2011) Adenosine and blood platelets. *Purinergic signalling* 7(3):357-365.

190. Dorris SL & Peebles RS, Jr. (2012) PGI2 as a regulator of inflammatory diseases. *Mediators of inflammation* 2012:926968.

191. Neitzel KL & Hepler JR (2006) Cellular mechanisms that determine selective RGS protein regulation of G protein-coupled receptor signaling. *Seminars in cell & developmental biology* 17(3):383-389.

192. Andreeva AV, Kutuzov MA, & Voyno-Yasenetskaya TA (2007) Scaffolding proteins in G-protein signaling. *Journal of molecular signaling* 2:13.

193. Hoefen RJ & Berk BC (2006) The multifunctional GIT family of proteins. *Journal of cell science* 119(Pt 8):1469-1475.

194. Calebiro D, Nikolaev VO, Persani L, & Lohse MJ (2010) Signaling by internalized G-protein-coupled receptors. *Trends in pharmacological sciences* 31(5):221-228.

195. Kelly E, Bailey CP, & Henderson G (2008) Agonist-selective mechanisms of GPCR desensitization. *British journal of pharmacology* 153 Suppl 1:S379-388.

196. Mohle R & Drost AC (2012) G protein-coupled receptor crosstalk and signaling in hematopoietic stem and progenitor cells. *Ann N Y Acad Sci* 1266:63-67.

197. Jalink K & Moolenaar WH (2010) G protein-coupled receptors: the inside story. *BioEssays : news and reviews in molecular, cellular and developmental biology* 32(1):13-16.

198. Ma L & Pei G (2007) Beta-arrestin signaling and regulation of transcription. *Journal of cell science* 120(Pt 2):213-218.

199. Gantz I*, et al.* (1997) Cloning and chromosomal localization of a gene (GPR18) encoding a novel seven transmembrane receptor highly expressed in spleen and testis. *Genomics* 42(3):462-466.

200. Kohno M*, et al.* (2006) Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochem Biophys Res Commun* 347(3):827-832.

201. Bradshaw HB, Lee SH, & McHugh D (2009) Orphan endogenous lipids and orphan GPCRs: a good match. *Prostaglandins & other lipid mediators* 89(3-4):131-134.

202. McHugh D, Wager-Miller J, Page J, & Bradshaw HB (2012) siRNA knockdown of GPR18 receptors in BV-2 microglia attenuates N-arachidonoyl glycine-induced cell migration. *Journal of molecular signaling* 7(1):10.

203. McHugh D*, et al.* (2010) N-arachidonoyl glycine, an abundant endogenous lipid, potently drives directed cellular migration through GPR18, the putative abnormal cannabidiol receptor. *BMC neuroscience* 11:44.

204. McHugh D, Page J, Dunn E, & Bradshaw HB (2012) Delta(9) -Tetrahydrocannabinol and N-arachidonyl glycine are full agonists at GPR18 receptors and induce migration in human endometrial HEC-1B cells. *British journal of pharmacology* 165(8):2414-2424.

205. Burstein SH, McQuain CA, Ross AH, Salmonsen RA, & Zurier RE (2011) Resolution of inflammation by N-arachidonoylglycine. *Journal of cellular biochemistry* 112(11):3227-3233.

206. Takenouchi R, Inoue K, Kambe Y, & Miyata A (2012) N-arachidonoyl glycine induces macrophage apoptosis via GPR18. *Biochem Biophys Res Commun* 418(2):366-371.

207. Yuan S & Sun Z (2009) Microinjection of mRNA and morpholino antisense oligonucleotides in zebrafish embryos. *Journal of visualized experiments : JoVE* (27).

208. Corey DR & Abrams JM (2001) Morpholino antisense oligonucleotides: tools for investigating vertebrate development. *Genome biology* 2(5):REVIEWS1015.

209. Ekker SC (2000) Morphants: a new systematic vertebrate functional genomics approach. *Yeast* 17(4):302-306.

210. Eisen JS & Smith JC (2008) Controlling morpholino experiments: don't stop making antisense. *Development* 135(10):1735-1743.

211. Moulton HM (2012) In Vivo Delivery of Morpholino Oligos by Cell-Penetrating Peptides. *Current pharmaceutical design*.

212. Kim S, Radhakrishnan UP, Rajpurohit SK, Kulkarni V, & Jagadeeswaran P (2010) Vivo-Morpholino knockdown of alphaIIb: A novel approach to inhibit thrombocyte function in adult zebrafish. *Blood Cells Mol Dis* 44(3):169-174.

213. Draper BW, Morcos PA, & Kimmel CB (2001) Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis* 30(3):154-156.

214. Jagadeeswaran P, Paris R, & Rao P (2006) Laser-induced thrombosis in zebrafish larvae: a novel genetic screening method for thrombosis. *Methods Mol Med* 129:187-195.

215. Jagadeeswaran P, Carrillo M, Radhakrishnan UP, Rajpurohit SK, & Kim S (2011) Laser-induced thrombosis in zebrafish. *Methods Cell Biol* 101:197-203.

216. Nakahata N (2008) Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacology & therapeutics* 118(1):18-35.

217. Michelson AD (2003) How platelets work: platelet function and dysfunction. *Journal of thrombosis and thrombolysis* 16(1-2):7-12.

218. Fabre JE*, et al.* (2001) Activation of the murine EP3 receptor for PGE2 inhibits cAMP production and promotes platelet aggregation. *The Journal of clinical investigation* 107(5):603-610.

219. Gross S, Tilly P, Hentsch D, Vonesch JL, & Fabre JE (2007) Vascular wall-produced prostaglandin E2 exacerbates arterial thrombosis and atherothrombosis through platelet EP3 receptors. *J Exp Med* 204(2):311-320.

220. Honda Z, Ishii S, & Shimizu T (2002) Platelet-activating factor receptor. *Journal of biochemistry* 131(6):773-779.

221. Ishii S, Nagase T, & Shimizu T (2002) Platelet-activating factor receptor. *Prostaglandins & other lipid mediators* 68-69:599-609.

222. Rivera J, Lozano ML, Navarro-Nunez L, & Vicente V (2009) Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica* 94(5):700-711.

223. Hepler JR & Gilman AG (1992) G proteins. *Trends in biochemical sciences* 17(10):383-387.

224. Lambert NA (2008) Dissociation of heterotrimeric g proteins in cells. *Science signaling* 1(25):re5.

225. Van Geet C, Izzi B, Labarque V, & Freson K (2009) Human platelet pathology related to defects in the G-protein signaling cascade. *J Thromb Haemost* 7 Suppl 1:282-286.

226. Cimmino G & Golino P (2013) Platelet Biology and Receptor Pathways. *Journal of cardiovascular translational research*.

227. Lundstrom K (2009) An overview on GPCRs and drug discovery: structure-based drug design and structural biology on GPCRs. *Methods Mol Biol* 552:51-66.

228. Zhang R & Xie X (2012) Tools for GPCR drug discovery. *Acta pharmacologica Sinica* 33(3):372-384.

229. De Meyer SF, Vanhoorelbeke K, Broos K, Salles, II, & Deckmyn H (2008) Antiplatelet drugs. *Br J Haematol* 142(4):515-528.

230. Barker DP, Latty BW, & Rutter N (1994) Heel blood sampling in preterm infants: which technique? *Archives of disease in childhood. Fetal and neonatal edition* 71(3):F206-208.