

ORIGIN AND ROLE OF FACTOR VIIa

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Factor VII, the initiator of the extrinsic coagulation cascade, circulates in human plasma mainly in its zymogen form, Factor VII and in small amounts in its activated form, Factor VIIa. However, the mechanism of initial generation of Factor VIIa is not known despite intensive research using currently available model systems. Earlier findings suggested serine proteases Factor VII activating protease, and hepsin play a role in activating Factor VII, however, it has remained controversial. In this work I estimated the levels of Factor VIIa and Factor VII for the first time in adult zebrafish plasma and also reevaluated the role of the above two serine proteases in activating Factor VII *in vivo* using zebrafish as a model system. Knockdown of factor VII activating protease did not reduce Factor VIIa levels while hepsin knockdown reduced Factor VIIa levels. After identifying role of hepsin in Factor VII activation in zebrafish, I wanted to identify novel serine proteases playing a role in Factor VII activation. However, a large scale knockdown of all serine proteases in zebrafish genome using available knockdown techniques is prohibitively expensive. Hence, I developed an inexpensive gene knockdown method which was validated with *al1b* gene knockdown, and knockdown all serine proteases in zebrafish genome. On performing the genetic screen I identified 2 novel genes, *hepatocytes growth factor like* and *prostasin* involved in Factor VII activation.

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CHAPTER 1

INTRODUCTION

1.1 Hemostasis

Hemostasis is the body's defense to control bleeding. Organisms with closed vascular systems have to maintain a constant flow of blood. Any injury to the vascular system would result in bleeding and would be considered defective hemostasis. This maintenance of uninterrupted blood flow is done by a complex array of procoagulant and anticoagulant factors. A disrupted balance of these factors can lead to either prothrombotic events resulting in complex conditions like pulmonary embolism, myocardial infarction or bleeding disorders such as hemophilia (1-3). Hemostasis consists of a pathway in which several proteins that are activated in a sequential manner after a threat to the vascular system, resulting in a complex array of physical, cellular and biochemical processes. The hemostatic system immediately responds to tissue injury by three mechanisms: vasoconstriction, primary hemostasis and secondary hemostasis. Under normal circumstances, the endothelium maintains the vascular tone by secreting vasodilators like prostaglandins, endothelium derived relaxing factor (nitric oxide) etc.. Dysfunction or injury to the endothelium results in its secretion of factors such as endothelium-derived constricting factor, endothelins that result in vessel constriction (4). Primary hemostasis, on the other hand, consists of the formation of a platelet plug. Finally, the secondary hemostasis requires activation of coagulation factors which lead to fibrin formation. The coagulation cascade occurs on the surface of the platelets and, thus, all three mechanisms contribute to the overall hemostasis. The clot is subsequently dissolved by the fibrinolytic mechanism which involves plasmin (5). Thus, a balance is maintained by the hemostatic pathways to prevent hyper or hypocoagulability states and maintain normal blood flow.

1.1.1 Primary Hemostasis

In mammals, primary hemostasis consists of the platelet response to the damaged endothelium. Platelets are fragments of anucleated cells derived from mature megakaryocytes (6). Platelets lack genomic DNA but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery needed for protein synthesis. They are produced in the bone marrow and circulate in blood with a half life of 10 days and are destroyed by the reticulo-endothelial system in the spleen (7). Other species like fish and birds have nucleated thrombocytes which are equivalent to mammalian platelets, and are anucleated (8). In case of an injury the subendothelial matrix exposes the collagen to which the circulating platelets adhere and get activated. The initial attachment of platelets to collagen at the site of injury is mediated by glycoprotein Ib/V/IX receptor and a ligand called the von Willebrand factor. This attachment results in secretion of chemicals stored in their granules like ADP, thromboxane which activate more platelets via signal transduction pathways. This platelet activation results in change in conformation of an integrin $\alpha\text{IIb}\beta\text{3}$ that facilitates binding of fibrinogen which act as a bridge between platelets. Furthermore, this fibrinogen binding results in outside-in-signaling in platelets. Thus, via a complex mechanism of platelet activation, amplification of the platelet signaling results in the aggregation of platelets at the site of injury resulting in the formation of a platelet plug which is further stabilized with the help of fibrin (9, 10). Fibrin is a product of the secondary hemostasis which occurs on the surface of these platelets. Thrombin a product of secondary hemostasis and also activates platelets by a subset of G protein-coupled receptors known as protease-activated receptors (PARs) (6).

1.1.2 Secondary Hemostasis

Secondary hemostasis consists of the activation of coagulation factors. The coagulation factors are zymogen proteins that are activated by proteolytic cleavage and subsequently activate other zymogen proteins in a cascade-like manner. The coagulation factors are by convention represented as Roman numerals assigned per the order in which they were discovered (11). The sub processes of the coagulation cascade consist of initiation of the procoagulant response, propagation by the enzymatic complexes and finally the termination of clot formation followed by its elimination. The clot formation can be achieved by two mechanisms: the intrinsic mechanism and the extrinsic mechanism as shown in Fig. 1.1. The intrinsic pathway is activated *in vitro* when factor XII (FXII) comes in contact with a negatively charged surface. The intrinsic pathway is also called the contact activation pathway. Activated FXII (FXIIa) then activates coagulation factor XI (FXI) to XIa, which then cleaves factor IX (FIX) to IXa which along with factor VIIIa (FVIIIa) finally activates factor X (FX) to Xa. Factor IXa/VIIIa is called intrinsic tenase complex. Activation of FX is a common step between both the intrinsic and extrinsic coagulation cascades (12). The extrinsic pathway of blood coagulation is initiated when there is a vascular injury resulting in the exposure of tissue factor (TF) present in the sub-endothelium. The circulating factor VIIa (FVIIa) in plasma normally present at 1% level to its zymogen, factor VII (FVII) levels, binds to exposed TF and forms the TF-FVIIa complex (13). This This complex then activates more of FVII and FX (14). FXa is also a predominant physiological FVII activator and activates FVII at a 30% higher rate than the TF-FVIIa (15). The TF-FVIIa requires Ca^{+2} for its action and the complex together is sometimes referred to as the extrinsic tenase. The extrinsic tenase also activates FIX of the intrinsic coagulation cascade (16). FXa, Ca^{+2} and another cofactor factor Va (FVa) form the prothrombinase complex that activates prothrombin to

thrombin. The cofactor FVa, an activated form of factor V (FV), is a single chain molecule activated by FXa and also thrombin (17). However, thrombin generates FVIIIa from factor VIII (FVIII) while FXa inhibits FVIII (18). Thrombin is also a potent activator of FVII and participates in a positive feedback mechanism to up regulate its production. Thus, thrombin, apart from activating FVII, also activates FV, FVIII, FXI and FXIII. Furthermore, thrombin converts soluble fibrinogen into an insoluble fibrin matrix that is stabilized by FXIIIa resulting in formation of blood clot ceasing blood loss (19-22). The termination of clot depends on the inhibitory system which consists of the proteins that inhibit the procoagulant enzymatic complexes either by direct inhibition of proteases or their cofactor proteins. The antithrombin III, protein C, tissue factor pathway inhibitor (TFPI), plasminogen systems are the inhibitory pathways for termination of clot formation. Antithrombin inhibits thrombin and other proteases of the intrinsic pathway and also some proteases of the extrinsic coagulation pathway in the presence of heparin (23). Protein C, on the other hand, is activated by thrombin/thrombomodulin complex and the activated protein C (APC) inhibits FVa and FVIIIa (24). Tissue factor pathway inhibitor, a member of serpins family, inhibits the FXa and TF-VIIa complex (25). The dissolution of the clot is done by plasmin which proteolytically cleaves fibrinogen. The plasmin is generated from its precursor plasminogen by tissue plasminogen activator (26).

1.1.2.1 Clot Based Assay Methods

Clot based assays are routinely used to study bleeding abnormalities in patients. The technique is usually based on testing citrated plasma and analyzing for defects in fibrin clot formation.

- Prothrombin Time

The prothrombin time (PT) test is performed to analyze the function of the extrinsic clotting cascade. In this test, human citrated plasma thromboplastin reagent that contains TF (obtained from brain, lung, placental extract or recombinant form) is added along with calcium and the clotting time is determined. The Quick method and the Owren method are two different reagent systems used to measure the PT. The Quick method to measure PT depends entirely on coagulation proteins present in the patient plasma sample and hence, measures all the extrinsic pathway components (FVII, FX, FV, prothrombin and Fibrinogen). The Owren method uses exogenous fibrinogen and FV in the test reagents hence, the test only measures FVII,FX and prothrombin (27). The PT for normal individuals usually ranges between 10 and 14 seconds. The PT is usually prolonged by deficiencies of coagulation factors participating in the extrinsic coagulation cascade. Deficiencies of FVII, FX, and FV, prothrombin, or fibrinogen and by antibodies directed against these factors are usually determined by the Quick's test (28). Another use of this test is to diagnose vitamin K deficiencies and is most frequently used to monitor warfarin therapy (29).

- Activated Partial Thromboplastin Time

The activated partial thromboplastin time (aPTT) is a test used to determine the deficiencies of the intrinsic pathway or the common pathway. The aPTT test is performed by obtaining citrated plasma from the patient and adding a surface activator, diluted phospholipid and calcium to the citrated plasma and then measuring the clotting time. (30). Since the TF is absent in the phospholipid this test is called partial thromboplastin time. The aPTT is used to diagnose deficiencies of contact factors like FIX, FVIII, FX, or FV and also prothrombin and fibrinogen. Use of anticoagulants e.g. direct thrombin inhibitors and heparin prolong aPTT (28).

- Thrombin Clotting Time

The thrombin clotting time (TCT) test is usually used to diagnose defects in fibrin formation. The test involves addition of excess thrombin to platelet poor plasma of the patients and time to form a clot is monitored. This test does not involve recalcification of plasma (28). Reptilase test is a modification of the TCT where the enzyme reptilase, an enzyme isolated from Bothrops snakes, that has activity similar to thrombin is used instead of thrombin (31). The TCT is prolonged by use of thrombin inhibitors and dysfunction in fibrin formation (28).

- Activated Clotting Time

Activated clotting time (ACT) is an assay used to determine the function of the intrinsic pathway. This test is based on addition of fresh blood into a tube containing a negatively charged activator like celite, glass or kaolin and the formation of blood clot is analyzed. This test is less precise than the aPTT (28, 32).

- Ecarin Clotting Time

The ecarin clotting time (ECT) is a test that is used to measure the activity of direct thrombin inhibitors. Ecarin is a metalloprotease isolated from viper venom that cleaves prothrombin to generate meizothrombin, an intermediate produced during prothrombin to thrombin conversion, which is inhibited by hirudin but unaffected by heparin. It is used in drug-monitoring method in r-hirudin therapy (28, 33).

- Chromogenic or Fluorogenic Assays

Many chromogenic or fluorogenic assays are now available to estimate levels of individual factors. These assays include use of individual factor-specific chromogenic or fluorogenic substrates as well as ELISA based chromogenic or fluorogenic assays using monoclonal antibodies or inhibitors specific to the coagulation factors. Assays to measure FXa, FVIIa, FVIII etc. are used to analyze their levels in plasma and diagnose coagulopathies (34-36) .

1.1.2.2 Evolution of the Coagulation Cascade

Coagulation system has evolved in organisms with closed vascular systems to prevent loss of blood or intravascular fluid and in organisms with an open circulatory system, the coagulation system protects against invasion of microorganisms. The formation of a protein clot is a common feature seen in vertebrates, crustaceans, arachnids (5). Many genes in the coagulation cascade on comparing with other systems like fish or ascidians reveals the duplication of existing genes in the vertebrate lineage after the divergence of urochordates (37). Aquatic vertebrates need efficient hemostatic mechanisms to protect them against vascular damage owing to their delicate gills and blood supply in close proximity with the environment.. Many important factors in hemostasis appeared in fish for the first time. The fish evolved into three major groups namely, Agnatha (which includes the hagfish and lampreys), Chondrichthyes (which includes the sharks, skates, and rays), and Actinopterygii (that includes the bony fish). It has been found that the jawless vertebrates (hagfish and lampreys) seem to possess only the extrinsic cascade but the teleost fish has both, intrinsic and extrinsic cascades (38). Studies on hemostasis in fish report differences in the clotting times of different species. Blood coagulation in teleost fish is usually faster while in trout it is much shorter as compared to mammals. Dogfish

blood usually clots slowly while lamprey blood forms a poor clot after 24 hours (39). The most studied fish for clotting analysis is zebrafish, *Danio rerio* and is found to carry all genes of the hemostatic pathway (40). *Fugu rubripes* or puffer fish also has orthologous genes for coagulation on comparison with mammalian genomes (41). Human FV and puffer fish FV are 41% identical while the FVIII are 42 % identical. Thrombin, a proteolytic enzyme that is formed from prothrombin is also well established in all groups of fish; cyclostomes, elasmobranches, and the teleosts (41). However, the total absence of clotting factors in basal chordates and insufficient information on other lower vertebrates makes it difficult to track the evolution of the coagulation cascade (42). Sequence alignments and phylogenetic studies suggest that the clotting cascade evolved before the divergence of tetrapods and teleosts and was benefited by gene duplications. However, genomic analysis of organisms like cnidarians and porifera can help track the evolution of proteins, especially serine proteases (43).

Examination of the coagulation factors revealed homology of clotting factors to cell receptors and cytoskeletal proteins. For example TF evolved from a cytokine receptor (44). Primitive form of fibrinogen was derived from cytotoxic or from a protein specific to cytotoxic T lymphocytes (45). There is also considerable convergent evolution between the blood clotting factors, hemolymph and the proteins playing role in innate immunity (46). The coagulation serine proteases are believed to have evolved from trypsin like digestive enzymes (47). Also one of the common features of the vertebrate coagulation is that the reactions are localized to anionic membrane surface (5). Gene duplication is a major mechanism for evolution of proteins and this mechanism is also responsible in case of coagulation proteins. The present serine proteases are a result of domain accretion and mutations in the serine proteases domain. Thus the present proteins of blood coagulation are a result from shuffling, addition and deletion of domains (5).

Homology and divergence studies on the sequences of coagulation proteins indicate the sequence of appearance of these proteins in evolution. According to these studies fibrinogen, prothrombin and TF appeared earlier and existed 600 million years ago. Gene duplications may have resulted in Gla and EGF domains leading to the evolution of FVII and FX around 500 million years ago along with FV and FXII. FVIII, FIX and FXI evolved later around 450 million years ago followed by the divergence of FXI and prekallikrein about 120 million years ago. Thus, the jawless vertebrates which consist of the hagfish and lamprey may have a blood coagulation network involving tissue factor, prothrombin and fibrinogen but without intrinsic system (48).

1.1.2.3 Tissue Factor

Tissue factor is a transmembrane glycoprotein of 263 amino acids. It has three domains: an extracellular domain (residue 1-9), a transmembrane domain (residues 220-242) and a carboxy terminal cytoplasmic domain (residues 243-263) (49). TF activity is usually present in the sub-endothelium and its contact with plasma results in activation of extrinsic coagulation pathway (50). The expression of TF in the cells is usually selective. It is highly expressed in brain, lungs, placenta; intermediately expressed in heart, kidney, uterus and testes and its low levels are reported in spleen, thymus, liver and skeletal muscles. The higher levels of TF may protect these organs which are vital (51). Low levels of TF are also found in blood, however, it is not known if this low level contributes to clotting. Appearance of low levels of TF in platelets is also controversial (52). Many cell types can form circulating TF containing micro particles. TF containing neutrophils and monocytes were also identified in blood in the same study (53). Circulating TF plays a role in venous thrombosis not resulting from vessel damage (14). In case

of vascular damage the tissue factor in sub-endothelium is exposed to the blood and facilitates the binding of activated FVII to it (54).

1.1.2.4 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor, an inhibitor for extrinsic pathway is mainly present in the endothelium. Tissue factor pathway inhibitor activity is also seen in the vascular smooth muscle cells, megakaryocytes, macrophages, microglia of brain, atherosclerotic plaques etc. (5). The TFPI gene is found on the long arm of chromosome 2(q32). The protein has a 24-28 amino acid long signal peptide. The mature protein has a molecular weight of 32 kDa and has three Kunitz type protease inhibitor domains (5). The TFPI protein regulates the extrinsic cascade by inhibiting the TF-FVIIa in a FXa dependent manner. The anticoagulant effect of TFPI takes place in two steps: the second Kunitz type domain binds to factor Xa (FXa) and deactivates it while the first Kunitz domain rapidly binds to TF-FVIIa and further prevents activation of FX (55). The binding of the TFPI to FXa is reversible and is not dependent on Ca^{+2} , while the binding of TFPI to TF-FVIIa is irreversible and calcium dependent (56-58).

1.1.2.5 Factor VII

FVII is a vitamin K dependent serine protease and is the first protein of the extrinsic coagulation cascade. It is expressed in the liver as a zymogen. It is a single chain glycoprotein of 50kDa which is secreted into blood stream in its zymogen form. In its activated form FVII binds to TF expressed on the sub-endothelium in case of a vascular injury and activates the extrinsic cascade by activating FX (59).

1.1.2.5.1 Structure of FVII

FVII is synthesized mainly by liver, and has been observed in macrophages or monocytes as well. It is secreted as a zymogen mainly by liver cells (60). It is a single chain protein molecule that contains several distinct structural domains. At the N-terminus, the first 40 residues are called Gla domain because it has ten γ -carboxyglutamic acids (Gla residues). Immediately after the Gla domain a short hydrophobic sequence is located. Residues 43-83 and 84-130 constitute the epidermal growth factor (EGF) domains and residues 131-167 forms serine protease domain (SP) (5). The serine protease domain contains the Arg 152-Ile 153 site that is cleaved in FVII activation. Zymogen FVII has two distinct β -barrel domains. Structural studies on FVII and FVIIa show that the difference lies in second β -barrel domain of these two forms of factor VII. The FVII zymogen protein has no active substrate binding cleft (61). The structure of the catalytic domain of FVII contains the catalytic triad just like other serine proteases (59). However, the intrinsic catalytic activity in FVII is absent.

Zymogen FVII requires post-translational modification before being released into the plasma. This occurs in the endoplasmic reticulum of the hepatocytes where carboxylase, an integral membrane glycoprotein, uses vitamin K to γ -carboxylate ten glutamic acid residues near the amino terminus of FVII. During the carboxylation process vitamin K is oxidized to its 2, 3-epoxide form while the enzyme vitamin K epoxide reductase catalyses the reduction of the epoxide (5, 62). FVII is then secreted into circulation and in human it is usually at a plasma concentration of 500ng/ml (63).

1.1.2.5.2 Factor VII Deficiency

1.1.2.5.2.1 Genetics and Molecular Basis

Human FVII cDNA was isolated from human liver cDNA libraries and human liver hepatocellular carcinoma cell line (Hep G2 cells) (59) and its gene structure was established. Humans FVII gene (*f7*) is located on the long arm of chromosome 13 (q34) adjacent to FX (*f10*) gene (1, 2). Its organization is similar to other vitamin K dependent factor genes (5). The major transcriptional start site for FVII gene is located 50bp upstream of the initiation codon. The hepatocytes nuclear factor-4 (HNF-4) and Sp1 are the important transcription factors binding to FVII promoter. Mutations within the HNF-4 and Sp1 binding sites can result in severe FVII deficiency (5). Inherited FVII deficiency is of autosomal recessive fashion and is rare with a frequency of occurrence of 1:500,000 and the disease manifestations are similar to a patient suffering from hemophilia (64). The frequency of FVII deficiency is higher in countries that approve consanguineous marriages (65). FVII deficiencies can be both quantitative and qualitative. Congenital FVII deficiency is classified in two types. Type I deficiency is characterized by a deficiency of both FVII activity and FVII antigen while Type II deficiency is characterized by a normal FVII antigen levels but a low FVII activity (66). FVII Padua1 is a result of Arg304Gln mutation wherein the prothrombin time is abnormal when thromboplastin derived from rabbit brain is used but normal when thromboplastin is derived from ox brain (67). FVII Padua2 is a disorder wherein the prothrombin time is abnormal when thromboplastin derived from ox brain is used but normal when thromboplastin is derived from rabbit brain (68). FVII Verona is another disorder associated with abnormal form of FVII (69). Mutations in exon 8 which is the largest exon in the gene and encodes the catalytic domain result in affected FVII function and stability and clinical bleeding tendency (70). FVII activity is influenced by

mutations in *f7* gene. So far more than 130 mutations have been isolated scattered along the gene (71). Six different polymorphisms in FVII gene have been identified that affect the plasma FVII levels (5, 72, 73). Large complex intragenic re-arrangement in the FVII gene including a discontinuous deletion involving two distinct portions of FVII has been characterized (74).

1.1.2.5.2.2 Clinical Manifestations

The clinical manifestations associated with congenital deficiency of FVII are heterogeneous ranging from severe hemorrhages to mild bleeding. Tissues rich in tissue factor i.e. brain, bowel, uterus, placenta, lung and heart where the hemostasis is mainly dependent on extrinsic coagulation are the main bleeding sites in FVII deficient patients. In congenital FVII deficiency bleeding is severest within the first 6 months of an infant, however, later in life spontaneous or provoked bleeding is usually seen. Severe bleeding encompasses life threatening hemorrhages such as intracranial and gastrointestinal hemorrhages. Hemarthrosis is another manifestation found in congenital FVII deficiency when infants begin crawling (75). Homozygous individuals have severe hemorrhages while compound heterozygous have moderate to mild hemorrhages. Symptomatic individuals exhibit easy bruising, epistaxis and menorrhagia. In women with FVII deficiency menorrhagia is frequently observed. It may be associated with hemoperitoneum related to ovarian cysts, metrorrhagia due to uterine fibromatosis or postpartum hemorrhage.

1.1.2.5.2.3 Diagnosis

FVII deficiency is suspected by an abnormal PT and with a normal aPTT. ELISA test specific for FVII detection are also now available for diagnosing FVII deficiency. Molecular

diagnosis of FVII deficiency is based on conventional techniques like the Polymerase Chain Reaction (PCR) or more recent semi-quantitative multiplex PCR techniques. Prenatal diagnosis is done by genetic analysis on cord blood usually obtained by either the trans-abdominal or trans-amniotic approach or by amniocentesis during the early stages of pregnancy (75).

1.1.2.5.2.4 Disease Management

For patients with inherited FVII deficiency in severe form, substitution therapy is the main therapeutic option. Fresh frozen plasma and prothrombin complex concentrate therapy has been used in the past. However, recombinant FVIIa, NovoSeven from Novo Nordisk (Copenhagen, Denmark) is the most used therapeutic option for inherited FVII deficiency. Interestingly, recombinant FVIIa is routinely given to control bleeding episodes in patients with inhibitors suffering from all kinds of Hemophilia (76). For example, patients suffering from Hemophilia A, a hereditary bleeding disorder caused by a lack of FVIII, are treated by repeated infusions of microgram quantities of pure human FVIIa (77). Recombinant FVIIa is synthesized and commercially available by extracting the human FVII cloned in baby hamster kidney cells (BHK cells) and converting it to FVIIa by autocatalysis. Recombinant FVIIa is also used in treating patients on warfarin who have traumatic intracranial haemorrhage and other hemorrhages (78, 79). Recombinant FVIIa known as NovoSeven is now an approved therapy for use in patients suffering from congenital hemophilia and acquired hemophilia with inhibitors against coagulation factors in the United States of America, European Union and Japan and its efficacy rate in severe bleedings and in major surgeries has been found to be around 90% with no serious safety concerns (80). Bio-distribution studies of pharmacologically administered recombinant FVIIa show its localization to the endothelium lining of the large blood vessels and

other TF-expressing cells. Within an hour the recombinant FVIIa bound to endothelial cells is transferred and diffused throughout the perivascular tissue surrounding the blood vessels. The recombinant FVIIa is localized to sinusoidal capillaries in liver and accumulated in hepatocytes. In bone recombinant FVIIa is found in the zone of calcified cartilage (81).

1.1.2.5.3 Elevated Levels of FVII and FVIIa

FVIIa levels are known to increase with age in both sexes. Post-menopausal women have a higher FVII and FVIIa plasma concentration compared to pre-menopausal women (82). Plasma FVII levels are also high in pregnant women and patients suffering from systemic lupus erythematosus (83). FVIIa levels have also been associated with recurrent fetal loss (84). Increased levels of FVIIa are known to be associated with many pathologic conditions. FVIIa and FVII clotting activity (FVIIc) levels are higher in patients suffering from acute cerebral infarction (85). Patients with idiopathic thrombocytopenic purpura have higher FVIIa levels (83). Arterial cardiovascular disease is common in people with higher FVIIa levels and FVIIa may be an independent risk factor for cardiovascular disease (86). Increased FVIIa and FVIIc levels in plasma may also contribute to coronary artery thrombosis (87). However, the role of FVIIa in heart disease is still under debate (88-91).

