The present invention relates to novel proteins and methods of using chimeric Factor VIIa polypeptides.
CHIMERIC FACTOR VII MOLECULES WITH
ENHANCED HALF LIFE AND METHODS OF
USE

STATEMENT OF PRIORITY

[0001] This application is a continuation-in-part application of, and claims priority to, U.S. application Ser. No. 12/823,382, filed Jun. 25, 2010 (pending), which claims the benefit, under 35 U.S.C. §119 (e), of U.S. Provisional Application Ser. No. 61/220,278, filed Jun. 25, 2009, the entire contents of each of which are incorporated by reference herein.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant number 5-P01-HL06350 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates to methods of treatment using chimeric human coagulation Factor VII (FVII) polypeptides having a prolonged hemostatic protective effect, or extended half-life and delayed clearance from the body as compared with available Factor VII polypeptides, as well as polynucleotide constructs encoding such polypeptides, and vectors and host cells comprising and expressing the polynucleotides. In addition, the present invention relates to methods of clearing proteins present in the extravascular space more slowly and increasing the functional half-life by reducing the interaction of Factor VIIa with anti-thrombin III or other inhibitors such as alpha 2 macroglobulin or tissue factor pathway inhibitor (TFPI). The present invention also relates to methods of clearing proteins such as chimeric FVII polypeptides in the extravascular space more quickly. The present invention also relates to methods of targeting proteins such as chimeric FVII polypeptides to the extravascular tissue where they may have an extended functional lifetime.

BACKGROUND OF THE INVENTION

[0004] Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components, which participate in what has been referred to as the coagulation "cascade," are enzymatically inactive proteins (proenzymes or zymogens) that are converted to proteolytic enzymes by the action of an activator (which itself is an activated clotting factor). Coagulation factors that have undergone such a conversion are generally referred to as "active factors," and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g., activated Factor VII is designated as Factor VIIa or FVIIa).

[0005] Initiation of the hemostatic process has been thought to be mediated by the formation of a complex between tissue factor and Factor VIIa. This complex then converts Factors IX (FIX) and X (FX) to their active forms. Factor Xa (FXa) converts limited amounts of prothrombin to thrombin on the tissue factor-bearing cell. Thrombin activates platelets and Factors V (FV) and VIII (FVIII) into Factors Va (FVa) and VIIIa (FVIIa), both cofactors in the further process leading to the full thrombin burst. Some of the generated thrombin feeds back and activates more FIX, which further sustains thrombin generation to allow a clot to form. This process includes generation of Factor Xa (FXa) by Factor IXa (FIXa) (in complex with Factor VIIIa) and occurs on the surface of activated platelets. Thrombin finally converts fibrinogen to fibrin, resulting in formation of a fibrin clot. Factor VIII and tissue factor have been found to be the main initiators of blood coagulation. The pharmacological use of Factor VIIa has rapidly expanded and it is often used off-label in trauma. Although administered FVIIa is rapidly removed from the circulatory system, maintaining it in the circulatory system or extravascular spaces, especially around blood vessels, may be valuable. Thus, the lifetime of FVII and/or FVIIa may be a complex question because it is cleared rapidly from circulation but may be present outside of the circulatory system as well. Factor VIIa is a plasma glycoprotein that is known to circulate in blood as a single-chain zymogen. The zymogen has marginal catalytic activity. Single-chain Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIa, Factor IXa, Factor VIIa or thrombin in vitro. Factor Xa is believed to be the major physiological activator of Factor VII, although other proteases may also be able to cleave FVII into FVIIa. Like several other plasma proteins involved in hemostasis, Factor VII is dependent on Vitamin K for its activity, which is required for the gamma-carboxylation of multiple glutamic acid residues that are clustered in the amino terminal domain of the protein, the so-called “Gla-domain.” These gamma-carboxylated glutamic acids are required for the efficient secretion of FVII and its metal ion-induced interaction with phospholipids. The conversion of zymogen Factor VII into the activated two-chain molecule occurs by cleavage of an internal Arg_{52}Leu_{55} Peptide bond. Additionally, it is well known that high concentrations of Factor VII lead to autoactivation in vitro; which is also true of the chimeric proteins of this invention. In the presence of tissue factor, phospholipids and calcium ions, the two-chain Factor VIIa rapidly activates Factor X or Factor IX by limited proteolysis.

[0006] The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al. Hum Genet. 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O’Hara et al. Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organization and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding signal sequence; exon 2 the prepropeptide and Gla domain; exons 3 and 4 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al. Biochemistry 1985; 24: 3736-3750).

[0007] Factor IX (Christmas factor) is the zymogen of a serine protease active in normal hemostasis. Its enzymatic activity also requires carboxylation of specific glutamic acid residues in its N-terminal Gla domain. Factors IX, X, VII and protein C are closely related paralogs of the same family of serine proteases, with a high degree of amino acid sequence identity and intron-exon arrangement of the genes coding for these proteins. These closely related proteins have a similar structure of functional domains from the amino to carboxyl terminus, including a γ-carboxyglutamic acid (GLA) domain, two epidermal growth factor-like (EGF) domains, an activation peptide and the catalytic domain. Protein S (UniProt Accession No. P07225) is a 676 amino acid, vitamin K-dependent protein with a GLA domain, 4 EGF-like domains, a thrombin sensitive region and 2 laminin domains.

[0008] The vitamin K dependent coagulation plasma proteins contain a GLA domain that functions as the site of
protein attachment to phospholipids or certain cell membranes and the GLA domain is highly conserved among the various coagulation proteins. Despite their similarity, the GLA domains exhibit a wide range of affinities for phospholipid, with the GLA domain of Protein S having the highest affinity for phospholipids. (Ellison et al. Biochemistry 1998; 37:7997-8003; McDonald et al. Biochemistry 1997; 36:5120-27).

[0009] It is often desirable to stimulate or improve the coagulation cascade in a subject. Factor VIIa has been used to control bleeding disorders that result from several causes such as clotting factor deficiencies (e.g., hemophilia A and B or deficiency of coagulation Factors XI or VII) or clotting factor inhibitors. Factor VIIa has also been used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). Such bleeding may, for example, be caused by a defective platelet function, thrombocytopenia or von Willebrand’s disease.

[0010] Bleeding is also a major problem in connection with surgery and other forms of trauma. For example, Factor VII has been used extensively for treating soldiers wounded in Iraq and Afghanistan. (Perkins et al. The Journal of Trauma 2007; 62:1095-9; discussion 9-101). Its use has been credited with saving many lives, but as with most medical treatments, there are side effects, such as stroke or other thrombotic events after treatment. The overall impression of physicians using FVIIa, however, is that its use has saved many more lives than it has lost. Perhaps the best indication of this is that in previous wars approximately 30 percent of the wounded died of their injuries, while the number in the Gulf war has been reduced to about 10 percent. (Gawande et al. N Engl J Med. 2004; 351:2471-5).

[0011] Studies of transgenic hemophilia B mice expressing factor VIIa have demonstrated that continuous Factor VIIa expression at low levels (below 1.5 μg/ml) restores clotting activity in hemophilia B mice. Levels of factor VIIa in wild type or hemophilia B mice above 2 μg/ml, however, led to thromboses in the heart and lungs; both the heart and lungs are sites of high tissue factor expression. This suggests that the high levels of factor VIIa in the circulation induce thrombosis when they contact tissue factor exposed upon vessel injury in the heart and lungs (Margaritas et al. J Clin Invest. 2004; 113:1025-31). Furthermore, studies have shown that transfected canine factor VIIa in hemophilic dogs is both safe and effective in the short and medium term (Margaritas et al. Gene Therapy 2009; 113:1025-31).

[0012] Warnings relating to treatment with Factor VII have been proposed for products seeking regulatory approval. For example, The European Medicines Agency, Human Medicines Evaluation Unit recommends that current Factor VIIa therapies carry a warning of the risk of thrombosis and disseminated intravascular coagulation, particularly in situations where the Factor VII is to be administered to patients with a history of coronary heart disease or liver disease, post-operative patients, neonates and those at risk from thrombosis and disseminated intravascular coagulation. See, e.g., Core SPC for Human Plasma Derived Coagulation Factor VII Products (CPMP/BPWG/2048/01), July, 2004.

[0013] It has been shown that the EGF-1 domain of Factor VIIa plays a role in the affinity of Factor VIIa for tissue factor (Chang et al. J Clin Invest 1997; 100(4):886-892; Jin et al. Biochemistry 1999; 38(4):1185-1192; Chang et al. Biochemistry 1995; 34(38):12227-12232). Using both a synthetic substate and Factor X (FX), in both the presence and absence of tissue factor, Factor VIIa polypeptides in which the EGF-1 domain had been substituted with a Factor IX EGF-1 domain had catalytic activity in vitro similar to the wild type Factor VIIa. However, the activity of Factor VIIa polypeptides in which the EGF-1 domain had been substituted with a Factor IX EGF-1 domain was substantially less than that of wild-type FVIIa when both molecules were assayed in vitro in the presence of tissue factor. At first glance, this would seem to obviate the use of chimeric constructs for treating bleeding; however, Monroe (British Journal of Haematology 1997; 99:542-549) has proposed that the physiological mechanism of FVIIa in treating hemophilia and bleeding is tissue factor independent. Additionally, it has been reported that up to 65% of the total body clearance of Factor VIIa function is mediated through antithrombin Ill/Factor VIIa complexes (Agero et al. J. Thromb Haemost. 2011; 9:333-338). This clearance, however, has been demonstrated in vitro to be dependent upon the Factor VIIa being bound to tissue factor (Rao et al. Blood 1993; 81:2600-07).

[0014] Commercial preparations of human recombinant FVIIa (rFVIIa), NovoSeven® and NovoSeven® RT, are indicated for the treatment of bleeding episodes in hemophilia A or B patients and are the only rFVIIa preparations for treatment of bleeding episodes available on the market. Also, it has been demonstrated that NovoSeven® may bind to rehydrated lyophilized platelets, which could be administered in combination to localize the Factor VII to a site of injury (Fischer et al. Platelets 2008; 19:182-91). Recently, a higher specific activity analogue of NovoSeven® has been shown to have a shorter plasma half-life than NovoSeven® because of its interaction with antithrombin. Thus, the functional half-life of both NovoSeven® and this analogue will be considerably shortened by in vivo interaction with antithrombin compared to the chimeric molecules described in this invention. (Petersen et al. British Journal of Haematology 2010; 152:99-107). The functional half-life of both NovoSeven® and this analogue may also be shortened because of binding with alpha 2 macroglobulin and/or tissue factor pathway inhibitor (TFPI), whereas the functional half-life of the chimeric molecules of this invention may not. Additionally, it has been shown that selective PEylation of Factor VII may increase plasma half-life, (Stennicke et al. Thromb Haemost 2008; 100:920-28) and that a recombinant human Factor VII with 3 amino acid substitutions has an increased activity on the surface of platelets (Moss et al. J. Thromb. Haemost. 2009; 7:299-305). PEylation of the chimeric Factor VIIa molecules of the present invention is expected to work in a similar manner to that described for PEylated wild type FVIIa to increase functional half-life. Likewise, other modifications of proteins known in the art, such as covalent attachment of non-polypeptide moieties to form conjugates, e.g., glycosylation, are expected to function in a similar manner in the chimeric Factor VIIa molecules of the present invention, i.e., the properties imparted by a protein by the covalent attachment of a non-polypeptide moiety are expected to be imparted to the chimeric Factor VII a molecules.

[0015] There is a need for variants of Factor VIIa having a prolonged, or extended functional half-life and a reduced clearance rate from the body.

SUMMARY OF THE INVENTION

[0016] The present invention provides a method of treating a bleeding disorder in a subject in need thereof, comprising
administering to the subject an effective amount of a chimeric Factor VIIa polypeptide with a prolonged functional lifespan in vivo or prolonged functional half-life in vivo as compared with a non-chimeric Factor VIIa polypeptide.

Further provided herein is a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric Factor VIIa polypeptide with a prolonged protective effect against bleeding due to the bleeding disorder or trauma, as compared with a non-chimeric Factor VIIa polypeptide.

Also provided herein is a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has a reduced affinity for tissue factor as compared with a non-modified Factor VIIa polypeptide.

The present invention further provides a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has an increased binding affinity for a basement membrane component and/or increased ability to mediate binding of a protein to a structural molecule of the extracellular tissue such as a basement membrane component as compared with a non-modified Factor VIIa polypeptide.

Also provided herein is a method of targeting or sequestering a modified Factor VIIa polypeptide to extracellular tissue in a subject in need of treatment for a bleeding disorder, comprising administering to a subject an effective amount of a modified Factor VIIa polypeptide that has increased targeting or sequestering to extracellular tissue as compared with a non-modified Factor VIIa polypeptide.

Additionally provided herein is a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has a reduced clearance rate from the body of the subject or reduced binding to Factor VIIa protease inhibitors that inactivate FVIIa protease activity or facilitate the clearance of bound FVIIa in the body of the subject as compared with a non-modified Factor VIIa polypeptide.

The present invention also provides a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric FVIIa polypeptide less frequently during bleeding incidences or in between bleeding incidences, with longer intermittent duration during a bleeding incidence, at lower doses during or before bleeding incidences and/or with longer duration prior to a bleeding incidence (e.g., as a prophylactic treatment), as compared with a non-chimeric Factor VIIa polypeptide.

In the methods of this invention a chimeric Factor VIIa polypeptide can be employed, wherein the chimeric Factor VIIa polypeptide, comprises a catalytic domain of Factor VII and/or an EGF-2 domain of Factor VII.

Such a chimeric Factor VIIa polypeptide can further comprise a domain that mediates binding of a protein to a structural molecule of the extracellular tissue such as a basement membrane component. Nonlimiting examples of such a domain include a) a single chain antibody Fab fragment or single chain variable fragment (scFv) that specifically binds a structural molecule of the extracellular tissue such as a basement membrane component; b) a laminin binding domain of a laminin binding receptor; c) a laminin binding domain of a proteoglycan; d) a laminin binding domain of a bacterial adhesin protein; e) a nidogen/entactin binding domain; f) a collagen type 4 binding domain; and g) any combination of (a)-(f) above.

In further embodiments, the chimeric Factor VIIa polypeptide of this invention can comprise, in addition to a FVII catalytic domain and a FVII EGF-2 domain, a domain that mediates binding of a protein to a structural molecule of the extravascular tissue such as a basement membrane component, and/or a GLA domain of a vitamin K dependent coagulation protein, including but not limited to Factor IX GLA domain or Protein S GLA domain.

The chimeric Factor VIIa polypeptide described above can further comprise an EGF-1 domain of a vitamin K dependent coagulation protein (e.g., that binds more poorly to tissue factor than non-chimeric Factor VIIa), including but not limited to a Factor IX EGF-1 domain, a Protein S EGF-1 domain, a Factor X EGF-1 domain, a Factor VII EGF-1 domain, a Protein C EGF-1 domain or a Gas 6 EGF-1 domain. In particular embodiments, the chimeric Factor VIIa polypeptide can comprises an EGF-1 domain of Factor IX or an EGF-1 domain of Protein S or an EGF-1 domain of Factor X.

In some embodiments of the chimeric Factor VIIa polypeptide of this invention, the GLA domain can be a Factor IX Gla domain. In some embodiments of the chimeric Factor VIIa polypeptide of this invention, the GLA domain can be a Factor IX Gla domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by arginine. In other embodiments of the chimeric Factor VIIa polypeptide of this invention, the GLA domain is a Factor IX Gla domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by alanine and/or a substitution of valine at residue 56 in the amino acid sequence of SEQ ID NO:19 with another amino acid (e.g., lysine, leucine, isoleucine and methionine as non-limiting examples). An exemplary embodiment is a chimeric Factor VIIa polypeptide comprising a Factor IX EGF-1 domain and a Factor IX Gla domain, wherein the FIX Gla domain comprises a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by alanine. This mutation in the FIX GLA domain reduces the affinity for collagen Type IV. Lowering the affinity of the FVIIa chimera for basement membrane collagen (Type IV) is expected to yield an advantage over other FVIIa molecules. For example, if some FVIIa chimeras described herein turn out to be hypercoagulable, then using a chimeric FVIIa that binds less tightly will result in less FVIIa protein bound to the basement membrane (collagen Type IV) and thereby reduce the thrombotic risk.

In additional embodiments of the chimeric Factor VIIa polypeptide of this invention, a) the EGF-1 domain can be a Factor VII EGF-1 domain comprising a substitution of isoleucine at residue 129 in the amino acid sequence of SEQ ID NO:18 by alanine, 2) the EGF-1 domain can be a Factor VII EGF-1 domain comprising a substitution of arginine at residue 139 in the amino acid sequence of SEQ ID NO:18 by alanine, 3) the catalytic domain of Factor VII can comprise a substitution of methionine at residue 366 of the amino acid sequence of SEQ ID NO:18 with valine, valine or isoleucine, 4) the catalytic domain of Factor VII can comprise a substitution of valine at residue 218 of the amino acid sequence of SEQ ID NO:18 with aspartate, 5) the catalytic domain of Factor VII can comprise a substitution of glutamate at residue 356 of the amino acid sequence of SEQ ID NO:18 with valine,
and/or the catalytic domain of Factor VII can comprise a substitution of methionine at residue 358 with glutamine, in any combination.

[0029] In the methods of this invention, a bleeding disorder can be but is not limited to, a clotting factor deficiency; defective platelet function; thrombocytopenia; von Willebrand’s disease; inhibition of clotting factors; bleeding induced by surgery; bleeding induced by trauma and any combination thereof. In some embodiments, the bleeding disorder is a deficiency of a clotting factor or its clotting function, which can be hemophilia.

[0030] The present invention further provides a method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a nucleic acid molecule comprising a nucleotide sequence encoding the chimeric Factor VIIa polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] FIG. 1 shows a diagrammatic representation of chimeric FVIIa molecules useful in the present invention. FVII: Factor VII; FIX: Factor IX; Pro S: Protein S.

[0032] FIG. 2 shows the thrombin generation (nM) in normal conditions and hemophilic conditions (removal of Factor IX or Factor VIII) in a cell based model system of hemophilia for varying concentrations of wild-type Factor VIIa.

[0033] FIG. 3 shows the thrombin generation (nM) in normal conditions and hemophilic conditions (removal of Factor IX or Factor VIII) in a cell based model system of hemophilia with administration of 50 nM wild-type Factor VIIa, or 10 nM chimeric Factor VIIa. 1063: Factor VIIa chimera with GLA domain and EGF-1 domain of Factor IX; CT54: Factor VIIa chimera with GLA domain of Protein S and EGF-1 domain of Factor IX.

[0034] FIG. 4 shows the peak thrombin, relative to the control value, produced in a cell based model system of hemophilia following administration of 50 nM wild-type Factor VIIa, or 10 nM chimeric Factor VIIa. 1063: Factor VIIa chimera with GLA domain and EGF-1 domain of Factor IX; CT54: Factor VIIa chimera with GLA domain of Protein S and EGF-1 domain of Factor IX.

[0035] FIG. 5 shows the disruption of hemostasis time in a clotting assay for a hemophiliacs B mouse with no treatment, a hemophiliacs B mouse administered with 2 mg/kg of NovoSeven®, a hemophiliacs B mouse administered with 2 mg/kg of a chimeric FVIIa molecule and a wild-type mouse with no treatment. 1063: Factor VIIa chimera with GLA domain and EGF-1 domain of Factor IX.

[0036] FIG. 6 shows the amino acid sequence of an exemplary chimeric Factor VII of the present invention, comprising the signal, pro peptide, GLA and EGF 1 domains of Factor IX (underlined) (SEQ ID NO:1).

[0037] FIG. 7 shows the amino acid sequence of an exemplary chimeric Factor VII of the present invention, comprising the signal, pro peptide, and EGF 1 domains of Factor IX (underlined), and the GLA domain of Protein S (bold) (SEQ ID NO:2).

DESCRIPTION OF THE INVENTION

[0038] The present invention will now be described more fully hereinafter. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0039] The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used in the description of the invention and the appended claims, the singular forms “a,” “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0040] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the present application and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference herein in their entirety.

[0041] Also as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

[0042] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination.

[0043] Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be included or omitted.