1.1.2.5.4 Activation of FVII

Zymogen FVII is activated to form FVIIa by proteolytically cleavage of the conserved arginine 152-isoleucine 153 bond. The proteolysis results in the activated form of FVII consisting of the amino terminal light chain and carboxyl terminal heavy chain linked to each other by a single disulphide bond between cysteine 135 and cysteine 262 residues. The heavy

chain of FVIIa contains the catalytic serine protease domain while the light chain contains the Gla domain and the EGF domains. The catalytic domain of FVIIa consists of serine residue at 344, aspartate at 242 and histidine at position 193 (5). The catalytic triad is similar to trypsin and chymotrypsin where in presence of the substrate the aspartate deprotonates. The histidine facilitates a proton transfer to aspartate and grabs one from the hydroxyl group of serine to activate it. The serine attacks the substrate and an oxyanion hole is formed in the tetrahedral intermediate and the amino product is released. Water then binds and the nucleophilic attack of water results in the collapse of the tetrahedral intermediate resulting in the carboxyl product release (92). However, the catalytic domain in free FVIIa is not readily accessible and upon Ca^{+2} and TF binding, it undergoes conformational change to expose the catalytic site. Studies have shown that the leucine 287 and methionine 298 residues help in maintaining the zymogen form before the activation. Zymogen FVII-TF complex cannot activate the extrinsic coagulation pathway (93). TF-FVIIa interactions stabilize the amino-terminal Ile-153 and induce allosteric changes involved in zymogen to enzyme conversion in FVIIa bound to TF. TF interaction with FVIIa is possible as it promotes loss of hydrogen bonding between residue 154 and residues 21 and 22. This also favors a salt bridge between Ile 16-Asp 194 which further stabilizes the activation domain (20, 94).

The total concentration of this circulating FVIIa in healthy humans is around 5ng/ml. Circulating FVIIa does not activate zymogen FVII in absence of TF. Many activators of FVII *in vitro* are known. However, the activators resulting in 1% FVIIa levels circulating in plasma are still unknown (95). Since during FVIIa catalysis the arginine-isoleucine bond is broken, the candidates for FVII activation are proteins that have the serine protease domain. The most potent activator of zymogen FVII is membrane bound FXa (15). FVII is activated *in vitro* efficiently by

FXa. In presence of phospholipid and Ca^{+2} the FVIIa activity increases to an initial rate of 20-fold per minute (20). FIXa also is an activator of FVII when the reaction is carried out in a glass tube. Though the efficiency of activation for both FXa and FIXa is decreased in absence of phospholipid and Ca^{+2} , FXa continues to activate 800-fold more than FIXa in absence of phospholipids and in presence of Ca^{+2} (96). Thrombin is another potent activator of FVII (20). FXII or Hageman factor is activated to FXIIa when the protein comes in contact with a negative surface. This activated Hageman factor is also thought to activate FVII *in vitro*. Activation of FVII by Hageman factor fragments promoted rapid proteolysis of one-chain FVII to an activated two chain form. The activation by FXa, FXIIa and thrombin reflects the control of intrinsic mechanism over the extrinsic pathway (96, 97). Tissue plasminogen activator, another serine protease involved in dissolution of clots is also reported to activate FVII. FVIIa levels were found to increase when purified FVII was incubated tissue plasminogen activator along with plasminogen. FVIIa levels were also found to be increased when purified FVII was incubated with plasmin (98). In an independent study, it was found that FVII was not activated in FXII, high-molecular-weight kininogen, FXI, and FIX deficient plasmas supporting the previous *in vitro* findings of their role in FVII activation (99). FVII activity was also enhanced in presence of purified kallikrein; However, this role of kallikrein is still under debate (99, 100). In patients suffering from FVIII congenital deficiency a substantial decrease in total FVII and low circulating FVIIa levels are observed (75). However, role of FVIII in FVII activation is not reported.

TF FVII dependent autoactivation has also been reported. Fully carboxylated mutant FVII was found to be auto activated when complexed under physiological conditions with either soluble TF or cell-surface TF provided by a human bladder carcinoma cell line (J82). Studies

with isotherms for ¹²⁵I-labeled FVII and ¹²⁵I-labeled S344A FVII (FVII isolated from BHK cells stably transfected with a plasmid containing the Ser344→Ala FVII sequence) suggest the possibility of trace concentrations of circulating FVIIa serving as the catalyst to rapidly convert a TF-FVII complex to an active TF-FVIIa complex at the site of vascular injury (101).

Purification of single-chain human recombinant FVII produced by transfected baby hamster kidney cells in the presence of benzamidine spontaneously activated FVII in the absence of inhibitor. The activation reaction was found to be enhanced in the presence of a positively charged surface provided either as an anion-exchange matrix or as poly(D-lysine) in process of purification (102).

Apart from the activation of FVII *in vitro* by coagulation factors and autoactivation two other serine proteases have been reported to activate FVII *in vitro*. Factor VII activating protease (FSAP) was isolated from prothrombin concentrate and was found to activate FVII *in vitro* (103). Transfection of baby hamster kidney cells with a plasmid containing the cDNA for human hepsin activated FVII along with other coagulation factors (104). However, the role of hepsin and FSAP in activating FVII is still an enigma (105, 106).

1.1.2.5.5 Factor VII Studies in Animals

Most of the FVII studies have been done using human FVII. However, since gene knockout and manipulation of genes cannot be performed in humans animal models are essential to carry out such studies.

Mus musculus is a well established model for coagulation studies and FVII (107). FVII gene in mice is located on chromosome 8. FVII deficient mice (FVII^{-/-}) were engineered by deleting the entire coding sequence for the mature FVII protein. The FVII deficient embryos

develop normally with normal vasculature development. However, they experience lethal post natal bleeding making FVII essential for prenatal hemostasis (108). The FVII $-/-$ neonates die from intra-abdominal and intra-cranial hemorrhage within 24 hours of or a few days after birth (109, 110).

Danio rerio (zebrafish) a teleost fish is another model used to study FVII. FVII gene is identified on the chromosome 1 of the zebrafish genome and is a well characterized protein in zebrafish. On comparing with human FVII, zebrafish FVII is found to be conserved (40, 111). Apart from FVII in zebrafish, a gene encoding a protein homologous to FVII has also been isolated. However, comparison of this gene to FVII gene in zebrafish shows the lack of RIV sequence required for activation and replacement of the serine residue of the catalytic triad by aspartate in FVIIi making the protein lack FVII activity. However, studies have shown this protein to have inhibitor like activities and this gene could serve as an inhibitor to FVII (40). Apart from zebrafish, FVII has also been identified in the puffer fish, *Fugu rubripes*. Four genes were identified in the genome of puffer fish coding for FVII: *f7B*, a homolog, codes for FVII protein which is 46% identical with mammalian FVII, two other homologs of FVII gene in puffer fish represented as *f7A* and *f7C* are 42% and 43% identical with the mammalian FVII, while the homolog ψ F7 lacks the catalytic site and is non functional (41). Liver Expressed Sequence Tag studies and 5'- and 3'-rapid amplification of cDNA ends (RACE) analysis have identified FVII in the lamprey, *Lethenteron japonicum* as well (37).

1.1.2.6 Zebrafish as a Genetic Model

Zebrafish is a teleost fish that belongs to class Actinopterygii. It is a small tropical fish that originated in India. They have a life span of 4 years and they reach sexual maturity at about

three months. They undergo external fertilization wherein the female lays over hundreds of eggs which are fertilized by males. This characteristic greatly facilitates genetic analysis. The fertilized eggs are transparent which allows easy staging of cells and study development (112). Zebrafish are small and easy to maintain and breed in large numbers (113). Francis Hamilton, first described zebrafish in 1822, followed by phage geneticist George Streisinger who used zebrafish as a model system to study genetic basis of vertebrate neural development (114). Zebrafish is now becoming a more favorite model because of the transparency and external development of embryos which makes it easy to study development.

The zebrafish genome is entirely sequenced and analyzed at the Wellcome Trust Sanger Institute. It is 1.4 Gb in size and contains 25 linkage group. The project has now reached the maintenance stage making its genetic information easily available (115). Both forward and reverse genetic approaches are successfully used in zebrafish. Ethylnitrosourea mutagenesis and inbreeding over two generations is performed to search for point mutations disrupting the required phenotype. This forward genetic screening is routinely performed in zebrafish to study genetic architecture (116). Anisense morpholinos technology (MO), is a powerful reverse genetics technique routinely used in identifying gene function (117, 118). Apart from these, over expression of gene of interest by microinjection of genes under powerful promoters or mRNAs to test gene function is a commonly used technique in zebrafish (119). Even though the low efficiency of recombination in zebrafish results in difficulty to create a knockout zebrafish, emerging new technologies have a solution to this problem. New techniques like Zinc Finger Nuclease Technology (ZFN) and Targeting Induced Local Lesions in Genomes (TILLING) are now being used to create knockout zebrafish (120, 121). Easy genetic manipulation and assessment of phenotype and a short generation time makes it a favorite genetic model. Apart

from being a successful model system for studying early development, zebrafish has also evolved as an excellent model system to study human diseases (122). It is being used for the study of cancer, age dependent diseases, cardiovascular diseases, hemostasis, toxicity and drug screening studies. (123-128).

1.1.2.6.1 Zebrafish as a Model to Study Hemostasis

In last two decades zebrafish emerged as a successful model to study genes involved in hemostasis. They have a closed circulatory system and the hemostatic pathways, anatomy of developing vasculature and the molecular mechanisms are highly conserved (10, 129). Studies on zebrafish vasculature show that their circulation begins at 24-26 hours post fertilization (hpf). In the beginning the blood flows through a single circulatory loop and exits the heart through the bulbus arteriosis and ventral aorta (129). The zebrafish heart consists of serially connected sinus venosus, atrium, ventricle, and bulbus arteriosus. The venous blood from the ventral aorta leads to the gill arches and after oxygenation is supplied to the rest of the body. The zebrafish heart is also structurally similar to mammalian heart (130). Shortly after initiation of circulation a second loop emerges and the blood exiting the first aortic arches begins to flow bidirectionally and after 1.5 days post fertilization (dpf) intersegmental arteries and veins begin to develop. By 2 dpf most of the trunk and tail lumenize and a basic vasculature pattern is established along with an active circulation. This rapid development of this teleost fish highlights the need to escape predation due to external fertilization (129).

Molecular programming of hematopoiesis in zebrafish is highly conserved (131). In a developing zebrafish embryo there are four distinct waves in the hematopoietic program. The first two waves start to appear in a region called intermediate cell mass (ICM) 30 hpf resulting in

primitive hematopoiesis and generating macrophages and erythrocytes. Definitive hematopoiesis comprises of the third and fourth waves and produce erythromyeloid progenitors and hematopoietic stem cells (HSCs), respectively. These HSCs then reside in the caudal hematopoietic tissue (CHT) and adult hematopoietic organs, the kidney and thymus shown in shown in Fig. 1.2. Thus, the primitive hematopoiesis generates the erythroid lineages while the definitive hematopoiesis generates erythroid, monocyte, granulocyte, and thrombocyte lineages (10). Characterization of the vascular patterning and hematopoiesis greatly aids in the use of zebrafish as a model to study hemostasis.

Although zebrafish is a fairly new model to study hemostasis, a lot of the hemostatic aspects are studied and already established.. The main steps of primary and secondary hemostasis are conserved in zebrafish when compared with mammalian hemostasis (132). Zebrafish shares most factors to its human counterpart. They have thrombocytes, a nucleated equivalent of mammalian platelet, which also demonstrate the mechanisms of platelet adhesion, activation and aggregation as well as many signaling mechanisms similar to human (10, 128, 133-136). Coagulation factors like FVII, TF, prothrombin are known to be present in zebrafish (111, 137-139). Genetic studies in zebrafish have confirmed the conservation of the major hemostatic pathways (140). Effect of warfarin on zebrafish yielded similar results in human showing the presence of vitamin K pathways. Characterization of hemostatic pathways in zebrafish have demonstrated the presence of both the intrinsic and extrinsic pathways of coagulation and presence of the natural anticoagulants protein C, antithrombin and heparin cofactor II (141). However, in the teleost fish including zebrafish, gene duplication is observed when compared with other vertebrates (142). One result of this duplication is the presence of the *f7i* gene, an inhibitor of coagulation (40). However, the ease of knockdowns of multiple genes

alleviates the difficulties in studying the duplicated genes (132, 143, 144). Indeed knockdowns and the application of this model system in studying hemostatic genes has been accomplished successfully (145).

1.1.2.6.2 FVII Studies in Zebrafish

Comparison of the gene structure of coagulation FVII, FIX, and FX and protein C indicates paralogous nature of these genes suggesting a common origin via gene duplication. Zebrafish FVII was identified and analyzed to be similar to human FVII. The size of zebrafish FVII protein is 50 kilo Dalton (kDa) and the structure consists of a prepropeptide, a putative signal peptide, a conserved vitamin K-dependent carboxylase recognition peptide and a dibasic amino acid processing site. A high degree of conservation is observed between the human FVII and zebrafish FVII domains shown in shown in Fig. 1.3. The Gla domains show 62% identity and 83% similarity, the Epidermal Growth Factor-1 (EGF-1) domain shows 55 identify and 66% similarity, EGF-2 domain shows 43% identity and 67% similarity and serine protease domains shows 48% identity and 62% similarity. Overall the human FVII and zebrafish FVII protein share 48% identity shown in Fig. 1.4. The zebrafish FVII is activated at the single activation site at Arg-195–Ile-196, a site that is conserved. Cleavage of this bond results in FVIIa consisting of a 158 amino acids long light chain from residues 38–195 and a 238 amino acid long heavy chain from residues 196–433. Aspartate residue 284, histidine residue 236, and serine residue 381 forms the catalytic triad of the zebrafish FVIIa (111). Gene expression studies in zebrafish FVII demonstrate the presence of Factor VII mRNA in both the liver and kidney (111).

1.1.2.6.3 Coagulation Assays in *Danio rerio*

The power of zebrafish as a model system for coagulation assays is the ability to perform *in vivo* assays. The following *in vivo* and *in vitro* assays are now developed for studying coagulation in zebrafish.

1.1.2.6.3.1 Vascular Occlusion

Ablation of vein with the help of laser and recording the time of occlusion is a technique to measure coagulation function in zebrafish. This technique is used in zebrafish larvae and consists of immobilizing the anaesthetized 4- 5 days post fertilization (dpf) larvae in agarose and exposing the caudal vein with pulsed nitrogen laser light pumped through coumarin 440 dye at 445 nm at seven pulses/second for five seconds (sec). The time to occlusion (TTO) of the vessel is then recorded for the larvae through a microscope (146). Since arterial clots are platelet-rich and fibrin-poor the TTO in arteries is usually an indicator of thrombocyte (equivalent of mammalian platelets) function while the TTO in veins is an indicator of coagulation function (147).

1.1.2.6.3.2 Modified Coagulation Assays

Modified coagulation assays are usually performed in zebrafish. These include:

- Modified Prothrombin Time

Modified PT also called kinetic PT (kPT) has been performed on zebrafish plasma using either rabbit brain thromboplastin or zebrafish thromboplastin isolated from muscle as the clotting reagent. The method involves preparing citrated plasma from adult zebrafish and adding

thromboplastin and CaCl_2 to the plasma and monitoring the clot formation. This method is used to determine extrinsic activity of zebrafish coagulation (141).

- Modified Activated Partial Thromboplastin Time

The modified aPTT also called kinetic PTT (kPTT) is performed by incubating the citrated zebrafish plasma with rabbit brain partial thromboplastin for 10 min followed by recalcification and analysis of clot formation. This method is used to determine intrinsic and common pathway activity of zebrafish coagulation (141).

- Modified Russell Viper Venom Time

The modified Russell viper venom time (RVVT) also called kinetic RVVT (kRVVT) is usually performed by addition of purified FX activator from Russell viper venom as the clotting reagent. The clot formation is then analyzed. This method is used to determine FX activity in zebrafish plasma (141).

- Other Assays

Many other assays have also been used to estimate zebrafish clotting factors. Assays for estimating FX, protein C, heparin and dermatan sulfate cofactor activity have been used before (141).

1.2 Overall Aims and Hypothesis

Despite advances in the field of hemostasis mechanisms of FVII activation *in vivo* resulting in constant levels of circulating FVIIa levels is still unknown. It is difficult to address

this issue biochemically and hence, gene knockdown could be a useful approach to answer this. Genetic studies using mouse model are not trivial (148). Since zebrafish is a vertebrate model system with conserved hemostatic pathways and a conserved FVII, I thought of using this organism to find answers with respect to origin of FVIIa in plasma. Since the activation of FVII involves cleavage between an arginine and isoleucine bond the role of a serine protease in this cleavage is suspected. The goal of this research study is to identify the serine protease involved in FVII activation *in vivo* using zebrafish as a model system. The following specific aims were addressed to test the above stated hypothesis:

Research Aim 1: Estimation of circulating FVIIa and FVII plasma levels in *Danio rerio*.

Hypothesis: Circulating FVIIa and FVII levels can be estimated in zebrafish plasma using ELISA techniques.

Plasma was isolated from wild type adult zebrafish and was immediately analyzed for FVIIa and FVII levels. The levels were analyzed by using enzyme-linked immunosorbent assay (ELISA) specific for FVIIa and FVII. Estimation of these levels and the assays helped to address the controversial issue on the identity of the serine protease involved in initial FVIIa generation by gene knockdown approach. Furthermore, it aided in expanding our knowledge in understanding the hemostasis in a teleost fish.

Research Aim 2: Verification of the role of FSAP and hepsin in activating FVII *in vivo* by using zebrafish reverse genetics.

Hypothesis: Knockdown of *habp2* and *hpn* should lead to decreased FVIIa levels in zebrafish plasma if these gene products are involved in activation of FVII.

Morpholinos, modified antisense oligonucleotides, were designed for the *habp2* and *hpn* genes which code for factor VII activating protease and hepsin respectively. The gene knockdown was performed by injecting Vivo morpholinos into adult zebrafish and the morpholinos in one to four cell stage embryos and the FVIIa levels were analyzed compared to

their respective controls. Extrinsic coagulation cascade was also assayed by kinetic assays to assess the hemostatic phenotype. *f7* gene knockdowns were also performed similarly to compare the efficiency of the knockdowns.

Research Aim 3: Identification of novel serine proteases playing a role in extrinsic cascade and FVII activation in zebrafish.

Hypothesis: Large scale gene knockdowns of all genes coding for serine proteases in zebrafish will identify novel serine proteases in FVII activation.

In order to understand the role of any serine proteases other than hepsin and FSAP in FVII activation I decided to comprehensively knockdown all genes coding for serine proteases in zebrafish genome and assess FVIIa levels. A novel technology was developed by me along with two other colleagues to perform large scale genetic knockdowns using a Non toxic oligonucleotide permeabilizer (NTOP) and a gene specific standard antisense oligonucleotide for delivery of these oligonucleotides into zebrafish cells. Using this technology I performed knockdown all serine proteases in zebrafish genome and assessed the kinetic coagulation to measure the extrinsic coagulation efficiency and analyzed FVIIa levels.

This study helped me to identify novel serine proteases responsible for activating FVII *in vivo*.

1.3 Significance of Research Study

Origin of circulating FVIIa levels at a constant level in human plasma as well as other vertebrates has been an unanswered question. In human, FVIIa circulate at 5ng/ml levels. Elevated levels of FVIIa compared to normal levels are responsible for many conditions like the arterial cardiovascular disease, thrombosis etc.. Hence it is important to understand the *in vivo* mechanism of FVII activation. To answer this I decided to use the well studied *Danio rerio*,

zebrafish, as my model organism. The practical applications of my investigations are as follows:

Aim 1 of my study deals with estimating FVII and FVIIa levels in adult and larval zebrafish populations. Even though zebrafish is a well characterized model for hemostasis and zebrafish FVII is identified, the concentrations of the FVIIa and FVII have not yet been studied in this model system. It is a known fact that teleost fish blood clots faster than human. However, the reasons were never accounted for. Estimation of coagulation FVII levels in zebrafish helps us understand this phenomenon. Furthermore, the assays developed in this aim are important to understand the FVIIa levels after knockdown of genes whose products may be involved in FVII activation. Aim 2 of my research study tries to resolve a controversy with respect to the role of two serine proteases FSAP and hepsin in FVII activation. Both the proteases have been shown to activate FVII *in vitro* using human cell lines. However, studies from other laboratories contradicted these results. Hence, it is important to analyze the role of these proteases *in vivo* using other model system in order to address the controversy. In the Aim 3 of my research study, I conducted a large scale gene knockdown of all serine proteases in the zebrafish genome. This could identify novel genes playing a role in extrinsic coagulation and FVII activation and thus hemostasis as a whole. Identification and characterization of such genes may help furthering our knowledge on hemostatic pathways and treatment of hemostatic disorders associated with extrinsic coagulation.

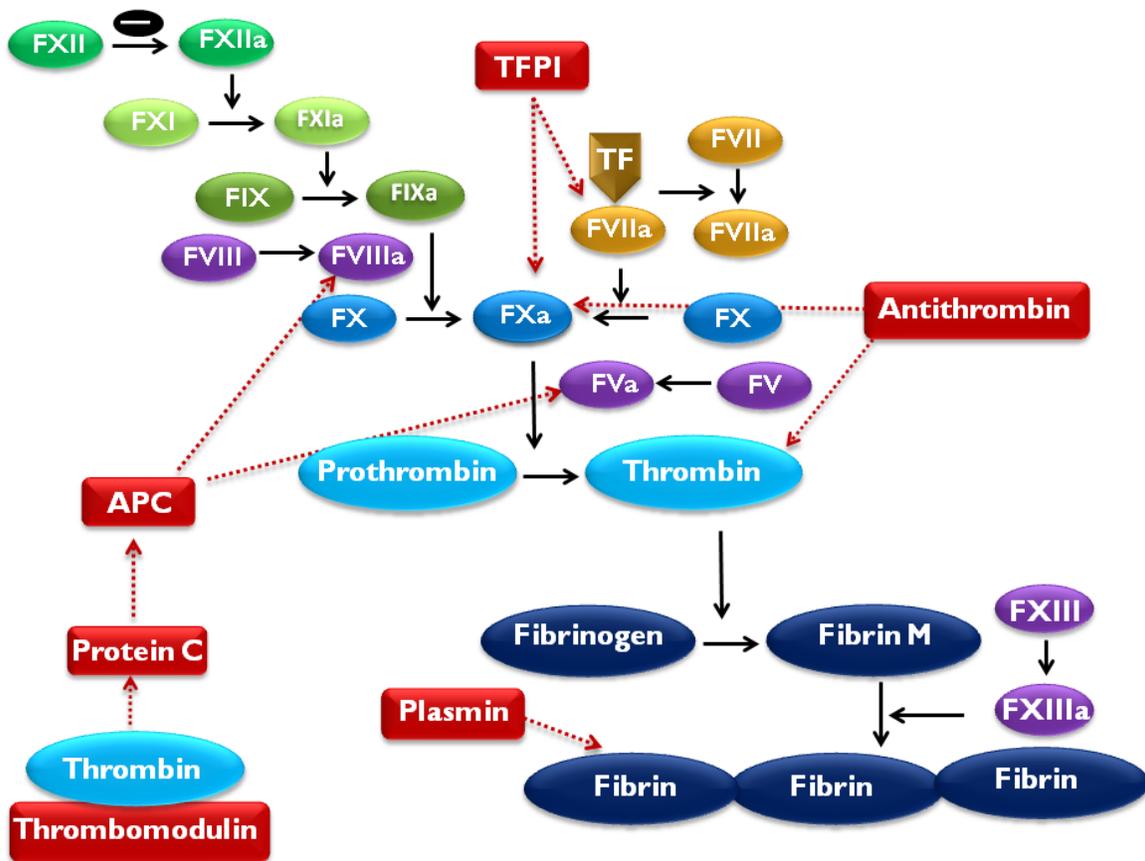


FIG. 1.1 Schematic representation of the coagulation system. The proteins highlighted grey belong to the procoagulant system while the proteins highlighted red belong to the anticoagulant system (Redrawn from *Thrombosis and Hemorrhage*, 3rd edition) (5).

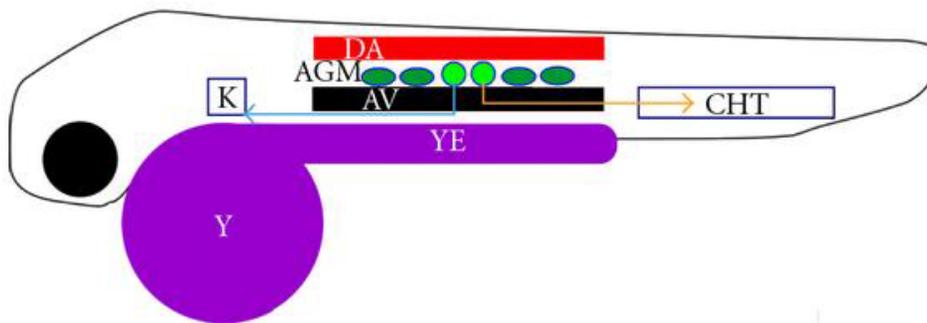


FIG 1.1 Schematic representation of definitive hematopoiesis in zebrafish larva. The hematopoietic stem cells reside in the caudal hematopoietic tissue (CHT), kidney (K) and thymus (not shown). DA, dorsal aorta; AV, axial vein; AGM, area corresponding to mammalian aorta- gonad- mesonephros; Y, yolk; YE, yolk extension; filled small circles and ovals represent blood cells, respectively . The yellow and blue lines with arrows correspond to the routes of immigration of these cells (10).