[0044] To illustrate, if the specification states that a complex comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

[0045] As used herein, the transitional phrase “consisting essentially of” (and grammatical variants) is to be interpreted as encompassing the recited materials or steps “and those that do not materially affect the basic and novel characteristic(s)” of the claimed invention. See, In re Herz, 557 F.2d 549, 551-52, 190 U.S.P.Q. 461, 463 (CCPA 1976) (emphasis in the original); see also MPEP §2111.03. Thus, the term “consisting essentially of” as used herein should not be interpreted as equivalent to “comprising.”

[0046] The term “about,” as used herein when referring to a measurable value such as an amount or concentration and the like, is meant to encompass variations of 20%, 10%, 5%, 1%, 0.5%, or even 0.1% of the specified amount.

[0047] The present invention provides methods of extending the lifetime of FVIIa in the body and of extending the effective treatment of a bleeding disorder in a subject having the bleeding disorder by administering one or more of the chimeric FVIIa polypeptides described herein. The method of treating the bleeding disorder may include a method of administering to the subject a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric Factor VIIa polypeptide and/or a method of administering to the subject the chimeric Factor VIIa protein that the nucleotide sequence encodes, as described herein. The methods of the current
invention use chimeric FVIIa molecules, and in particular embodiments, employ chimeric hFVIIa molecules comprising hFVIIa domains and domains from one or more proteins of the coagulation system, providing one or more desired benefits. While not wishing to be constrained by the inventors’ present theory of activity, it is believed that by locating the chimeric FVII or FVIIa molecules extravascularly or on a basement membrane component such as type IV collagen, they will be exposed when the endothelium lining the blood vessel is breached and will accumulate in the vicinity of the coagulation proteins and activated platelets such that a response to a bleeding episode is more effective and a number of benefits are achieved as described below.

[0048] The chimeric FVIIa and FVII molecules of the present invention, therefore, have one or more improved properties as compared to commercially available rFVIIa or rFVII or other non-chimeric FVII polypeptides, including having a prolonged, or extended functional half-life and a reduced clearance rate from the body. Consequently, medical treatment with a FVIIa chimera of the invention offers advantages over the currently available rFVIIa compound, such as requiring potentially lower doses and/or providing extended duration of protection against further bleeding episodes between intermittent doses and/or following the end of treatment. Other advantages include accumulating the chimeric Factor VIIa molecules in the vicinity of other coagulation proteins and activated platelets such that a response to a bleeding episode is quicker and/or more effective.

[0049] Thus in one embodiment, the present invention provides a method of treating a bleeding disorder in a subject (e.g., a subject in need thereof), comprising administering to the subject an effective amount of a chimeric Factor VIIa polypeptide with a prolonged functional lifespan in vivo or a prolonged functional half-life in vivo (e.g., prolonged by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, 100% or more) as compared with a non-chimeric Factor VIIa polypeptide. The determination of the functional lifespan in vivo or prolonged functional half life in vivo of the polypeptides of this invention can be made by carrying out protocols as described herein and as are known in the art.

[0050] In another embodiment, the present invention provides a method of treating a bleeding disorder in a subject (e.g., a subject in need thereof), comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has an increased binding affinity for a structural molecule of the extravascular tissue such as a basement membrane component (e.g., increased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, or 100%) as compared with a non-modified Factor VIIa polypeptide. The determination of binding affinity for a structural molecule of the extravascular tissue such as a basement membrane component of the polypeptides of this invention can be made by carrying out protocols as described herein and as are known in the art.

[0051] Also provided herein is a method of treating a bleeding disorder in a subject (e.g., a subject in need thereof), comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has a reduced clearance rate from the body of the subject and/or reduced binding to Factor VIIa protease inhibitors that inactivate FVIIa protease activity or facilitate the clearance of bound FVIIa in the body of the subject (e.g., prolonged by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, or 100%) as compared with a non-modified Factor VIIa polypeptide. The determination of the clearance rate or reduced binding to Factor VIIa protease inhibitors that inactivate FVIIa protease activity or facilitate the clearance of bound FVIIa of the polypeptides of this invention can be made by carrying out protocols as described herein and as are known in the art.

[0052] Representative chimeric FVIIa polypeptides useful in the methods of the invention include a chimeric FVIIa polypeptide comprising the EGF-2 and catalytic domains of FVII and the GLA domain of a vitamin K-dependent coagulation protein and the EGF-1 domain of a vitamin K-dependent coagulation protein (e.g., an EGF-1 domain that binds more poorly to tissue factor than a non-chimeric Factor VIIa polypeptide). Other representative chimeric FVIIa polypeptides useful in the methods of the invention include a chimeric FVIIa comprising the EGF-1, EGF-2 and catalytic domains of FVII and the GLA domain of a vitamin K-dependent coagulation protein. In particular embodiments of the invention chimeric FVIIa polypeptides useful in the methods of the invention include 1) a chimeric FVIIa comprising the GLA and EGF-1 domains of FIX and the EGF-2 and catalytic domains of FVII; 2) a chimeric FVIIa comprising the EGF-1 domain of FIX and the GLA, EGF-2 and catalytic domains of FVII; 3) a chimeric FVIIa comprising the GLA domain of Protein S, the EGF-1 domain of FIX and the EGF-2 and catalytic domains of FVII; 4) a chimeric FVIIa comprising the GLA and EGF-1 domains of Protein S and the EGF-2 and catalytic domains of FVII; 5) a chimeric FVIIa comprising the EGF-1 domain of Protein S and the GLA, EGF-2 and catalytic domains of FVII; 6) a chimeric FVIIa comprising the EGF-1 domain of Protein S, the GLA domain of FIX and the EGF-2 and catalytic domains of FVII; 7) a chimeric FVIIa comprising the EGF-1 domain of factor X, the Glu domain of FIX and the EGF2 and catalytic domain of FVII; 8) a chimeric FVIIa comprising the EGF-1 domain of factor X, the Glu domain of Protein S and the EGF2 and catalytic domain of FVII; 9) a chimeric FVIIa comprising the GLA and EGF-1 domains of FIX and the EGF-2 and catalytic domains of FVII, where the lysine at residue 51 in the FIX GLA domain (i.e., at residue 51 in the amino acid sequence of SEQ ID NO:19) has been substituted with an arginine, thereby improving or increasing binding to extravascular tissue components (e.g., basement membrane components such as collagen type IV); and 10) a chimeric FVIIa comprising the EGF-1 domain of Protein S, the GLA domain of FIX and the EGF-2 and catalytic domains of FVII, where the lysine at residue 51 in the FIX GLA domain (i.e., at residue 51 in the amino acid sequence of SEQ ID NO:19) has been substituted with an alanine and/or the valine at residue 56 of the amino acid sequence of SEQ ID NO:19 has been substituted with another amino acid (e.g., lysine, leucine, isoleucine, methionine). A further embodiment of this invention includes a chimeric FVIIa comprising the EGF-1 domain of Protein S, the GLA domain of FIX and the EGF-2 and catalytic domains of FVII, where the lysine at residue 51 in the
FIX GLA domain (i.e., at residue 51 in the amino acid sequence of SEQ ID NO:19) has been substituted with an alanine and/or the valine at residue 56 of the amino acid sequence of SEQ ID NO:19 has been substituted with another amino acid (e.g., lysine, leucine, isoleucine, methionine). Naturally, this invention also contemplates chimeric forms of FVII in the aforementioned embodiments also. Naturally, this invention also contemplates chimeric forms of FVII in the aforementioned embodiments also.

[0053] Representative FVIIa polypeptides useful in the methods of the invention also may include wild-type FVIIa or any of the above described chimeric FVIIa polypeptides, which have amino acid substitutions in the FVII EGF-1 domain or the FVII GLA domain. The substitutions can be conservative substitutions and/or non-conservative substitutions. Such substitutions may include the substitution of the isoleucine at residue 129 by alanine and/or a substitution of the arginine at residue 139 by alanine in the amino acid sequence of the FVII EGF-1 domain shown as SEQ ID NO:18. Additional substitutions may include, in a FVIIa chimera comprising a FIX GLA domain, the substitution of the lysine at residue 51 by arginine in the amino acid sequence of the FIX GLA domain as shown in SEQ ID NO:19, which causes the GLA domain to have a higher binding affinity for collagen type IV but does not appear to affect platelet binding (Guo et al. J Biol Chem 1992; 267:20529-31; Melton et al. Blood Coagulation and Fibrinolysis 2001; 12:227-43). Further substitutions in such a chimera comprising a FIX GLA domain include a substitution of the lysine at residue 51 by alanine in the amino acid sequence of the FIX GLA domain as shown in SEQ ID NO:19 and/or a substitution of the valine at residue 56 of the GLA domain as shown in SEQ ID NO:19 with another amino acid (e.g., lysine, leucine, isoleucine, methionine, etc.).

[0054] The FVII molecules of this invention can comprise a substitution of the methionine at residue 366 in the amino acid sequence of the FVII catalytic domain shown as SEQ ID NO:18 by another amino acid, which in some embodiments can be a conservative amino acid substitution, which can be, for example alanine, valine, leucine or isoleucine, which further reduces the affinity for tissue factor; and, the substitution of the valine at residue 218 in the amino acid sequence of the FVII catalytic domain as shown in SEQ ID NO:18 by aspartate, the substitution of the glutamate at residue 356 in the amino acid sequence of the FVII catalytic domain shown as SEQ ID NO:18 by valine and/or the substitution of the methionine at residue 358 in the amino acid sequence of the FVII catalytic domain shown as SEQ ID NO:18 by glutamine, which result in a Factor VIIa with higher specific activity.

[0055] Additionally, the invention provides a method of treating a bleeding disorder in a subject having the bleeding disorder by administering a modified FVII polypeptide or modified FVII polypeptide, wherein the modified polypeptide is targeted to and/or accumulates in the extravascular space, thereby shielding the modified polypeptide from the clearance mechanisms in a subject’s body that act on a non-modified FVII or FVII polypeptide (e.g., the currently available Factor VII or Factor VIIa polypeptides) or protecting the modified polypeptide from inactivation regardless of whether it is cleared from the body. Examples of such modified polypeptides are FVIIa polypeptides comprising a GLA domain of any coagulation protein that binds collagen type IV in the basement membrane (or another basement membrane component), or the phospholipid membrane of cells. Nonlimiting examples of such GLA domains include the GLA domain of FIX and the GLA domain of Protein S, which can further comprise substitutions and/or other modifications as described herein that enhance binding to basement membrane components such as collagen type IV. Nonlimiting examples of a modified polypeptide of this invention include a recombinant protein comprising a GLA domain of FIX or a GLA domain of Protein S, a chimeric protein comprising a GLA domain of FIX or a GLA domain of Protein S and/or a chimeric FVIIa polypeptide of this invention comprising a GLA domain of FIX or a GLA domain of Protein S.

[0056] In another embodiment, the present invention provides a method of treating a subject having a bleeding disorder by administering a chimeric Factor VIIa polypeptide of the invention that comprises a catalytic domain derived from a Factor VII polypeptide and a domain that mediates binding of a protein to a structural molecule of the extracellular tissue such as a basement membrane component. Such a domain can be, for example, a collagen type IV targeting domain.

[0057] Such collagen type IV targeting domains include domains from proteins that interact with or bind to collagen type IV (see for example, UniProt Accession Nos. P02462, P08572, Q01955, P53420, P29400, QQP375 and Q14031). Nonlimiting examples of such domains include a single chain antibody Fab fragment or single chain variable fragment (scFv) (see, e.g., Colcher et al. “Pharmacokinetics and biodistribution of genetically engineered antibodies” Q J Nucl Med 1998; 42(4):225-241) that specifically binds a structural molecule of the extracellular tissue such as a basement membrane component. Further nonlimiting examples of domains that bind basement membrane components include the laminin binding domain or laminin-binding protein sequences, such as from laminin-binding receptors (see, for example, UniProt Accession No. P08865) or the laminin binding proteoglycan, agrin (see, e.g., UniProt Accession No. 000468; Kroger & Schroeder “Agrin in the developing CNS: New roles for a synapse organizer” News Phys Sci 2002; 17:207-212; Mascarenhas et al. “Mapping of the laminin-binding site of the N-terminal agrin domain (NTA)” EMBO J 2003; 22(3): 529-36, each incorporated by reference herein in its entirety), or laminin-binding adhesions from bacteria (see Linke et al. “The laminin-binding protein LBP from Streptococcus pyogenes is a zinc receptor” Journal of Bacteriology 2009; 191: 5814-23; Soares de Lima et al. “Mapping the laminin-binding and adhesion domain of the cell surface associated Hlp/LBP protein from Mycobacterium leprae” Microbes Infect 2005; 7(9-10):1097-1109; Terra et al. “Novel laminin-binding protein of Streptococcus pyogenes, Lbp, involved in adhesion to epithelial cells” Infect Immun 2002; 70(2):993-997; UniProt Accession NO. Q7DJ15, each incorporated by reference herein in its entirety), or the laminin-associated protein, nidogen/entactin (See, e.g., UniProt Accession No. P14543).

[0058] In various embodiments, a domain that mediates binding of a protein to a structural molecule of the extracellular tissue such as a basement membrane component can be present in the chimeric Factor VIIa polypeptide of this invention at the carboxy terminus, in between the GLA and EGF-1 domains, as a substitution for the GLA domain and any combination thereof.

[0059] As used herein the term “basement membrane component” includes protein molecules present in the thin layer of connective tissue that typically underlies endothelial cells, including those that form the wall of a blood or lymphatic
vessel of any type, or an epithelial surface or boundary. Non-limiting examples of basement membrane components include proteins such as laminin, nidogen, entactin, collagens of several types, including Type IV and Type XVIII, proteoglycans (such as Perlecian) and glycosaminoglycans (such as heperan sulfate).

0060 Also as used herein, a “domain that mediates binding of a protein to a basement membrane component” or a domain “that mediates binding of a protein to a structural molecule of the extravascular tissue such as a basement membrane component” is a part (e.g., fragment or subsection) of a protein that can fold, function, and exist independently of the rest of the protein sequence or structure. In this definition, a protein domain may (but does not necessarily have to) comprise the entire sequence or structure of a protein. A basement membrane binding domain is defined as a protein domain that can bind to, or mediate binding of the protein to, molecular components in the basement membrane or subendothelial matrix. Such targeting of aggregation proteins to the extravascular space using a domain that binds to a basement membrane component or mediates binding of a protein to a structural molecule of the extravascular tissue such as a basement membrane component and has reduced affinity for tissue factor has the additional, but unexpected benefit of protecting the chimeric FVIIa polypeptides from the normal circulatory clearance mechanisms present in a subject’s body while prolonging the functional half-life of the chimeric Factor VIIa polypeptide. This approach can also be used to target other therapeutically useful polypeptides or other molecules to extravascular storage sites. Such therapeutically useful molecules may include other coagulation cascade proteins and the like as are known in the art.

0061 Such targeting of aggregation proteins to the extravascular space using a domain that binds to a basement membrane component and has reduced affinity for tissue factor has the additional, but unexpected benefit of protecting the chimeric FVIIa polypeptides from the normal circulatory clearance mechanisms present in a subject’s body while prolonging the functional half-life of the chimeric Factor VIIa polypeptides. This approach can also be used to target other therapeutically useful polypeptides or other molecules to extravascular storage sites. Such therapeutically useful molecules may include other coagulation cascade proteins and the like as are known in the art.

0062 The present invention further provides a method of treating a bleeding disorder in a subject having the bleeding disorder by administering to the subject a modified polypeptide (e.g., a modified FVII or FVIIa polypeptide) with reduced binding affinity for tissue factor (e.g., reduced at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, or 100%) as compared with the binding affinity for tissue factor of a non-modified polypeptide (e.g., a non-modified Factor VII or Factor VIIa polypeptide). A modified polypeptide with reduced binding affinity for tissue factor as compared with a non-modified polypeptide can be, for example, a chimeric Factor VII polypeptide of this invention or active fragment thereof. Non-limiting examples of such polypeptides include a chimeric FVIIa polypeptide comprising the GLA and EGF-1 domains of FIX and the EGF-2 and catalytic domains of FVII; and a chimeric FVIIa polypeptide comprising the GLA domain of Protein S, the EGF-1 domain of FIX and the EGF-2 and catalytic domains of FVII. The reduction or elimination of binding to tissue factor by the chimeric FVIIa polypeptide should also extend the useful half-life of the chimeric FVIIa polypeptide because the interaction with anti-thrombin III should be much reduced (Rao et al. *Blood* 1993 81(10):2600-2607; Broze et al. *Blood* 1993 82(5):1679-1681).

0063 As used herein, the terms “chimeric polypeptide” or “chimeric Factor VII polypeptide” or “chimeric Factor VIIa polypeptide” refer to a polypeptide comprising the EGF-2 and catalytic domains from Factor VII in combination with one or more domains from another protein. The terms “non-chimeric polypeptide” or “non-chimeric Factor VII polypeptide” or “non-chimeric Factor VIIa polypeptide” thus refer to a Factor VII protein whose domains are Factor VII derived and not derived from other proteins.

0064 Also as used herein the term “modified Factor VII polypeptide” or modified Factor VIIa polypeptide” can include a Factor VII polypeptide or Factor VIIa polypeptide that has been modified (e.g., by substitution, deletion, insertion, etc., of one or more amino acids), resulting in a Factor VII polypeptide or Factor VIIa polypeptide that has altered properties (e.g., enhanced or diminished binding affinity, enhanced or diminished activity, etc.) as compared with a non-modified Factor VII polypeptide or Factor VIIa polypeptide, which is understood to be a native or wild type Factor VII or Factor VIIa, which can include naturally or non-naturally occurring variants that may differ in amino acid sequence from “wild type” Factor VII or Factor VIIa but do not have the particular altered properties as described herein.

0065 The term “activity” as used herein means the ability of a Factor VII polypeptide to convert its substrate Factor X to the active Factor Xa or Factor IX to Factor IXa with or without the ability to bind tissue factor, or any other function of Factor VII.

0066 FVIIa includes a product consisting of the two-chain form of FVII, FVIIa may also indicate the active pro-form of a FVII polypeptide.

0067 The term “inherent activity” includes the ability to generate thrombin on the surface of activated platelets in the absence of tissue factor.

0068 The term “GLA domain” or “N-terminal GLA-domain” includes the amino acid sequence from about amino acid residue 61 to about amino acid residue 105 of the amino acid sequence of SEQ ID NO:18 (Factor VII precursor sequence, see, e.g., UniProt Accession No. P08709), the amino acid sequence from about amino acid residue 47 to about amino acid residue 92 of the amino acid sequence of SEQ ID NO:19 (Factor IX precursor sequence, see, e.g., UniProt Accession No. P00740), the amino acid sequence from about amino acid residue 41 to about amino acid residue 85 of the amino acid sequence of SEQ ID NO:22 (Factor X precursor sequence, see, e.g., UniProt Accession No. P00742), the amino acid sequence from about amino acid 43 to about amino acid 88 of SEQ ID NO:23 (Protein C precursor sequence, see, e.g., UniProt Accession No. P04070), the amino acid sequence from about amino acid residue 53 to about amino acid 94 of SEQ ID NO:24 (Growth arrest-specific protein 6 (GAS6 or Gas 6) precursor sequence, see, e.g., UniProt Accession No. Q14393), any post-translational modifications to the identified amino acid sequences, any conservative amino acid substitutions in the identified amino acid sequences, addition of amino acid residues to the identified amino acid sequences, deletions of amino acid residues...
from the identified amino acid sequences, or any other amino acid sequence from a coagulation cascade protein that binds to phospholipid membranes.

The term “EGF-1” describes a region of 30-40 amino acids having structural homology to epidermal growth factor (EGF) and typically containing six cysteine residues found originally in EGF (epidermal growth factor) and also in a range of proteins involved in cell signaling and in coagulation proteins, with nearly all known EGF-like domains containing disulfide bonds 1-2, 2-3, and 4-5. The EGF-1 domain of Factor VII is from about amino acid residue 106 to about amino acid residue 142 of the amino acid sequence of SEQ ID NO:18. The EGF-1 domain of Factor IX is from about amino acid residue 93 to about amino acid residue 129 of the amino acid sequence of SEQ ID NO:19. The EGF-1 domain of Protein S is from about amino acid residue 117 to about amino acid residue 155 of the amino acid sequence of SEQ ID NO:20. The EGF-1 domain of Factor X is from about amino acid residue 86 to about amino acid residue 122 of the amino acid sequence of SEQ ID NO:22. The EGF-1 domain from Protein C is from about amino acid residue 97 to about amino acid residue 132 of the amino acid sequence of SEQ ID NO:23. The EGF-1 domain from GAS6 is from about amino acid residue 116 to about amino acid residue 154 of the amino acid sequence of SEQ ID NO:24.