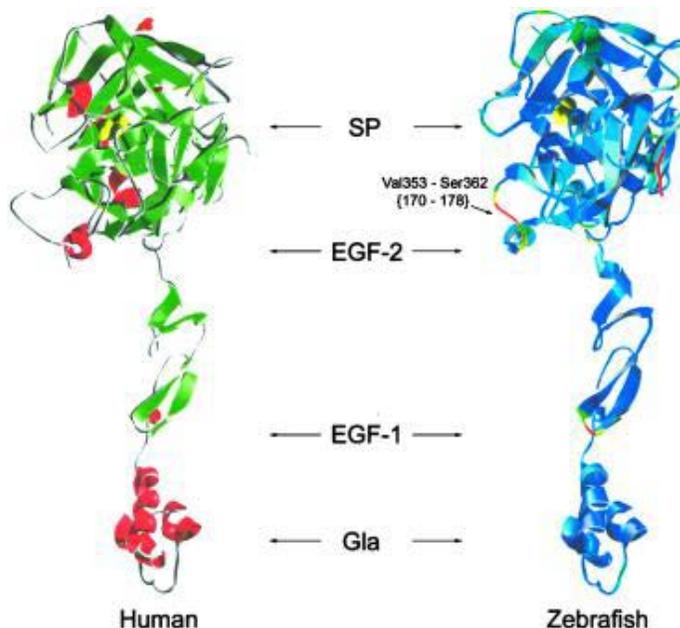


FIG 1.2 Homology model of FVIIa. The crystal structure of Human FVIIa and zebrafish FVIIa show presence of serine protease (SP), epidermal growth factor (EGF) 1 &2 and Gla domains. Copyright (2001) National Academy of Sciences, U.S.A. (11).

Score	Expect	Method	Identities	Positives	Gaps
380 bits(976)	1e-131	Compositional matrix adjust.	200/413(48%)	264/413(63%)	14/413(3%)
Query 21	VFVHRDEAHEVLIRSKRANS	GWFEELKTGNLERECLEEKCSYEEAREVFEHTEATNEFWK	80		
Sbjct 44	VFV ++EAH VLR + RAN+ + EEL+ G+ LEREC EE+CS+EEARE+F+ E T FW		102		
Query 81	IYDVKDHCASSPCEHDGLCTTQNADSYMCLCAPGFSGRHCEQSIGDVLDSCLHDNGGCEH	140			
Sbjct 103	Y D CASSPC++ G C Q SY+ C C P F GR+CE D L C+++NGGCE	160			
Query 141	FCTEQDG-RRNCSCADGYILDNSGQKCRSHEVFPCGKVPLLQAGKAADHQVDLRSRIVGG	199			
Sbjct 161	+C++ G +R+C C +GY L G C +PCGK+P+L+ A+ Q RIVGG	216			
Query 200	SECPKGHCPWQVLLKYGEKGFCCGGVIYKPTW ILTAAHCLEKLV-KFLRIVAGEHDLEVD	258			
Sbjct 217	CPKG CPWQVLL CGG + W+++AAHC +K+K + L V GEHDL	276			
Query 259	EGTEQLIQVDQMFTHPAYVSETADSDIALLRLRTPIVYSVYAVPVCLPLREMAERELWAY	318			
Sbjct 277	+G EQ +V Q+ YV T + DIALLRL P+V + + VP+CLP R +ER L V	336			
Query 319	SKHTVSGWGKRSEDGPTSRLLRRLVPRITQECVQVS-----NLTLTSMFCAGYIEGR	373			
Sbjct 337	VSGWG+ + G T+ L L VPR+TQ+C+Q S + +T MFCAGY +G	396			
Query 374	QDSCKGDSGGPLVTRYRDTAFLLGIVSWGKGCARPGSYG IYTRVSNYLQWIRQ	426			
Sbjct 397	+DSCKGDSGGP T YR T +L GIVSWG+GCA G +G+YTRVS Y++W+++	449			

FIG 1.3 BLAST analysis. Comparison of the zebrafish and human FVII protein sequences using Basic Local Alignment Search Tool shows 48% identity between the two protein sequences. The identical amino acid residues are highlighted.

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CHAPTER 2

ESTIMATION OF CIRCULATING FVIIa AND FVII PLASMA LEVELS IN *Danio rerio**

2.1 Introduction

FVII is a vitamin K dependent plasma protein that plays an important role in initiating the extrinsic pathway of blood coagulation in mammals. Zymogen form of FVII is synthesized by the liver and secreted into blood as a single glycoprotein (1). Activated form of FVII binds to the TF exposed to circulating blood after an injury and the TF-FVIIa complex initiate thrombin generation shown in Fig. 2.1 (2). In normal adults most of the FVII circulates in its zymogen form and small but considerable amount accounts for the activated form FVIIa (3). The mechanisms of the activation of FVII resulting in circulating FVIIa levels in plasma are poorly understood. Zebrafish have a conserved extrinsic coagulation pathway and the FVII cDNA is orthologous to human FVII and is used as a successful model to study FVII (4). However, in order to understand the activation of FVII using zebrafish model, levels of FVIIa and FVII need to be measured in a zebrafish population. Studies in zebrafish have revealed the presence of FVII in plasma and its expression in liver (4). However, levels of the zymogen protein and its activated form have never been described before due to the lack of a proper assay. It is a known fact that teleost fish blood coagulates faster. However, there have never been any studies to understand this (5). Estimation of the FVII and FVIIa levels will also give an understanding of the concentration of the extrinsic factors in teleost fish. The main objective of this research is to estimate the concentration of zymogen and activated levels of FVII in an adult zebrafish population. To do this I took the ELISA approach to estimate the coagulation factor levels. ELISA is an enzyme linked immune sorbent assay that incorporates use of monoclonal

* This chapter is presented partly from Khandekar G, Jagadeeswaran P. 2014. Role of hepsin in factor VII activation in zebrafish. *Blood Cells Mol Dis* 52:76-81.

antibodies specific to a protein to capture it. The reaction is then analyzed by a secondary antibody against the primary antibody tagged to a fluorescent or chromogenic moiety. The reaction is subsequently detected using a suitable substrate. Comparisons with the standard protein-antibody reaction can help quantitate the assay (6). The goal of my research is to estimate the zymogen and activated levels of FVII using a monoclonal antibody specific to FVII/FVIIa. I plan to estimate FVIIa and FVII using monoclonal antibodies raised against FVII/FVIIa and use FVIIa specific inhibitor to estimate FVIIa, whereas use Gla antibody to estimate total FVII levels. Analysis of the levels of activated FVII and zymogen FVII will not only serve as a measure of FVII in zebrafish but will also give an idea of the extrinsic coagulation in teleost fish in general.

2.2 Materials and Methods

All experiments described below were approved by the University of North Texas Institutional Animal Care and Use Committee.

2.2.1 Human Plasma

Human blood samples were collected under institutional review board–approved protocols that included written informed consent from donors. Blood was collected into citrated tubes and the plasma was immediately used for the experiments.

2.2.2 Zebrafish Aquaculture

The zebrafish maintenance and breeding was performed as per mentioned in *The Zebrafish Book* and previous techniques mentioned in published papers (7). The adult zebrafish

were maintained at 28.5⁰C in deionized water supplemented with Instant Ocean. The zebrafish were maintained in a facility with 14 hour light - 10 hour day dark cycle and were fed with rich food like live brine shrimps and dry flake food.

2.2.3 FXa Experiments in Adult Zebrafish

5 µg and 0.5 µg of FXa (New England BioLabs, INC) were injected in the adult zebrafish intravenously as mentioned in previous papers (8). We chose these higher concentrations of FXa because we are using human FXa to cleave zebrafish FVII since; zebrafish and human FVII have only 48% identity at the protein level. The adult zebrafish were anesthetized in 2mM MS-222 (tricaine methanesulfonate) . For injection, a 27G1^{1/4} needle attached to 1 ml syringe was placed in the region closer to the anal pore near the inferior vena cava. The FXa solution was then gently injected into the vessels. The fish were then placed in individual fish tanks in deionized water preheated to 28.5⁰C and supplemented with Instant Ocean. The control fish were injected with 0.05 mM phosphate buffered saline, pH 7.4 and treated in a similar fashion. The fish were analyzed after 5, 10, 15, 20, 25 and 30 min for FVIIa activity by performing ELISA on their plasma using antibodies raised against zebrafish FVII/FVIIa.

2.2.4 *Xenopus* Injections

To obtain oocytes, *Xenopus* female was injected with 0.3 ml Human chorionic gonadotropin (Sigma) and anesthetized with 10% benzocaine the next day. An incision was made from the groin to the middle of the belly and the ovarian tissue was obtained. The ovarian tissue was minced and placed in a collagenase medium for defolliculation at 20⁰C for 2-3 hours. The oocytes were washed and then sorted under an optical microscope to select oocytes in stage

IV. The selected oocytes were washed in Barths medium and stored in Barths medium supplemented with gentamycin for injection (9). The good *Xenopus* oocytes were injected with the 50 nl cRNA using the picospritzer III (Parker Precision Fluidics; Hollis, NH). The injected eggs were retained at 16°C for 48 hours in Barths medium and used for Western blot analysis.

2.2.5 Zebrafish Plasma Collection

Blood was collected from adult zebrafish as described previously in citrated buffer (10). Blood was collected from wild type or adult injected fish by making a lateral incision posterior to the dorsal fin in the region of the dorsal aorta with the help of dissecting scissors and preventing the puncture of the gastrointestinal tract. The blood was immediately collected by a micropipette tip and put in citrated buffer in a ratio of 1:4. The citrated plasma was obtained by centrifugation of the blood at 1000 g for 3 min and was immediately used for ELISA assays.

2.2.6 ELISA Assays

2.2.6.1 FVIIa Standard Curve

The standard curve for FVIIa estimation was prepared using FVIIa standard provided in the kit (American Diagnostic, Inc). The FVIIa standard was diluted as per instructions from the manufacturer and used immediately. The standard was serially diluted to generate concentrations of 100, 50, 25, 12.5, 6.25 and 3.12 ng/ml. The FVIIa standards generated were captured by antibody raised against human FVIIa and detected by the enzyme inhibitor both provided in the kit. A standard curve was prepared each time with the zebrafish plasma samples.

2.2.6.2 ELISA Assay to Detect FVIIa Using Antibody Specific to Human FVII/VIIa

The ELISA kit to detect human FVIIa was tried on zebrafish plasma to analyze for specificity. The procedure was followed as per the manufacturer's instructions. The plasma from adult zebrafish or the supernatant from larval extract was incubated with the biotinylated FVIIa inhibitor provided in the kit and then loaded onto a microtiter plate pre-coated with monoclonal antibody specific to human FVII/ VIIa. The FVII/FVIIa was allowed to be captured by the antibody coated onto the wells of the microplate and was detected by the FVIIa specific inhibitor. Steptavidin-conjugated horse raddish peroxidase (HRP) was added and the reaction was detected by using 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate provided in the kit. The enzymatic reaction was quenched by addition of 50 μ L of 0.5 N H₂SO₄. Color development was quantified using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at 405 nm. Human FVIIa and the relative zebrafish FVIIa concentrations were interpolated from the human FVIIa standard curves which were generated each time the assay was performed as per the kit manual.

2.2.6.3 ELISA Assay to Detect FVIIa Using Antibody Specific to Zebrafish FVII/FVIIa

Rabbit anti-zebrafish FVII peptide antibody used in previous studies was used to detect zebrafish FVII (4).

2.2.6.3.1 Bradford Assay

A Bradford assay was performed to estimate the concentration of the antibody. The bovine serum albumin was used as a standard to measure the concentration. A standard curve

was generated for 0-1.0 mg/ml of bovine serum albumin using the Bradford reagent as described in the previous studies and the antibody concentration was extrapolated from it (11).

2.2.6.3.2 Coating of the Microplates

Microtiter plates (96-wells/plate) were coated with the rabbit anti-zebrafish FVII peptide antibody at 10 µg/ml in 100 µl antibody coating buffer (ImmunoChemistry Technologies, LLC) overnight at 4°C followed by blocking with SynBlock Buffer (ImmunoChemistry Technologies, LLC) followed by washing with the Universal ELISA Wash Buffer (ImmunoChemistry Technologies, LLC). The plates were then dried and stored at 4°C until further use.

2.2.6.3.3 ELISA Assay

FVIIa activity was assayed using the inhibitor and buffers from the IMUBIND® FVIIa ELISA (American Diagnostic, Inc) and captured with the rabbit anti-zebrafish FVII peptide antibody. The procedure was followed as mentioned in the kit manual except for the capture antibody. Briefly, the zebrafish plasma was incubated with the biotinylated enzyme inhibitor of FVIIa and the samples were added to the microtiter plate coated with rabbit anti-zebrafish FVII peptide antibody and incubated at 4°C for 1 hour. After brief washing, the streptavidin-conjugated to HRP was added and incubated for 30 min at 25°C and this was followed by three washes. The reaction was measured using TMB substrate provided in the kit. The enzymatic reaction was quenched by the addition of 50 µL of 0.5 N H₂SO₄. Color development was quantified using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at 405 nm. FVIIa concentration was interpolated from the standard curves generated each time the assay was performed as per the manufacturer's instructions.

2.2.6.4 FVII Standard Curve

The standard curve for FVII estimation was generated using human FVII standard. The FVII standard was diluted serially to generate additional concentration of 100, 50, 25, 12.5, 6.25 and 3.12 ng/mL. The FVII standards generated were captured by antibodies raised against human FVII and the Gla antibody was used to detect FVII concentration. A standard curve was run each time with the zebrafish plasma samples.

2.2.6.5 ELISA Assay to Detect FVII Using Antibody Specific to Zebrafish FVII/VIIa

Rabbit anti-zebrafish FVII peptide antibody used in previous studies was used to detect zebrafish FVII (4). Microtiter plates (96-well plate) were coated with the antibody at 10 µg/ml in 100 µl as mentioned above. The zebrafish plasma from wildtype fish was added to the coated plate and incubated at 4°C for 1 hour. After brief washing, the anti Gla antibody raised against Gla domain in mouse were used to detect the total FVII. After brief washing, the anti mouse antibody-conjugated to HRP was added and incubated for 30 minmin at RT followed by three times wash. The reaction was detected using 3, 3', 5, 5'- TMB. The enzymatic reaction was quenched by addition of 50 µL of 0.5 N H₂SO₄ and the color development was quantified as mentioned above using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at 405 nm. Total FVII concentration was interpolated from the standard curves generated each time the assay was performed.

2.2.7 Expression of FVII in *Xenopus* Eggs and Western Blot

FVII cRNA was transcribed from a previously engineered pCDNA3.1+ vector construct containing the zebrafish *f7* cDNA subcloned into the EcoRI site using T7 polymerase (Roche

Applied Science) (4). A solution containing 50 nl of cRNA was injected into *Xenopus* oocytes using pizospitzer. Since FVII is gamma carboxylated post translationally using gamma-glutamyl carboxylase which requires vitamin K, 10 µg/ml of vitamin K was also injected into the *Xenopus* oocytes. The oocytes were retained at 16°C for 48 hours in Barths medium and were washed and homogenized in Tris-10 mM EDTA (pH 8.0) followed by a brief centrifugation to remove insoluble material of the oocytes extracts. Similar extracts were prepared from 1X PBS-injected oocytes. The 10 µl extracts from 1X PBS-injected oocytes and cRNA-injected whole cell extract of *Xenopus* oocytes were incubated with 5 µl of 0.5 µg FXa and 100 mmol/l of CaCl₂ for 20 min. The controls were incubated without FXa .The whole cell extracts (both control and experimental) were then boiled for 10 min in equal volumes of 2X Laemmli Sample Buffer (BIO-RAD) and were used immediately for Western analysis. The samples were run on SDS/9% PAGE at 140 V for 90 min until complete separation of the prestained protein marker was obtained and transferred on nitrocellulose membrane for Western blotting. Biotinylated enzyme inhibitor of FVIIa (American Diagnostic, Inc) was used as a primary probe in place of the primary antibody and was diluted 1:40. Streptavidin-conjugated HRP antibody was diluted 1:1000.

2.2.8 Statistical Analysis

Statistical analysis was performed using Sigma Plot 10[®] with Sigma Stat integration software. Statistical significance was assessed by ANOVA and a P value <0.05 was considered significant. Dunnett's test was used for post-hoc analysis.

2.3 Results

Previous studies from our laboratory identified FVIIa in zebrafish and homology modeling suggested conservation in the overall three-dimensional structure between zebrafish and human FVIIa (4). However, the levels of FVIIa in zebrafish plasma were never determined. In order to quantitate the levels of activated FVII and total FVII we used ELISA approach.

2.3.1 Estimation of FVIIa in Zebrafish Population

2.3.2 ELISA Assay to Measure FVIIa Using Antibodies Against Human VII/VIIa

To estimate the levels of FVIIa in zebrafish population I decided to use the Immubind[®] FVIIa kit (American Diagnostic, Inc) available to estimate FVIIa levels in human. The principle behind the kit is the use of an FVIIa enzyme inhibitor that will covalently bind only to FVIIa and not FVII. The assay also makes use of antibodies specific to human FVII/FVIIa to capture the coagulation factor in the ELISA. Using this kit, I estimated FVIIa levels in adult zebrafish plasma. The principle of this assay is based on capture of FVII/ VIIa in the plasma by the monoclonal antibodies specific to FVII/FVIIa and detection of only FVIIa by the enzyme inhibitor shown in Fig. 2.2. A standard curve is generated each time and the FVIIa levels in the plasma were extrapolated from the curve. The standard curve was generated with FVIIa standard provided in the kit and incubated with the enzyme inhibitor and captured by the antibodies to human FVIIa provided in the kit shown in Fig. 2.3. On using the ELISA to estimate FVIIa concentration in adult zebrafish plasma the relative FVIIa levels were found to be 7 ng/ml as shown in Fig. 2.4. The human plasma was found to have 5.3 ng/ml of circulating activated FVII in plasma and was used as a positive control for the assay as shown in Fig. 2.4.

2.3.3 ELISA Assay to Measure FVIIa Using Antibodies Against Zebrafish FVII/VIIa

Since zebrafish blood clots faster than mammalian blood which might be due to high concentration of activated coagulation factors, I thought the estimation may not be the complete estimation owing to the lack of specificity of the antibody towards zebrafish FVIIa (12). Hence to make the assay more specific I decided to use antibodies raised against zebrafish FVIIa. The rabbit anti-zebrafish FVII peptide antibody was previously used to detect zebrafish FVII (4). I did a Bradford assay to estimate the concentration of the antibodies. I did a Bradford assay, to estimate the concentration of the antibodies shown in Fig. 2.5, before coating the 96-welled microtiter plate with the antibody. Using the enzyme inhibitor and anti-zebrafish FVII peptide antibody we estimated the FVIIa concentration in adult zebrafish population. The relative FVIIa concentration in adult zebrafish plasma was found to be 108 ng/ml shown in Fig. 2.6. Since the enzyme inhibitor provided by the American Diagnostica is specific to FVIIa and does not bind to FVII, the estimated concentration of FVIIa is of the FVIIa circulating in plasma and FVIIa bound to TF complex.

2.3.4 Western Blot Analysis for Enzyme Inhibitor Specificity

Since the specificity of the enzyme inhibitor has to be determined for zebrafish FVIIa I did Western blot analysis on binding of enzyme inhibitor to zebrafish FVIIa. For this, a previously cloned pCDNA3.1+ vector construct containing the zebrafish cDNA was transcribed and the cRNA was injected in *Xenopus* oocytes along with vitamin K. Since FVIIa is post-translationally modified protein that requires vitamin K for the gamma carboxylation of the glutamine acid residues in the Gla domain we injected vitamin K along with the cRNA. The oocytes were lysed after 48 hr and the cell extract was run on a SDS PAGE gel. Part of the

Xenopus oocytes extract was also incubated with Factor Xa and 100 mmol/l CaCl₂ to confirm the presence of FVIIa. FXa will activate FVII and this increase would be estimated in Western blots. The uninjected oocytes with and without incubation of FXa and 100 mmol/l CaCl₂ served as a control. On performing Western analysis with the help of enzyme inhibitor as the primary detector and streptavidin-conjugated to HRP as the secondary detector, a band of 50 kDa was obtained, confirming the specificity of the enzyme inhibitor to zebrafish FVIIa. In the cell extracts incubated with FXa increased intensity for the 50 kDa band was seen confirming the FVIIa activity. No other bands were observed shown in Fig. 2.7.

2.3.5 Injection of FXa into Adult Zebrafish to Validate the ELISA Assay

FXa is known to activate FVII *in vivo* (13). Injection of FXa in adult zebrafish will lead to increase in FVIIa levels and this increase would be measured by the assay. To prove this 5 µg and 0.5 µg of FXa was injected intravenously into adult zebrafish. The blood was collected from the injected adult fishes at 5, 10, 15, 20, 25 and 30 min and the plasma was analyzed for factor VIIa activity using the ELISA. Factor VIIa levels were found to increase with the increasing time, however, after 20 min the fish were found to be thrombotic and blood collection was difficult. At 30 min most of the fish died and hence, all later experiments were performed after 20 min incubation shown in Fig. 2.8. After 20 min the blood was collected and the ELISA assay was performed. FVIIa levels were found to be increased by 1.8 times more in 0.5 µg of FXa injected zebrafish while the zebrafish injected with 5 µg of FXa had their FVIIa levels increase 2.6 times proving the estimation of FVIIa in the developed assay shown in Fig. 2.9.

2.3.6 Estimation of FVII in Adult Zebrafish Population

To estimate the total FVII levels in zebrafish plasma I decided to use rabbit anti-zebrafish FVII peptide antibody used in previous studies and antibodies raised against the Gla domain. The principle behind this sandwich ELISA is the estimation of FVII/VIIa by capturing with antibodies raised against zebrafish FVII and detecting the captured FVII/VIIa with antibodies raised against the Gla domain shown in Fig. 2.10. A standard curve was run with human FVII standard and the antibodies to human FVIIa along with the Gla antibodies shown in Fig. 2.11. Human plasma was run as a control by capturing with antibodies against human FVII. The concentration of FVII in human plasma was found to be 491 ng/ml and is shown in Fig. 2.12. On performing the sandwich ELISA in adult zebrafish plasma by using antibodies against zebrafish FVII the levels of total FVIIa were found to be 958 ng/ml as shown in Fig. 2.12. Since the Gla domain is present both in FVII and its activated form this assay does not differentiate between FVII and FVIIa and estimates the total levels of zymogen FVII and its activated form.

2.4 Discussion

The FVII levels were estimated in order to get an understanding of the levels of FVII/VIIa circulating in zebrafish plasma as well as to use this assay in the knockdown of genes that may potentially play a role in FVIIa generation. In the earlier studies in our laboratory we analyzed presence of zebrafish FVII and its high similarity with human FVII. However, it is also important to know the circulating levels of FVII/ VIIa in order to understand completely understand the extrinsic coagulation in zebrafish. To do this, I used the ELISA chromogenic assay routinely used in estimations of FVIIa levels in human. On performing the ELISA the relative concentration of FVIIa in adult zebrafish was found to be 7 ng/ml. Since zebrafish like

the other teleost fish clots faster which could be due to increased coagulation factors or increased FVIIa levels, I wondered whether the estimated FVIIa levels in zebrafish plasma obtained by using human antibody may be lower than the values I obtained. This made me question the specificity of the antibody to human protein with the zebrafish FVII. Hence, I decided to use the antibody raised against zebrafish FVIIa to capture it in the ELISA assay. The specificity of the zebrafish FVIIa enzyme inhibitor was confirmed using a Western blot assay. To do a Western blot, I decided to express zebrafish FVII in *Xenopus* oocyte expression system. Since the inhibitor only binds to FVIIa and not FVII, the protein recognized by the Western analysis is FVIIa and not FVII. To further confirm that this band obtained was due to specificity to FVIIa and not FVII, I incubated the *Xenopus* extracts with FXa and CaCl₂. FXa activates FVII in presence of Ca⁺² and phospholipids (14). So the *Xenopus* extracts were incubated with FXa and Ca⁺² and the increase in FVIIa was reflected as an increase in the intensity of the 50 kDa band during Western analysis confirming specificity of the inhibitor to zebrafish FVIIa. After confirmation of the specificity of the inhibitor with the Western analysis the ELISA assay was performed on adult zebrafish injected with FXa to measure the increase in FVIIa levels after *in vivo* activation. A time course experiment was performed to determine the optimal concentration of FVIIa after FXa injections. Twenty min after FXa injections I found the fish to be dying of thrombosis and therefore I analyzed FVIIa levels at 20 min for the ELISA assay. Analysis of the plasma FVIIa levels in adult zebrafish showed an increase in FVIIa levels in zebrafish injected with FXa as compared to the controls. Thus, the Western blot and ELISA after FXa treatment provided support for the specificity of the assay in estimating FVIIa levels in zebrafish.