The term “EGF-2” means the second EGF-like domain in a series (of two or more EGF-like domains). The EGF-2 domain of Factor VII is from about amino acid residue 147 to about amino acid residue 188 in the amino acid sequence of SEQ ID NO:18. The EGF-2 domain of Factor IX is from about amino acid residue 130 to about amino acid residue 171 in the amino acid sequence of SEQ ID NO:19. The EGF-2 domain of Protein S is from about amino acid residue 157 to about amino acid residue 200 in the amino acid sequence of SEQ ID NO:20. The EGF-2 domain of Factor X is from about amino acid residue 125 to about amino acid residue 165 in the amino acid sequence of SEQ ID NO:22. The EGF-2 domain from Protein C is from about amino acid residue 136 to about amino acid residue 176 in the amino acid sequence of SEQ ID NO:23. The EGF-2 domain from GAS6 is from about amino acid residue 156 to about amino acid residue 196 of the amino acid sequence of SEQ ID NO:24.

The term “catalytic domain” as used herein means a domain in a protein that may mediate cleavage of peptide bonds. The catalytic domain of Factor VII is from about amino acid residue 213 to about amino acid residue 452 in the amino acid sequence of SEQ ID NO:18. The catalytic domain of Factor IX is from about amino acid residue 227 to about amino acid residue 459 in the amino acid sequence of SEQ ID NO:19. The catalytic domain of Factor X is from about amino acid residue 235 to about amino acid residue 467 in the amino acid sequence of SEQ ID NO:22. The catalytic domain from Protein C is from about amino acid residue 212 to about amino acid residue 450 of the amino acid sequence of SEQ ID NO:23.

The amino acid sequences described herein can include any post-translation modifications to the identified amino acid sequences, any conservative amino acid substitutions in the identified amino acid sequences, any addition of amino acid residues to the identified amino acid sequences and/or any deletions of amino acid residues from the identified amino acid sequences.

The three-letter indication “GLA” (or “Gla”) means 4-carboxyglutamic acid (\(\gamma\)-carboxyglutamate).

The term “protease domain” means a domain in a protein that may mediate cleavage of peptide bonds, generally considered to be from about amino acid residue 213 to the carboxy terminal amino acid residue of the amino acid sequence of SEQ ID NO:18. (the heavy-chain of Factor VIIa). Generally speaking, the terms “catalytic domain” and “protease domain” are recognized as the same and are interchangeable.

The term “Factor VII polypeptide” as used herein means any protein comprising the amino acid sequence encompassing residues 61-466 of native human Factor VII (SEQ ID NO:18) or variants or fragments thereof. This includes but is not limited to human Factor VII, human Factor VIIa and variants thereof.

The term “Factor VII” as used herein is intended to comprise the inactive one-chain zymogen Factor VII molecule as well as the activated two-chain Factor VII molecule (Factor VIIa). This includes proteins that have the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa. One of skill in the art recognizes that minor sequence changes would be expected to perform in a similar manner and that the domains, polypeptides and Factor VII involved could be slightly shortened or lengthened without departing from the invention. Thus, the definition also encompasses proteins and peptides with a slightly modified amino acid sequence, for instance, a modified N-terminal end including N-terminal amino acid deletions or additions so long as those proteins substantially retain the activity of Factor VIIa (e.g., retain about 50%, 60%, 70%, 80%, 90%, 95%, 100%, etc. of the activity of native Factor VIIa). The term “Factor VIIa,” or “FVIIa” as used herein means a product consisting of the activated form (Factor VIIa), “Factor VII” or “Factor VIIa” within the above definition also includes natural allelic variations that may exist and occur from one individual to another. Also, degree, specific monosaccharide composition and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells, tissue, or animal species of expression and the nature of the host cell or tissue environment.

The term “domain” as used herein is intended to encompass a part of a protein sequence and structure that can evolve, function, and exist independently of the rest of the protein chain. A domain is capable of forming a compact three-dimensional structure and often can be independently stable and cooperatively folded. One domain may appear in a variety of evolutionarily related proteins. Domains can vary in length from between about 25 amino acids up to about 500 amino acids in length. A “domain” can also encompass a domain from a wild-type protein that has had an amino acid residue, or residues, replaced by conservative substitution. Because they are independently and cooperatively folded and self-stable in a protein milieu, domains can be “swapped” by genetic engineering between one protein and another to make chimeric proteins.

The term “variant” or “variants,” as used herein, is intended to designate Factor VII having the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in the protein and/or wherein one or more amino acids have been added to the parent. Such addition can take place either at the N-terminal
end or at the C-terminal end of the parent protein or both, as well as internally. The "variant" or "variants" within this definition still have FVII activity in their activated form. For example, the EGF-1 domain of FVII has 65.7% identity with the EGF-1 domain of FIX and the GLA domain of FVII has 58.6% identity with the GLA domain of FIX and 51% identity with the GLA domain of Protein S.

Thus, in some embodiments, a variant is at least 40%, 50%, 60%, 70%, 80%, 90% or 95% identical with the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa. In one embodiment a variant is at least 80% identical with the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa. In another embodiment a variant is at least 90% identical with the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa. In a further embodiment a variant is at least 95% identical with the amino acid sequence encompassing residues 61-466 (SEQ ID NO:18) of native human Factor VII or Factor VIIa.

The term "any other amino acid" as used herein means one amino acid that is different from that amino acid naturally present at that position. This includes but is not limited to amino acids that can be encoded by a polynucleotide. Preferably the different amino acid is in natural L-form and can be encoded by a polynucleotide. A specific example is L-cysteine (Cys). This also includes non-naturally occurring amino acids, such as synthetic or modified amino acids, as are well known in the art.

As used herein, the term "operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence of interest; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation into the protein or peptide of interest. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and in the case of a secretory leader, contiguous and in reading phase. Linking is most easily accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term "vector," as used herein, means any nucleic acid entity capable of amplification in a host cell. Thus, the vector may be an autonomously replicating vector, i.e., a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The choice of vector will often depend on the host cell into which it is to be introduced. Vectors include, but are not limited to plasmid vectors, phage vectors, viruses or cosmids vectors. Vectors usually contain a replication origin and at least one selectable gene, i.e., a gene which encodes a product which is readily detectable or the presence of which is essential for cell growth.

The term "host cell," as used herein, represents any cell, including hybrid cells, in which heterologous DNA can be expressed. Typical host cells include, but are not limited to insect cells, yeast cells, mammalian cells, including human cells, such as BHK, CHO, HEK, and COS cells. In practicing the present invention, the host cells being cultivated are preferably mammalian cells, more preferably an established mammalian cell line, including, without limitation, CHO (e.g., ATCC CCL 61), COS-1 (e.g., ATCC CRL 1650), baby hamster kidney (BHK) and HEK293 (e.g., ATCC CCL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A suitable BHK cell line is the tk- tk13 BHK cell line (Waeckerle and Baserga, Proc. Natl. Acad. Sci. USA 70:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110, under ATCC accession number CRL 10314. A tk-ts15 BHK cell line is also available from the ATCC under accession number CRL 1632. Other suitable cell lines include, without limitation, Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980). Also useful are 3T3 cells, Namalwa cells, myelomas and fusions of myelomas with other cells.

As used herein the term "appropriate growth medium" means a medium containing nutrients and other components required for the growth of cells and the expression of the nucleic acid sequence encoding the Factor VII polypeptide of the invention.

The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e., soluble in physiological fluids such as blood. Examples of conjugated polypeptides of the invention include glycosylated and/or PE-glylated polypeptides.

The term "covalent attachment" means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. The term "non-conjugated polypeptide" can be used to refer to the polypeptide part of the conjugate.

When used herein, the term "non-polypeptide moiety" means a molecule that is capable of conjugating to an attachment group of the polypeptide of the invention. Suitable examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents. When used in the context of a conjugate of the invention it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugate through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined...
to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties.

[0088] The “polymer molecule” is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term “polymer” can be used interchangeably with the term “polymer molecule.” The term is intended to encompass carbohydrate molecules attached by in vitro glycosylation, i.e., a synthetic glycosylation performed in vitro normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide, optionally using a cross-linking agent.

[0089] A carbohydrate molecule attached by in vivo glycosylation, such as N- or O-glycosylation (as further described below) is referred to herein as a “sugar moiety.” Except where the number of non-polypeptide moieties, such as polymer molecule(s) or sugar moieties in the conjugate is expressly indicated or referred to as “a non-polypeptide moiety” contained in a conjugate or otherwise used in the present invention shall be a reference to one or more non-polypeptide moieties, such as polymer molecule(s) or sugar moieties.

[0090] The term “attachment group” is intended to indicate a functional group of the polypeptide, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar moiety or an organic derivatizing agent.

[0091] For in vivo N-glycosylation, the term “attachment group” is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present.

[0092] Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by N-glycosylation, the term “amino acid residue comprising an attachment group for the non-polypeptide moiety” as used in connection with alterations of the amino acid sequence of the polypeptide of interest is to be understood as meaning that one or more amino acid residues constituting an N-glycosylation site are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

[0093] In the present context, the term “treat” or “treatment” is meant to include both control and/or prevention of an expected or unexpected bleeding, such as in surgery, and regulation of an already occurring bleeding, such as in trauma or in on demand treatment for hemophilia patients, with the purpose of inhibiting or minimizing the bleeding. Prophylactic administration of the chimeric or modified Factor VIIIa polypeptide according to the invention is thus included in the term “treatment.”

[0094] The term “bleeding episode” is meant to include uncontrolled and/or excessive bleeding. Bleeding episodes may be a problem or complication both in connection with surgery and other forms of tissue damage. Uncontrolled and/or excessive bleeding may occur in subjects having a normal coagulation system and subjects having coagulation or bleeding disorders.

[0095] Half-life customarily means when 1/2 of the currently present material has been removed from circulation. In view of the discoveries and inventive contributions disclosed herein, the amount of molecule in circulation is not of exclusive importance, rather, it is the activity present in the amounts localized in the extravascular space, bound to cell surfaces, bound to the basement membrane or present in the extracellular fluid. Thus, the clearance process is complex and does not follow a typical profile of removal of a molecule in circulation by the kidneys or liver or other cellular uptake mechanisms. Protection from bleeding or other aspects of the FVII bypass activity of functions is what is meant by half-life irrespective of the level or amount in circulation.

[0096] As used herein the term “bleeding disorder” includes any defect, congenital, acquired or induced, of cellular, physiological, or molecular origin that is manifested in bleeding or bleedings. Examples are clotting factor deficiencies (e.g., hemophilia A and B or deficiency of coagulation Factors XI or VII), clotting factor inhibitors, defective platelet function, thrombocytopenia, von Willebrand’s disease, or bleeding induced by surgery or trauma. This term is also intended to encompass a situation where the patient has normal levels of a clotting factor protein, but this protein is not functional due, for example, to neutralization by circulating antibodies (e.g., Factor VIII inhibitor antibodies) or because the patient may have been administered an overdose of warfarin (which could cause the endogenous clotting factor to be synthesized in a non-functional form), or cases where the person has normal levels of FVII in the blood but it has a genetic mutation that renders it dysfunctional. For example, there are several clinical conditions where a person bleeds because of a deficiency in the function of some clotting factor in the blood but not necessarily a deficiency in the amount of the actual dysfunctional clotting factor.

[0097] Excessive bleeding also occurs in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors) and may be caused by a defective platelet function, thrombocytopenia or von Willebrand’s disease. In such cases, the bleedings may be likened to those bleedings caused by hemophilia because the haemostatic system, as in hemophilia, lacks or has abnormal essential clotting “compounds” (such as platelets or von Willebrand factor protein), causing major bleedings. In subjects who experience extensive tissue damage in association with surgery or trauma, the normal haemostatic mechanism may be overwhelmed by the demand of immediate hemostasis and they may develop bleeding in spite of a normal haemostatic mechanism. Achieving satisfactory hemostasis also is a problem when bleedings occur in organs such as the brain, inner ear region and eyes, with limited possibility for surgical hemostasis. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumor tissue, gastrointestinal tract) as well as in laparoscopic surgery. Common for all these situations is the difficulty to provide hemostasis by surgical techniques (sutures, clips, etc.), which also is the case when bleeding is diffuse (hemorrhagic gastric and profuse uterine bleeding). Acute profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective hemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to
be counteracted rapidly. Radical retropubic prostatectomy is a commonly performed procedure for subjects with localized prostate cancer. The operation is frequently complicated by significant and sometimes massive blood loss. The considerable blood loss during prostatectomy is mainly related to the complicated anatomical situation, with various densely vascularized sites that are not easily accessible for surgical hemostasis, and which may result in diffuse bleeding from a large area. Also, intracerebral hemorrhage is the least treatable form of stroke and is associated with high mortality and hematoma growth in the first few hours following intracerebral hemorrhage. Treatment with rFVIIa can limit the growth of the hematoma, reduce mortality, and improve functional outcomes at 90 days. With currently available rFVIIa, however, there is a risk of thromboembolic adverse events. The chimeric Factor VII molecules of the present invention, with lower thrombogenicity, can overcome this problem in stroke therapy. Another situation that may cause problems in the case of unsatisfactory hemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

[0098] In one embodiment of the invention, the bleeding is associated with hemophilia. In another embodiment, the bleeding is associated with hemophilia with acquired inhibitors. In another embodiment, the bleeding is associated with thrombocytopenia. In another embodiment, the bleeding is associated with von Willebrand’s disease. In another embodiment, the bleeding is associated with severe tissue damage. In another embodiment, the bleeding is associated with severe trauma. In another embodiment, the bleeding is associated with surgery. In another embodiment, the bleeding is associated with laparoscopic surgery. In another embodiment, the bleeding is associated with hemorrhagic gastritis. In another embodiment, the bleeding is profuse uterine bleeding. In another embodiment, the bleeding is occurring in organs with a limited possibility for mechanical hemostasis. In another embodiment, the bleeding is occurring in the brain, inner ear region or eyes. In another embodiment, the bleeding is associated with the process of taking biopsies. In another embodiment, the bleeding is associated with anticoagulant therapy.

[0099] The term “subject” as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term “patient.” A subject “in need thereof” is a subject for whom the administration of the chimeric FVII or FVIIa polypeptides and/or modified Factor VII or Factor VIIa polypeptides and/or nucleic acid molecules encoding the chimeric FVII or FVIIa polypeptides and/or modified Factor VII or Factor VIIa polypeptides of this invention is beneficial, i.e., imparts a beneficial or therapeutic effect. Such a subject in need thereof can be a subject having a bleeding disorder, a subject suspected of having a bleeding disorder or a subject not having a bleeding disorder.

[0100] The term “enhancement of the normal haemostatic system” means an improvement of the ability to generate thrombin or to generate a functional clot.

[0101] The term “prolonged protective hemostatic effect” means an increase in the time a molecule remains in the body of a subject, even if not in the circulation, and remains functional in protecting against bleeding, including preventing new bleeds. The term can also mean that a molecule that is functionally different in protecting against bleeding—including diminishing the number or extent of new bleeding episodes—can be administered in lower doses, the dosing regimen can be less frequent, there is an increased interval between doses and/or bleeding can be prevented for a longer period of time following cessation of pharmaceutical administration.

[0102] The term “functional lifetime” or “functional lifespan” means the length of time that a protein is functionally active in the body (i.e., in vivo), regardless of where the protein is located. Examples of the functionality of the protein may include protease activity, the ability to protect against bleeds, prevent new bleeds, and/or bind to cell surfaces. And/or protease activity for a longer period than presently available FVIIa preparations.

[0103] The term “functional half-life” means the amount of time that is required for the loss of half of the function of the protein in vivo or in vitro.

[0104] The term “clearance rate” means the rate at which a coagulation protein or other protein is removed from the body, including from the extravascular space or the rate at which the activity of a coagulation protein is lost from the body, including the extravascular space.

[0105] The term “gene therapy” refers to a method of changing the expression of an endogenous gene by exogenous administration of a gene. As used herein, “gene therapy” also refers to the replacement of a defective gene encoding a defective protein, or replacement of a missing gene, by introducing a functional gene corresponding to the defective or missing gene into somatic or stem cells of an individual in need. Gene therapy can be accomplished by ex vivo methods, in which differentiated or somatic stem cells are removed from the individual’s body followed by the introduction of a normal copy of the defective gene into the explanted cells using, e.g., a viral vector as the gene delivery vehicle. In addition, in vivo direct gene transfer technologies allow for gene transfer into cells in the individual in situ using a broad range of viral vectors, liposomes, protein DNA complexes and/or naked DNA in order to achieve a therapeutic outcome. The term “gene therapy” also refers to the replacement of a defective gene encoding a defective protein by introducing a polynucleotide that functions substantially the same as the defective gene or protein should function if it were not defective into somatic or stem cells of an individual in need.

[0106] In the present invention, gene therapy may be employed in the context of administering to a subject a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric FVII of this invention. Thus, the present invention can utilize an isolated nucleic acid molecule comprising a nucleotide sequence encoding a chimeric FVII protein of this invention. Such a nucleic acid molecule can be present in a nucleic acid construct (e.g., a vector or plasmid). Such a nucleic acid construct can be present in a cell. Further provided herein is a method of delivering a chimeric FVII protein of this invention to a cell, comprising introducing into the cell a nucleic acid molecule comprising a nucleotide sequence encoding the chimeric FVII protein under conditions whereby the nucleotide sequence is expressed to produce the chimeric FVII protein in the cell. The cell can be a cell that is introduced into a subject and/or the cell can be a cell already present in the subject.

Preparation of Chimeric Factor VII

[0107] The chimeric Factor VII polypeptides described herein may be produced by means of recombinant nucleic
acid techniques. In general, a cloned wild-type Factor VII nucleic acid sequence is modified to encode the desired protein. This modified sequence is then inserted into an expression vector, which is in turn transformed or transfected into host cells. Higher eukaryotic cells, in particular cultured mammalian cells, are suitable as host cells. The complete nucleotide and amino acid sequences for human Factor VII are known (see U.S. Pat. No. 4,784,950, where the cloning and expression of recombinant human Factor VII is described, and GenBank® Accession Nos. J00293 and AAS51983 and SwissProt Accession No. P08700-1) and the amino acid sequence of the precursor form is provided herein as SEQ ID NO:18. The hovine Factor VII sequence is described in Takeya et al., J. Biol. Chem. 263:14868-14872 (1988)). The complete nucleotide and amino acid sequences for Factor IX are known (see Davie et al., Proc. Natl. Acad. Sci. U.S.A. 1982; 79:6461-6464; Jaye et al., Nucleic Acids Res. 1983; 11:2325-2335; and, McGraw et al., Proc. Natl. Acad. Sci. U.S.A. 1985; 82:2847-2851 and GenBank® Accession Nos. J00136 and AAC98726 and SwissProt Accession No. P00740) and the amino acid sequence of the precursor form is provided herein as SEQ ID NO:19; as are the complete nucleotide and amino acid sequences for Protein S (see Hoskins et al., Proc. Natl. Acad. Sci. U.S.A. 1987; 84:349-353 and GenBank® Accession Nos. M15036 and AAX6479 and SwissProt Accession No. P07225) and the amino acid sequence of the precursor form is provided herein as SEQ ID NO:20. The complete nucleotide and amino acid sequences for other coagulation proteins are also known and can be viewed using SwissProt or at the NCBI web page.

[0110] The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the nucleic acid sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described in, for example, Zoller and Smith (DNA 3:479-488, 1984) or Horton et al. (“Splicing by extension overlap” Gene 77:61-68, 1989). Thus, using the nucleotide and amino acid sequences of Factor VII, one may introduce the alteration(s) of choice. Likewise, procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (see, PCR Protocols, 1990, Academic Press, San Diego, Calif., USA). Additionally, the chimeric Factor VII polypeptides useful in the methods of the present invention may be preparued by introducing unique restriction sites into the nucleotide sequences encoding the various polypeptides, which can be used to isolate fragments of nucleotide sequences that encode entire domains of various polypeptides. Alternatively, the chimeric Factor VII polypeptides useful in the methods of the present invention may be made by constructing a complete nucleotide sequence to encode the entire chimeric polypeptide based on the known nucleotide sequences of the domains of various polypeptides. Such complete nucleotide sequences can be optimized for expression in a system of choice and are available from companies such as Blue Heron Biotechnology, Bothell, Wash. Subsequently, these various nucleotide sequences can be recombined to produce the various chimeric Factor VII polypeptides useful in the methods of the present invention.

[0119] For example, unique restriction sites may be introduced into the nucleotide sequences encoding various coagulation proteins so that the GLA, EGF-1, EGF-2 and catalytic domains may be freely interchanged between the various coagulation proteins. The EGF-1 domain can rotate freely around the EGF-2 domain, (Pike et al. Proc. Natl. Acad. Sci. 1999; 96:8925-8930) whereas, the GLA and EGF-1 domains may not rotate freely around each other when calcium is present (Sunnerhagen et al. Nature Structural Biology 1995; 2:504-509). In this embodiment, the GLA and EGF-1 domains may be from the same coagulation protein, but from a different protein than the EGF-2 and catalytic domains. The chimeric Factor VIIa polypeptides useful in the methods of the present invention, may therefore, comprise domains from any of the coagulation proteins, provided at least 50% of one domain is a Factor VII domain. Further, the chimeric Factor VIIa polypeptides of the present invention may include GLA and EGF-1 domains from the same protein, or from different proteins.

[0111] The nucleic acid construct encoding the chimeric Factor VII polypeptides may also be prepared synthetically by established standard methods, e.g., the phosphoamidite method described by Beaucage and Caruthers. Tetrahedron Letters 22 (1981), 1859-1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

[0112] Furthermore, the nucleic acid construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleic acid construct, in accordance with standard techniques.

[0113] DNA sequences for use in producing chimeric Factor VII polypeptides useful in the methods of the present invention will typically encode a pre-pro polypeptide at the amino-terminus of Factor VII to obtain proper posttranslational processing (e.g., gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro polypeptide may be that of Factor VII or another vitamin K-dependent plasma protein, such as Factor IX, Factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can be made in the amino acid sequence of the chimeric Factor VII polypeptides where those modifications do not significantly impair the ability of the protein to act as a coagulant.

[0114] Expression vectors for use in expressing Factor VIIa variants will comprise a promoter capable of directing the transcription of a cloned gene or cDNA. Suitable promoters for use in cultured mammalian cells include viral promoters and cellular promoters. Viral promoters include the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981) and the CMV promoter (Boshart et al., Cell 41:521-530, 1985). A particularly suitable viral promoter is the major late promoter from adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982). Another particularly preferable promoter is the Chinese Hamster elongation factor-1-alpha
(CHEF1) promoter. Cellular promoters include the mouse kappa gene promoter (Bergman et al. Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983) and the mouse V_γ promoter (Loh et al. Cell 33:85-93, 1983). A particularly suitable cellular promoter is the mouse metallothionein-I promoter (Palminteri et al. Science 222:809-814, 1983). Expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the chimeric Factor VII sequence itself. Suitable RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly suitable polyadenylation signals include the early or late polyadenylation signal from SV-40 (Kaufman and Sharp), the polyadenylation signal from the adenovirus 5 E1b region, the human growth hormone gene terminator (DeNoto et al. Nucl. Acids Res. 9:3719-3730, 1981) or the polyadenylation signal from the human Factor VII gene or the bovine Factor VII gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

[0015] Cloned DNA sequences are introduced into cultivated mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725-732, 1978; Carso and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and Van der Eb, Virology 52:456-467, 1973) or electroporation (Neumann et al., EMBO J. 1:841-845, 1982). To identify and select cells that express the exogenous DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into cells along with the gene of cDNA of interest. Suitable selectable markers include genes that confer resistance to drugs such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amphiplifiable selectable marker. A suitable amplifiable selectable marker is a dihydrofolate reductase (DHFR) sequence. Another suitable selectable marker is histidinol. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass., incorporated herein by reference). The person skilled in the art will easily be able to choose suitable selectable markers.

[0016] Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Pat. No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

[0017] After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically for 1-2 days, to begin expressing the gene of interest. The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., in catalogues of the American Type Culture Collection). The media are prepared using procedures known in the art (see, e.g., references for bacteria and yeast; Bennett, J. W. and LaSure, L., editors, More Gene Manipulations in Fungi, Academic Press, CA, 1991). Growth media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, proteins and growth factors. For production of gamma-carboxylated chimeric Factor VII polypeptides, the medium will contain vitamin K, preferably at a concentration of about 0.05 ng/ml to about 100 μg/ml, which can vary dependent upon the uptake or sequestering of vitamin K by the cells. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of the desired chimeric Factor VII polypeptide.

[0018] Suitable mammalian cell lines include the CHO (ATCC CCL 61), COS-1 (ATCC CRL 1650), baby hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines or derivative cell lines thereof. A suitable BHK cell line is the tk-ap-s-tsl3 BHK cell line (Waeckert and Baserga, Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982), hereinafter referred to as BHK 570 cells. The BHK 570 cell line is available from the American Type Culture Collection, 12501 Parklawn Dr., Manassas, Va. 20852, under ATCC accession number CRL 10314. A tk- BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980).

[0019] The chimeric Factor VII polypeptides useful in the methods of the invention may be conjugated to prolong the half-life of the polypeptide in vivo. Such conjugation may decrease renal clearance and increase the amount of Factor VII present in vivo, while decreasing the frequency of administration of the chimeric Factor VII polypeptides. Suitable conjugates and methods of preparing conjugated Factor VII polypeptides are described in U.S. Pat. No. 7,442,524, which is hereby incorporated by reference in its entirety.

**Transgenic Animals**

[0020] Transgenic animal technology may be employed to produce the chimeric Factor VII polypeptides useful in the methods of the invention. It is preferred to produce the proteins within the mammary glands of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and biochemically well characterized. Furthermore, the major milk proteins are present in milk at high concentrations (typically from about 1 to 15 g/l). The chimeric Factor VII polypeptides useful in the methods of the present invention may also be produced in the urine of a host animal, which has the advantage over production in milk in that both male and female animals produce urine and that there are less proteins in urine than in milk for isolation purposes.

[0021] From a commercial point of view when considering use of milk as a source of the chimeric Factor VII polypeptides useful in the methods of the present invention, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof of principle stage), it is
preferred to use livestock mammals including, but not limited to, pigs, goats, sheep and cattle. Sheep are particularly
suitable due to such factors as the previous history of transgenesis in this species, milk yield, cost and the ready availability of
equipment for collecting sheep milk (see, for example, PCT Publication No. WO 88/00239 for a comparison of factors
influencing the choice of host species). It is generally desir-
able to select a breed of host animal that has been bred for
dairy use, such as East Friesland sheep, or to introduce duty
stock by breeding of the transgenic line at a later date. In any
event, animals of known, good health status should be used.

[0122] To obtain expression in the mammary gland, a trans-
scription promoter from a milk protein gene is used. Milk
protein genes include those genes encoding caseins (see U.S.
Pat. No. 5,304,489), beta-lactoglobulin, alpha-lactalbumin,
and whey acidic protein. The beta-lactoglobulin (BLG) promoter
is suitable. In the case of the ovine beta-lactoglobulin gene,
a region of at least the proximal 406 bp of 5' flanking sequence
of the gene will generally be used, although larger portions
of the 5' flanking sequence, up to about 5 kbp, are suitable,
as about 4.25 kbp DNA segment encompassing the 5' flanking
promoter and non-coding portion of the beta-lactoglobulin
gene (see Whitelaw et al., Biochem. J. 286: 31-39 (1992)).
Similar fragments of promoter DNA from other species are
also suitable.

[0123] To obtain expression in urine, a urothelium-specific
promoter is used. For example, a promoter that drives expres-
sion of uroplakin-related genes can be used. (See, e.g., U.S.
Pat. No. 5,601,646, which is incorporated by reference herein
in its entirety).

[0124] Other regions of the beta-lactoglobulin gene may
also be incorporated in constructs, as may genomic regions
of the gene to be expressed. It is generally accepted in the art
that constructs lacking introns, for example, express poorly
in comparison with those that contain such DNA sequences (see
(1988); Palmiter et al., Proc. Natl. Acad. Sci. USA 88: 478-
482 (1991); Whitelaw et al., Transgenic Res. 1: 3-13 (1991);
PCT Publication No. WO 89/01343; and PCT Publication
No. WO 91/02318, each of which is incorporated herein by
reference). In this regard, it is generally preferred, where
possible, to use genomic sequences containing all or some of
the native introns of a gene encoding the protein or polypep-
dide of interest, thus the further inclusion of at least some
introns from, e.g., the beta-lactoglobulin gene, is preferred.
One such region is a DNA segment that provides for intron
splicing and RNA polyadenylation from the 3' non-coding
region of the ovine beta-lactoglobulin gene. When substitut-
ed for the natural 3' non-coding sequences of a gene, this ovine
beta-lactoglobulin segment can both enhance and stabilize
expression levels of the protein or polypeptide of interest.
Within other embodiments, the region surrounding the initia-
tion ATG of the chimeric Factor VIIa sequence is replaced with
corresponding sequences from a milk specific protein gene.
Such replacement provides a putative tissue-specific initia-
tion environment to enhance expression. It is convenient to
replace the entire chimeric Factor VII pre-pro and 5' non-
coding sequences with those of, for example, the BLG gene,
although smaller regions may be replaced.

[0125] For expression of chimeric FVIIa polypeptides in
transgenic animals, a DNA segment encoding chimeric Fac-
tor VIIa is operably linked to additional DNA segments
required for its expression to produce expression units. Such
additional segments include the above-mentioned promoter,
as well as sequences that provide for termination of transcrip-
tion and polyadenylation of mRNA. The expression units will
further include a DNA segment encoding a secretory signal
sequence operably linked to the segment encoding the chimer-
ic Factor VIIa. The secretory signal sequence may be a
native Factor VII secretory signal sequence or may be that of
another protein, such as a milk protein (see, for example, von
Heijne, Nucl. Acids Res. 14: 4683-4690 (1986); and Meade et
al., U.S. Pat. No. 4,873,316, which are incorporated herein by
reference).

[0126] Construction of expression units for use in trans-
genic animals is conveniently carried out by inserting a chimir-
ic Factor VII sequence into a plasmid or phage vector
containing the additional DNA segments, although the
expression unit may be constructed by essentially any
sequence of ligations. It is particularly convenient to provide
a vector containing a DNA segment encoding a milk protein
and to replace the coding sequence for the milk protein with
that of a chimeric Factor VII polypeptide whereby creating a
gene fusion that includes the expression control sequences of
the milk protein gene. In any event, cloning of the expression
units in plasmids or other vectors facilitates the amplification
of the chimeric Factor VII sequence. Amplification is conve-
niently carried out in bacterial (e.g., E. coli) host cells, thus
the vectors will typically include an origin of replication and a
selectable marker functional in bacterial host cells. The
expression unit is then introduced into fertilized eggs (includ-
ing early-stage embryos) of the chosen host species. Intro-
duction of heterologous DNA can be accomplished by one
of several routes, including microinjection (e.g., U.S. Pat.
No. 4,873,191), retroviral infection (Jaenisch, Science 240:
1468-1474 (1988)) or site-directed integration using embryonic
stem (ES) cells (reviewed by Bradley et al., Bio/Technology
10: 534-539 (1992)). The eggs are then implanted into the
oviducts or uteri of pseudopregnant females and allowed to
develop to term. Offspring carrying the introduced DNA in
their germ line can pass the DNA on to their progeny in the
normal, Mendelian fashion, allowing the development of
transgenic herds. General procedures for producing trans-
genic animals are known in the art (see, for example, Hogan et
al., Manipulating the Mouse Embryo: A Laboratory
Manual, Cold Spring Harbor Laboratory, 1986; Simons et al.,
32: 645-651 (1985); Buhler et al., Bio/Technology 8: 140-143
(1990); Ebert et al., Bio/Technology 9: 835-838 (1991); Krin-
penfort et al., Bio/Technology 9: 844-847 (1991); Wall et
191; U.S. Pat. No. 4,873,316; PCT Publication No. WO
88/00239, PCT Publication No. WO 90/05188; PCT Publi-
cation No. WO 92/11757; and Great Britain Publication No.
GB 87/00458). Techniques for introducing foreign DNA
sequences into mammals and their germ cells were originally
developed in the mouse (see, e.g., Gordon et al., Proc. Natl.
Acad. Sci. USA 77: 7380-7384 (1980); Gordon and Ruddle,
Science 214: 1244-1246 (1981); Palmiter and Brinster, Cell
USA 82: 4438-4442 (1985); and Hogan et al.). These
techniques were subsequently adapted for use with larger ani-
mals, including livestock species (see, e.g., PCT Publi-
cation Nos. WO 88/00239, WO 90/05188, and WO 92/11757;
and Simons et al., Bio/Technology 6: 179-183 (1988)). To
summarize, in the most efficient route used to date in the gen-
eration of transgenic mice or livestock, several hundred linear
molecules of the DNA of interest are injected into one of the

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injection of DNA into the cytoplasm of a zygote can also be employed.

Production in transgenic plants may also be employed. Expression may be generalized or directed to a particular organ, such as a tuber (see, e.g., Holm, *Nature* 344:469-479 (1990); Edelbaum et al., *J. Interferon Res.* 12:449-453 (1992); Sijmons et al., *Bio/Technology* 8:217-221 (1990); and EIB 255:378).

Production of the chimeric Factor VII polypeptide useful in the methods of the present invention may also be achieved through in vitro translation, such as for example by rabbit reticulocyte lysate, wheat germ extract and *E. coli* cell free systems. The in vitro translation may also be linked or coupled. Linked in vitro translation is based on transcription with a bacteriophage polymerase followed by translation in the rabbit reticulocyte lysate or wheat germ lysate systems. Coupled in vitro translation is based upon the *E. coli* cell free system.

Recovery and Activation

The chimeric Factor VII polypeptides useful in the methods of the invention are recovered from cell culture medium or the milk, urine and/or plasma of a transgenic animal. The chimeric Factor VII polypeptides useful in the methods of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, pseudo-affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCII Publishers, New York, 1989). Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including precipitation by barium salts, are known in the art, and may be applied to the purification of the chimeric Factor VII polypeptides described herein (see, for example, Scopes, R., *Protein Purification*, Springer-Verlag, N.Y., 1982).

For therapeutic purposes it is preferred that the chimeric Factor VII polypeptides of the invention are substantially pure. Thus, in one embodiment of the invention the Factor VII variants of the invention are purified to at least about 90 to 95% purity, preferably to at least about 98% purity. Purity may be assessed by, e.g., gel electrophoresis, amino-terminal amino acid sequencing and reverse-phase HPLC.

The chimeric Factor VII polypeptide is cleaved at its activation site in order to convert it to its two-chain form. Activation may be carried out according to procedures known in the art, such as those disclosed by Osterud et al., *Biochemistry* 11:2853-2857 (1972); Thomas, U.S. Pat. No. 4,456,591; Hedner and Kisiel, *J. Clin. Invest.* 71:1836-1841 (1983); or Kisiel and Fujikawa, *Behring Inst. Mitt.* 73:29-42 (1983). Alternatively, the chimeric Factor VII polypeptide may be activated by concentrating the chimeric Factor VII polypeptide and contacting a positively charged surface or resin, for example, as described by Bjorn et al. (*Research Disclosure*, 269 September 1986, pp. 564-565). Factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q® (Pharmacia fine Chemicals) or the like. The chimeric Factor VII polypeptide may also be activated in solution by obtaining a solution comprising a substantially purified preparation of the chimeric Factor VII polypeptide; adding to the solution an amine compound, Ca²⁺ to a final concentration of about 5 mMM to about 50 mMM (such as about 10 mMM to about 30 mMM), and adjusting the final pH of the solution to about 7.2 to 8.6 (such as about 7.6 to about 8.2); incubating the resulting activation mixture at between about 26°C and about 25°C for an amount of time sufficient to convert at least 90% of the chimeric FVIIa polypeptide to chimeric FVIIa polypeptide; and, optionally, isolating the FVIIa from the activation mixture. The chimeric Factor VII polypeptide may also be activated by increasing the concentration of Factor VII in solution so that the Factor VII autoactivates. This typically occurs at concentrations greater than 5 mg/ml. Alternatively it can be activated in the cell while being synthesized. The resulting activated chimeric Factor VII polypeptide may then be formulated and administered as described below. Alternatively, the Factor VII polypeptide can be administered in the inactive form by gene therapy vectors and become activated intracellularly. Such intracellular activation may be accomplished by including an RKKRRKR cleavage motif at the activation site, as disclosed in (Margariths et al. *J. Clin. Invest* 2004 113:1025-31), which is incorporated by reference herein in its entirety, and Example V below. Thus the material can be activated by any known method including polyamines, polycations, high concentrations of the molecule, concentrations on a cell or tissue surface, or through gene therapy.

Gene Therapy

The chimeric Factor VII polypeptides useful in the methods of the invention may also be used in methods of treating bleeding disorders by way of gene therapy. In this embodiment of the invention, the chimeric Factor VII polypeptides useful in the methods of the invention are encoded by nucleic acid molecules that may be introduced into cells of the subject by ex vivo transfer or by in vivo transfer.


The polynucleotides encoding the chimeric Factor VII polypeptides can be inserted into an appropriate cloning vector. Vectors suitable for gene therapy include viruses, such as adenoviruses, adeno-associated virus (AAV), vaccinia, herpesviruses, baculoviruses and retroviruses, parvovirus, lentivirus, bacteriophages, cosmids, plasmids, fungal vectors and other recombinant vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.
In one embodiment, the vector is a viral vector. Viral vectors, especially adenoviral vectors can be complexed with a cationic amphiphile, such as a cationic lipid, poly-L-lysine (PLL), and diethylaminoethyl-dextran (DELAE-dextran), which provide increased efficiency of viral infection of target cells (see, e.g., PCT Publication No. PCT/US97/21496 filed Nov. 20, 1997, incorporated herein by reference). Suitable viral vectors for use in the present invention include vectors derived from vaccinia, herpesvirus, AAV and retroviruses. For a review of viral vectors in gene therapy, see Mah et al., Clin. Pharmacokinet. 2002; 41(12):901-11; Scott et al., Neuromuscular. Disord. 2002; 12 Suppl 1:S23-9.

In one embodiment, a vector is used in which the coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for expression of the construct from a nucleic acid molecule that has integrated into the genome (Koller and Smithies, Proc. Natl. Acad. Sci. USA 1989, 86:8932-8935; Zigler et al., Nature 1989, 342: 435-438; U.S. Pat. No. 6,244,113 to Zurlang et al.; and U.S. Pat. No. 6,200,812 to Pati et al., each of which is incorporated herein in its entirety by reference).

Delivery of the vector into a patient may be either direct, in which case the patient is directly exposed to the vector or a delivery complex, or indirect, in which case, cells are first transformed with the vector in vitro, and then transplanted into the patient. These two approaches are known, respectively, as in vivo and ex vivo gene therapy.

In one embodiment, the vector is directly administered in vivo, where it enters the cells of the subject and mediates expression of the gene. This can be accomplished by any of numerous methods known in the art and discussed above, e.g., by constructing it as part of an appropriate expression vector and administering it so that it becomes intracellular, e.g., by infection using a defective or attenuated retroviral or other viral vector (see, U.S. Pat. No. 4,980,286, or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, DuPont); or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in biopolymers (e.g., poly-beta-1-6-N-acetylglucosamine polysaccharide; see U.S. Pat. No. 5,635,493), encapsulation in liposomes, microparticles, or microcapsules; by administering it in linkage to a peptide or other ligand known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem. 1987 62:4429-4432), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusing viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation, or cationic 12-mer peptides, e.g., derived from antennapedia, that can be used to transfer therapeutic DNA into cells (Mi et al., Mol. Therapy. 2000 2:339-47). In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publication Nos. WO 92/06180, WO 92/22635, WO 92/20316 and WO 93/14188). Additionally, a technique referred to as magnetofection may be used to deliver vectors to mammals. This technique associates the vectors with superparamagnetic nanoparticles for delivery under the influence of magnetic fields. This application reduces the delivery time and enhances vector efficacy (Scherer et al., Gene Therapy 2002 9:102-9).