On comparing the circulating plasma FVIIa levels in zebrafish with those found in human, the FVIIa plasma levels in a wildtype adult zebrafish population are about 10 times more

than those found in human plasma (3). These high levels of FVIIa do explain the phenomenon of blood clotting faster in teleost fish. Studies in fresh water fish, like the cichlid fish and the teleost, have shown blood coagulation similar to mammals but with a much reduced clotting time which is attributed to highly active intrinsic and extrinsic coagulation pathways (5, 12, 15). Our results also indicate that the high levels of activated FVII can contribute to the presence of a highly efficient hemostatic mechanism in *Danio rerio*, a teleost fish, to cope up with the vascular damage.

On comparing the total FVII levels in adult zebrafish population, the concentration of FVII in plasma was found to be 958 ng/ml. The total FVII concentration in human plasma is around 491 ng/ml (16). Thus, comparison of total FVII levels and activated FVII levels in zebrafish and human studies reflects that the threshold levels are maintained in zebrafish. However, due to increased activated form of FVII the blood may clot faster in zebrafish. Finally, the assays developed in my Aim 1 should be useful in identifying the serine protease activity responsible for FVIIa generation by using genetic knockdown approaches.

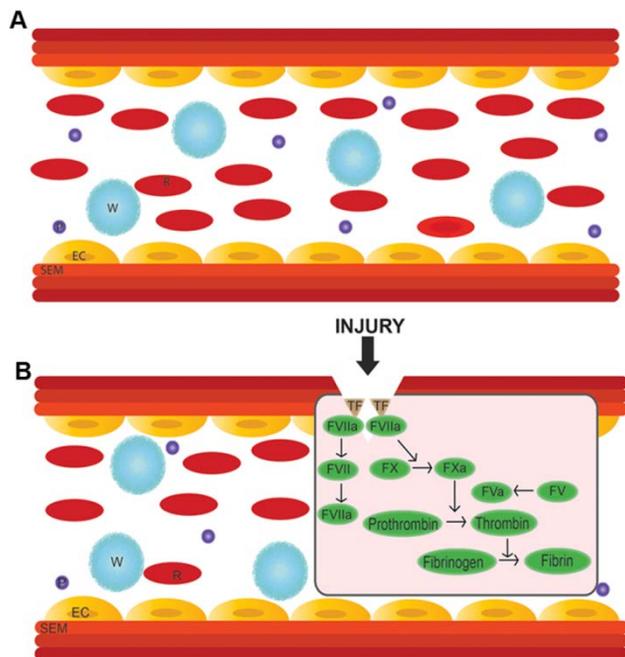


FIG 2.1 Extrinsic pathway of blood coagulation. Schematic representation of the clot formation after an injury inside a vein. A. Normal blood flow inside a vein. B. Activation of extrinsic pathway after an injury. The red blood cells (R) are represented by red circles, white blood cells (W) are represented by blue circles, platelets (P) are represented by purple circles; subendothelial membrane (SEM) and endothelial cells (EC) are also shown (Thrombosis and Hemorrhage, 3rd edition) (17) .

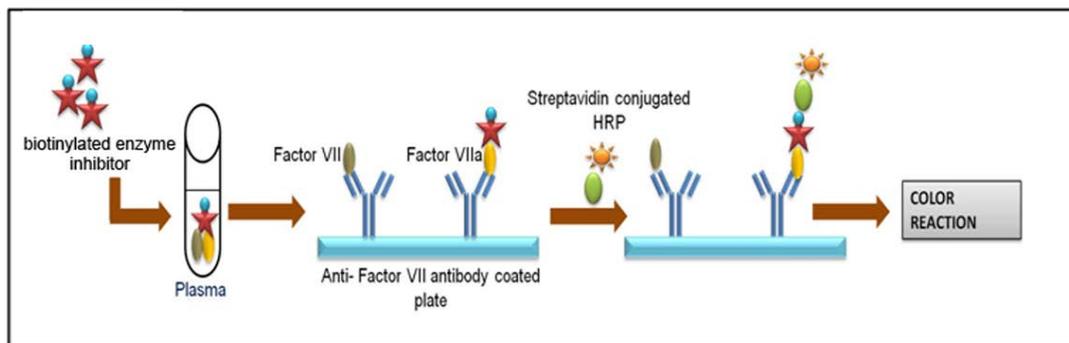


FIG 2.2 Schematic representation of FVIIa ELISA. Figure drawn from the procedure mentioned in IMUBIND® Factor VIIa kit, American Diagnostica, Inc..

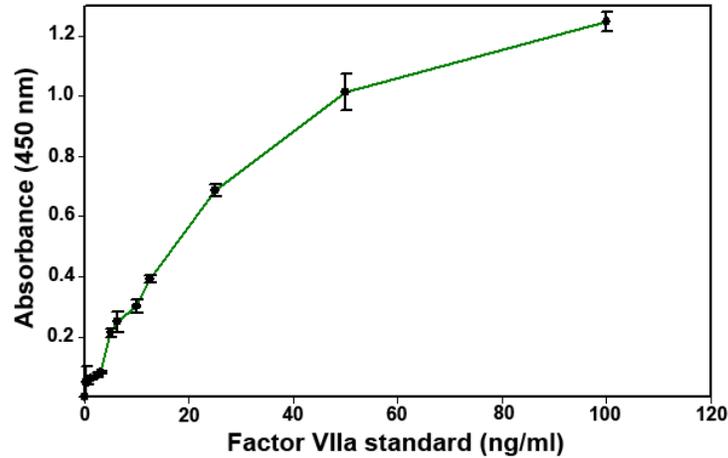


FIG 2.3 FVIIa standard curve. The standard curve was generated for 0-100ng/ml concentration of FVIIa incubated with the enzyme inhibitor and captured by antibodies to human FVIIa.

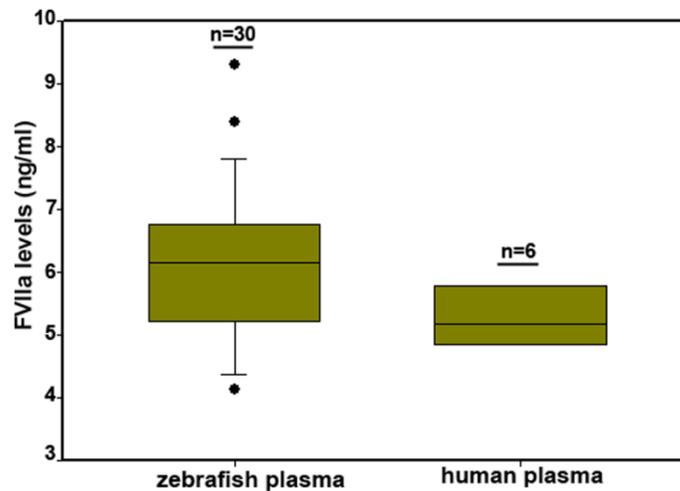


FIG 2.4 FVIIa estimation using antibodies to human FVIIa. Plot 1 shows FVIIa levels in adult zebrafish plasma. The zebrafish blood was collected in 3.8% sodium citrate and the plasma was analyzed for FVIIa levels by performing ELISA using anti FVII antibodies against human FVIIa. n= 30, Median= 4.5, SE= 1.1. Plot 2 shows FVIIa levels in human plasma. The FVIIa levels were analyzed by performing ELISA using anti FVII antibodies against human FVIIa. n= 6, Median = 5.2, SE= 0.2. Units are in ng/ml.

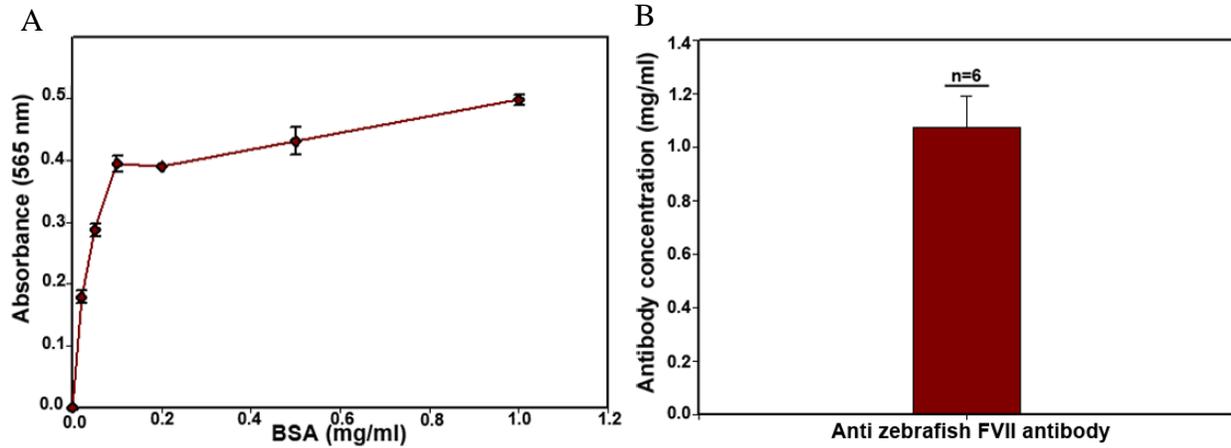


FIG 2.5 Bovine serum albumin standard curve. (A) Standard curve generated using 0-1.0 mg/ml of bovine serum albumin by Bradford assay (B) Antibody concentration estimation.

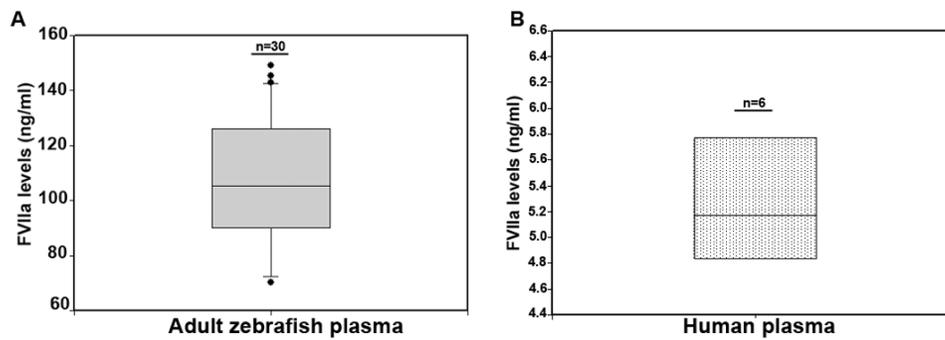


FIG 2.6 FVIIa estimation using antibodies to zebrafish FVIIa. (A) ELISA using antibodies against zebrafish Factor VII and biotinylated enzyme inhibitor of FVIIa to estimate the zebrafish FVIIa levels. n= 30, Median = 105.3, SE= 4.3. (B) ELISA using antibodies against human Factor VII and biotinylated enzyme inhibitor of FVIIa to estimate the human FVIIa levels. N= 6, Median= 5.2, SE= 0.2. Units are in ng/ml.

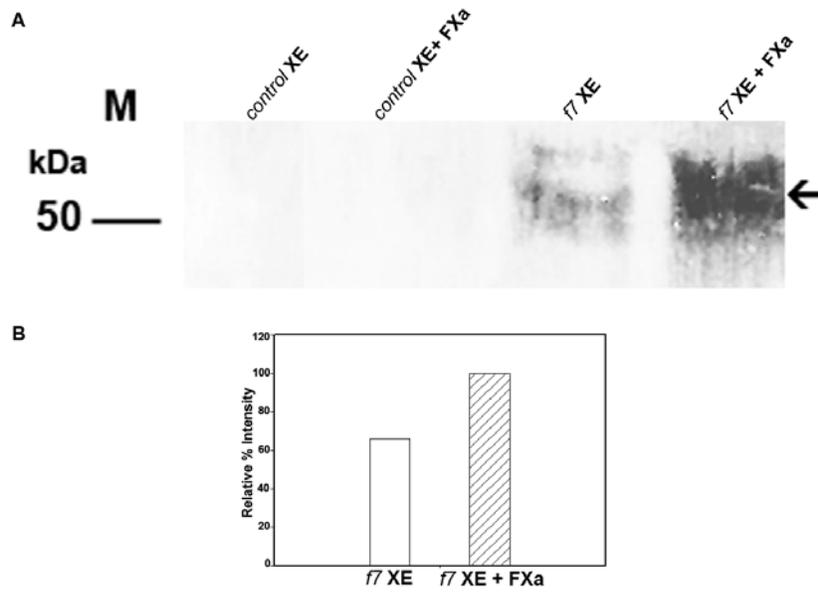


FIG 2.7 Detection of zebrafish FVIIa expressed in *Xenopus* oocytes by Western analysis. Detection of zebrafish FVIIa expressed in *Xenopus* oocytes by Western analysis. (A) Western blot detecting FVIIa. Lane 1, 1X PBS-injected *Xenopus* oocyte extracts (control XE); Lane 2, 1X PBS-injected *Xenopus* oocyte extracts incubated with FXa (control XE + FXa); Lane 3, *Xenopus* oocyte extracts injected with *f7* cRNA (*f7* XE); Lane 4, *Xenopus* oocyte extracts injected with *f7*cRNA and incubated with FXa (*f7* XE + FXa). Molecular mass markers (M) are indicated on the left. Arrows point to the 50 kDa band. (B) The graph showing comparison of the relative percentage intensities of the observed FVIIa bands in panel A using ImageJ[®] analysis on equal areas of the bands after normalizing to their respective controls.

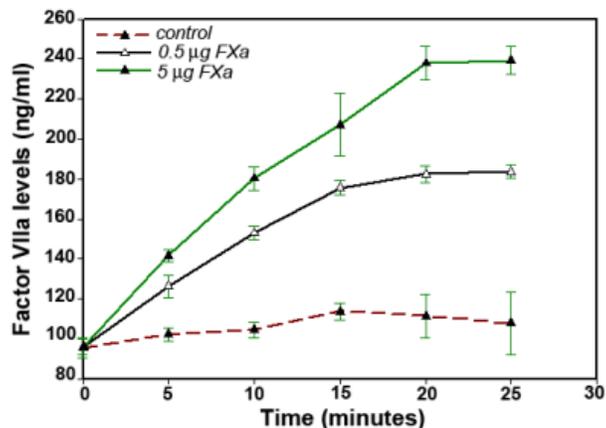


FIG 2.8 Time line for increasing FVIIa levels after injecting FXa. Adult zebrafish were injected with 5 µg and 0.5 µg of FXa. The zebrafish blood was collected in 3.8% sodium citrate and the plasma was analyzed for FVIIa levels at 5 minute time intervals by performing ELISA using anti FVII antibodies against zebrafish FVIIa. The fish that were incubated over 25 min were found to be thrombotic and died. Control fish were injected with 1X PBS (n= 3).

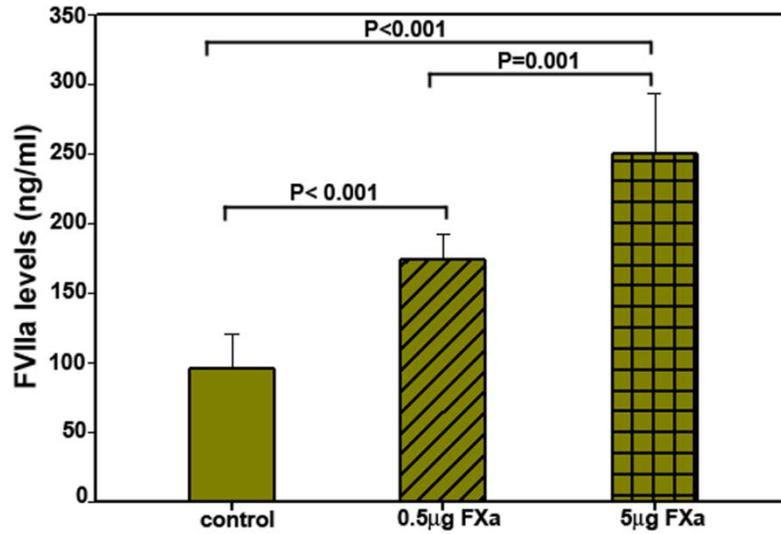


FIG 2.9 FVIIa levels in adult zebrafish injected with FXa. Estimation of FVIIa levels by ELISA after FXa injections in adult zebrafish plasma. The mean for: 1X PBS-injected control zebrafish plasma (control) is 96 ng/ml; 0.5 µg of FXa-injected zebrafish plasma (0.5µg FXa) is 174 ng/ml and 5 µg of FXa-injected zebrafish plasma (5µg FXa) is 250 ng/ml. n= 12 and the statistical significance is shown by P values in the graph.

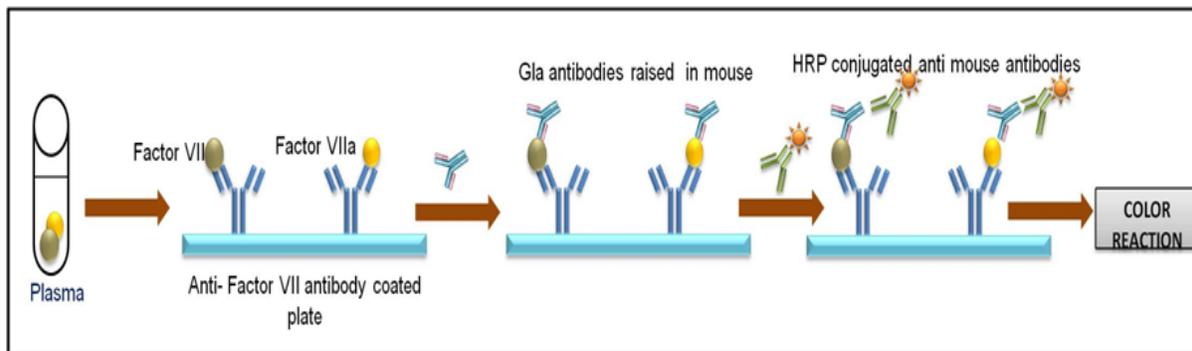


FIG 2.10 Schematic representation of sandwich ELISA to estimate total FVII.

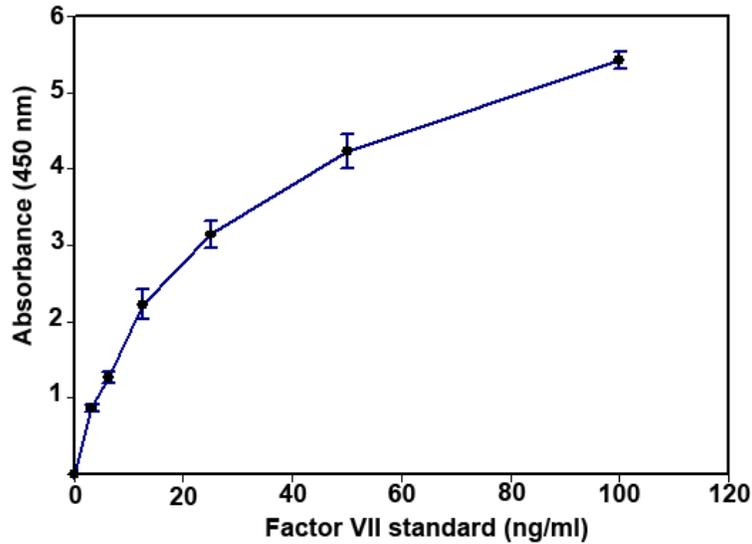


FIG 2.11 FVII standard curve. The standard curve was generated for 0-100 ng/ml concentration of FVII after capturing with anti FVII antibodies against human FVII/FVIIa and using antibodies to the Gla domain as the secondary antibody.

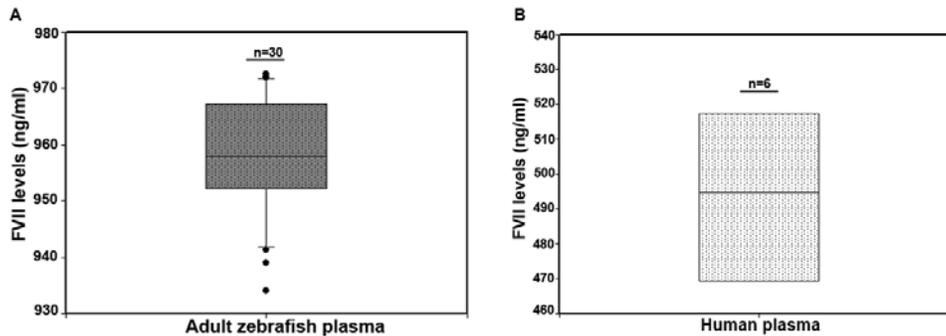


FIG 2.12 FVII estimation using antibodies to zebrafish FVII/FVIIa. (A) Sandwich ELISA using antibodies against zebrafish Factor VII to capture FVII+FVIIa and anti GLA antibodies to detect FVII levels in zebrafish plasma. n= 30, Median= 957. 9, SE= 1.9. (B) Sandwich ELISA using antibodies against human Factor VII to capture FVII+VIIa and anti GLA antibodies to detect FVII levels in human plasma. n= 6, Median= 489.6, SE= 9.3. Units are in ng/ml.

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CHAPTER 3

VERIFICATION OF THE ROLE OF FSAP AND HEPSIN IN ACTIVATING FVII IN VIVO BY USING ZEBRAFISH REVERSE GENETICS[†]

3.1 Introduction

The mechanisms of the activation of FVII resulting in FVIIa levels in plasma are poorly understood. Human FVIIa being a serine protease is activated by a cleavage at its arginine 152 and isoleucine 153 bond. Human FVIIa cleavage results in the formation of a light chain polypeptide of 152 amino acids and a heavy chain polypeptide of 254 amino acids held together by a disulfide bond as shown in Fig. 3.1 (1). On the other hand zebrafish FVII is cleaved between arginine 195 and isoleucine 196 resulting in a protein containing a 158 amino acid light chain and a 238 amino acid heavy chain (2). FVII is secreted as a zymogen in the liver and gets activated by unknown factors to release FVIIa into plasma. Circulating plasma FVIIa levels are maintained as a part of normal hemostasis. The circulating FVIIa levels in human are 5 ng/ml, while in zebrafish I found them to be about 108 ng/ml (3). However, to date many activators of FVII are known *in vitro*. However, their mechanism of action *in vivo* remains poorly understood. FXa is a known FVII activator both *in vitro* and *in vivo* (4, 5). FVII is activated *in vitro* efficiently by FIXa, FXIIa, thrombin, tissue plasminogen activator, plasmin etc. (4-7). Apart from these, serine proteases which were never shown to be participants in the coagulation cascade earlier are now shown to activate FVII *in vitro*. FSAP, also called hyaluronan binding protein 2, is an extracellular serine protease synthesized as a single chain peptide which undergoes activation to form a functionally active heterodimer. It was first isolated from human plasma in a prothrombin complex concentrate and was thought to be 'thrombin-like' due to its

[†] This chapter is presented partly from Khandekar G, Jagadeeswaran P. 2014. Role of hepsin in factor VII activation in zebrafish. *Blood Cells Mol Dis* 52:76-81. Other part of the results from this chapter are submitted to a peer reviewed journal.

amidolytic properties (8). It binds to hyaluronic acid and is mainly involved in cell adhesion (9). FSAP was found to activate FVII independent of TF *in vitro* (8). It was also found to have a specificity for activating pro-urokinase (pro-uPA) (10). Interestingly, experiments with recombinant FSAP failed to activate FVII *in vitro* (11). Analysis of FSAP substrates in human plasma using sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed fibrinogen and fibronectin as the major substrates. It did not play any role in initiation of fibrin clot nor did it cause the fibrinolysis directly. It also did not cleave prothrombin and plasminogen but was found to activate single chain urinary plasminogen activator (9).

Another serine protease called hepsin was also found to activate FVII *in vitro*. hepsin is a membrane bound serine protease highly expressed in hepatocytes while low levels are found in several human tissues including the kidney, prostate and thyroid tissues (12, 13). hepsin was found to play an important role in cell growth and function and also regulate liver morphology (13, 14). *In vitro* studies have shown hepsin to activate FVII, FIX, pro-uPA and pro-hepatocyte growth factor (pro-HGF) (15, 16). Hepsin is also found to be strongly up-regulated in prostate cancer and few other cancers like ovarian cancer, renal cell carcinomas, breast tumors etc. (17). Transfection of BHK cells with a plasmid containing the cDNA for human hepsin activated FVII along with other coagulation factors (15). However, studies on hepsin-knockout mice showed these mice viable and found them to be fertile with no defects in the liver when assayed by biochemical and histological methods The blood clotting was analyzed by tail bleeding times and was found to be normal indicating no role of hepsin in hemostasis or liver function (18). To date, the physiological function of hepsin remains unknown. Thus the role of hepsin and FSAP in activating FVII *in vivo* is still controversial (11, 19). In this part of my project I decided to

reinvestigate the role of FSAP and hepsin in activating FVII *in vivo* using zebrafish as a model system.