In one embodiment, the nucleic acid can be administered using a lipid carrier. Lipid carriers can be associated with naked nucleic acids (e.g., plasmid DNA) to facilitate passage through cellular membranes. Cationic, anionic, or neutral lipids can be used for this purpose. However, cationic lipids are suitable because they have been shown to associate better with DNA which, generally, has a negative charge. Cationic lipids have also been shown to mediate intracellular delivery of plasmid DNA (Feinberg and Ringold, Nature 1989; 337:387). Intravenous injection of cationic lipid-plasmid complexes into mice has been shown to result in expression of the DNA in lung (Braithwaite et al., Am. J. Med. Sci. 1989; 298:278). See also, Osaka et al., J. Pharm. Sci. 1996; 85(6): 612-618; San et al., Human Gene Therapy 1993; 4:781-788; Senior et al., Biochimica et Biophysica Acta 1991; 1070:173-179; Kabanov and Kabanov, Bioconjugate Chem. 1995; 6:7-20; Liu et al., Pharmaceut. Res. 1996; 13; Remy et al., Bioconjugate Chem. 1994; 5:647-654; Behr, J-P., Bioconjugate Chem. 1994; 5:382-389; Wyman et al., Biochem. 1997; 36:3008-3017; U.S. Pat. No. 5,939,401 to Marshall et al.; U.S. Pat. No. 6,331,524 to Scheule et al. Representative cationic lipids include those disclosed, for example, in U.S. Pat. No. 5,283,185, and e.g., U.S. Pat. No. 5,767,099, the disclosures of which are incorporated herein by reference. In one embodiment, the cationic lipid is N₂-spermine cholesterol carbamate (GL-67) disclosed in U.S. Pat. No. 5,767,099. Additional suitable lipids include N₂-spermidine cholesterol carbamate (GL-53) and 1-(N₂-spermine)-2,3-dilaurglycerol carbamate (GL-89).

For in vivo administration of viral vectors, an appropriate immunosuppressive treatment can be employed in conjunction with the viral vector, e.g., adenovirus vector, to avoid immuno-deactivation of the viral vector and transplanted cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-gamma (IFN-gamma), or anti-C4D antibody, can be administered to block humoral or cellular immune responses to the viral vectors. In that regard, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

Somatic cells may be engineered ex vivo with a construct encoding a chimeric Factor VII polypeptide of the invention using any of the methods described above, and re-implanted into an individual. This method is described generally in PCT Publication No. WO 93/09222 to Seldén et al. In addition, this technology is used in Cell Based Delivery’s proprietary IMPACT technology, described in Payumo et al., Clin. Orthopaed. and Related Res. 2002; 405S: S228-S242. In such a gene therapy system, somatic cells (e.g., fibroblasts, hepatocytes, or endothelial cells) are removed from the patient, cultured in vitro, transfected with the gene (s) of therapeutic interest, characterized, and reintroduced into the patient. Both primary cells (derived from an individual or tissue and engineered prior to passing), and secondary cells (passed in vitro prior to introduction in vivo) can be used, as well as immortalized cell lines known in the art. Somatic cells useful for the methods of the present invention include but are not limited to somatic cells, such as fibroblasts, keratinocytes, epithelial cells, endothelial cells, hepatocytes, formed elements of the blood, muscle cells, other somatic cells that can be cultured, and somatic cell precursors. In one embodiment, the cells are hepatocytes.

Constructs that include the polynucleotide encoding the chimeric FVIIa polypeptides useful in the methods of the invention and, optionally, nucleic acids encoding a selectable
marker, along with additional sequences necessary for expression of the chimeric FVIIa in recipient primary or secondary cells, are used to transfect primary or secondary cells in which the encoded product is to be produced. Such constructs include but are not limited to infectious vectors, such as retroviral, herpes, adenovirus, adeno-associated virus, mumps and poliovirus vectors, can be used for this purpose.

[0143] Transdermal delivery is especially suited for indirect transfer using cell types of the epidermis including keratinocytes, melanocytes, and dendritic cells (Pfitzner et al., Expert Opin. Investig. Drugs 2000; 9:2069-83).

[0144] In one embodiment, the vector for gene therapy is a single-stranded self-complementary adeno-associated virus. Additionally, this self-complementary adeno-associated virus may contain the small transshretin promoter-enhancer, introns from the mouse minute virus and the polyadenylation signal from bovine growth hormone.

[0145] The vector may also contain the RRRKR sequence described by Margaritis et al. that causes the Factor VII to be secreted in the active form, i.e., Factor VIIa. (Margaritis et al., J. Clin. Invest. 2004; 113:1025-31).

Administration and Pharmaceutical Compositions

[0146] The chimeric Factor VII polypeptides useful in the methods of the present invention may be used to control bleeding disorders which have several causes such as clotting factor deficiencies (e.g., hemophilia A and B or deficiency of coagulation factors X or VII) or clotting factor inhibitors, or they may be used to control excessive bleeding occurring in subjects with a normally functioning blood clotting cascade (no clotting factor deficiencies or inhibitors against any of the coagulation factors). The bleedings may be caused by a defective platelet function, thrombocytopenia or von Willebrand’s disease. They may also be seen in subjects in whom an increased fibrinolytic activity has been induced by various stimuli.

[0147] In subjects who experience extensive tissue damage in association with surgery or trauma, the haemostatic mechanism may be overwhelmed by the demand of immediate hemostasis and they may develop bleedings in spite of a normal haemostatic mechanism. Achieving satisfactory hemostasis is also a problem when bleedings occur in organs such as the brain, inner ear region and eyes and may also be a problem in cases of diffuse bleedings (hemorrhagic gastritis and profuse uterine bleeding) when it is difficult to identify the source. The same problem may arise in the process of taking biopsies from various organs (liver, lung, tumor tissue, gastrointestinal tract) as well as in laparoscopic surgery. These situations share the difficulty of providing hemostasis by surgical techniques (stitches, clips, etc.). Adequate and profuse bleedings may also occur in subjects on anticoagulant therapy in whom a defective hemostasis has been induced by the therapy given. Such subjects may need surgical interventions in case the anticoagulant effect has to be counteracted rapidly. Another situation that may cause problems in the case of unsatisfactory hemostasis is when subjects with a normal haemostatic mechanism are given anticoagulant therapy to prevent thromboembolic disease. Such therapy may include heparin, other forms of proteoglycans, warfarin or other forms of vitamin K-antagonists as well as aspirin and other platelet aggregation inhibitors.

[0148] For treatment in connection with deliberate interventions, the chimeric Factor VII polypeptides useful in the methods of the invention will typically be administered up to about 3 days or within about 24 hours prior to performing the intervention, and for periods of about 3, 7, or 14 days or more thereafter. Administration as a coagulant can be by a variety of routes as described herein.

[0149] The dose of the chimeric Factor VII or Factor VIIa polypeptides ranges from about 0.05 mg/day to about 500 mg/day, preferably from about 1 mg/day to about 200 mg/day, and more preferably from about 10 mg/day to about 175 mg/day for a 70 kg subject as loading and maintenance doses, depending on the weight of the subject and the severity of the condition. Alternatively this could be calculated as 90 mg/kg to 270 mg/kg of the patient. A dosage range for the administration to a subject of a nucleic acid molecule comprising a nucleotide sequence encoding a chimeric Factor VIIa polypeptide, chimeric Factor VII polypeptide, modified Factor VIIa polypeptide or modified Factor VII polypeptide in the form of a viral vector can be, for example, from about 5x10^12 viral genomes per kg to about 5x10^15 viral genomes per kg. One of skill in the art would be able to determine the optimal dose for a given subject and a given condition.

[0150] The pharmaceutical compositions are primarily intended for parenteral administration for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly, or it may be administered by continuous or pulsatile infusion. Alternatively, the pharmaceutical compositions may be formulated for administration in various ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intrasally, transdermally, intraperitoneally, intramuscularly, intrapulmonally, vaginally, rectally, intracutorally, or in any other acceptable manner.

[0151] The compositions for parenteral administration comprise the chimeric Factor VII polypeptide useful in the methods of the invention in combination with, preferably dissolved in, a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, such as water, buffered water, 0.4% saline, 0.3% glycine and the like. The chimeric Factor VII polypeptides useful in the methods of the invention may also be formulated with compositions that prolong stability and storage, such as methionine and sucrose. The chimeric Factor VII polypeptides useful in the methods of the invention can also be formulated into liposome preparations for delivery or targeting to the sites of injury. Liposome preparations are generally described in, e.g., U.S. Pat. Nos. 4,837,028, 4,501,728, and 4,975,282. The compositions may be sterilized by conventional, well-known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The compositions may also contain preservatives, isotonicifiers, non-ionic surfactants or detergents, antioxidants and/or other miscellaneous additives.

[0152] The concentration of chimeric Factor VII polypeptide in these formulations can vary widely, i.e., from less than about 0.5% by weight, usually at or at least about 1% by
weight to as much as about 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer’s solution and 10 mg of the chimeric Factor VII polypeptide. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington’s Pharmaceutical Sciences, 18th ed., Mack Publishing Company, Easton, Pa. (1990).

[0153] The compositions containing the chimeric Factor VII polypeptides useful in the methods of the present invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a subject in need of the methods of this invention, as described above, in an amount sufficient to cure, alleviate or partially arrest the disorder and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective amount" or "effective amount." As will be understood by the person skilled in the art amounts effective for this purpose will depend on the severity of the disease or injury as well as the weight and general state of the subject. In general, however, the effective amount will range from about 0.05 mg up to about 500 mg of the chimeric Factor VII polypeptide per day for a 70 kg subject, with dosages of from about 1.0 mg to about 200 mg of the chimeric Factor VII polypeptide per day being more commonly used.

[0154] It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and general lack of immunogenicity of human Factor VII in humans, it is possible and may be felt desirable by the treating physician to administer a substantial excess of these chimeric Factor VII polypeptide compositions.

[0155] In prophylactic applications, compositions containing the chimeric Factor VII polypeptide useful in the methods of the invention are administered to a subject susceptible to or otherwise at risk of a disease state or injury to enhance the subject’s own coagulative capability. Such an amount is defined to be a "prophylactically effective dose." In prophylactic applications, the precise amounts once again depend on the subject’s state of health and weight, but the dose generally ranges from about 0.05 mg to about 500 mg per day for a 70-kilogram subject, more commonly from about 1.0 mg to about 200 mg per day for a 70-kilogram subject.

[0156] Single or multiple administrations of the compositions can be carried out with dose levels and patterns being selected by the treating physician. For ambulatory subjects requiring daily maintenance levels, the chimeric Factor VII polypeptides may be administered by continuous infusion using e.g., a portable pump system.

[0157] The chimeric Factor VII polypeptides useful in the methods of the present invention may also be formulated in sustained, or extended release formulations. Methods of formulating sustained, or extended release compositions are known in the art and include, but are not limited to, semi-permeable matrices of solid hydrophobic particles containing the polypeptide.

[0158] Local delivery of the chimeric Factor VIIa polypeptides useful in the methods of the present invention, such as, for example, topical application may be carried out, for example, by means of a spray, perfusion, double balloon catheters, stent, incorporated into vascular grafts or stents, hydrogels used to coat balloon catheters, or other well established methods. In any event, the pharmaceutical compositions should provide a quantity of Factor VII variant sufficient to effectively treat the subject.

[0159] The following examples have been included to illustrate the invention. Certain aspects of the following examples are described in terms of techniques and procedures found or contemplated by the present co-inventors to work well in the practice of the invention. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following examples are intended to be exemplary only and that numerous changes, modifications and alterations may be employed without departing from the scope of the invention.

EXAMPLES

Example I

Construction and Expression of Chimeric Factor VIIa Polypeptides

[0160] The cDNA of both human Factor VII and human Factor IX were obtained and the following unique restriction sites were introduced to facilitate domain exchange:

[0161] BstEI site at the 5' end of EGF-1 domain (residues 47-49 of Factor VII; residues 48-50 of Factor IX);

[0162] SalI site at the junction of the EGF-1 and EGF-2 domains (residues 82-83 of Factor VII; residues 83-84 of Factor IX);

[0163] NotI site at the 3' end of the EGF-2 domain (residues 135-137 of Factor VII; residues 132-134 of Factor IX).

[0164] Various chimeras were constructed using these restriction sites to exchange domains between Factor VII and Factor IX and are depicted in FIG. 1. The same, or similar restriction sites can be used to exchange domains between Factor VII and Protein S.

[0165] The amino acid sequence of the resulting chimeras are set forth in SEQ ID NO:1 (Factor IXsignal, propeptide, GLA and EGF1 domains, with Factor VII EGF2 and catalytic domains) and SEQ ID NO:2 (Factor IXsignal and propeptide domains, Protein S GLA domain, Factor IX EGF1 domain, with Factor VII EGF2 and catalytic domains). A selection of these recombinant DNA's were subcloned into the expression vector pCMV5 for mammalian cell transfection.

[0166] The resulting recombinant constructs were then co-transfected with pSV2neo and pCMVhGC into the 293 human kidney cell line (ATCC CRL-1573). A clone expressing a high level of each construct was selected and screened as previously described (Toomey et al. 1991, J. Biol. Chem., 266:19198-19202). Each screened clone was expanded to 900 cm² roller bottles for large scale production and recombinant proteins were purified by a pseudo-affinity chromatographic method using Fast Flow Q-Sepharose and elution with a calcium gradient, followed by a NaCl gradient.

Example II

Thrombin Generation of Factor VIIa Polypeptides

[0167] The thrombin generation of Factor VIIa was determined using an in vitro model of hemophilia. Monocytes were used as a source of tissue factor and combined with unactivated platelets and synthetic plasma containing plasma concentrations of Factors V, VII, IX, VIII, and XI, and plasma
concentrations of antithrombin and TFPI. Hemophilic conditions were created by omitting factors VIII and IX.

Monocytes were prepared by drawing 4 ml of blood from a healthy individual and placing in a sodium citrate tube. The blood was carefully layered on top of 3 ml of Accuprep™ Lymphocyte separation medium (Accurate Chemicals, NY, USA) in a 15 ml conical tube and centrifuged at 1500 rpm for 30 minutes. The mononuclear layer was then removed and added to an equal volume of Versene (Lineberger Tissue Culture) at 4°C and centrifuged at 800 rpm for 10 minutes. The resulting pellet was resuspended in 5 ml of Versene at 4°C and centrifuged at 800 rpm for 10 minutes. The resulting pellet was resuspended in 4 ml of macrophage SFM media (Life Technologies, CA, USA), supplemented with 500 ng/ml of lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA) and cells were plated at 200 µl/well of a 96-well plate. The plate was incubated at 37°C, 5% CO₂, for two hours and then washed three times with macrophage SFM media and then incubated overnight.

Platelets were prepared by drawing 4 ml of blood from a healthy individual and placing in a sodium citrate tube. The blood was carefully layered on top of 5 ml of Accuprep™ Lymphocyte separation medium (Accurate Chemicals, NY, USA) in a 15 ml conical tube and centrifuged at 1500 rpm for 30 minutes. The platelet layer was then removed and added to an equal volume of citrate-glucose-saline with 10 µg/ml of prostaglandin E₁ (PGE₁) (Sigma-Aldrich, MO, USA) and centrifuged at 800 rpm for 10 minutes. The resulting pellet was discarded and the platelets were isolated from plasma proteins by Sephasose gel filtration in calcium free Tyrodes buffer supplemented with 1 mg/ml ovalbumin. The recovered platelets were stored at 37°C.

Synthetic plasma for the in vitro assay that mimics hemophilia was generated by using Factor XI C-1-esterase inhibitor at a concentration of 5 µg/ml with the addition after 1 hour incubation with the previously prepared monocytes of 200 µg/ml antithrombin, 0.07 µg/ml of TFPI, 100 mg/ml of prothrombin, 8 µg/ml of Factor X and 0.5 µg/ml of Factor VII. Following an overnight incubation, Factor V was added at a concentration of 7 µg/ml. For normal conditions, Factor IX was added after 1 hour incubation at a concentration of 4 µg/ml and following the overnight incubation, Factor VIII was added at 1 U/ml.

260 µl of platelets were added to the above synthetic plasma solutions and the resulting suspension was added to the monocytes to start the reaction. At timed intervals 10 µl samples were removed and added to 90 µl of thrombin assay buffer containing 1 nM EDTA and 0.5 mM Gly-Pro-Arg-pNA (Centecem Inc., CT, USA). 100 µl of 50% acetic acid was added to stop synthetic substrate cleavage and the OD was measured at 405 nm. Thrombin concentration was determined using the following formula:

\[
\text{Thrombin concentration} = \frac{\text{OD}_{405} \times \text{Volume}}{\text{Conversion factor}}
\]

wherein the conversion factor is 1 nM thrombin gives a rate of 0.0117 OD/min at 405 nm.

Thrombin generation in normal conditions (Factors VIII and IX included) shows peak thrombin production with a short lag phase (FIG. 2). Increasing concentrations of wild-type Factor VIIa improved thrombin production in the hemophilic condition. (FIG. 2).

Example III
Thrombin Generation of Chimeric Factor VIIa Polypeptides

The thrombin generation of the chimeric proteins produced in Example I was determined using an in vitro model of hemophilia described in Example II. The addition of 10 nM of a chimeric Factor VIIa containing the EGF-2 and catalytic domains of Factor VII and the GLA and EGF-1 domains of Factor IX, or a chimeric Factor VIIa containing the GLA, EGF-2 and catalytic domains of Factor VII and the EGF-1 domain of Factor IX had a similar activity to 50 nM of wild-type Factor VIIa. (FIG. 3).

Looking at peak thrombin production in relation to the control, neither wild-type Factor VIIa, nor the chimeric Factor VIIa polypeptides restored peak thrombin production to the level of the control. However, 10 nM of a chimeric Factor VIIa containing the EGF-2 and catalytic domains of Factor VII and the GLA and EGF-1 domains of Factor IX, or a chimeric Factor VIIa containing the GLA, EGF-2 and catalytic domains of Factor VII and the EGF-1 domain of Factor IX restored peak thrombin production to a level similar to that produced by 50 nM of wild-type Factor VIIa. (FIG. 4).

Example IV
Clotting Assay of Chimeric Factor VIIa Polypeptides

The clotting activity of the chimeric Factor VIIa polypeptides was assessed in vivo, in a clotting assay, as described by Boyue, Y., et al. 2008 Blood 112:3234-3241. Briefly, Factor VII was administered to a hemophilia B mouse (2 mg/kg of NovoSeven®, 2 mg/kg of a chimeric Factor VIIa containing the GLA and EGF-1 domains of Factor IX and the EGF-2 and catalytic domains of Factor VII, or 1.4 mg/kg of a chimeric Factor VIIa containing the GLA domain of Protein S, the EGF-1 domain of Factor IX and the EGF-2 and catalytic domains of Factor VIIa), through a catheter inserted into a vein in the leg. A wild-type mouse was used as a control. The mice were anesthetized and the saphenous vein of the other leg was transected by pushing a small gauge needle through it. The distal portion is then cut by inserting the tip of a pair of scissors into the vein and snipping to create a small cup. A clot is formed and the time until bleeding stops was recorded. The clot was then removed using a 30 gauge needle and the time until bleeding stops was again recorded. This process was repeated for 30 minutes. The number of clots was recorded as the number of disruptions. The times recorded were averaged and recorded as an average time. The experiment was conducted on one mouse for the wild-type mouse and the chimeric Factor VIIa containing the GLA domain of Protein S, the EGF-1 domain of Factor IX and the EGF-2 and catalytic domains of Factor VIIa. For the chimeric Factor VIIa containing the GLA and EGF-1 domains of Factor IX and the EGF-2 and catalytic domains of Factor VIIa the experiment was conducted on two hemophilia B mice with the same results. For the hemophilia B mouse that was not administered any Factor VII, the 0.25 disruptions recorded in Table 1 below indicate that only one out of four mice did not bleed out with the initial injury.

| TABLE 1 |
|-------------------|-----------------|-----------------|-----------------|---------------|
| Hemophilia    | B NovoSeven® | C1 (1063) | C2 Mouse | WT Mouse |
| Disruptions | 0.25 | 20 | 29 | 13 | 25 |
| Average time (sec) | 1712 | 71 | 51 | 125 | 54 |
[0177] The experiment was repeated with one hemophilia B mouse with no treatment, one hemophilia B mouse administered 2 mg/kg of NovoSeven®, one hemophilia B mouse administered 2 mg/kg of a chimeric Factor VIIa containing the GLA and EGF-1 domains of Factor IX and the EGF-2 and catalytic domains of Factor VIIa and one wild-type mouse with no treatment. The hemophilia B mouse administered the 2 mg/kg of chimeric Factor VIIa containing the GLA and EGF-1 domains of Factor IX and the EGF-2 and catalytic domains of Factor VIIa had a shorter average time to cessation of bleeding than the hemophilia B mouse administered 2 mg/kg of NovoSeven® and the wild-type mouse. (FIG. 5).

Example V

Thrombogenicity of Chimeric Factor VIIa Polypeptides

[0178] Thrombogenicity of the chimeric Factor VIIa polypeptides of the invention can be tested in a mouse model that expresses high levels of these proteins. Recently, it has been shown that 50% of mice expressing greater than 2 μg/ml recombinant wild type mouse factor VIIa died from thromboses within 16 months. When backcrossing into the C57BL/6J mouse strain to generate Hemophilia B mice expressing similar levels of factor VIIa, these mice had the same mortality rates and thromboses occurred much earlier (<4 months). (Aljamali M N, et al. J Clin Invest. 2008; 118:1825-1834).

[0179] A hemophilia B mouse (strain C57BL/6J) expressing 2-10 μg of more of the chimeric FVIIa polypeptides has been produced. If expression of the chimeric Factor VIIa polypeptides would have caused thromboses in these mice similar to that observed in mice expressing wild type FVIIa, then it would have been expected that these chimeric expressing mice would have died from thrombosis early on (<4 months). However the chimeric FVIIa-expressing mice have remained healthy throughout the observation period of ~6 months, and show no bleeding events.

[0180] The mouse strain can be developed by using a new self-complemented adeno associated vector (AAV) developed by Dr. Paul Monahan of the Gene Therapy Center at UNC. (Wu Z, et al. Mol. Ther. 2008; 16:280-289). This vector contains a small transgene promoter-enhancer (TTR promoter), introns from the mouse virus of mouse, the MVM and the bovine growth hormone aP a signal. It has been shown that injection of 1 x 10^11 vector genomes, based on this vector, into the portal vein resulted in sustained expression of Factor IX of 7 μg per ml within 8 weeks of infection. (Wu Z, et al. Mol. Ther. 2008; 16:280-289).

[0181] The cDNA of both mouse and human factor VIIa, as well as the chimeric Factor VII polypeptides of the invention may be used to assess thrombogenicity. The RKKRRK (SEQ ID NO:21) sequence described by Margaritis et al. that causes the Factor VII to be secreted in the active form, i.e., Factor VIIa. (Margaritis et al., J. Clin. Invest., 2004; 113:1025-31) can also be used.

[0182] To further test thrombogenicity of wild-type and chimeric Factor VIIa polypeptides, mouse polypeptides can be generated for injection into mice. It has been reported that mouse tissue factor does not react well with human Factor VII, so mouse polypeptides (both wild type and chimeric Factor VIIa) are used when utilizing a murine model to demonstrate the thrombogenicity of the chimeric versus wild-type FVIIa polypeptides. It is thought that the hemostatic function of FVIIa when used pharmacologically is independent of tissue factor; but, based upon observations that thrombosis occurs in tissues rich in tissue factor, it is likely that the thrombotic events are tissue factor dependent. The mouse polypeptides that can be used to test this hypothesis include a wild-type mouse Factor VII (SEQ ID NO:3), a wild-type mouse Factor VII with an RKKRRK sequence to cause the polypeptide to be secreted in the two-chain, active form (FVIIa) (SEQ ID NO:4), a mouse Factor IX signal, propeptide, GLA and EGF1 domains, with Factor VII EGF2 and catalytic domains and an RKKRRK sequence (SEQ ID NO:5) and a mouse Factor IX signal and propeptide domains, Protein S GLA domain, Factor IX EGF1 domain, with Factor VII EGF2 and catalytic domains and an RKKRRK sequence (SEQ ID NO:6). The chimeric mouse Factor VII polypeptides (SEQ ID NO:5 and 6) will have a reduced affinity for tissue factor (perhaps as much as 100 fold lower when compared to the wild-type FVII molecule) and this should be sufficient to prevent unwanted thromboses.

[0183] The chimeric Factor VII polypeptides may also be additionally modified to further reduce the affinity for tissue factor. An example of such a mutation is to change the methionine at residue 366 in the amino acid sequence of SEQ ID NO:18 to alanine. Other modifications of the chimeric Factor VII polypeptides may be included that result in a higher specific activity of Factor VIIa. Examples of such modifications include the following equivalent to residues 218, 356 and 358 of the human sequence (SEQ ID NO:18): V218D, E356V and M358Q.

[0184] The wild-type and chimeric mouse Factor VII polypeptides described above can be constructed by using mouse Factor VII cDNA as a template and using oligonucleotide primers to amplify the various domains and then inserting the constructs into a pSC-TTR-mvm vector, the construction of which is described in Wu et al., Molecular Therapy 2008 16:280-289. Specifically, the wild-type mouse Factor VII can be amplified from mouse Factor VII cDNA using the following primers: 5'-TGAGGATCCCCACCATGGTTCCACAGCGCATGGGCT3' (SEQ ID NO:7) and 5'TTCCCCAGCATGCTACAGTAGTGGGAGTCGGAAAAC3' (SEQ ID NO:8). The amplified fragment can then be digested with BamHI/SphI and inserted into the pSC-TTR-mvm vector between the BglII/SphI sites. Subsequently, the BglI polyadenylation sequence can be inserted into the resulting vector at the SphI site.

[0185] The wild-type mouse Factor VII construct with the RKKRRK sequence can be constructed in a similar fashion. Specifically, the wild-type mouse Factor VII with the RKKRRK sequence can be amplified from mouse Factor VII cDNA using the following primers:

5'-TGAGGATCCCCACCATGGTTCCACAGGCCATGGGCT3' (SEQ ID NO:7) and 5'ACAAATGCGTTTTTCCGGCCTTACGCAGCCCTGCGGCTTCTGAGTGGATTTGCGCTTGAGCAGATCGGAAAAC3' (SEQ ID NO:9), to generate a first fragment; and,

5'-TTCCCCAGCATGCTACAGTAGTGGGGAGTCGGAAAAC3' (SEQ ID NO:7) and 5'GGCCGCGTGAAGAGCGGAAAACGCATGCTGAGGAGCGAACAGTGTGCGCC3' (SEQ ID NO:10) to generate a second fragment. An overlapping PCR is then performed using these two fragments as template and the following primers:

5'-TGAGGATCCCCACCATGGTTCCACAGGCCATGGGCT3' (SEQ ID NO:7) and 5'TTCCCCAGCATGCTACAGTAGTGGGGAGTCGGAAAAC3' (SEQ ID
NO:8). The resulting amplified fragment can then be digested with BamHI/SphI and inserted into the pSCT-TR-mvm vector between the BglII/SphI sites. Subsequently, the BGH polyadenylation sequence can be inserted into the resulting vector at the Sphl site.

[0189] The construct containing the mouse Factor IX signal, propeptide, GLA and EGF-1 domains, with Factor VII EGF-2 and catalytic domains and an RRRKR sequence (SEQ ID NO:5) is similarly constructed. Specifically, a first fragment is generated using mouse Factor IX cDNA as a template and the following primers:

[0190] 5’AGGCCCTGAAGATCTCCACCCTGAAAGCACA-CTGAACACCCTC3’ (SEQ ID NO:11) and 5’GATCCAGCTTCAATTTTGTATTATGATGAGATC3’ (SEQ ID NO:12). A second fragment is generated using mouse Factor VII as a template and the following primers:

[0191] 5’GATTGGAAGGAAGAAGCTG- GAATAAGCAGAACATTGACGCTGATC3’ (SEQ ID NO:13) and 5’ACATGGGTTTTTCGGGGTACCCTGCTGGAATC3’ (SEQ ID NO:9).

[0192] A third fragment can be generated using mouse Factor VII as a template and the following primers:

[0193] 5’TCCCCCaCGATCCATGCAGTTATGGA- GACGGCTAAGAAC3’ (SEQ ID NO:8) and 5’CCGGCCGTAAAGGGCAGAAGCAGAT- TGTGAGACAGCTGCCCGCC3’ (SEQ ID NO:10). An overlapping PCR can then be performed using these three fragments as template and primers:

[0194] 5’AGCCCTGAAGATCTCCACCCTGAAAGCACA- CTGAACACCCTC3’ (SEQ ID NO:11) and 5’TCCCCCaCGATCCATGCAGTTATGGA- GACGGCTAAGAAC3’ (SEQ ID NO:8). The resulting amplified fragment is then digested with BamHI/SphI and inserted into the pSCT-TR-mvm vector between the BglII/SphI sites. Subsequently, the BGH polyadenylation sequence is inserted into the resulting vector at the Sphl site.

[0195] A similar process can be used to generate the construct containing the mouse Factor IX signal and propeptide domains, Protein S GLA domain, Factor IX EGF-1 domain, with Factor VII EGF-2 and catalytic domains and an RRRKR sequence (SEQ ID NO:6). Specifically, a first fragment can be generated using mouse Factor IX cDNA and the following primers:

[0196] 5’AGGCCCTGAAGATCTCCACCCTGAAAGCACA- CTGAACACCCTC3’ (SEQ ID NO:11) and 5’TCCCCCaCGATCCATGCAGTTATGGA- GACGGCTAAGAAC3’ (SEQ ID NO:8). An overlapping PCR is then performed using these three fragments as template and primers:

[0199] 5’AGGCCCTGAAGATCTCCACCCTGAAAGCACA- CTGAACACCCTC3’ (SEQ ID NO:11) and 5’TCCCCCaCGATCCATGCAGTTATGGA- GACGGCTAAGAAC3’ (SEQ ID NO:8). An overlapping PCR is then performed using these three fragments as template and primers:

[0200] The wild-type mouse Factor VII (SEQ ID NO:3) and wild-type mouse Factor VII with the RRRKR sequence (SEQ ID NO:4) construct were each injected into two male hemophilia B mice of matched age (5×10^11 vector genomes per mouse). The mouse Factor IX signal, propeptide, GLA and EGF1 domains, with Factor VII EGF2 and catalytic domains and an RRRKR sequence (SEQ ID NO:5) and mouse Factor IX signal and propeptide domains, Protein S GLA domain, Factor IX EGF1 domain, with Factor VII EGF2 and catalytic domains and an RRRKR sequence (SEQ ID NO:6) vector constructs were each injected into three male hemophilia B mice of matched age (5×10^11 vector genomes per mouse). Approximately three weeks post-injection, all mice injected with either wild-type mouse Factor VII (SEQ ID NO:3) or wild-type mouse Factor VII with the RRRKR sequence (SEQ ID NO:4) died. The mice injected with either mouse Factor IX signal, propeptide, GLA and EGF1 domains, with Factor VII EGF2 and catalytic domains and an RRRKR sequence (SEQ ID NO:5), or mouse Factor IX signal and propeptide domains, Protein S GLA domain, Factor IX EGF1 domain, with Factor VII EGF2 and catalytic domains and an RRRKR sequence (SEQ ID NO:6) were alive and showed no adverse symptoms approximately five weeks post-injection. Blood was collected once a week from the mice and after six to eight weeks following injection the level of mouse Factor VII polypeptide can be measured. Also, antigen levels to the mouse Factor VII polypeptides can be determined indirectly by measuring the clotting activity in vitro from blood samples of the injected mice (Margaritas et al., Gene Therapy 2001; 113:3682-3689).

Example VI

Binding of Collagen and Accumulation of Chimeric Factor VIIa Polypeptides in the Basement Membrane

[0201] Binding of collagen type IV by the chimeric Factor VIIa polypeptides and accumulation of the chimeric Factor VIIa polypeptides can be shown by injecting the polypeptides into mice and subsequently taking tissue samples from the mice and staining with antibodies labeled for collagen type IV and the chimeric polypeptides. For example, 2 mg/kg of the chimeric Factor VIIa polypeptides can be administered through a catheter inserted into a vein in the leg of a hemophilia B mouse strain. The animals can be sacrificed at predetermined time points and tissue sections can be taken and preserved by placing in OCT embedding compound, snap-freezing in liquid nitrogen cooled isopentane and transferred to liquid nitrogen. The cryosections of the tissue can then be fixed for 15 minutes in 3% parafomaldehyde in PBS, pH 7.4, rinsed in PBS for 5 minutes, incubated in methanol for 10 minutes, rinsed three times in PBS and then blocked in 3% BSA/PBS for 1 hour. Following this, the tissue sections
can be incubated overnight with an affinity purified antibody directed to the chimeric Factor VIlA polypeptides and then a secondary antibody directed to the primary antibody, which is labeled with a fluorochrome, such as FITC. Another antibody directed to collagen type IV can also be used during the incubation step and a further antibody directed to the collagen type IV-antibody complex can be labeled with another fluorochrome such as rhodamine. Following these incubation steps, the stained tissue sections can be viewed on a fluorescence microscope, such as a Nikon FXA. Binding of the chimeric Factor VIlA polypeptides can then be visualized and detected with the presence of a green signal in the interstitial spaces, collagen type IV can be visualized and detected with the presence of a red signal, and the colocalization of the chimeric Factor VIlA polypeptides with collagen type IV and the accumulation of the polypeptides in the extravascular space can be visualized and detected with the presence of a yellow signal in the interstitial spaces.

Example VII

Affinity of Chimeric Factor VIlA Polypeptides for Tissue Factor

Tissue Factor at various concentrations (0-400 nM) can be distributed into 96 well plates. Factor VIlA or chimeric Factor VIlA polypeptides of the invention can be added to the wells at a final concentration of 10 nM. Alternatively, varying concentrations of Factor VIlA or the chimeric Factor VIlA polypeptides of the invention (0-128 nM) can be added to a fixed concentration of Tissue Factor (10 nM). Reaction buffer (Tris-HCl 20 mM, p17.4, NaCl 100 mM, and PEG 0.1%) can be added to the wells and incubated for 1 hour at room temperature. Synthetic substrate S-2288 can be added to the reaction mix at a concentration of 2 mM. The absorbance at 405 nm can be measured on a microplate reader for 30 minutes and the initial rate of hydrolysis of the synthetic substrate can be used for analysis. The data collected can be analyzed by a quadratic solution to the equilibrium equation and the affinity of the chimeric FVIIa polypeptides for Tissue Factor, relative to wild type FVIIa, can be estimated.

Example VIII

Clearance Rate of Chimeric Factor VIlA Polypeptides

The functional half-life and clearance rate of the chimeric Factor VIlA polypeptides, compared to wild-type Factor VII can be determined by taking blood samples from subjects at specific time periods following administration of the polypeptides and assaying for the total Factor VII, the active Factor VII, and the Factor VII associated with anti-thrombin. For example each participant can have a peripheral line inserted and receive, for example, 90 µg/kg body weight of NovoSeven® or the chimeric Factor VIlA polypeptides intravenously. Blood specimens can be collected at baseline and at 0.5, 1.0, 2.0, 4.0 and 6.0 h post-dose in 3% sodium citrate evacuated containers to document Factor VII coagulant activity, total Factor VII concentration and Factor VII-anti-thrombin complex concentration at each time point.

Because the interaction between Factor VIIa and anti-thrombin III is essentially irreversible, just measuring total Factor VIIa antigen is not sufficient to measure active circulating Factor VIIa, it is also necessary to measure the amount of Factor VIIa-anti-thrombin III complex. Total Factor VII concentration and Factor VII-anti-thrombin complex concentration can be measured by Enzyme Immunosorbent Assay. Factor VII coagulant activity can be analyzed using the Staclot® VIIa-rTF assay (Diagnostica Stago). The inability of the chimeric Factor VIlA polypeptides to bind tissue factor should be demonstrated by a decrease in the Factor VII-antithrombin complex concentration at each time point compared to NovoSeven®. This should translate into a reduced clearance rate for the chimeric Factor VIlA polypeptides and an increase in the functional half-life of the chimeric Factor VIlA polypeptides when compared to NovoSeven®.

Example IX

Studies to Demonstrate that Both Human and Mouse Versions of FIXGlu-Ego1FVIIa and ProSGluFIX_Ego1FVIIa Chimeras can Stop Bleeding in Hem B Mice

These results are based upon a tail bleeding model and a bleeding model treated with the saphenous vein is severed and the time to clot formation is measured. The clot is then removed and the time to cessation of bleeding is measured.

Two parameters are produced: 1) the number of times that bleeding stops in a 30 minute period, and 2) the average time until bleeding stops after clot removal. For hem B mice, the average bleeding time is the 1800 second limit of the observational period.

In the experiments described herein, the method is demonstrated by infusing human FIX into a hem B mouse. Increasing concentrations of FIX (Mononine) were infused via catheter in the contralateral saphenous vein, and the average time for bleeding to stop was measured for different mice at different concentrations of FIX. The response curve is similar to that expected from a hemophilic patient: 0.1 U/mL gives similar values as seen in wt mice, 0.01 U/mL gives similar values as seen in hemophilic animals, and 0.02 and 0.05 U/mL give dose-dependent movement toward the wt values. The sharp dose dependence of this assay allows for assessment of effects of molecules with enhanced (or reduced) clotting activity.

In another experiment, the activity of human rFVIIa in mice was compared to the activity of arFVIIa with enhanced activity in biochemical assays (NN1731). Hemo- stasis was obtained with lower doses of the super-active rFVIIa, indicating it has about 10-fold greater hemostatic efficacy.

This same analysis has been applied to certain chimeric constructs of this invention. Experiments were done in two mice that were each given a single dose (2 mg/mL) of either human rFVIIa or human FIX_Glu-Ego1FVIIa. As shown in Table 2, this dose did not completely restore hemostasis with wtFVIIa, but the same dose of chimeric FIX_Glu-Ego1FVIIa bled less than normal mice. This finding indicates that the chimera is more hemostatic even though it has reduced affinity for tissue factor (TF).

Additional information about the hemostatic capability of chimeric FVIIa molecules of this invention is available from observations on the tail-bleeding characteristics of hem B mice expressing the murine chimera FIX_Glu-Ego1FVIIa. For gene therapy experiments, a self-complementary adeno associated vector (AAV) that causes a very high level of expression was used. With the use of this vector, mice expressing ~11 µg/mL murine FVIIa at 2 weeks of age
have been produced. The very high expressing mice died within the first three weeks. However, twenty weeks later the chimeric mice were still alive and healthy, but the chimeras had less recombinant protein in the circulation (~1.3 μg/mL for FIX<sub>Glu<sub>529</sub>EGF</sub> in vivo). Despite the decreased plasma levels, chimeric mice are protected from bleeding in a tail clip model.

[0211] It was thought that the apparent change from 2 weeks to 22 weeks in expression of FIX<sub>Glu<sub>529</sub>EGF</sub> in vivo was due to chimeric binding to collagen IV outside the vasculature. A bolus of FIX was injected 10 minutes prior to drawing blood for ELISA measurements of expressed chimera and a 4-fold increase in the chimeric protein was measured, indicating that the chimeric FIX<sub>VIIa</sub> is likely bound to type IV collagen.

[0212] Testing of in vivo hemostatic efficacy of additional FIX<sub>VIIa</sub> chimeras with different TF-, phospholipid-, and collagen IV-binding properties. Murine wt and chimeric FIX<sub>VIIa</sub> protein will be produced for infusion into hemophilic mice and the hemostasis model involving transection of the sphenous vein will be used to characterize hemostasis. Gene therapy technologies will also be used to produce mice expressing wt<sub>VIIa</sub> and FIX<sub>VIIa</sub> chimeras and hemostasis will be measured in these mice. These studies will be carried out with the expectation that 1) FIX<sub>VIIa</sub> chimeras that do not bind TF will be as hemostatic as wt<sub>VIIa</sub> in bleeding models and 2) FIX<sub>VIIa</sub> chimeras that have enhanced platelet- or collagen IV-binding will be more hemostatic than wt<sub>VIIa</sub>. Preliminary data suggest the FIX<sub>VIIa</sub> chimeras support hemostasis and that FIX<sub>VIIa</sub> penta-chain activity is TF-independent. These experiments will extend this finding, and show the relative roles of phospholipid and collagen IV binding in hemostasis.