The main objective of this research is to knockdown the function of genes coding for FSAP and hepsin and analyze the effect on FVIIa levels and extrinsic clotting cascade function as a whole. To do this I took the knockdown approach using antisense oligonucleotides called Morpholinos to ablate the gene function. Morpholino oligos (Morpholinos) are widely used to knock down gene expression in zebrafish and are nonionic DNA analogs with altered sugar and backbone linkages. The Morpholinos (MO) are designed antisense to the mRNA for specific gene either for a translational start site so that no protein is synthesized or for a pre-mRNA splice site such that an important functional domain in the protein is lost by exon skipping. The resulting RNA-MO hybrids either prevent translation or produce a non functional protein in RNase H independent manner (20). These MOs are injected into the yolks of one to four celled stage zebrafish embryos. It is believed that the cytoplasmic bridges connecting the early embryonic cells aid in rapid diffusion of the MOs and result in efficient and ubiquitous delivery into the cells (21). This technology is now commonly used to study gene function during early developmental stages as well as in larvae (20, 22). However, MOs cannot be delivered into later stage embryonic cells or adult tissues. To address this issue recently Vivo-Morpholino (VMO) were developed. These VMOs are MOs that are linked covalently to a molecular scaffold containing a guanidinium group on each of its eight tips. The free guanidinium groups form two hydrogen bonds with the phosphate's oxygen atoms and this strong interaction distorts the endosomal membrane and renders it permeable to cells. Thus the VMO can easily enter the cytosol and nuclei of cells in a tissue from the extracellular space. The VMOs have been successfully used in gene knockdown experiments in adult mice particularly in treating Duchene

muscular dystrophy (23, 24). Recently our laboratory has introduced for the first time to knockdown the genes in adult zebrafish to study thrombocyte function. Since then a number of laboratories have used VMOs to knockdown several genes in adult zebrafish tissues including brain. (25-27). Therefore I hypothesized that using the VMO and MO designed for splice sites for *habp2* and *hpn* (genes coding for FSAP and hepsin, respectively) pre-mRNAs should result in abnormal and nonfunctional FSAP and hepsin, which will affect FVIIa levels if these proteins are involved in FVII activation.

3.2 Materials and Methods

3.2.1 Morpholino Design

VMOs were designed for FVII (*f7*), FSAP (*habp2*) and hepsin (*hpn*) genes and were purchased from Gene Tools; Philomath, OR. VMO for *f7* 5'-TCTGTGCTCCTCTGCTGTACCTGCC-3', was designed at the donor splice site of the exon 6. VMO for *habp2* 5'-CAAATAAATCCAACCTACATGCAGT-3', was designed at the donor splice site of the exon 10 while VMO for *hpn* 5'-AAATGTGTGTGTCACCTCACTCAGGA-3', was designed at the donor splice site of the exon 8. A control Vivo-MO 5'-CCTCTTACCTCAGTTACAATTTATA-3' was also purchased from Gene-Tools. Regular MOs were also designed for *f7*, *habp2* and *hpn* with the same sequence as the *f7* VMO, *habp2* VMO, *hpn* VMO and the control VMO, respectively (Gene Tools; Philomath, OR).

3.2.2 Morpholino Injections

5 μ l of the 0.5mM of VMOs were injected into the adult zebrafish using a 27G1^{1/4} needle intravenously as previously described (25). The zebrafish were analyzed after 48 hr for FVIIa

levels using ELISA assay and kPT. The regular MOs were microinjected into the yolk of the one to four celled zebrafish embryos. Microinjections were performed using picospritzer III (Parker Precision Fluidics; Hollis, NH) and an Olympus inverted microscope equipped with left and right hand Leitz micromanipulators. The embryos were placed on a 2% agarose bed with grooves that hold the embryos together. The injection pipette was loaded with 1 μ l of the MO and 1-2 nl was injected into the yolk sac with a short stabbing motion (26, 28).

3.2.3 ELISA Assays

The ELISA assays were performed on VMO-injected zebrafish with the technique developed in my Aim 1 using rabbit anti-zebrafish FVII peptide antibody to estimate FVIIa levels. The blood was collected from both the control VMO and gene specific VMO-injected zebrafish after 2 days and the plasma was separated by centrifugation and used for ELISA to detect FVIIa levels in zebrafish plasma. The one to four celled embryos were also injected with regular MOs. ELISA assays were also performed on 5 dpf on larvae by crushing the larvae in sodium citrate and centrifuged at 1000 g for 5 min. The supernatant was analyzed for FVIIa levels using the ELISA.

3.2.4 Coagulation Assays

3.2.4.1 Kinetic Prothrombin Time

The thromboplastin reagent was made from zebrafish muscle as described before (29). The zebrafish muscle tissue was suspended in 0.1 M NaCl, 50 mM Tris, pH 7.5, on ice and homogenized for 30 sec. The homogenate was stirred at 37°C for 30 min followed by centrifugation at 11,000 g for 15 min. EDTA was then added to the supernatant to achieve a

concentration of 20 mM followed by centrifugation at 48,000 g for 60 min. The resulting pellet was washed twice followed by and was finally resuspended in 1.5 ml of buffer containing 0.1 M NaCl, 50 mM Tris, pH 7.5. The reagent was stored at -25°C for use in the kinetic assays.

The kinetic prothombin time (kPT) was performed as described previously (29). The blood was collected from the VMO-injected zebrafish and was centrifuged to collect plasma. 0.25 µl of the zebrafish plasma was assayed for coagulation by adding 5 µl of the above prepared thromboplastin reagent and 10 µl of fibrinogen to the plasma in a 96-well microtiter plate. The plasma was recalcified with 100mM CaCl₂ and the fibrin generation over time was detected by absorbance at 405 nm using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). 1X PBS instead of thromboplastin was used as a negative control.

3.2.4.2 Time to Occlusion Assay

Time to occlusion (TTO) was measured in 4 day old zebrafish larvae generated after injection with the MO at the one to four cell stages. The larvae were placed in 250 µl of water and anesthetized with 10 µl of 0.3 mM of tricaine solution. The larvae were then immobilized in 1% low-melting agarose solution. The caudal vein area around 5-6 somites posterior to the anal pore was then ablated using a nitrogen laser light pumped through coumarin 440 dye using a MicroPoint Laser System (Photonic Instruments Inc.; St. Charles, IL) connected to a Nikon Optiphot microscope and the time to occlude was measured in seconds as previously described (30).

3.2.5 RNA Extraction

3.2.5.1 Zebrafish Liver

Total RNA was extracted from whole liver of VMO-injected adult zebrafish using the Direct-zol™ RNA MiniPrep (Zymo Research). For liver RNA extractions, the whole zebrafish liver was isolated from the VMO-injected zebrafish and homogenized in TRI REAGENT®. Briefly one volume of 90% ethanol was added to the homogenized TRI REAGENT® and the mixture was vortexed for 30 seconds. The mixture was loaded into the Zymo-Spin™ IIC Column2 in a Collection Tube provided in the kit and was centrifuged for 1 min. The flow through was transferred into the column into a new Collection Tube and 400 µl of Direct-zol™ RNA PreWash (provide in the kit) was applied followed by centrifugation. The column was then washed thrice with RNA Wash Buffer followed by centrifugation. The RNA was then eluted by adding DEPC treated water in the column and centrifugation at maximum speed for 1 minute. The eluted RNA was stored at -80°C until further use.

3.2.5.2 Whole Larvae

The morpholino injected zebrafish larvae at 4 dpf were homogenized in TRI REAGENT® and processed as mentioned above for RNA extraction.

3.2.6 Reverse Transcriptase PCR

The RNA was amplified using the SuperScript® One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). The primer sets used were *f7*: forward primer 5'GCGCAGACGGCTATTACCTA 3' and reverse primer: 5' GATCCAGGTGGGCTTGTAGA 3', *habp2* forward primer 5' TCCAGATGGAGACCGAGAAC 3' and reverse primer: 5'

GAGGGCGTCAAAAAGTCTCTG 3', *hpn* forward primer 5' AGGGCAGATTCTTGAGGTGA 3' and reverse Primer: 5' GAACTGCAGAGGTTTCGTCA 3' For the amplification of the *efl* α the primers sets used were forward primer 5'-CGGTGACAACATGCTGGAGG-3' and reverse primer 5'-ACCAGTCTCCACACGACCCA-3' were used. The extracted RNA was converted to cDNA at 42°C for 30 min followed by initial denaturation at 94°C for 2 min followed by 0.15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, with 10 min extension at 72°C on the last cycle. The product was amplified for 45 cycles and the products were separated using 1.5 % agarose gel electrophoresis.

3.2.7 Statistical Analysis

Statistical analysis was performed using Sigma Plot 10[®] with Sigma Stat integration software. Statistical significance was assessed by ANOVA and a P value <0.05 was considered significant. Dunnett's test was used for post-hoc analysis.

3.3 Results

After estimating the concentration of FVIIa and total FVII levels in zebrafish plasma I decided to analyze role of FSAP and hepsin in FVII activation in adult zebrafish. Because of the controversy regarding role of these two serine proteases in activating FVII, I decided to use a gene knockdown approach in zebrafish to see its effect on FVII activation *in vivo*. By this it will be the first study of *in vivo* effects of FSAP and hepsin in zebrafish. To accomplish this goal, VMOs were designed at the spliced site of the serine protease domain of both the FSAP and hepsin genes to knockdown their function in adult fish. FVII knockdown was also performed as a control to compare the loss of hemostatic function.

3.3.1 Efficiency of Gene Knockdown

Since FSAP, hepsin and FVII are all synthesized in the liver I extracted RNA from the liver of zebrafish injected with the VMOs to analyze the efficiency of the knockdown of the protein.

To analyze the efficiency of *f7* gene knockdown, the liver RNA extracted from the *f7* VMO-injected fish, was analyzed for alternative splicing using primers designed from exon 5 and exon 7 of *f7* gene. As the *f7* VMO targets the exon 6 of the *f7* gene if normal splicing occurs an amplification of 236 bp is expected and if exon skipping occurs a product of 114 bp is expected. RT-PCR analysis of the liver RNA yielded a 114 bp band as shown in Fig. 3.2a. For analysis of *habp2* gene knockdown a 454 bp band is expected for normal splicing and 222 bp in case of exon skipping using primers that target exon 9 and exon 11. RT-PCR analysis of the liver RNA extracted from the *habp2* VMO-injected fish yielded a 222 bp band as shown in Fig. 3.2b. In case of *hpn* gene knockdown a 388 bp band is expected for normal splicing and 159 bp in case of exon skipping using primers that target exon 7 and exon 9. RT-PCR analysis of the liver RNA extracted from the *hpn* VMO-injected fish yielded a 159 bp band as shown in Fig. 3.2c. Amplification of *ef1 α* yielded a band of 220 bp from all RNA's as shown in Fig. 3.2. Thus, this provided proof that the VMOs worked in targeted gene knockdowns.

The knockdown in MO-injected zebrafish embryos was also analyzed by extracting RNA from the whole larvae at 4 dpf using the same primers and results similar to those of VMO experiments were obtained.

3.3.2 ELISA Assay to Estimate FVIIa Levels in the Vmorphants

To investigate the role of FSAP and hepsin in FVII activation we measured FVIIa levels in *habp2* and *hpn* knockeddown zebrafish and also tested their extrinsic pathway activity using the kinetic coagulation assay. The VMO for *habp2* and *hpn* and the control VMO were injected in adult zebrafish and the plasma of the injected fish was assayed for the FVIIa levels using the developed ELISA assay. *f7* gene knockdown was also performed as a control to compare the loss of hemostatic function. After injection with the VMO followed by 48 hr of incubation, the plasma of the injected fish was assayed for FVIIa levels by performing the ELISA assay. The zebrafish injected with *f7* VMO showed a considerable reduction in FVIIa levels. The *hpn* gene knockdown also showed a considerable reduction in FVIIa plasma levels. However, *habp2* gene knockdowns did not show any reduction in FVIIa levels as shown in Fig. 3.3. To test whether whole larval crushing could be used to detect the decreased levels of FVIIa, the genes were subjected to knockdown by injecting MO's for *habp2*, *hpn* and *f7* in one to four celled zebrafish embryos. On performing the ELISA on the 5 dpf after injections, FVIIa levels in hepsin knockdown larvae were considerably lower than the control and FSAP knockdown larvae Shown in as shown in Fig. 3.4.

3.3.3 Kinetic Prothrombin Time of the Morphants

To assay the hemostatic function of hepsin and FSAP, kPT assays were performed on the plasma of *hpn* and *habp2* VMO-injected adult zebrafish. The kPTs of *habp2* and *hpn* vmorphants' plasma were compared with *f7* VMO-injected zebrafish plasma to check for any delay in fibrin generation initiated by zebrafish thromboplastin. The plasma obtained from *f7* and

hpn vmorphant showed a considerable delay in fibrin generation. However, the plasma obtained from *habp2* vmorphant did not show any delay in fibrin formation as shown in Fig. 3.5.

3.3.4 Laser- Induced Venous Thrombosis in Morphant Larvae

To test whether TTO is also affected in *habp2* and *hpn* morphants we analyzed these morphants in larval stages for hemostatic function. We injected MOs for *f7*, *habp2* and *hpn* genes into one to four celled stage the zebrafish embryos. The embryos were analyzed on the 4 dpf for their hemostatic function by performing laser-induced venous thrombosis assay. We found that the larvae with *f7* and *hpn* gene knockdown had an increased TTO compared with the control. *habp2* gene knockdown larvae did not show any significant difference in their TTO when compared with the controls and that the results were consistent with the experiments performed in adult fish as shown in Fig. 3.6.

3.4 Discussion

As part of my earlier aim I developed an assay to estimate the FVIIa levels in zebrafish population and estimated activated FVII and total FVII levels in zebrafish population both adult and larvae. In this aim I investigated the role of FSAP and hepsin in activating FVII.

In the last two decades, *Danio rerio* has evolved as a powerful genetic model and we decided to use this powerful model to investigate role of FSAP and hepsin in activating FVII *in vivo* (22, 31). Hepsin and FSAP are two serine proteases that have been amidst controversy with their role in activating FVII. FSAP, also known as hyaluronan binding protein for its ability to bind to hyaluronic acid, was isolated from human plasma fractions and was shown to activate FVII *in vitro* (8, 32). However, studies involving recombinant FSAP, found negligible role of

this protease, in activating FVII (11). Hepsin, a transmembrane serine protease was also found to activate FVII. Transfection studies in BHK cells with a plasmid containing the cDNA for human hepsin showed activation of FVII (15). Hepsin was also thought to be essential in cell growth and morphology (33). However, characterization of mice deficient in *hpn* (-/-) did not find any role of hepsin in hemostasis, embryonic development or maintenance of normal liver function. In the same study the *hpn* knockout mice (-/-) did not show any prolongation in PT, aPTT and tail bleeding time. However, due to the unavailability of FVIIa assay the levels of FVIIa were not reported in the study (19). In order to reevaluate the role of these proteins *in vivo* I decided to knockdown the *habp2* and *hpn* and analyze its effect on FVII activation and the extrinsic cascade. My results indicated that the knockdown of *habp2* did not influence the FVIIa levels nor alter the function of the extrinsic coagulation as measured using different clotting assays, making me skeptical of the putative role of FSAP in activating FVII or in the extrinsic coagulation. However, based on my results hepsin does play a role in FVII activation and hemostasis *in vivo* in zebrafish. Knockdown of *hpn* in zebrafish had lowered the FVIIa levels by 70% and prolonged the kPT suggesting its role in FVII activation and in hemostasis in general. Studies from other laboratories showed that deletion of hepsin (-/-) in mice affected hepatocytes size. However, studies in hepsin (-/-) mice from another laboratory found no abnormalities in the liver (14, 19). According to a study by *Vu et al.*, hepsin could be the first serine protease expressed during mammalian development and, thus, could play potential role in development (34). In order to evaluate the role of hepsin in early hemostasis, we decided to knockdown the gene in zebrafish embryos and assay the gene function as early as 4 dpf. The TTO in *hpn* knockdown larvae was highly affected. On performing laser-induced venous thrombosis delayed clotting as late as two min was observed while in some the blood did not clot over five min.

Thus, hepsin seems to play a prominent role in FVII activation and extrinsic coagulation in general and hence, the lack of its role in hemostasis in mouse model may be species specific.

Our hepsin data are consistent with the results obtained in BHK cells; however, it is contradicting the results obtained with knockout mice. This discrepancy is probably due to the species specific variations. Such species specific differences have been observed in many other systems. Since there is compelling genetic evidence in human that FVIIa is important, the zebrafish system may be closer to humans with respect to FVIIa generation, when compared to mice.

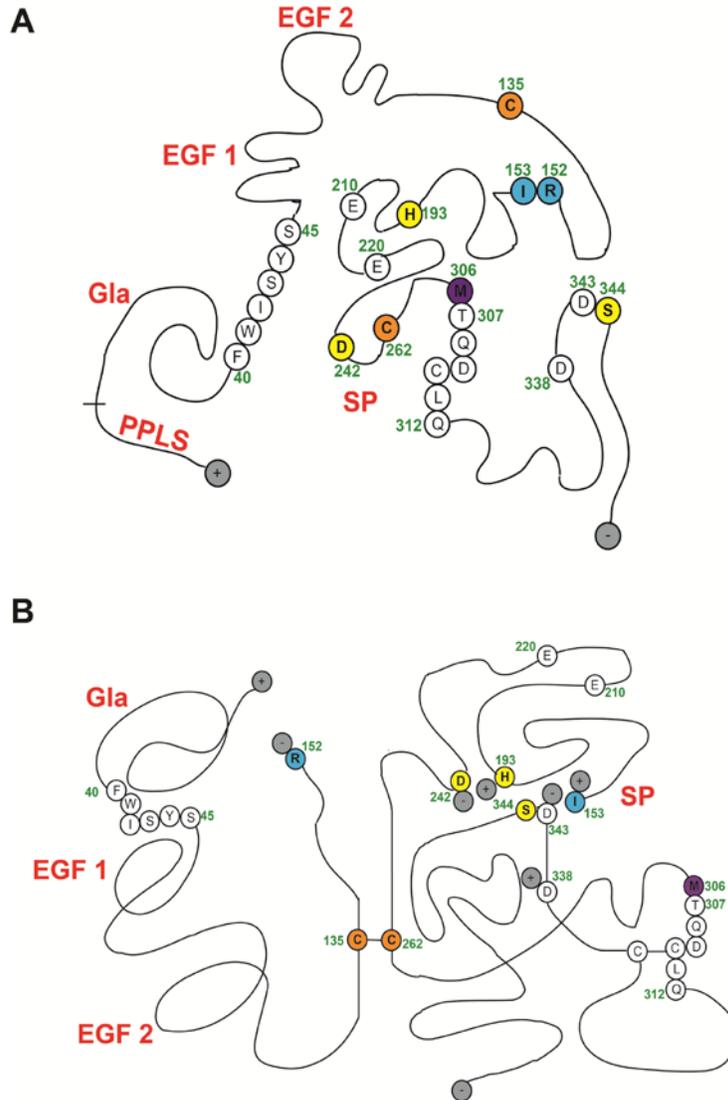


FIG 3.1 Schematic representation of FVII activation. (A) Human zymogen FVII structure. (B) Human activated FVII structure. The important amino acids participating in activation or catalytic activity are labeled. Both activated and zymogen form shows presence of vitamin K-dependent carboxylation/gamma-carboxyglutamic domain (Gla), epidermal growth factor domain 1 & 2 (EGF 1 & 2), serine protease domain (SP) (35).

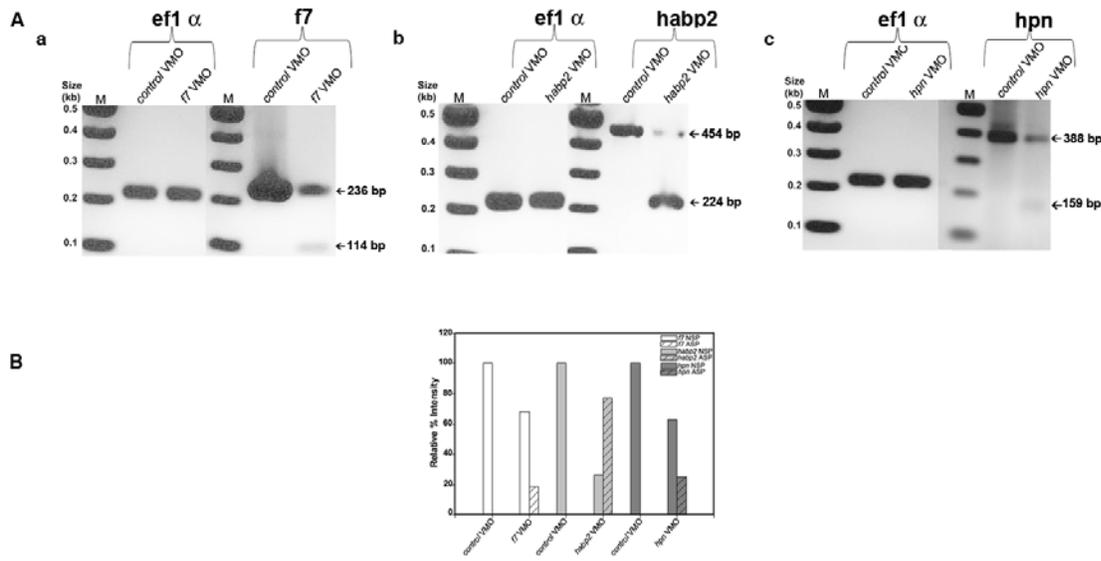


FIG 3.2 RT-PCR analysis of the knockdown efficiency of the VMOs. (A) Agarose gels showing the RT-PCR products amplified from liver RNA prepared from zebrafish injected with (a) *control* VMO and *f7* VMO, (b) *control* VMO and *habp2* VMO and (c) *control* VMO and *hpn* VMO. DNA size markers are shown in the left lane (M). Arrows show the normal and alternatively spliced products. (B) The graph showing comparison of the relative percentage intensities on equal areas of the bands of the observed normal spliced products (NSP) and alternatively spliced products (ASP) using ImageJ[®] analysis. Inset boxes describe the bars for *f7*, *habp2* and *hpn* genes, respectively.

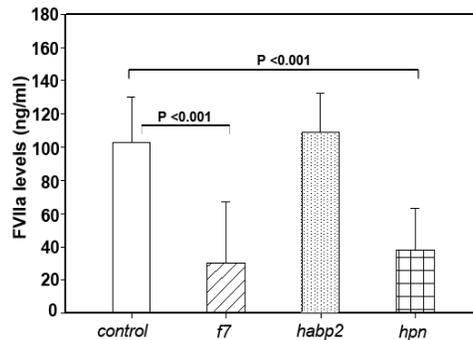


FIG 3.3 Estimation of FVIIa levels in morpholino injected adult zebrafish. Bar graph shows FVIIa levels in plasma obtained from zebrafish injected with control VMO (*control*), *f7* VMO (*f7*), *habp2* VMO (*habp2*) and *hpn* VMO (*hpn*) zebrafish plasma and their means are 103, 30, 109 and 38 ng/ml, respectively. n= 48 and the statistical significance by Dunnett's post-hoc test is shown by P values in the graph.

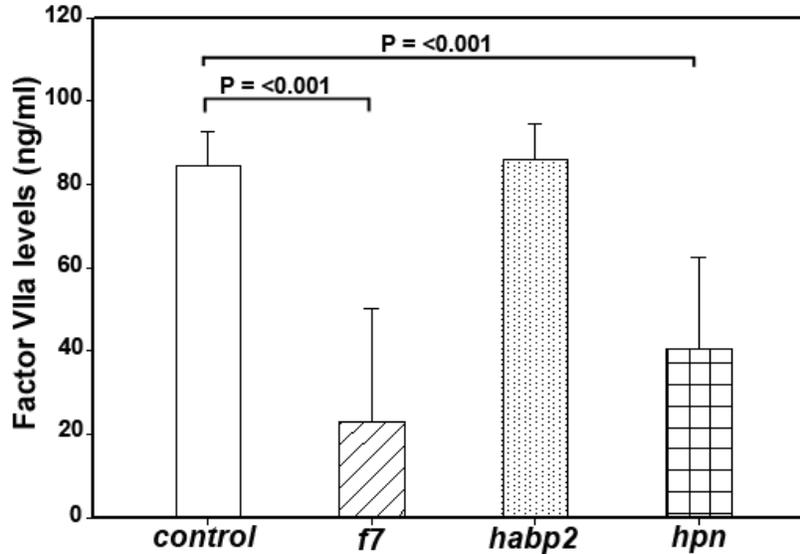


FIG 3.4 Estimation of FVIIa levels in morpholino injected 5 dpf zebrafish larva. *habp2* and *hpn* MO were injected in zebrafish embryos and the FVIIa levels were estimated on 5 dpf zebrafish larvae. *f7* MO was also injected to compare the loss of hemostatic function. Control MO was used as a control. The mean for the control MO-injected zebrafish was 84, *f7* MO-injected zebrafish was 23.006, *habp2* MO-injected zebrafish was 86 and *hpn* MO-injected zebrafish was 40. n= 30 and the statistical significance by dunnett's test is shown by P-values in the graph. Units are in ng/ml.