[0213] Measurement of hemostasis in vivo in mice infused with FIX<sub>VIIa</sub> or chimeric FIX<sub>VIIa</sub> protein. Hemostasis will be compared in wt mice, hem B mice, and hem B mice infused with different murine chimeric proteins using the bleeding model described herein, which involves puncture of the full thickness vessel wall, has been shown to provide dose-response information on hemostatic agents in hemophilic mice, and requires fewer animals to achieve statistical significance than the tail bleeding model. This model is a better model of real vascular injury than traditional thrombosis models because it does not depend on chemical, oxidant, or heat-induced endothelial injury, or result in intravascular thrombosis. The bleeding model described herein will be used.

[0214] Statistical: Pilot data show mean SD TTH of 71±12 s for rF<sub>VIIa</sub> versus 51±8 s for FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub>. A two sample t-test will detect a mean difference of 20 with SD of 90 between wt<sub>VIIa</sub> and FIX<sub>VIIa</sub> chimeras at a level of 0.005 with 90% power with 9 mice/group.

[0215] These experiments will show the immediate roles of TF-, platelet-, and collagen IV-binding for chimeric hemostatic activity. To determine the role of TF binding, hemostasis in mice infused with chimeras that lack TF binding ability (any of the molecules containing FIX’s EGF domain) will be compared with normal and hemophilic mice, and mice infused with wt<sub>VIIa</sub>. The efficacy of the non-TF-binding FIX<sub>VIIa</sub> chimeras in the tail clip model suggests FIX<sub>VIIa</sub>’s mechanism of action is TF-independent.

[0216] To determine the role of platelet binding, hemostasis in mice infused with chimeras that have higher affinity for phospholipids (ProS<sub>Glu</sub>FIX<sub>EGF</sub> FIX<sub>VIIa</sub>) or lower affinity for platelets (FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub> and Pro<sub>S<sub>Glu</sub>FIX<sub>EGF</sub> FIX<sub>VIIa</sub>) will be compared with normal and hemophilic mice, and mice infused with wt<sub>VIIa</sub>. It is anticipated that the chimera with reduced affinity for platelets will require higher doses to support hemostasis, whereas the chimera with increased phospholipid binding will support hemostasis similar to FIX<sub>VIIa</sub>, or will support hemostasis at lower levels. To determine the role of collagen IV binding, hemostasis in mice infused with chimeras that have normal (FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub>), reduced (FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub>), or higher (FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub>) collagen IV binding will be compared with normal and hemophilic mice, and mice infused with wt<sub>VIIa</sub>. It is anticipated that collagen IV binding will localize the chimera to vessel periphery, where it can be most effective upon vessel transsection. If this is correct, it is anticipated that FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub> will be hemostatic at the lowest doses in this assay.

[0217] Measurement of hemostasis in vivo in mice that express FIX<sub>VIIa</sub> or FIX<sub>VIIa</sub> chimeras. A comparison will be made of hemostasis in mice genetically engineered to express murine FIX<sub>VIIa</sub> and FIX<sub>VIIa</sub> chimeras. Mice expressing FIX<sub>VIIa</sub> and two of the chimeras, FIX<sub>Glu<sub>529</sub>EGF</sub> FIX<sub>VIIa</sub> and ProS<sub>Glu</sub>FIX<sub>EGF</sub> FIX<sub>VIIa</sub> have been produced and mice expressing all three constructs are being maintained. The same gene therapy technologies will be used to produce additional constructs. Hemostasis will be assessed using the bleeding model described herein with similar groups as described above.

[0218] Because the FIX<sub>VIIa</sub> chimeras have been engineered to have different TF-, platelet-, and collagen IV-binding properties, it is expected that they will equilibrate differently between the plasma and extravascular space. These experiments will show the hemostatic effects of the FIX<sub>VIIa</sub> chimeras equilibrated in these compartments. Comparisons will be performed as described above to interrogate the effects of TF-, platelet-, and collagen IV-binding on hemostatic activity. It is anticipated that the results with the genetically-manipulated mice will be similar to findings with the infused mice. However, it is not feasible to measure hemostatic efficiency of very low levels of FIX<sub>VIIa</sub> that are found in vivo.

[0219] Testing of thrombogenic propensity of FIX<sub>VIIa</sub> chimeras with different TF-, phospholipid, and collagen IV-binding properties. In mice expressing the chimeras and in mice infused with chimeric protein, circulating thrombin-antithrombin (TAT) and D-dimer levels will be measured, the time to vessel occlusion (TTO) in a ferric chloride (FeCl<sub>3</sub>) thrombosis model will be determined, and the stability of intravascular thrombi produced will be assessed. These studies are expected to demonstrate that 1) thrombotic potential of rF<sub>VIIa</sub> is related to its TF-dependent activity and 2) compared to wt<sub>VIIa</sub>, FIX<sub>VIIa</sub> chimeras with reduced TF binding will produce lower TAT and D-dimer levels and/or demonstrate a longer TTO in hemophilic mice, even when expressed at high levels.

[0220] Measurement and comparison of circulating prothrombotic biomarkers (TAT, D-dimer) and fibrin formation in hemophilic mice expressing FIX<sub>VIIa</sub> and FIX<sub>VIIa</sub> chimeras having different TF-, phospholipid- and collagen IV-binding properties. It is well accepted in human clinical medicine that elevated levels of TAT and D-dimer are correlated with thrombosis, and that both these markers and thrombotic risk increase with age. In mice, elevated levels of TAT have been associated with early death due to fibrin deposition in animals.
expressing high levels of FVIIa. Blood will be drawn from chimeric mice after 1, 2, and 4 weeks, and then monthly. TAT and D-dimer levels will be measured by ELISA.  

[0221] Statistical: Baseline TAT in wt mice is 17.4±2.3 ng/mL (mean±SD). A 2- to 5-fold increase in TAT would be biologically meaningful. Assuming means of 17.5 and 35 and SD of 7.5, a two sample t-test at level 0.005 has at least 90% power with 9 mice/group. Baseline D-dimer in wt mice is 94±4.39 ng/mL, and D-dimer levels in hypercoagulable mice are 236.7±72 ng/mL. An increase of even 10-20% above baseline would be considered biologically significant. A two sample t-test can detect a mean difference of 100 ng/mL with standard deviation 50 ng/mL between wtFVIIa and FVIIa chimera at a level of 0.005 with 90% power with 9 mice/group.  

[0222] These experiments will show effects of expressing elevated levels of FVIIa or FVIIa chimera molecules in hemophilic mice. Mice expressing FVIIa (wild type or chimeric) will be expected to have increased TAT and D-dimers relative to hemophilic mice and TAT levels will be expected to positively correlate with expression of FVIIa (wild type or chimeric). Importantly, however, TAT and D-dimer measurements in mice expressing chimeric FVIIa will be compared to FVIIa. If the thrombogenic potential of FVIIa is related to its TF-binding properties, it is anticipated that TAT and D-dimer levels will be lower in mice expressing FVIIa chimera that do not bind TF than in mice expressing wtFVIIa. It is also anticipated that mice expressing FIX*Gla,Rk*ESG,FVIIa chimera with reduced TF binding and increased collagen IV binding will show similar or lower TAT and D-dimer levels than similar chimeras without collagen IV binding.  

[0223] Measurement of circulating prothrombic biomarkers (TAT, D-dimer) in wt mice infused with FVIIa and FVIIa chimera having different TF-, phospholipid-, and collagen IV-binding properties. Wt mice will be infused with different levels of wt or FVIIa chimera as described above, and development of circulating TAT and D-dimer will be measured in blood samples (5, 15, and 30 min, 1, 2, 4 hours) by ELISA.  

[0224] These experiments will show immediate effects of FVIIa and FVIIa chimera infusion on biomarkers of thrombosis in hemostatically-intact mice. It is expected that these biomarkers will be increased in a dose-dependent fashion following infusion of FVIIa (wild type or chimeric), but that mice infused with chimeras that do not bind TF or platelets will show lower increases in TAT and D-dimer levels than mice infused with wtFVIIa. Similar relative increases in TAT and D-dimer for the FVIIa chimera as observed above are also anticipated.  

[0225] Measurement of thrombogenic potential of FVIIa chimera using murine models of intravascular thrombosis and thrombolysis. Resistance to fibrinolysis has been associated with increased thrombotic risk and worse outcomes in patients with deep vein thrombosis. Therefore, in addition to monitoring thrombus formation in response to injury, a model of thrombolysis will be used to assess clot structure in response to FVIIa or FVIIa chimera.  

[0226] To determine the effect of these molecules on TTO and thrombus stability, thrombosis and thrombolysis models in transgenic chimera-expressing hem A mice and in wt mice infused with wtFVIIa or FVIIa chimera will be used. First the common carotid artery will be injured by applying a filter strip soaked in 10% FeCl₃ solution for 3 minutes and the loss of blood flow (time to occlusion, TTO) will be measured by Doppler flow probe. This protocol has been optimized for the FeCl₃ thrombosis model in C57BL/6, but the FeCl₃ concentration and/or time of injury can be altered to maximize differences between wt and hemophilic mice. Blood will be obtained via the inferior vena cava for measuring plasma TAT after vessel occlusion. In a subset of mice, after 5 minutes of stable occlusion, thrombolytic drug will be infused via saphenous vein catheter, the occluded carotid artery will be monitored until resumption of flow. The recombining anti tPA analog tenecteplase (TNKase) will be used, because compared to tPA, it exhibits greater resistance to inactivation by PAI-1 and higher fibrin specificity, and it can be administered via bolus infusion.  

[0227] Statistical: In the FeCl₃ thrombosis model, mean±SD TTO for wt mice (normal coagulation) is 15±1.3 min. A 3-5 minute shortening would suggest hypercoagulability. To detect a 3 minute reduction with a standard deviation of 1.5 minutes, a sample size of 9 is needed for 90% power with two sample t-tests at level 0.005. The time to flow return in wt mice is 50±10 minutes (mean±SD). Resistant animals may never have flow return, that is, very large times. For detecting a difference with means of 50 and 100 minutes, sample size 9 gives power which is at least 80% based on a logrank test with level 0.005, assuming exponentially time to flow return is exponentially distributed.  

[0228] Previous studies have demonstrated the TF-dependence of the FeCl₃ thrombosis model, and that hemophilic mice show prolonged TTO compared to wt mice. Thus, it is anticipated that hem B mice expressing wtFVIIa or FVIIa chimera will show a shortened TTO compared to hem B. However, for both hem B and wt mice, relative thrombogenicity of the chimeras will be assessed by comparing their TTO with that of wtFVIIa. If the thrombogenic propensity of rFVIIa is related to its TF-binding properties, then it is expected that chimeras with reduced TF binding will exhibit longer TTO and decreased resistance to lysis following FeCl₃ injury as compared to FVIIa. These experiments will also allow for the identification of potential prothrombotic consequences of FVIIa-binding to collagen IV in the extracellular space. It is expected that collagen IV binding will not promote FVIIa thrombogenic activity; therefore shortened TTO in FVIIa chimeras that bind collagen IV compared with FVIIa itself is not anticipated.  

[0229] Comparison of bio-distribution and activity of transgene-expressed wtFVIIa and FVIIa chimera in wt mice having altered TF-, phospholipid-, and collagen IV-binding properties. It is anticipated that chimeric FVIIa will have fundamentally altered bio-distribution because of altered binding to collagen IV. These bioengineered differences will be confirmed by comparing chimeric and wtFVIIa in plasma and tissues where wtFVIIa is known to be sequestered. Circulating plasma antigen (total FVIIa and chimera-AF complexes) and activity will be measured by quantitative western blotting and calibrated automated thrombography (CAT), respectively, and extravascular sequestration of FVIIa chimeras will be identified by immunohistochemistry. These studies are expected to demonstrate that 1) molecules that express the collagen IV-binding region of the FIX Gla domain will partition to the extracellular space, co-localized with collagen IV and 2) FVIIa chimeras that bind collagen IV or do not bind TF will have a longer functional half-life.  

[0230] Measurement of expression levels of the murine FVII chimera and determination of their distribution in the plasma and extracellular space. Protein expression will be measured in the plasma and the extracellular space at equi-
librium in mice expressing FVIIa and FVIIa chimeras. Plasma determinations will be made by reducing and non-
reducing SDS-PAGE and western blotting using fluorescently-labeled anti-FVII and anti-FIX monoclonal antibo-
dies. These antibodies have been used routinely in ELISAs for FIXGla-EGF-FVIIa; comparing images from individually-la-
belled blots with merged images will allow for discrimination between endogenous FVII and the FVIIa/FIX chimeras. A determination will be made of whether the FVIIa chimeras are sequestered in the extravascular space by immunohisto-
chemistry on paraffin-embedded tissues harvested from chimeric mice. For these experiments, the same antibodies
used in preliminary ELISAs will be used. Extravascular chimeras will be competed into blood by injecting a 2 mg/kg
bolus of human FIX into mice and measuring chimeras after 10 minutes. The inventors’ observations suggest that injecting
2 mg/kg FIX displaces most chimeric FVIIa from the extravascular space, enabling its detection in plasma.

[0231] Statistical: Large differences are anticipated between chimeric and index groups. A sample size of 9/group
gives 90% power to detect an effect size of 2 for two sample t-test at level 0.005. While preliminary ELISA data on the
chimeras are not available, these effect sizes are realistic given the bioengineered design of the chimeric animals. For
immunohistochemistry, a categorical scale of low to high staining will be used; previous experience has shown means±SD of
1.0±0.5 versus 2.5±0.5 for similar measurements. In this scenario, the effect size is (2.5–1)/0.5–3, which gives adequate
power.

[0232] It is anticipated that chimeras bearing the FIX Gla domain will be bound extravascularly. These observations
will be used to reconcile any differences in in vivo and ex vivo hemostasis measures of the chimeric-expressing mice. For
example, if mice expressing FIXGLa-EGFFVIIa show normal hemostasis in the bleeding model but no plasma procoagulant
activity or antigen, it would be expected to observe significant chimeric bound in the extravascular space.

[0233] Measurement of thrombin generation and clot stab-
ility ex vivo in plasmas from normal and hemophilic mice
and mice expressing chimeric FVIIa. CAT will be used to
measure plasma thrombin generation. Briefly, CAT assays
will be performed by diluting platelet-rich plasmas (PRP)
from chimeric mice 1:3 and triggering clotting with TF. Recombinant murine TF will be used for these assays. Read-
outs (lag time, peak thrombin generated, and endogenous thrombin potential) will be compared to wt plasma, hem B
plasma, and hem B plasma spiked to normal (100%) levels of FIX. Fibrinolysis assays will be performed similarly, but
in the presence of plasmin. The readouts, time to peak turbidity and peak turbidity indicate the procoagulant potential and the
peak amount of fibrin produced during the reaction, respectively, and will be compared to wt and hem B plasmas, as
described above.

[0234] Statistical: For CAT, pilot data show the mean±SD for peak thrombin in normal plasma is 146±59 nM, and with
50 nM wtFVIIa in hemophilic plasma is 37±1 nM. A two sample t-test can detect a mean difference of 110 with standard
deviation of 40 at a level of 0.005 with at least 90% power with 9 mice/group. For fibrinolysis experiments, pilot
data show mean±SD for peak turbidity in normal plasma is 800±300 sec, and with 2 nM wtFVIIa in hemophilic plasma is
1700±300 sec. A two sample t-test can detect a mean difference of 900 with standard deviation 450 sec at a level of 0.005
with 90% power with 9 mice/group.

[0235] CAT and plasma fibrinolysis assays have demonstrated utility for detecting both hemostatic and prothrom-
botic activity, and abnormal readouts have been correlated with hemostatic and thrombotic disorders in epidemiologic
studies. The role of FVIIa TF binding will be determined by comparing PRP from mice whose chimeras lack TF bind-
ing ability FVIIagla-FIXEGF-FVIIa with FIXGLa-EGF-FVIIa with PRP from normal, hemophilic, and FIXGLa chimeric mice. It is
anticipated that the non-TF-binding chimeras will support similar thrombin generation and fibrinolytic stability as wt
plasma and FVIIa. To determine the role of platelet binding, activity in plasmas from mice whose chimeras have higher
affinity for phospholipids ProSGlu-FIXEGF-FVIIa, lower affinity for platelets FIXGLa-EGF-FVIIa will be compared with
plasmas from normal, hemophilic, and FIXGLa chimeric mice. At equivalent levels of protein, it is anticipated that plasmas
from mice expressing ProSGlu-FIXEGF-FVIIa will show higher thrombin generation and clot stability than plasmas from
mice expressing chimeras with the FVIIa or FIX Gla domains. Plasma from FIXGLa-EGF-FVIIa mice (reduced platelet binding)
will show reduced thrombin generation and clot stability. It is anticipated that FIXGLa-EGFFVIIa, FIXGLaCys5-EGF-FVIIa,
and FIXGLaCys5-EGFFVIIa will have similar plasma activity because neither CAT nor the fibrin-
olysis assay is sensitive to collagen IV binding. However, if FIXGLaCys5-EGFFVIIa demonstrates higher in vivo activity, but
similar plasma hemostatic activity compared to FIXGLa-EGF-FVIIa, it will be demonstrated that binding to collagen IV
increases hemostatic activity in vivo.

[0236] Determination of whether part of the mechanism of FVIIa clearance is AT inhibition of TF-bound FVIIa. First,
levels of circulating FVIIa-AT complexes will be measured in plasma of mice expressing FVIIa chimeras. Second, mouse
chimeric protein will be infused via saphenous vein catheter into hem B mice, plasma FVIIa will be measured as a function
of time, and a determination will be made of what fraction of circulating FVIIa is in complex with AT. Antibodies for
mouse AT are commercially available (Haematologic Technologies) and the inventors have antibodies to mouse FVIIa
CAT domain that interact both with FVIIa and with all of the chimeras. Assay conditions and a standard curve will be
established by adding preformed mouse FVIIa-AT complexes (FVIIa expressed as described herein and AT from
Enzyme Research Labs) to mouse plasma. Background levels of FVIIa-AT will be measured in wt mice, and statistical
analysis will be performed as described herein.

[0237] These experiments will show circulating FVIIa-AT complexes at equilibrium in chimeric mice, and complexes
produced following chimerica infusion. If AT inhibition of FVIIa is mediated by TF binding, mice expressing FVIIa
molecules that cannot bind to TF (FIXGLa-EGF-FVIIa) would be expected to have lower circulating FVIIa-AT complexes
than mice expressing wtFVIIa, and may approach the background seen in mice that do not express FVIIa. It is similarly
anticipated that mice infused with FIXGLa-EGF-FVIIa will have reduced circulating FVIIa-AT complexes. If FVIIa-AT
levels are not reduced in these mice, it can be determined that FVIIa-AT formation in vivo is mediated by a TF-independent
mechanism.

[0238] Comparison of pharmacokinetics of chimeric and wtFVIIa in hem A dogs and comparison of hemostatic activ-
ity of chimeric and wtFVIIa. Human wtFVIIa and two of the FVIIa-kd chimeras will be prepared to infuse hem A dogs for
pharmacokinetic (PK) evaluation and assessment of pro-
thrombotic biomarkers and FVIIa inhibition by measuring TAT, D-dimer, and FVIIa-AT complex levels following infusion. An ARFI-monitored hemostatic challenge to measure the Time to Hemostasis (TTH) and area and rate of hemorrage will be used. It is anticipated that the FVIIa chimera will support novel ultrasound based ARFI-monitored TTH with minimal activation of coagulation (TAT or D-dimer).

[0239] It is anticipated that the studies described above will yield candidate FVIIa chimera with high hemostatic and yet low thrombogenic activity in murine models of hemostasis and thrombosis. FIX稳定剂-DGR/rFVIIa is currently the leading chimera because preliminary data show it exhibits similar hemostatic activity as wtFVIIa, but with reduced TF binding. Additional promising chimera for production are expected to be identified following the experiments described herein. These molecules will be expected to exhibit different PK profiles than wt FVII, due to their engineered, altered TF-, platelet-, and collagen IV-binding properties.