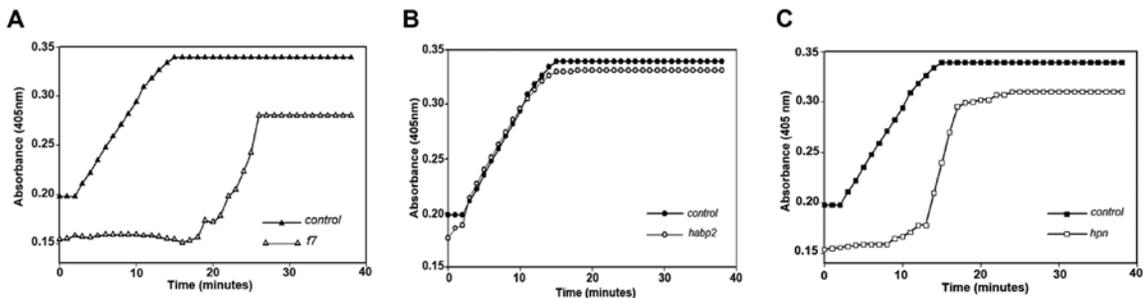


FIG 3.5 Kinetic coagulation assays in *habp2* and *hpn* knockdown in adult zebrafish. The fibrin formation over time in min was measured as an increase in absorbance at 405 nm after addition of zebrafish thromboplastin to plasma obtained from zebrafish injected with VMOs for (A) *f7*, (B) *habp2* and (C) *hpn* genes and their corresponding control plasma obtained after control VMO injections. Control- and gene-specific kPT curves are marked in the graph.

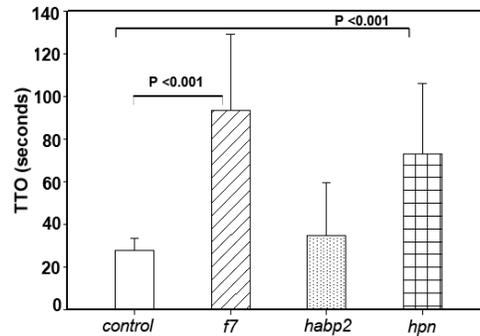


FIG 3.6 Time to occlusion analysis in *habp2* and *hpn* knockdown zebrafish larvae. Bar graph shows TTO in in 4 dpf larvae obtained from 1-4 cell stage embryos injected with control MO (*control*), *f7* MO (*f7*), *habp2* MO (*habp2*) and *hpn* MO (*hpn*) and their means are 28, 93, 35 and 73 seconds, respectively. n= 90 and the statistical significance by Dunnett's post-hoc test is shown by P values in the graph.

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CHAPTER 4

IDENTIFICATION OF NOVEL SERINE PROTEASES PLAYING A ROLE IN EXTRINSIC CASCADE AND FVII ACTIVATION IN ZEBRAFISH[‡]

4.1 Introduction

Coagulation is often represented as isolated set of sequential reactions but in reality it is often an overlapping continuum of events (1). The activation of FVII by hepsin could be single step activation or a sequential reaction involving number of other serine proteases. hepsin, a type II membrane bound serine protease expressed in liver consists of an extracellular region formed by two domains connected by a disulphide bond and noncovalent interactions. The large domain contains the serine protease domain similar to the (chymo)trypsin superfamily while the smaller domain belongs to the Scavenger Receptor Cysteine-Rich Family. The transmembrane and cytoplasmic domains of hepsin might be involved in transmembrane signaling (2, 3). Based on the amino acid sequence, hepsin seems to be expressed as a zymogen (4). Hepsin like most other type II transmembrane serine protease is not functionally extensively characterized and hence, it is difficult to establish its mechanism of FVII activation (5). It is likely that other serine proteases could play a role in hepsin activation. Furthermore, hepsin may not directly activate FVII and may be involved in a cascade of serine proteases in the ultimate activation of FVII in order to maintain plasma FVIIa levels at 1% zymogen concentration. Thus, to find out the serine proteases involved in FVII activation standard biochemical methods may not be efficient. Therefore, I hypothesized that a whole genome knockdown of all serine proteases and subsequent estimation of FVII levels in zebrafish plasma may identify any other serine protease involved in FVII activation. However, with the current existing VMO technology large scale

[‡] Part of the results from this chapter are submitted to a peer reviewed journal and the manuscript describing other part of the results is under preparation.

gene knockdown approach is prohibitively expensive and a need to develop a low cost knockdown technology was important.

Antisense oligonucleotides have been used to inhibit gene function in an RNaseH dependent degradation of mRNA (6). However, since they have a short half life due to their degradation by nucleases several modified oligonucleotides were devised such as phosphorothioates which also work in an RNaseH dependent manner (7-9). Attempts to deliver these phosphorothioates into cells either yielded low efficiencies or were toxic (10-12). We therefore developed a technology in our laboratory wherein we permeabilized a standard oligonucleotide (SO) onto a non toxic oligonucleotide permeabilizer (NTOP) which should enter the cell effectively and should bind to the target mRNA/pre-mRNA leading to the cleavage of the target mRNA as shown in Fig. 1. In addition, the SO/NTOP could also participate in splice and translation blocking using a mechanism similar to VMO targeting. In this Aim, I tested this hypothesis on *αIIb* gene and found that it is possible to deliver SO permeabilized to NTOP and knockdown gene function and that this complex leads to the degradation of mRNA. This technique can be applied for large scale knockdowns because of its cost effectiveness. Using this developed technology I performed knockdown of genes encoding for serine proteases available in zebrafish genome database and used kPT to screen the serine proteases responsible for activating the extrinsic coagulation cascade and then estimated their effect on FVIIa levels.

4.2 Materials and Methods

4.2.1 Zebrafish Aquaculture

Adult zebrafish, larvae and embryos were maintained at 28⁰C in deionized water supplemented with Instant Ocean. Embryos were collected and maintained as described (13).

4.2.2 Zebrafish Injections

Adult zebrafish were anaesthetized and injected intravenously with either gene specific-SO/NTOP or its control using a 27G1_{1/4} needle as described previously (14). For embryo injections the solutions were injected into the zebrafish fertilized eggs between 1-4 cell stages as described previously. Larval injections were performed by injecting the above solutions into the vascular system of the larvae in the common cardinal vein at 4 dpf (15, 16).

4.2.3 Knockdowns with SO/NTOP

We designed an SO that can bind both to NTOP and to *allb* pre-mRNA at the donor splice site of exon 20 (25 bp), 5'-GGAAGTGA CTAAACCCTCACCTCATNNNNNNNNNNNNNNNNN-3' where N could be any of the four deoxynucleotides. A control SO that can bind to NTOP and has a complementary sequence corresponding to the antisense sequence portion of the above SO, 5'-ATGAGGTGAGGGTTTAGTCACTTCCNNNNNNNNNNNNNNNNN-3' was designed. We also designed another SO for *allb* gene in the center of the coding sequence, 5'-CACGATAATCAGCAGCGCTGGATTCCNNNNNNNNNNNNNNNNN-3' and called it *allb1*. A control SO complementary to the *allb1* SO was also designed, 5'-GAATCCAGCGCTGCTGATTATCGAGNNNNNNNNNNNNNNNNN. All SOs and their controls were purchased either from Invitrogen, Carlsbad, CA or from Sigma Inc. St.Louis, MO. 4.5 µl of NTOP was mixed with 4.5 µl of 0.5 mM SO and 1 µl 1X PBS, pH 7.4. The SO and NTOP were mixed together and 5 µl of SO/NTOP was used to inject an adult zebrafish intravenously as described previously (14).

4.2.4 RT-PCR using Zebrafish Thrombocytes

Zebrafish blood was centrifuged at 500g and the white cell layer was used to amplify α IIB mRNA using the cell to cDNA kit (Agilent Technologies, LaJolla, CA) to amplify the α IIB mRNA. Forward 5'-AGTGCTGCATGGACAAAGTG-3' and reverse 5'-GGTTCTCCACCTGTTCCAGA-3' primers for exons 18 and 22, respectively were synthesized by Biosynthesis, Lewisville, TX and were used to amplify the 396 bp product that was resolved on 1.5% agarose gel. A product of 149 base pairs is expected if exon skipping occurs due to the splice blocking by the oligonucleotides. RNA was also extracted in a similar way from the adult zebrafish injected with α IIB1 -SO/NTOP. Three different primer sets were used to amplify the target sequence and the adjacent regions. Forward primer 5'-CTCGTGGACCAAGAAGAAGC-3' and reverse primer 5'-TCTTGGTGATGTCCGGTGGTA-3' were designed to amplify the 181 bp product left to the target sequence of α IIB1-SO. Forward primer 5'-GCGTCAACAGAACCAATCCT-3' and reverse primer 5'-ACACCAAGGCACTGAAATCC-3' were designed to amplify the 169 bp product, the region targeted by α IIB1-SO. Forward primer 5'-TGATGAATGTGCCCTCAAAA-3' and reverse primer 5'-GCAGCCAATAGAAGCAGTCC-3' were designed to amplify the 157 bp product right to the target sequence of α IIB1-SO. Forward primer 5'-CGGTGACAACATGCTGGAGG-3' and reverse primer 5'-ACCAGTCTCCACACGACCCA-3' were used to amplify 220 bp region of *efl* α gene as a control. In a separate experiment the mRNA degradation was quantified by amplifying the region targeted by α IIB1-SO using the above primers and comparing the band intensities using ImageJ[®] software (NIH) and normalized to levels of *efl* α .

4.2.5 Western Blotting

Blood was collected from adult zebrafish injected with control- SO/NTOP and α IIB1-SO/NTOP as described previously in citrated buffer (17). The thrombocyte enriched white cell fraction was isolated by centrifugation of the blood at 500 g for 2 min. The cell fraction was boiled in equal volumes of 2X Laemmli Sample Buffer (BIO-RAD) for 10 min and was used for Western blot analysis. The samples were resolved by SDS/ 9% PAGE and transferred on PVDF membrane for Western blotting. The protein was detected using anti- α IIB antibody raised against zebrafish α IIB heavy chain as a primary antibody and anti Rabbit IgG-conjugated to alkaline phosphatase as the secondary antibody. β -tubulin was used as a control antibody.

4.2.6 Thrombocyte Functional Gill Bleeding Assay

Adult zebrafish were maintained in our zebrafish facility as described earlier and were anesthetized by placing them in 2mM tricaine (Sigma-Aldrich, St. Louis, MO) for 3 min (14). Gill bleeding was then induced by placing these fish in a petridish containing 50ml of 50 μ M NaOH for 80 sec. The fish were photographed with a Nikon E995 Coolpix camera and the red pixels were counted by Adobe Photoshop[®] software 7.0 and used to quantify bleeding.

4.2.7 Immunostaining of Thrombocytes

Immunostaining was performed on freshly prepared blood smears from control-SO/NTOP and α IIB-SO/NTOP-injected zebrafish as previously described (14). Briefly the slides were fixed with 70% ethanol (cold) for 15 min followed by three washes with 1X PBS. The blood cells were incubated with 20 μ l of the rabbit polyclonal antisera against the zebrafish α IIB peptide (Alpha Diagnostic, Inc., San Antonio, TX) for 1.5 hr at 25°C followed by three washes

with 1X PBS. The slides were then incubated with FITC-conjugated rabbit anti-sheep IgG (Sigma-Aldrich, St. Louis, MO) for 1.5 hr followed by PBS wash and a brief rinse with water. The images were captured using a Nikon Eclipse 80i microscope and the intensities were measured by NIS-Elements AR 2.30 software from Nikon.

4.2.8 Time to Occlusion and Time to Aggregation Assays

TTO assays were performed on 5dpf larvae that were injected with control-SO/NTOP and SO/NTOP when they were at 4dpf using a MicroPoint Laser as previously described (15). The thrombocyte aggregation assay also called TTA was performed with addition of 1 μ l of citrated buffered blood from control-SO/NTOP and gene specific/NTOP-injected adult zebrafish to 8 μ l 0.63% sodium citrate in phosphate buffered saline (PBS) in a conical micro-welled microtiter plate and manually tilted every 5 min for 1hr as described earlier to determine the time to aggregation of thrombocytes.

4.2.9 Serine Protease Knockdown

4.2.9.1 Bioinformatics

The genes coding for all serine proteases in the zebrafish genome were scanned using the NCBI and ENSEMBL databases. Oligonucleotides were designed antisense to all 249 genes coding for proteins having serine protease like domain in the center of the coding sequence. The SO's were designed so that they could bind both to NTOP and to the pre-mRNA of target genes as described above. The list of genes and their sequences are mentioned in Table 4.1. The gene specific SO/NTOP's for all genes were prepared as mentioned above. The gene specific

SO/NTOP's were injected independently into adult zebrafish and the kPT was performed after 48 hr of injection.

4.2.9.2 Kinetic Prothrombin Time

The kPT was performed as described previously (18). The blood was collected from the gene specific-SO /NTOP-injected zebrafish and was centrifuged to collect plasma. 0.5 μ l of the zebrafish plasma was assayed for coagulation by adding 5 μ l of the zebrafish thromboplastin reagent to the plasma in a 96-well microtiter plate. The plasma was recalcified with 100 mmol/l CaCl_2 and the fibrin generation over time was detected using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) at 405 nm. 1X PBS instead of thromboplastin was used as a negative control. The genes positive in the kPT assay were screened for FVIIa levels. Delay in fibrin formation for more than 10 min was considered a positive phenotype for prolongation of kPT.

4.2.9.3 ELISA Assay to estimate FVIIa levels

The FVIIa plasma levels were estimated in the fish injected with gene specific-SO/NTOP that were screened positive in the kPT assay. The ELISA assay was performed as described in 2.8.1.2.

4.2.10 Statistical Analysis

Statistical analysis was performed using SigmaPlot 10[®] with SigmaStat integration software. Statistical significance was assessed by one way ANOVA and a P value <0.05 was considered significant. Dunnett's test was used a post-hoc test.

4.3 Results

The α IIB a platelet integrin plays a major role in platelet aggregation and has evolved as a primary target for many anti thrombotic agents (19). Moreover, *α IIB* gene is often used as a target to validate knockdown function in platelet studies (14, 20). Hence, to establish the proof of principle of our technology, we applied this method to knockdown *α IIB*. The SO (40 nt) for *α IIB* was synthesized in such a way that fifteen nucleotides of each SO at its 3'-end can bind to NTOP and the rest of 25 nucleotides were antisense to either target mRNA or pre-mRNA as shown in in Fig. 4.1. We also designed control-SO which were 40 nt long and out of which 15 nt were complementary to NTOP as described above but 25 nucleotides were in the sense direction of target mRNA or pre-mRNA. The α IIB-SO and its control were independently mixed with NTOP and incubated. This mixture was injected intravenously into wild-type adult zebrafish.

To knockdown α IIB, the α IIB -SO was designed at the donor splice site of exon 20 of the zebrafish *α IIB* gene as we have targeted this exon in our earlier work using VMO specific for α IIB (14). The α IIB -SO/NTOP or the corresponding control were injected separately into adult zebrafish intravenously and after 48 hr the RNA was extracted from the thrombocyte enriched white cell fraction of both the control and experimental fish and was analyzed for alternative splicing by using primers designed from exon 18 and 22. If normal splicing occurs, we expected a 396 bp product. If exon skipping occurs, we expected 149 bp of DNA. As predicted, we obtained a 149 bp band in the thrombocytes of zebrafish where α IIB-SO/NTOP was injected compared to control-SO/NTOP as shown in Fig. 4.2. However, interestingly the overall intensity of the 396 bp band was reduced in the alternative splicing analysis and, in fact, in some experiments the band in this region was completely nonexistent whereas the *ef1 α* amplified product showed no difference in the intensities. On comparing the extent of RNA degradation in

normal splice product obtained after injecting α IIB-SO/NTOP with the results obtained after injecting α IIB VMO we find that the extent of RNA degradation is more profound using our technology as shown in Fig. 4.3. However, the alternatively spliced product may or may not be obtained using our technology indicating target mRNA or pre-mRNA degradation.

Thrombocytes were analyzed for α IIB levels by immunostaining in α IIB-SO/NTOP injected fish. Thrombocytes derived from zebrafish injected with α IIB-SO/NTOP when probed with anti- α IIB antibody showed significantly reduced fluorescence compared to controls Shown in shown in Fig. 4.4. We also evaluated thrombocyte function using the gill bleeding assay as well as TTA (14, 15). The fish treated with the α IIB-SO/NTOP exhibited more bleeding compared to the fish treated with control-SO/NTOP Shown in Shown in Fig. 4.5). The thrombocytes from experimental fish also showed significantly greater TTA compared to controls Shown in Fig. 4.5. We then injected the α IIB-SO/NTOP intravenously into 4 dpf larvae and performed laser-induced arterial thrombosis assays on 5 dpf larvae which yielded prolongation of TTA when compared to the controls as shown in Fig. 4.5. All the above results established that the α IIB-SO/NTOP in fact is knocking down α IIB.

The extent of alternative splicing was not sufficient to explain the observed functional data in the RT-PCR analysis of α IIB-SO/NTOP-injected zebrafish thrombocyte RNA. Considerable reduction in normal spliced product suggested that in addition to the alternative splicing due to splice blocking, the pre-mRNA/mRNA may be degraded. To test this hypothesis we designed α IIB1-SO at a site complementary to the coding sequence only in exon 20 and not the splice junction. The α IIB1-SO/NTOP and its control were separately injected in the adult zebrafish and after 48 hr the RNA was extracted from the thrombocyte enriched white cell fraction of both the control and experimental fish and was analyzed by RT-PCR. We used three

sets of primers in RT-PCR amplifications one corresponded to exon 20 (α IIBc) where α IIB1-SO would bind and the other two sets were in the sections flanking the region corresponding to exon 20 (α IIBL and α IIBR). The results of RT-PCR analysis showed reduced intensities of all the three RT-PCR products compared to the controls suggesting overall degradation of mRNA. However, the α IIBc was considerably reduced compared to the α IIBL and α IIBR. To further quantitate the extent of pre-mRNA/mRNA degradation in the region targeted by α IIB1-SO we amplified the region using the above primers and the internal control in the same set and comparing the band intensities using ImageJ[®] software (NIH) and normalized to levels of *efl* α . On quantification the extent of RNA degradation in the region targeted by the α IIB1-SO is over 60 % as shown in Fig. 4.6).

The knockdown efficiency of α IIB at protein levels was also analyzed by performing a Western blot analysis on the thrombocyte enriched white fraction obtained from control-SO/NTOP and α IIB1-SO/NTOP-injected zebrafish. We found that the α IIB levels in the knockdown zebrafish were significantly lower than the controls as shown in Fig. 4.7. We also assayed for the hemostatic function of α IIB1-SO/NTOP-injected fish with a gill bleeding assay. The fish treated with α IIB1-SO/NTOP showed greater extent of bleeding compared to controls as shown in Fig. 4.8. We then injected the α IIB1-SO/NTOP intravenously into 4 dpf larvae and performed laser thrombosis assays on 5 dpf larvae which yielded prolongation of TTO compared to controls shown in Fig. 4.8. Thus, the NTOP permeabilized oligonucleotides appeared to create knockdown deficiency through RNA degradation.

After establishing the proof of principle of the developed technology I designed SOs to knockdown all serine proteases in the zebrafish genome to identify novel serine proteases playing a role in extrinsic coagulation cascade and FVII activation. To do this the genes coding

for serine proteases or proteins having an association with the serine proteases were scanned using the NCBI and ENSEMBL databases and SOs were designed for all 181 genes as shown in Table 1. 5 μ l of 0.5 mM gene specific-SO/NTOP were injected into zebrafish twice at 24 hr intervals and the gene function was analyzed by performing a kPT assay. On performing the knockdown of all 181 genes in adult zebrafish 22 genes were found to prolong kPT for more than 10 min as shown in Fig. 4.9. These 22 genes were rescreened using kPT assay to rule out background mutations and other sampling errors. On performing the secondary screening with kPT assay 15 genes were found to have a prolonged fibrin generation shown in Fig. 4.10. On performing the tertiary screening on these 15 genes with the kPT assay all 15 genes gave a similar result as shown in Fig. 4.11. Thus, out of 181 genes coding for serine proteases, 15 genes coding for serine proteases were found to be involved in the extrinsic coagulation cascade. The 15 genes were then screened for their FVIIa levels in order to elucidate their role in FVIIa activation. Six genes out of 15 were found to have statistically significant lower levels of FVIIa in their plasma of the fish where knockdown was performed as shown in Fig. 4.12.

In summary, knockdown of 181 genes coding for serine proteases using my new technology was performed and screened with kPT assay to analyze for extrinsic clotting cascade defects. On primary, secondary and tertiary screening with the kPT assay 15 genes were found to be involved in extrinsic coagulation. Out of these 15 genes, 6 genes were found to be involved in FVIIa activation. Out of these 6 genes 2 genes were found to be novel as shown in Table 4.2.

4.4 Discussion

In this Aim, I developed a methodology that is inexpensive and has the potential for large scale knockdowns in zebrafish. The technology builds on the use of NTOP that will bind to

standard oligonucleotides and help carry out targeted gene knockdowns. The knockdown of α IIb yielded a similar phenotype as with other successful knockdown methods making this system an equally efficient but cost effective method of gene knockdown.

Knockdown of α IIb in wild-type zebrafish explains the phenomenon of the circulating blood cells and/or precursor cells taking up the gene specific-SO/NTOP. However, it is possible to knockdown genes in tissues other than blood tissue. I applied this new technology to conduct large scale knockdowns on all genes coding for serine proteases to identify their role in FVII activation. Using bioinformatics tools, 181 genes in the zebrafish genome were found to be having serine protease domains. The SOs were designed antisense to these genes and the gene specific-SO/ NTOPs were injected into the zebrafish for targeted gene knockdown. Since hepsin results showed that its knockdown affects FVII activation and also affects the extrinsic cascade of coagulation, kPT assays were used as a primary screen. Delay in fibrin formation for more than 10 min when compared to control was considered to be a positive phenotype for extrinsic pathway defect. To rule out background mutations, the genes found positive in the primary screen were rescreened to finally identify serine proteases participating in extrinsic coagulation cascade. Fifteen genes were found to be actively participating in fibrin generation via the extrinsic coagulation mechanism.

Because these 15 genes are possible candidates for FVII activation, they were then screened for their knockdown effect on FVIIa levels, using the ELISA assay that I developed. Six genes were found to be involved in FVIIa generation which are, *f2*, *f7*, *f10*, *hpn*, *zgc: 92313*, *LOC100333596*. Out of these *f2*, *f7*, *f10* are known serine proteases involving in FVII activation and *hpn* was identified in my earlier aim to activate FVII. Thus, in this screen, I identified genes two novel genes, *zgc:92313*, *LOC100333596* as candidates involved in FVII activation. The

gene *zgc:92313*, is prostatic protein having a trypsin-like serine protease activity, while the gene *LOC100333596*, is a hepatocyte growth factor-like protein that lacks catalytic triad of serine protease. Prostatic is a membrane anchored protein which requires matriptase for its activation in skin epithelial cells (21). Matriptase on the other hand is inhibited by extrinsic antithrombin (22). Previous results from other laboratories in BHK cell lines and my results in zebrafish, it is evident that hepsin activates FVII (23). Hence prostatic seems to be activating FVII based on my results, it is possible that prostatic activates hepsin and hepsin in turn activates FVII. It has been already shown that hepsin activates prostatic (24). Thus it is possible that hepsin generated can back-activate prostatic to generate higher amounts of it, a phenomenon commonly seen in coagulation cascades. Thus, a whole cascade of membrane bound serine proteases could be responsible for circulating FVIIa levels in zebrafish plasma which may also occur in human. For this cascade to work, all these proteases must be expressed in the liver. hepsin, prostatic and matriptase-2 are all found to be expressed in liver (2, 25, 26). Hence it is possible that in the liver these membrane bound serine proteases can activate one another to eventually activating FVII. The protein hepatocyte growth factor-like was also identified in my screen to be activating FVII. However, hepatocyte growth factor-like protein, a heterodimeric protein, comprises of a disulfide-linked alpha chain and a serine protease-like beta chain which is inactive (27). The inactive serine protease domain makes it difficult to anticipate hepatocyte growth factor-like protein, a direct activator of FVII. However, hepatocyte growth factor-like protein has been shown to act on Ron, a tyrosine kinase receptor and signal the cells via MAP kinase and Akt pathways (28). Thus, it is likely that the hepatocyte growth factor-like protein may act through these signaling pathways to control FVII levels and, thus, may indirectly affect FVIIa levels. Also the 75 % inhibition of FVIIa levels observed in my ELISA assays after knockdown of

serine proteases in my screen suggest that all these proteases are involved in a cascade like events. Thus, the above proposed mechanism of action of the serine proteases affecting FVIIa levels in my screen seems exciting but need to be tested, in order to propose the role of membrane bound serine proteases in FVII activation.

Table 4.1 List of genes coding for serine protease with corresponding SO sequences.

Gene ID	Sequence 5' to 3'	Gene ID	Sequence 5' to 3'
GID641564	CAGCTCAGTGTACCAGCAGCATAGG	GID100330319	TTGTCTATCAACACAATATCTACTA
GID100034472	GAGTTCTGGAGTCTGATTGGCTGAT	GID259260	TCTGCAGGCAGAGCGCCGTCGCCAT
GID565078	GCACAGCTGGCTGGATGGTGGCACT	GID100002060	GCCAGAAAGTTCATCTACTGGACCAC
GID100538141	GTCCCCAGCCTGTGATGTGGCAGTA	GID558805	TCAGTGGTTTGTCTTGTCTGGGAT
GID100151400	TGGGCAAACCTTCAGGCTCTACCAG	GID561689	GTGTCAGTACTGATGGAGCTGGTGG
GID795978	TGTGCTGGACTGGATAATTTAAGCA	GID566809	GTCTGTGAGTGCAGTCAATGTTAAA
GID562139	ACACATGAGTGTGATCTTAGCAGG	GID562156	CCCCACTGAGAATGATCACAGTCCA
GID322451	ACACGTGAGTGTGATCTTAGCAGG	GID798775	TCAGAACTCCACCACAATGAAACCT
GID550419	CATGGGCATTTCAGAGAGGCTGGTGC	GID100034638	CCAATGTAACGCCTTTTCCATCCCG
GID553713	CTCATTCCGGCGGTTCTCTCGGCCA	GID445282	ACGTATGTGTTCAGGGAGCGTGTGG
GID325881	TCTCCTCCACAATTCTGCTTCCAG	GID100001065	GTTTGGGTAGATTATAAATATCCAC
GID359826	CACCAATTATACGACTTTTTAGGCC	GID100001138	CTTTGACAAAATTTGCTGATCTGGC
GID678552	CTCCTACAATGCGATACTTATTGTT	GID100000986	AAACTTTAACAATTTGTGATCTT
GID100004411	TTATCACCCAGCGCTGATTAATCAG	GID100001210	TTGTAATATTACAAAATGATTTAG
GID114423	CACAGAAACCTTCTCACCTACTT	GID563528	GACTCCCGAAGGACTTCTGTATCTC
GID282671	AGTGGGCCGAGTGATCAGCCAGGG	GID560704	AGCTGACGATGCCAGATGTGATGGT
GID282670	CCGGCTGTGGACACCACCGGTAGGA	GID797799	AAGGAAATCCCTGCTGTCACTTTCA
GID30604	GTTGGGTCTGAAGAAAGTCTTCTTT	GID562040	AGTACAGCTGACTCTTAAACTGTCT
GID563828	AAATCCACATCATGACTGTTAAAGC	GID100003428	CCAATGTAACGCCTTTTCGATCCCG
GID553553	GGAATTTCACTGGTTTGACTGCATC	GID100330113	CAGTAACCCAGCTCGCTGTCCCATC
GID403061	CAAGCAGGTGAAATCTTGTCAGTGA	GID322691	TTCTGAGGTGTTTTGGAATGTGAT
GID334304	ATGCAGGAGTGATGGTGTACCTAT	GID100320028	GGGCACTGGGGACATCACACAGTT
GID554107	GAAGTTTGTTCAGGAAGAGCACCACC	GID100008445	GCATTCTTTCCCAAGACGACAGAGT
GID100334908	TGCACATATGCGTTCAGATTGGCAT	GID100538108	GTCAGTATCCAGCAGGGTGAGATCA
GID100535584	CCTGCACGTATGAGCTCAGGGTGGC	GID553791	ACAGTCTTTCACATCGTGAAGGAA
GID100535157	CAGGAGGGAAGTTGCTTTGCTCATC	GID100005716	CAGTGTGTGTCTGTGCAATTCATT
GID100535114	CCCAGTCTCTGGTCCACAGGGGAA	GID100004460	TATCCATCACAGTTACAGTCATCAT
GID100536434	TGATTTTCCAGTATGCACACCGGCT	GID571235	CCAGTCTTTCACCCAGCAGTGAGT
GID571565	CCTGCGACCAGCTGTTGGTGGCAC	GID559556	AGTGCTCTGCTCCTTCACCATAGC
GID566557	GTGGTGAAGTGGGACGGTTGGACG	GID799308	CGTGTACCAATAAAGACTAGCCTG
GID566591	CACCATTGGTCTCCAGTCGTTTGT	GID794630	GAAAGGCAATGATTTATGAGCTCG
GID492770	AATGACTGACCAGACGCCATCAGCG	GID798402	AGCCACCCGGTGAATGCCATAAAT
GID567589	TTTGCTTTGGTAATGAAGTCAGATG	GID563403	AGAAAGCCCTGTGGTCTTCATGTC
GID795070	CTTTTCTTTTAGGGATTTTGTGA	GID436794	CAATGGGAATTTCTCTCCCTCAAT

ψ NNNNNNNNNNNNNN sequence was added 3' to all sequences, where N is any nucleotide.

Table 4.1 (cont'd.)

Gene ID	Sequence 5' to 3'	Gene ID	Sequence 5' to 3'
GID794999	CTTTTTCATTTTAGGGATTTTGTGA	GID799791	GGTTTATGAGAGGCCCTCCTGAATT
GID100536844	AGTGGGATGGGCAATATAGACTTAC	GID100004427	TTGAATAGCCCCATCCTGTGACCCA
GID493781	GCACTTTTGCGTCTGTGGTAAACA	GID557566	TGAGCACAAAATCCTCTCTTATCAA
GID100333596	ATGCTGCAGTCTGTACGGGACGCT	GID793282	ACCTTTACAGTCTCCATGGGACAGA
GID553472	TTCTTGCGCTATCATGCTAAATGC	GID556860	ATGATATCAGTCCAACATTGTTGCT
GID100034451	AAATCAGTCCAATATTGTGCTCAG	GID799537	TCATCACTCCAATGTAACGCCTTTT
GID556666	GATATCAGTCCAACGTTGTTGCTCA	GID335152	GCCCGAATATCTCCCCAGCCTGTGA
GID100034620	ATATTAGTCCAACATTGTCACTCAG	GID554458	AGGACAACAGTCCATCCTGTGGCAT
GID567711	ATATCAGTCCAACGTTGTTGCTCAG	GID541408	CCAGCTGAACATCTCCCAGCCTG
GID556598	ATATCAGTCCAACATTGTTGCTCAG	GID553673	TCCAGCTCTTGGTTCTCCAGCAA
GID100034618	ATGGAGATCCAACCTACAGTTTGGC	GID554144	ACCTGCACATATGAGCTCAGAGTGG
GID100034439	ATATCAGTCCAATGCTGTTGCTCAG	GID561476	ACCAATGCAATATCGTAGTCCAGTT
GID100034650	ATATCATTCCAACAATGTTGCTCAG	GID574008	GCACATATGAGTTACGCTGGCATC
GID100034605	ATATCAGTCCAACATTCTTGCTCAG	GID553249	TAGGATGGCAGTGTGGCCTCTGCA
GID100034478	GTAATGATATCAGTCTAATGTTTTT	GID641414	TTCCCCAACCCGTGGCCAGCAGG
GID100034483	TCATAAATTAATCCCCATCTATAAT	GID692313	ACACATGAGTGTGATCTTAGCAGG
GID100034660	GGGTGGTGTTTTCCCCCATCTGC	GID768292	CACCTTTACCAAGTGAAGTCCAGA
GID561111	GTAGTGATATCAGTCCAACATTGTG	GID555481	AAAGTAGAATCAGTGGTCCAGTGTG
GID100034636	GATATCAGTCCAACATTCTTGCTCA	GID792472	TACCACCATGTTGGCTTGACCATC
GID100034588	ATATTGGTCTTATCTTGTGTTCTCAG	GID799770	ATCCAGAACCAGTAGAGCATTACA
GID100034654	ACATGGAAATGATCTTGACGTTCTT	GID100038778	CGCATTTATACTGTCCGTCATACTC
GID100034628	TGTCTTTTTTCTGTGGTATTGATAT	ENSDARG00000088893	AGATGCGAGACGTACGTGGGCTGG
GID797859	TCATTTCTAAAAGGGCAATATTGTA	ENSDARG00000071386	CCCGTTTCATTCTTTTTCAGATCAT
GID100034437	ATATCAGTCCAATGCTGTTGCTCAG	ENSDARG00000090967	TTTCATTGTTTAGTCCACTGTAGCC
GID100034528	ATGGAGATCCAACCTACAGTTTGGC	ENSDARG00000092951	TGGGGGCGATTACTTTTGTGACGTGGA
GID100000024	AGAGACGAATAGCGAAGGAAATCCC	ENSDARG00000010925	TGGCAATGTCTGACTTCTGTCAAT
GID678603	ACATTTGATGAGTCCGAGACGAACC	ENSDARG00000052895	CTGGCAAACACCTTTAGAAAGTCGT
GID777629	CACTCATATCACCACAGTCAATTGTA	ENSDARG00000005943	TTACGCACCCATAACCCAGTTCCTG
GID557248	AGGATTATCCGAATGAAACTTTAC	ENSDARG000000045935	ACGGTGATGGTAATGAACTTAATCC
GID100331291	TACACGTGTTGCATTTGGCCTCATC	ENSDARG00000004925	TCGATCCCGATGACCCGTTTGATGA
GID100334526	TAAGATGAGGGAACCTTCTCTTAA	ENSDARG00000007988	CCCTCAGGCATGTAGCTTGATAAAA
GID378845	TTATTCATGTTAATAGTGGCTGTGA	ENSDARG00000014634	CGTCCTGTCACGCCATACCATTA
GID565459	CGGCTGTCGGTGTGTACATGCTGT	ENSDARG00000061830	ACAAGAATACAAAAGAGCGAATGGA
GID569688	AGCTGTCGACATACGAGTGTCCCA	ENSDARG00000076602	GTTTGGTGTCTTGAGGCTGGAAACG
GID798480	ATAATCTCATTGACTTCCACCAGC	ENSDARG00000055786	TCTCATACTGGCATCATAGAGAGC
GID65223	CGGTCTTAACGTAGCTGTTGATTTG	ENSDARG00000059081	GATGCCAGGATCTTCTGAACACGTT
GID560023	AACAGCGTGGCTGAACATACGCAT	ENSDARG00000030967	CGTCTGTTTCCAGCAAGCCATAAG
GID100535672	TACTTGTGAGGGTGGCAGGCTTGC	ENSDARG00000093100	CCCAGGCGATTCCCTTCTAATGCG
GID563795	GAATCGGCTGAACATACTGGTTGAA	ENSDARG0000007040	GAGCACTGAAGTGAAGCCGATTTGT
GID565163	GACCGGTGTTCTGTTGGGACGGG	ENSDARG00000077573	CGCTTCCCCAATAATCCTTGATGA
GID560086	GTGGCAGCGCACAGACACGTA	ENSDARG00000079393	ATGTGGAATGCAAAACAGTCACTCC

Ψ NNNNNNNNNNNNNNNN sequence was added 3' to all sequences, where N is any nucleotide.

Table 4.1 (cont'd.)

Gene ID	Sequence 5' to 3'	Gene ID	Sequence 5' to 3'
GID571843	CTCTCAAACACAGGCTTCTCTCT	ENSDARG00000045889	GTGCTCCGCCACAATCAGTACATT
GID100149563	TCATCACTCCAATGTAACGCCTTTT	ENSDARG00000036545	TCGACACATTCCCTCCTACAATACG
GID100333187	TTCATTGTGCCGTAGCCAGCCTGAA	ENSDARG00000090562	CTGGCTGATATACGGTGTACCTGT
GID100003308	CCAATGTAACGCCTTTTCGATCCTG	ENSDARG00000006693	GTATGCTGGCCGTATACTGCAAGC

Gene ID	Sequence 5' to 3'
ENSDARG00000012860	CGCTTCCCCAATAATCCTTGATGA
ENSDARG000000087717	TTCATATCCCGAAAGCTGCCCTTGA
ENSDARG000000086161	TTTTGCGCAGGAATACGGAAGACGG
ENSDARG00000029841	CTCGCATGTATTCAATGGTGCCGTC
ENSDARG00000074895	GCAGGAGCCGTGGTGCGGTACAAG
ENSDARG00000052064	CTACATCCCATGTATGGTGTGAAG
ENSDARG00000027196	TATTCACAGATCCAAAGCTTGGGCT
ENSDARG00000070710	CCAGCCTGAACATCTCCCCAGCCTG
ENSDARG00000055644	CCAGCCTGAACATCTCCCCAGCCTG
100334482	CACGCTTCCCCAATAATCCTTGAT
100330996	CTTCGATTATACTGGGGGTCAAAGT
100538108	GTCAGTATCCAGCAGGGTGAATCA
564182	TGGGCAAACCTTCAGGCTCTACCAG
100101646	TTGATCTCAATAATAACATCTCCTG
100141339	AGTGTAGAAGAAATGGAATCTCCAG
100003031	GGCTGGTGGAGCCAGACATACAGGA
100137109	TTTACCCATTGATCTCAATAATAA
393114	GACGACACCATCCACTGTCTGTAGT
322453	GTGTTTCCCCAGCCAGAGACTCTGC
436869	CACATCATGTCCGGCCGGATGTCAA
445056	CAGTGGTGCCAAGTGCACATATGCG
474322	GGATAGACAACTCCAGTGTGATCA
445032	CGCTGGACAGTTCAGAAAGCAAT

^ψ NNNNNNNNNNNNNN sequence was added 3' to all sequences, where N is any nucleotide.

Table 4.2 Compiled results of tertiary screening of serine protease.

Gene ID	Gene	kPT	FVIIa
641564	<i>zgc:123295</i>	+	-
100034472	<i>si:dkey-7814.10</i>	+	-
562139	<i>LOC562139</i>	+	-
325881	<i>f2</i>	+	+
114423	<i>f7</i>	+	+
282670	<i>f10</i>	+	+
100536434	<i>LOC100536434</i>	+	-
100333596	<i>LOC100333596</i>	+	+
ENSDARG00000090967	<i>hpn</i>	+	+
561689	<i>magi3</i>	+	-
563528	<i>LOC563528</i>	+	-
799308	<i>LOC799308</i>	+	-
100034451	<i>si:dkey-21e2.12</i>	+	-
ENSDARG00000093100	<i>tmprss12</i>	+	-
436869	<i>zgc:92313</i>	+	+

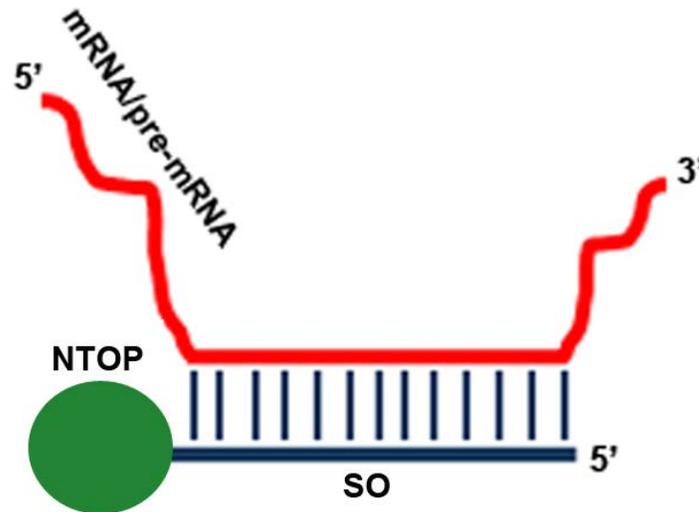


FIG 4.1 Schematic diagram of SO/NTOP action. Standard oligonucleotide (SO) /non toxic oligonucleotide permeabilizer (NTOP) binding to the mRNA/pre-mRNA is shown. Base pairing is indicated by small vertical bars. NTOP is shown by green circle. SO is shown by blue line. mRNA/pre-mRNA (mRNA or pre-mRNA) is shown by the red line.

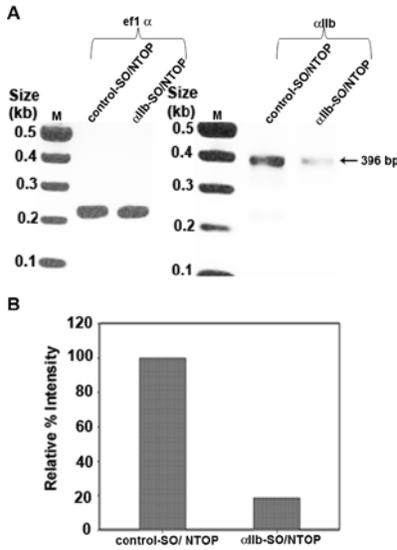


FIG 4.2 Analysis of transcripts of α IIB knockdown. A) Agarose gel showing the RT-PCR products of RNA from thrombocyte enriched white cell fraction obtained from adult zebrafish injected with control-SO/NTOP and α IIB-SO/NTOP. 2-log DNA Ladder was used as DNA size markers (M). (B) The graph shows comparison of the relative percentage intensities of the bands corresponding to the normal spliced product in control-SO/NTOP and α IIB-SO/NTOP.

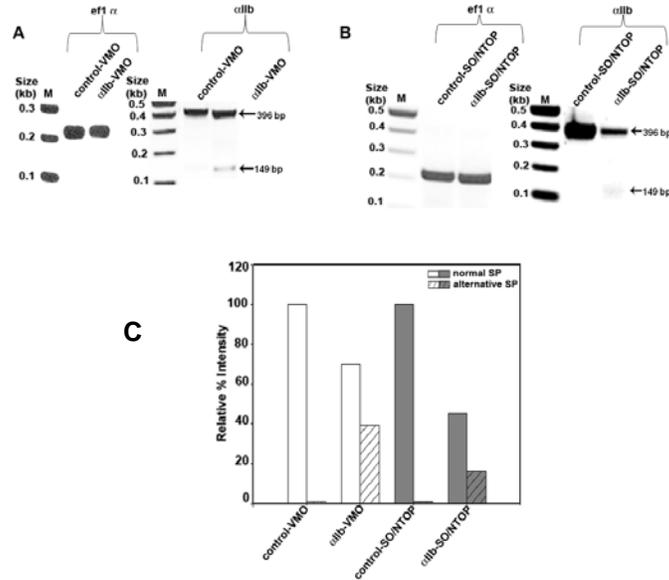


FIG 4.3 Comparative analysis of α IIB knockdown efficiency. (A) Agarose gels showing the RT-PCR products of RNA from thrombocyte enriched white cell fraction obtained from adult zebrafish injected with control VMO and α IIB VMO (B) control-SO/NTOP and α IIB-SO/NTOP. The agarose gel in this panel was overexposed to see the alternatively spliced product 2-log DNA Ladder was used as DNA size markers (M). (C) The graph shows comparison of the relative percentage intensities of the bands corresponding to the normal

spliced product (normal SP) and alternative spliced product (alternative SP) in the α IIB VMO and α Ib-SO/NTOP-injected zebrafish normalizing to levels of ef1 α using ImageJ[®] analysis.

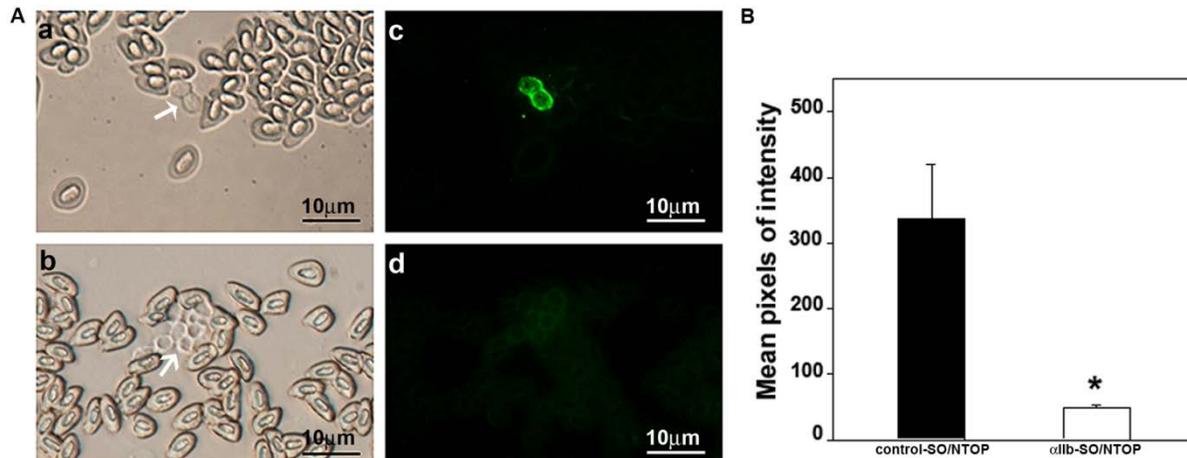


FIG 4.4 Immunostaining of thrombocytes in α Ib gene knockdown fish. (A) Immunostaining of a representative portion of blood smear probed with anti- α Ib antibody showing thrombocytes derived from zebrafish injected with control-SO/NTOP (Panels a & c) and α Ib-SO/NTOP (Panels b & d). Panels a & b and c & d are the bright field and epifluorescence images, respectively. Arrows show thrombocytes. (B) The graph shows the comparison of mean pixel intensity of thrombocytes from the blood smears probed with anti- α Ib antibody showing thrombocytes derived from zebrafish injected with control-SO/NTOP and α Ib-SO/NTOP. n= 17, P= 0.004 between the control and α Ib indicated by *.

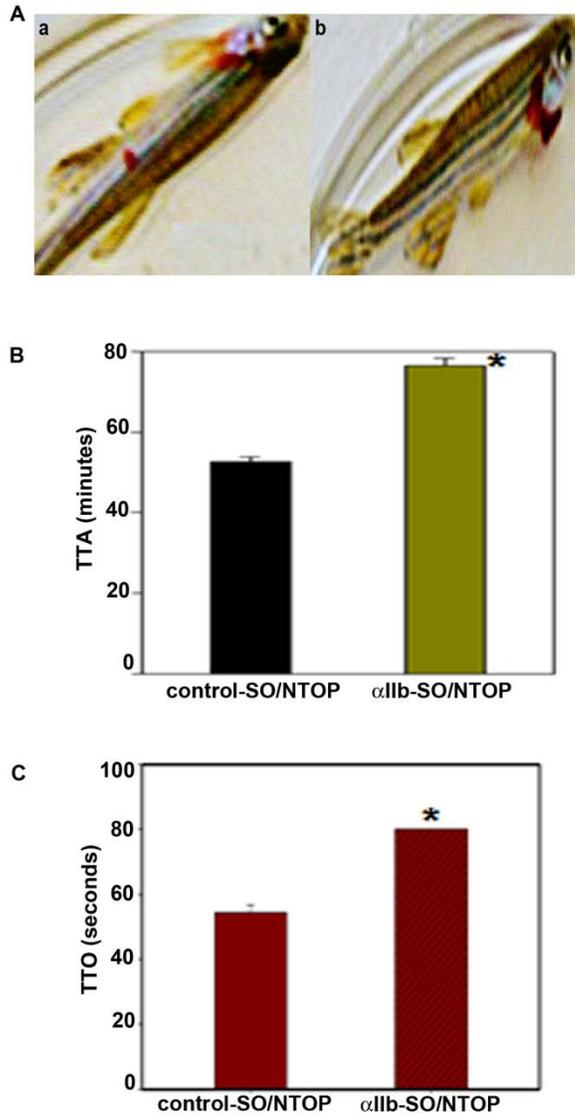


FIG 4.5 Coagulation assays in α IIb knockdown zebrafish. (A) Gill bleeding assay performed on zebrafish injected with control-SO/NTOP (Panel a) and α IIb-SO/NTOP (Panel b). The red color near the gills denotes the extent of bleeding. The small red spot on the middle of the body in panel a fish is the site of injection. In the panel b fish this injection spot is on the other side. (B) Time to aggregation (TTA) of whole blood from zebrafish injected with control-SO/NTOP and α IIb-SO/NTOP . n= 6; P= 0.003 between the control and α IIb indicated by *. (C) Time to occlusion (TTO) assay performed on control-SO/NTOP and α IIb-SO/NTOP- injected zebrafish larvae. Injections were done on 4dpf larvae and TTO assays were performed on 5dpf larvae. n= 6; P= 0.002 between the control and α IIb indicated by *.

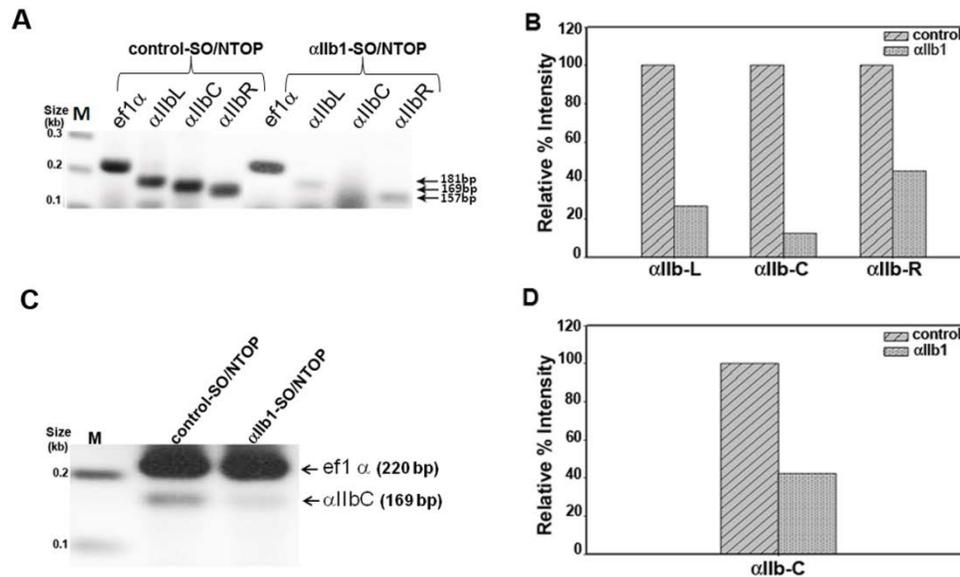


FIG 4.6 Knockdown by RNA degradation. (A) RT-PCR products of RNA from thrombocyte enriched white cell fraction prepared from adult zebrafish injected with control-SO/NTOP and α Iib1-SO/NTOP using three different primer sets, α IibL, α IibR and α IibC which yielded 181 bp, 157 bp and 169 bp bands, respectively (shown by arrows). ef1 α amplification is used as a control which yielded 220 bp product. (B) The graph shows comparison of the relative percentage intensities of the bands (shown in A) amplified using the α IibL, α IibR and α IibC primer sets in control-SO/NTOP and α Iib1-SO/NTOP-injected zebrafish normalizing to levels of ef1 α using ImageJ[®] analysis. (C) RT-PCR products of RNA prepared from adult zebrafish injected with control-SO/NTOP and α Iib1-SO/NTOP using α IibC and ef1 α primer sets in the same tube. Arrow shows the 169 bp band corresponds to α IibC amplification. (D) The graph shows comparison of the relative percentage intensities of the observed bands (shown in C) in control-SO/NTOP and α Iib1-SO/NTOP normalizing to levels of ef1 α using ImageJ[®] analysis. 2-log DNA Ladder was used as DNA size markers (M).

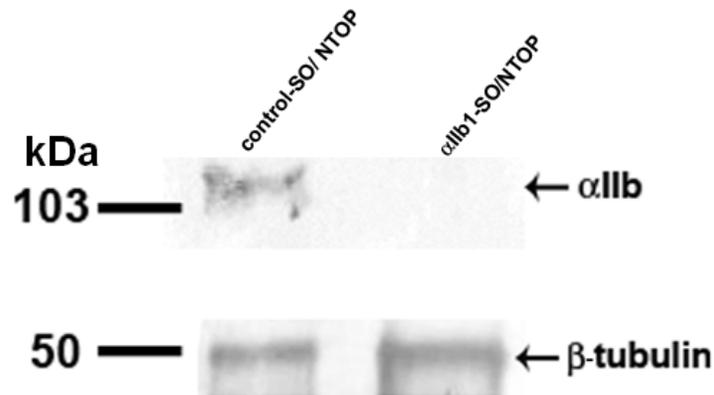


FIG 4.7 Western blot analysis of α IIb gene knockdown. Western blot of protein extracts of thrombocyte enriched white cell fraction prepared from adult zebrafish injected with control-SO/NTOP and α IIb1-SO/NTOP. Lanes 1 & 2 samples from control-SO/NTOP and α IIb1-SO/NTOP injections, respectively. Top and bottom panels show blots using anti- α IIb and anti- β -tubulin antibody, respectively. M represents the size of the protein markers in kilodaltons (kDa).

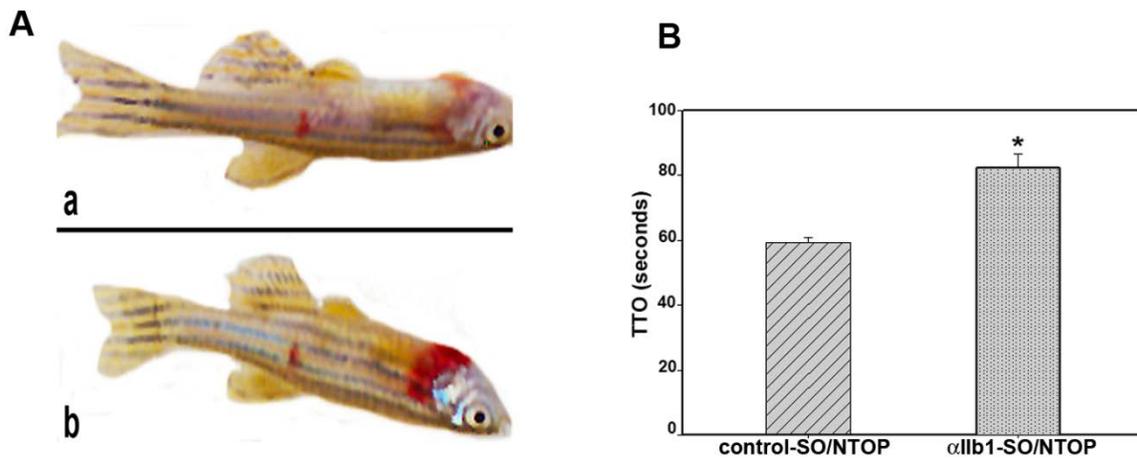


FIG 4.8 Coagulation assays in α IIb1 knockdown zebrafish. (A) Gill bleeding assay performed on zebrafish injected with control SO/NTOP (Panel a) and α IIb1-SO/NTOP (Panel b). The red color near the gills denotes the extent of bleeding. The small red spots on the middle of the fish body correspond to the sites of injection. (B) Time to occlusion (TTO) assay performed on control-SO/NTOP and α IIb1-SO/NTOP -injected zebrafish larvae. Injections were done on 4 dpf larvae and TTO assays were performed on 5 dpf larvae. n= 6; P= 0.002 between the control and α IIb1 indicated by *.

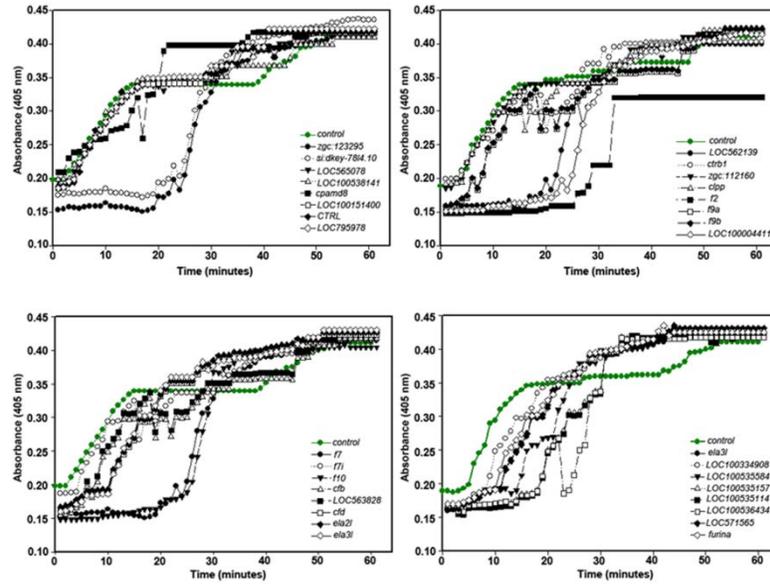


FIG 4.9 Primary screening of serine protease knockdown using kPT assay.

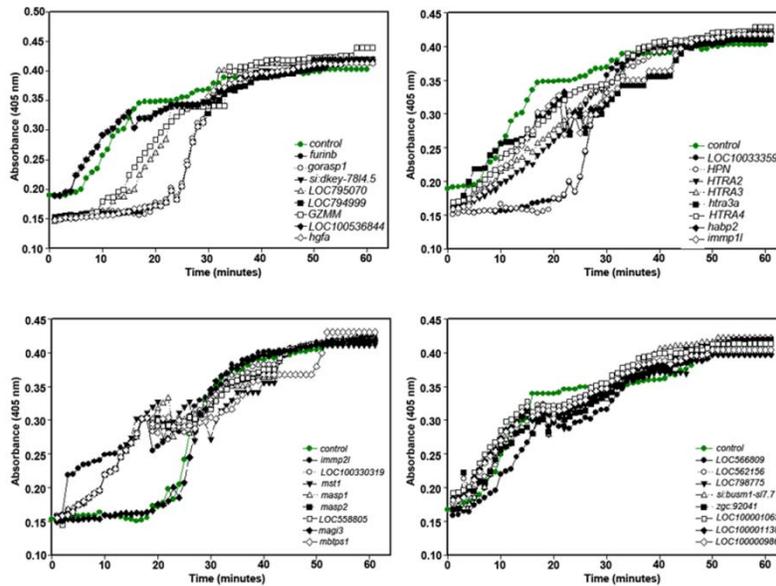


FIG 4.9a Primary screening of serine protease knockdown using kPT assay.

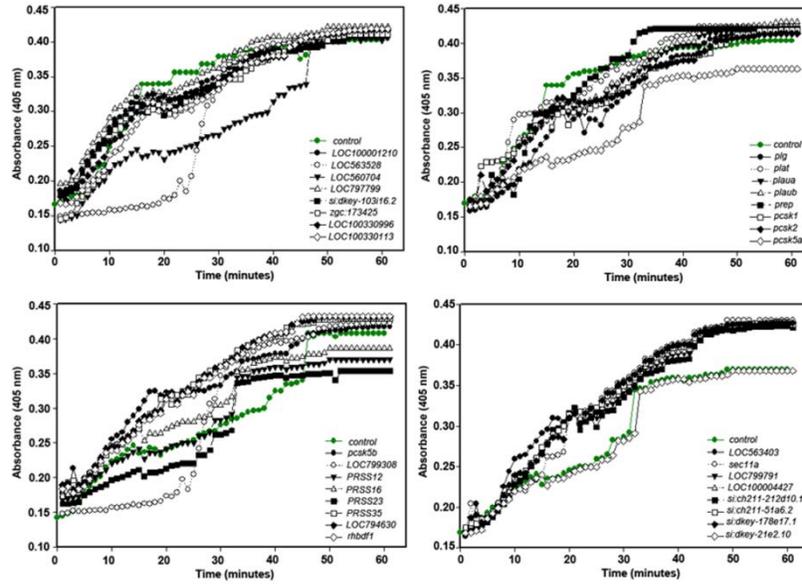


FIG 4.9b Primary screening of serine protease knockdown using kPT assay.

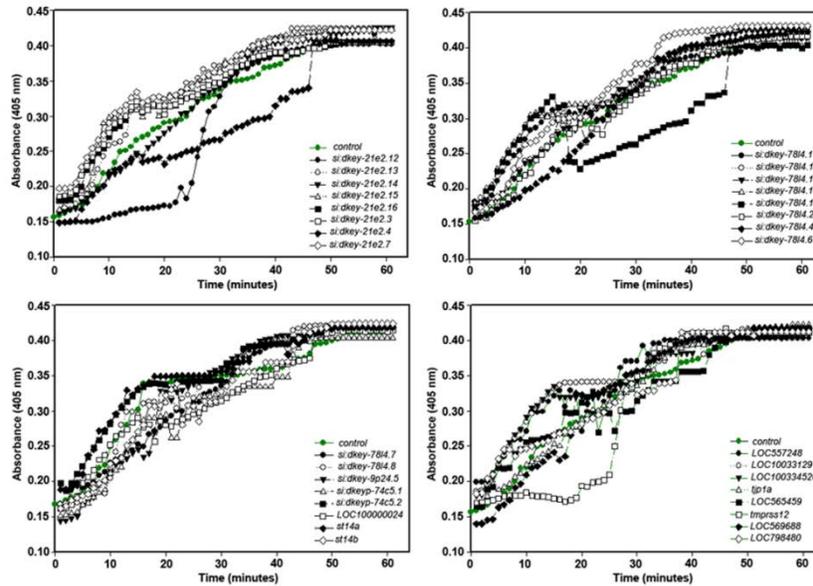


FIG 4.9c Primary screening of serine protease knockdown using kPT assay.

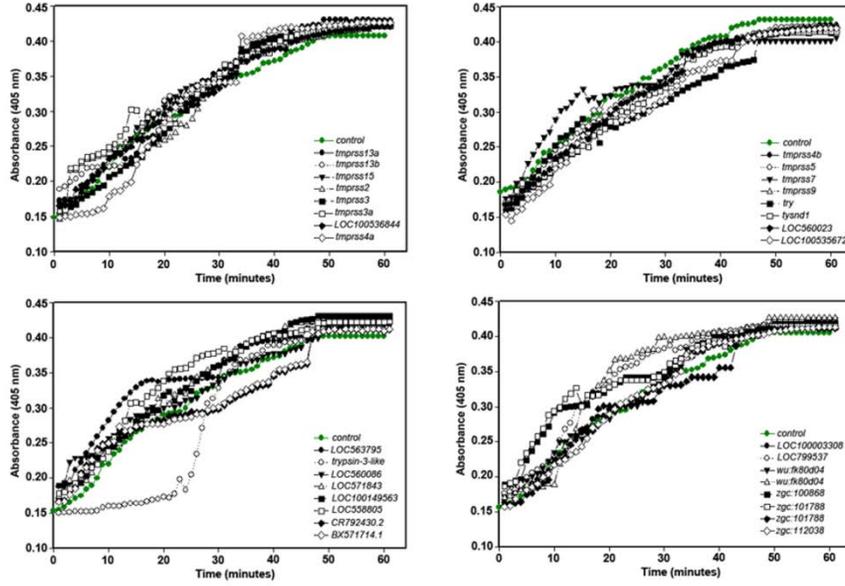


FIG 4.9d Primary screening of serine protease knockdown using kPT assay.

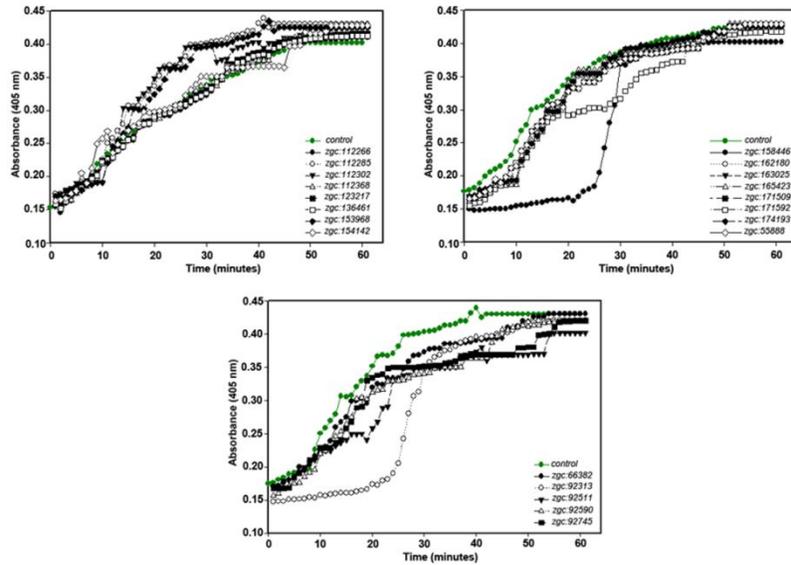


FIG 4.9e Primary screening of serine protease knockdown using kPT assay.

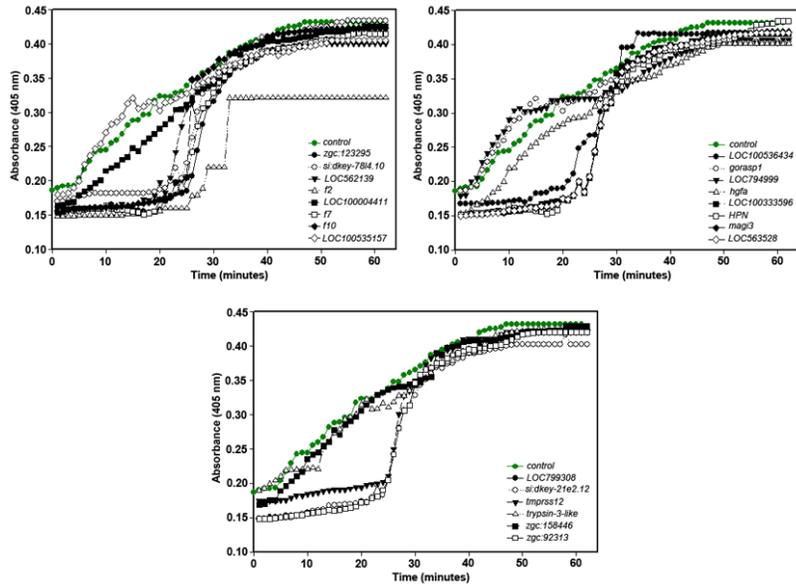


FIG 4.10 Secondary screening of serine protease knockdown using kPT assay.

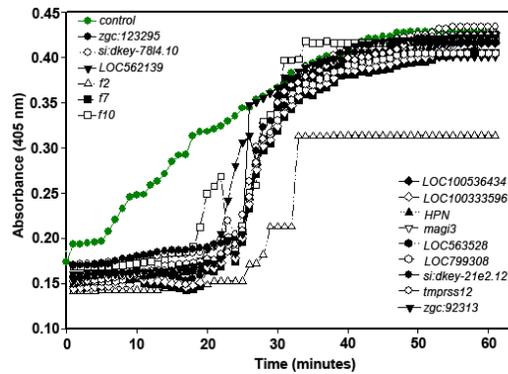


FIG 4.11 Tertiary screening of serine protease knockdown using kPT assay.

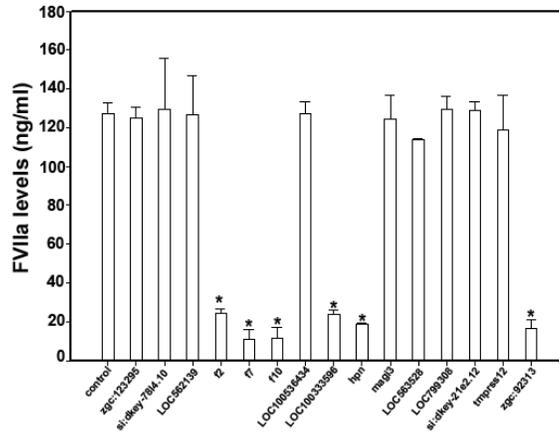


FIG 4.12 FVIIa concentration in zebrafish plasma. The genes that were found to delay fibrin formation were analyzed for FVIIa levels. FVIIa levels were estimated in zebrafish plasma injected with gene specific-SO/ NTOP. n= 3, P= <0.001 and the statistical significance is shown by * in the graph.

4.5 References

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CHAPTER 5

CONCLUSIONS

Over the past decade zebrafish has been shown to be an excellent model to study mammalian hemostasis (1). Since it has been shown that zebrafish coagulation cascade is conserved including the presence of FVII in zebrafish plasma we felt that it may be worth using zebrafish model to reevaluate the role of FSAP and hepsin in FVIIa generation (2). Both FSAP and hepsin have been in controversy with respect to FVII activation. Both the serine proteases are produced in the liver and were found to activate FVIIa (3, 4). However, studies with recombinant FSAP showed no role of this recombinant FSAP in FVII activation while hepsin knockout mouse model failed to show any role of hepsin in hemostasis (5, 6). In order to help us understand the role of these proteins *in vivo*, we decided to knockdown the *habp2* and *hpn* and analyze its effect on FVII activation and the extrinsic coagulation cascade. However, in order to determine the effect of knockdown of the genes *habp2* and *hpn* on FVIIa activity, a chromogenic assay to detect plasma FVIIa as well as FVII levels in zebrafish was needed. Unfortunately, neither FVIIa levels nor the technology to estimate FVIIa levels in zebrafish was available. Therefore, I developed a technique to estimate zebrafish FVIIa levels by modifying the existing FVIIa assay available for estimating human FVIIa. Since the assay was based on biotinylated enzyme inhibitor covalently binding to human FVIIa, its specificity to zebrafish FVIIa needed to be established. To prove the specificity of the enzyme inhibitor to zebrafish FVIIa, we expressed zebrafish FVII in *Xenopus* oocytes and showed that the recombinantly generated FVIIa had the ability to bind to the inhibitor. FXa treatment was important in proving that the FVIIa levels are increased upon incubation with FXa. This treatment was not only performed *in vivo* in zebrafish but also *in vitro* to activate FVII prepared using *Xenopus* system. After developing the above

technology, we analyzed the effect of knockdown of *habp2* and *hpn* on FVIIa levels. My results indicated that the knockdown of *habp2* did not influence the FVIIa levels nor altered the function of the extrinsic coagulation when measured using different clotting assays making us skeptical of the putative role of FSAP in activating FVII or in the extrinsic coagulation. However, based on my results hepsin did play a role in FVII activation and hemostasis *in vivo* in zebrafish. Knockdown of *hpn* in zebrafish had lowered the FVIIa levels and showed a prolongation in kPT suggesting its role in FVII activation and in hemostasis in general. Studies from other laboratories showed knockout of hepsin (-/-) in mice affected the hepatocyte size. However, those studies did not include study on coagulation (7). Using the same model another study showed that hepsin knockout had no effect on Prothrombin time in mice suggesting no role of hepsin in coagulation. Our results even though contradict the results found in hepsin knockout mice they support the *in vitro* findings of FVII activation using BHK cells. It is possible that mechanisms of initial generation of FVIIa might be different in mice. In fact several differences between mice and human hemostasis have been noted earlier (8-11). Nevertheless, the fact that BHK cells expressing human hepsin activated FVII *in vitro* and zebrafish hepsin activating FVII *in vivo* argues that in humans hepsin may be the protease that is involved in activation of FVII.

It has been shown that hepsin could be the first serine protease expressed during mammalian development and, thus, could play a potential role in development (12). However, in our studies using zebrafish embryos, hepsin knockdown did not affect the embryonic development but the TTO in *hpn* knockdown larvae was highly affected. These results are consistent with the mouse knockout experiments on hepsin genes as these knockouts did not affect any major development. Thus according to our results hepsin seems to play a prominent

role in FVII activation and extrinsic coagulation in general in zebrafish but does not play a role in development.

In my study I also estimated FVII and FVIIa levels in an adult zebrafish wildtype population. This study is first of its kind in early vertebrates. The estimated total FVII levels in the zebrafish plasma are comparable to those found in humans. However, the estimated FVIIa plasma levels using the ELISA assay in zebrafish is several-fold higher than those found in human plasma (13). These high levels of FVIIa do explain the rapid blood clotting in teleost fish. Studies in fresh water fish, like the cichlid fish and the teleost, have shown blood coagulation is similar to mammals but with a much reduced clotting time which is attributed to highly active intrinsic and extrinsic coagulation pathways (14-16). Our results also indicate that the high levels of activated FVII can contribute to the presence of a highly efficient hemostatic mechanism in zebrafish, a teleost fish, to cope up with the vascular damage under water.

Role of hepsin in activating FVII increases our understanding of its role in regulating hemostasis. However, because hepsin (like most other type II transmembrane serine proteases) is not functionally well characterized, it becomes difficult to speculate about its mechanism of action in FVII activation (17). It is likely that other serine proteases could play a role in FVII activation along with hepsin. It is also possible that a whole array of serine proteases would sequentially activate FVII and that hepsin could be one such protease in the cascade. Thus, to find out other serine proteases involved in FVII activation a whole genome knockdown of all serine proteases in zebrafish is required. However, given the expensive nature of the available gene knockdown technology a new low cost gene knockdown method was required. To accomplish this, I developed a technology that builds on the use of a non toxic oligonucleotide permeabilizer (NTOP) that will bind to standard oligonucleotides and help carry out targeted

gene knockdowns. Based on this technology I performed a knockdown α IIb, an integrin that plays a major role in platelet aggregation, a response very important in hemostasis (18). *α IIb* has often been used as a standard gene to show loss of function in knockdown experiments in hemostasis using antisense oligonucleotides (19, 20). Using my technology I found knockdown of *α IIb* to be over 60 % making it efficient as compared to available antisense technology in zebrafish but considerably cheaper. Many antisense oligonucleotides have been tried before however, the advantage of the technology is degradation of RNA. Even though phosphorothioates knockdown has been known to be more stable and mediated by RNase H activity, their backbone was found to induce toxic effects (21). In my study, the technology developed degraded the target mRNA which resulted in lower protein levels without any evidence of toxicity or inflammation. To date, MOs are a commonly used knockdown technology (22). Our results indicate that our technology has an equal efficiency as compared to the MO but has an advantage over the later with respect to cost which is an important factor for large-scale high throughput screens. The technology is equally efficient and the gene specificity relies on the chosen oligonucleotides sequence. The standard oligonucleotides used in this technology are 25 bp long which minimizes the “off-target” effects. However, the optimal length of the oligonucleotides required for knockdown requires further study.

Using the above technology, I performed knockdowns on all serine proteases present in the zebrafish genome to find out which novel genes are involved in FVII activation. Serine proteases involved in FVII activation will not only affect FVIIa levels but also affect fibrin generation initiated by thromboplastin which is measured in kPT assays. Therefore, I used the kPT assay as my screening assay. To rule out background mutation in zebrafish affecting clotting, I rescreened the genes that affected kPT. Using primary, secondary and tertiary

screening with the kPT assay, 15 serine proteases were found to have a function in hemostasis. These 15 serine proteases were then screened for their role in FVII activation using the ELISA assay that I developed. Out of these, 6 serine proteases resulted in decreased FVIIa levels. Out of these, 6 serine proteases, serine proteases hepatocyte growth factor like (*LOC100333596*) and prostaticin (*zgc:92313*) were identified as novel candidates for FVII activation in this study.

Thus, in summary, my graduate research studies involved estimation of FVII and FVIIa in zebrafish. This study was the first of its kind, as no elaborate study to estimate coagulation factor levels in teleost fish had been done before. This study also determined that hepsin is responsible for *in vivo* activation of FVII in zebrafish. In this dissertation I also developed a low cost gene knockdown technology. This technology is cost effective and has the potential to conduct genome-wide knockdowns of zebrafish gene expression. Using this technology I performed knockdowns of 181 serine proteases and identified 2 novel serine proteases, hepatocyte growth factor like and prostaticin. Interestingly, prostaticin is related to hepsin. What a coincidence!

5.1 References

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