[0240] Comparison of pharmacokinetics of wt and chimeric FVIIa in a dog model of hem A. This will be a cross over study in four naïve hem A dogs comparing chimeric FVIIa with wtFVIIa.22-25 The choice of hem A dogs is based upon knowledge that the PK and pharmacodynamics (PD) of human wtFVIIa in dogs are similar to those in humans.23-25 Equal amounts of novel FVIIa chimeras or wtFVIIa will be administered intravenously in a cross over study. Dogs will be dosed with wtFVIIa on day 1. After the whole blood clotting time (WBCT) and thromboelastography (TEG) parameters have returned to baseline, chimeric FVIIa will be administered (day 3 or 4). Plasma will be collected at 5, 15, and 30 min, and 1, 2, 3, 4, 6, 8, 12, and 24 hours after dosing and analyzed for FVIIa activity, antigen, and FVIIa-AT complexes.24,26 Plasma FVIIa activity, total antigen-, and FVIIa-AT complex-time data will be analyzed by noncompartmental methods to recover robust estimates of standard PK outcomes, including maximum concentration, area under the curve, systemic clearance (CI), volume of distribution at steady state, and terminal half-life (t1/2). PK/PD compartmental models will be developed using data collected from these studies to understand the plasma FVIIa exposure-response relationship.27 These analyses will employ Phoenix WinNonlin (Pharsight, Cary, N.C.) as the primary modeling software.

[0241] A previous study of a single dose of wtFVIIa (5.4 mg/kg) in male dogs demonstrated a total antigen CI of ~73 mL/h/kg and a terminal t1/2 of 2.1 hours.26 The difference between total antigen- and FVIIa activity-derived CI was explained by complex formation with AT (60-70% of total CI in the initial phase, resulting in a distribution t1/2 of 0.71 hours. 26 Similar differences are anticipated between total antigen- and activity-derived parameters for wt and chimeric FVIIa.

[0242] Comparison of hemostatic activity of wt and chimeric FVIIa in a dog model of hem A. In vivo methods will be used to compare hemostatic efficacy and ex vivo methods will be used to monitor safety of the FVIIa chimeras versus wtFVIIa. Biomarkers of procoagulant activity from whole blood and plasma will be measured. The biomarkers include a complete blood count (leukocytes, platelets, hemoglobin), chemistry panel, aPTT, prothrombin time, fibrinogen levels, TAT, CAT, D-dimer, and fibrin degradation products, and screening inhibitor assessments.

[0243] Information from studies described above will be used to determine the optimum time for performing the ARFI hemostasis study; the optimum time would be when either wtFVIIa or chimeric protein has decreased to very low plasma antigen levels. This optimum time is anticipated to be between 2-4 hours after infusion of chimeric FVIIa, when it should reside predominantly in the extravascular space. At the nadir determined in the studies described above, hem A dogs will be infused with wt or chimeric FVIIa in a cross over fashion and the ARFI-monitored hemostatic challenge will be performed. Specifically, ARFI ultrasound will be used to monitor the TTH and area and rate of bleeding.22

[0244] The expectation is that FVIIa chimeras with decreased TF binding or enhanced collagen IV binding properties will exhibit hemostatic activity in vivo, even when their levels cannot be detected in plasma. This expectation would be supported by findings that the ARFI endpoint of the TTH (min) is significantly shortened in treated dogs compared to naïve hem A dogs, even at the plasma nadir. The secondary endpoints of area (mm²) and rate (mm²/sec) of hemostasis at this time point will also be measured and it is similarly expected that these parameters will be reduced in treated compared to naïve hem A dogs.

[0245] The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein. All publications, patent applications, patents, patent publications, sequences identified by GenBank® database and/or SNP accession numbers, and other references cited herein are incorporated by reference in their entirety for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

**TABLE 2**

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**REFERENCES FOR EXAMPLE IX**


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Gly Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
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Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  400
Ala Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  410
Asp Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  420
Gly Thr Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  430
Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  440
Leu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin
  450
<210> SEQ ID NO 5
<211> LENGTH: 458
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Chimeric Factor VII protein sequence

<400> SEQUENCE: 5

Met Lys His Leu Asn Thr Val Met Ala Glu Ser Pro Ala Leu Ile Thr
1 5 10 15
Ile Phe Leu Leu Gly Tyr Leu Leu Ser Thr Glu Cys Ala Val Phe Leu
20 25 30
Asp Arg Glu Asn Ala Thr Lys Ile Leu Thr Arg Pro Lys Arg Tyr Asn
35 40 45
Ser Gly Lys Leu Glu Glu Phe Val Arg Gly Asn Leu Glu Arg Glu Cys
50 55 60
Ile Glu Glu Arg Cys Ser Phe Glu Glu Ala Arg Glu Val Phe Glu Asn
65 70 75 90
Thr Glu Lys Thr Thr Glu Phe Trp Lys Glu Tyr Val Asp Gly Asp Gln
85 90 95
Cys Glu Ser Asn Pro Cys Leu Asn Gly Glu Ile Cys Lys Asp Asp Ile
100 105 110
Ser Ser Tyr Glu Cys Trp Cys Glu Val Gly Phe Glu Gly Arg Asn Cys
115 120 125
Glu Phe Ser Lys Asn Glu Gln Leu Ile Cys Ala Asn Glu Asn Gly Asp
130 135 140
Cys Asp Gln Tyr Cys Arg Asp His Val G1y Thr Lys Arg Thr Cys Ser
145 150 155 160
Cys His Glu Asp Tyr Thr Leu Gln Pro Asp Glu Val Ser Cys Lys Pro
165 170 175
Lys Val Glu Tyr Pro Cys Gly Arg Ile Pro Val Glu Lys Arg Asn
180 185 190
Ser Ser Ser Arg Glu Gly Arg Arg Arg Lys Arg Ile Val Gly
195 200 205
Gly Asn Val Cys Pro Lys Gly Glu Cys Pro Trp Gln Ala Val Leu Lys
210 215 220
Ile Asn Gly Leu Leu Cys Gly Ala Val Leu Leu Asp Ala Arg Trp
225 230 235 240
Ile Val Thr Ala Ala His Cys Phe Asp Asn Ile Arg Tyr Trp Gly Asn
245 250 255
Ile Thr Val Val Met G1y His Asp Phe Ser G1uy Asp Gly Asp
260 265 270
Glu Gln Val Arg Arg Val Thr Gln Val Ile Met Pro Asp Lys Tyr Ile
275 280 285
Arg Gly Lys Ile Asn His Asp Ile Ala Leu Leu Arg Leu His Arg Pro
290 295 300
Val Thr Phe Thr Asp Tyr Val Val Pro Leu Cys Leu Pro Glu Lys Ser
305 310 315 320
Phe Ser Glu Asn Thr Leu Ala Arg Ile Arg Phe Ser Arg Val Ser Gly
325 330 335
Trp Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Gly Leu Met Ser
340 345 350
<210> SEQ ID NO: 6
<211> LENGTH: 457
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<222> OTHER INFORMATION: Chimeric Factor VII protein sequence

<400> SEQUENCE: 6

Met Lys His Leu Asn Thr Val Met Ala Asp Ser Pro Ala Leu Ile Thr
1  5   10  15
Ile Phe Leu Leu Gly Tyr Leu Leu Ser Thr Glu Cys Ala Val Phe Leu
20  25  30
Asp Arg Glu Asn Ala Thr Lys Ile Leu Thr Arg Pro Lys Arg Ala Asn
35  40  45
Thr Leu Phe Glu Glu Thr Met Lys Gly Asn Leu Arg Glu Arg Glu Cys Ile
50  55  60
Glu Glu Leu Cys Asn Lys Glu Glu Ala Arg Val Phe Glu Asn Asn
65  70  75  80
Pro Glu Thr Asp Tyr Phe Tyr Lys Glu Tyr Val Asp Gly Asp Gly Cys
85  90  95
Glu Ser Asn Pro Cys Leu Asn Gly Gly Ile Cys Lys Asp Arg Asp Ile Ser
100 105 110
Ser Tyr Glu Cys Trp Cys Glu Val Gly Phe Glu Gly Arg Asn Cys Glu
115 120 125
Phe Ser Lys Asn Glu Gln Leu Ile Cys Ala Asn Glu Gly Asp Cys
130 135
Asp Gln Tyr Cys Arg Asp His Val Gly Thr Arg Thr Cys Ser Cys
145 150 155 160
His Glu Asp Tyr Thr Leu Gln Pro Asp Glu Val Ser Cys Lys Pro Lys
165 170 175
Val Glu Tyr Pro Cys Gly Arg Ile Pro Val Val Lys Arg Asn Ser
180 185 190
Ser Ser Arg Gln Gly Arg Lys Arg Arg Lys Arg Ile Val Gly Gly
195 200 205
Asn Val Cys Pro Lys Gly Gly Cys Pro Trp Gln Ala Val Leu Lys Ile
210 215 220
Asn Gln Leu Leu Leu Cys Gly Ala Val Leu Leu Asp Ala Arg Trp Ile
225 230 235 240

<Continued>
Val Thr Ala Ala His Cys Phe Asp Asn Ile Arg Tyr Trp Gly Asn Ile
245 250 255
Thr Val Val Met Gly Glu His Asp Phe Ser Glu Lys Asp Gly Asp Glu
260 265 270
Gln Val Arg Arg Val Thr Gln Val Ile Met Pro Asp Lys Tyr Ile Arg
275 280 285
Gly Lys Ile Asn His Asp Ile Ala Leu Leu Arg Leu His Arg Pro Val
290 295 300
Thr Phe Thr Asp Tyr Val Val Pro Leu Cys Leu Pro Glu Lys Ser Phe
305 310 315 320
Ser Glu Asn Thr Leu Ala Arg Ile Arg Phe Ser Arg Val Ser Gly Trp
325 330 335
Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Ser Ile
340 345 350
Glu Val Pro Arg Leu Met Thr Gln Asp Cys Leu Glu His Ala Lys His
355 360 365
Ser Ser Asn Thr Pro Lys Ile Thr Glu Ann Met Phe Cys Ala Gly Tyr
370 375 380
Met Asp Gly Thr Lys Asp Ala Cys Lys Gly Asp Ser Gly Gly Pro His
385 390 395 400
Ala Thr His Tyr His Gly Thr Trp Tyr Leu Thr Gly Val Val Ser Trp
405 410 415
Gly Glu Gly Cys Ala Ala Ile Gly His Ile Gly Val Tyr Thr Arg Val
420 425 430
Ser Gln Tyr Ile Asp Trp Leu Val Arg His Met Asp Ser Lys Leu Gln
435 440 445
Val Gly Val Phe Arg Leu Pro Leu Leu
450 455

<210> SEQ ID NO 7
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<400> SEQUENCE: 7
tgaggatccc caccatggtt ccacaggggc atgggct

<210> SEQ ID NO 8
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<400> SEQUENCE: 8
ttcccacagc tgcctacagt agtggaagt gcgaaac

<210> SEQ ID NO 9
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<400> SEQUENCE: 9
aacaatgcttt ttcgcgcggct acgggccgct tcggctgc tiggagt

<210> SEQ ID NO 10
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 10

gccgcgctaa ggcgggaaaa cgcattgtgg gaggaaccggt gtgcc

<210> SEQ ID NO 11
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 11

agccgctgaag tctccacaca tgaagcaccg gcaccggtc

<210> SEQ ID NO 12
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 12

gtcagctgc tcattcttgct tttttcaca gttctttcct tcaactc

<210> SEQ ID NO 13
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 13

gatttgaggg aaggaactgt gasaaagca agaatgagca gctgatc

<210> SEQ ID NO 14
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 14

gttotctcg acaaggtatt tgtctctttt ggacggttaa gaattttg

<210> SEQ ID NO 15
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer

<400> SEQUENCE: 15

caaaaatcctt aacgcctcaca agagagcaaa tacctgtttc gaagaaac
-continued-

<210> SEQ ID NO 16
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<400> SEQUENCE: 16

gatctcactc ascatactgc ttataaaaat aatcogtctc gggatt

<210> SEQ ID NO 17
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCR primer
<400> SEQUENCE: 17

aatccgaga ggattatcttt ttaagcag tattgagag gatc

<210> SEQ ID NO 18
<211> LENGTH: 466
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 18

Met Val Ser Gln Ala Leu Arg Leu Leu Leu Leu Gln Val Leu Cys Val Leu Leu Gln Val Leu Cys Leu Ala Ala Gly Val Ala Lys Ala Ser Gly Gly Gly Glu Thr Arg Asp Met Pro Trp Lys Pro Gly Pro His Arg Val Phe Val Thr Gln Glu Glu Ala His Gly Val Leu His Arg Arg Arg Ala Asn Ala Phe 1 5 10 15
Gly Cys Leu Ala Ala Gly Val Ala Lys Ala Ser Gly Gly Glu Thr Arg Asp Met Pro Trp Lys Pro Gly Pro His Arg Val Phe Val Thr Gln Glu Glu Ala His Gly Val Leu His Arg Arg Arg Ala Asn Ala Phe 50 55 60
Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys Lys Glu Glu 65 70 75 80
Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp Ala Glu Arg 95 90 95
Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln Cys Ala Ser 100 105 110
Ser Pro Cys Gln Asn Gly Ser Ser Cys Lys Asp Gln Leu Gin Ser Tyr 115 120 125
Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys Glu Thr His 130 135 140
Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly Gly Cys Glu 145 150 155 160
Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg Cys His Glu 165 170 175
Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro Thr Val Glu 180 185 190
Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn Ala Ser Lys 195 200 205
Pro Gin Gly Arg Ile Val Gly Lys Val Cys Pro Lys Gly Glu Cys 210 215 220
Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gin Leu Cys Gly Glu 225 230 235 240
Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His Cys Phe Asp 245 250 255
Lys Ile Lys Asn Thr Arg Asn Leu Ile Ala Val Leu Gly Glu His Asp 260 265 270
Leu Ser Glu His Asp Gly Asp Glu Gin Ser Arg Val Ala Gin Val 275 280 285
Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His Asp Ile Ala 290 295 300
Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His Val Val Pro 305 310 315 320
Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu Ala Phe Val 325 330 335
Arg Phe Ser Leu Val Ser Gly Trp Gly Gin Leu Leu Asp Arg Gly Ala 340 345 350
Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu Met Thr Gln 355 360 365
Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro Asn Ile Thr 370 375
Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys 385 390 395 400
Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp 405 410 415
Tyr Leu Thr Gly Ile Val Ser Trp Gly Gin Gin Cys Ala Thr Val Gln 420 425 430
His Phe Gly Val Tyr Thr Arg Ser Gin Tyr Ile Glu Trp Leu Gln 435 440 445
Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro 450 455 460
Phe Pro 465

<210> SEQ ID NO 19
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 19
Met Glu Arg Val Asn Met Ile Met Ala Glu Ser Pro Ser Leu Ile Thr
1  5  10  15
Ile Cys Leu Leu Gly Tyr Leu Ser Ala Glu Cys Thr Val Phe Leu 20  25  30
Asp His Glu Asn Ala Asn Lys Ile Leu Asn Arg Pro Lys Arg Tyr Asn 35  40  45
Ser Gly Lys Leu Glu Phe Val Gin Gly Asn Leu Glu Arg Glu Cys 50  55  60
Met Glu Glu Lys Cys Ser Phe Glu Glu Pro Arg Glu Val Phe Glu Asn 65  70  75  80
Thr Glu Lys Thr Thr Glu Phe Trp Lys Gin Tyr Val Asp Gly Asp Gin 95  100  105  110
Cys Glu Ser Asn Pro Cys Leu Asn Gly Gly Ser Cys Lys Asp Asp Ile 115  120  125
Asn Ser Tyr Glu Cys Trp Cys Pro Phe Gly Phe Glu Gly Lys Asn Cys
Glu Leu Asp Val Thr Cys Asn Ile Lys Asn Gly Arg Cys Glu Gln Phe
130  135  140
Cys Lys Asn Ser Ala Arg Asn Val Val Cys Ser Cys Thr Glu Gly
145  150  155  160
Tyr Arg Leu Ala Glu Asn Glu Lys Ser Cys Glu Pro Ala Val Pro Phe
165  170  175
Pro Cys Gly Arg Val Ser Val Ser Gln Thr Ser Lys Leu Thr Arg Ala
180  185  190
Glu Ala Val Phe Pro Asp Val Asp Tyr Val Asn Pro Thr Glu Ala Glu
195  200  205
Thr Ile Leu Asp Arg Ile Thr Gln Gly Thr Gin Ser Phe Asp Arg Phe
210  215  220
Thr Arg Val Val Gly Gly Glu Asp Ala Lys Pro Gly Gin Phe Pro Trp
225  230  235  240
Gln Val Val Leu Asn Gly Lys Val Asp Ala Phe Cys Gly Gly Ser Ile
245  250  255
Val Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Glu Thr Gly
260  265  270
Val Lys Ile Thr Val Ala Gly Glu Hist Asn Ile Glu Glu Thr Glu
275  280  285
His Thr Glu Gin Lys Arg Asn Val Ile Arg Ile Pro His His Asn
290  295  300
Tyr Asn Ala Ala Ile Asn Lys Tyr Asn His Asp Ile Ala Leu Leu Glu
305  310  315  320
Leu Asp Glu Pro Leu Val Leu Asn Ser Tyr Val Thr Pro Ile Cys Ile
325  330  335
 Ala Asp Lys Glu Tyr Thr Asn Ile Phe Leu Lys Phe Gly Ser Gly Tyr
340  345  350
Val Ser Gly Trp Ala Arg Val Phe His Lys Gly Arg Ser Ala Leu Val
355  360  365
Leu Gin Tyr Leu Arg Val Leu Val Asp Arg Ala Thr Cys Leu Arg
370  375  380
Ser Thr Lys Phe Thr Ile Tyr Asn Asn Met Phe Cys Ala Gly Phe His
385  390  395  400
Glu Gly Gly Arg Asp Ser Cys Gin Gly Asp Ser Gly Gly Pro His Val
405  410  415
Thr Glu Val Glu Gly Thr Ser Phe Leu Thr Gly Ile Ile Ser Trp Gly
420  425  430
Glu Glu Cys Ala Met Lys Gly Lys Tyr Gly Ile Tyr Thr Lys Val Ser
435  440  445
Arg Tyr Val Asn Trp Ile Lys Glu Lys Thr Lys Leu Thr
450  455  460

<210> SEQ ID NO 20
<211> LENGTH: 676
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 20
Met Arg Val Leu Gly Gly Arg Cys Gly Ala Pro Leu Ala Cys Leu Leu 1  5  10  15
Leu Val Leu Pro Val Ser Glu Ala Asn Phe Leu Ser Lys Gln Gln Ala
Lys Val Glu Ser Glu Leu Ile Lys Pro Ile Asn Pro Arg Leu Asp Gly 435 440 445
Cys Ile Arg Ser Trp Asn Leu Met Lys Gln Gly Ala Ser Gly Ile Lys 450 455 460
Glu Ile Ile Gln Glu Lys Gln Asn Lys His Cys Leu Val Thr Val Glu 465 470 475 480
Lys Gly Ser Tyr Tyr Pro Gly Ser Gly Ile Ala Gln Phe His Ile Asp 485 490 495
Tyr Asn Asn Val Ser Ser Ala Glu Gly Trp His Val Asn Val Thr Leu 500 505 510
Asn Ile Arg Pro Ser Thr Gly Thr Gly Val Met Leu Ala Leu Val Ser 515 520 525
Gly Asn Asn Thr Val Pro Phe Ala Val Ser Leu Val Asp Ser Thr Ser 530 535 540
Glu Lys Ser Gln Asp Ile Leu Leu Ser Val Glu Asn Thr Val Ile Tyr 545 550 555 560
Arg Ile Gln Ala Leu Ser Leu Cys Ser Asp Gln Gln Ser His Leu Glu 565 570 575
Phe Arg Val Asn Arg Asn Leu Glu Leu Ser Thr Pro Leu Lys Ile 580 585 590
Glu Thr Ile Ser His Glu Asp Leu Gln Arg Gln Leu Ala Val Leu Asp 595 600 605
Lys Ala Met Lys Ala Lys Val Ala Thr Tyr Leu Gly Gly Leu Pro Asp 610 615 620
Val Pro Phe Ser Ala Thr Pro Val Asn Ala Phe Tyr Asn Gly Cys Met 625 630 635 640
Glu Val Asn Ile Asn Gly Val Val Leu Asp Leu Asp Glu Ala Ile Ser 645 650 655
Lys His Asn Asp Ile Arg Ala His Ser Cys Pro Ser Val Trp Lys Lys 660 665 670
Thr Lys Asn Ser 675

<210> SEQ ID NO 21
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Secretion signal sequence
<400> SEQUENCE: 21
Arg Lys Arg Arg Lys Arg

<210> SEQ ID NO 22
<211> LENGTH: 488
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 22
Met Gly Arg Pro Leu His Leu Val Leu Leu Ser Ala Ser Leu Ala Gly 1 5 10 15
Leu Leu Leu Gly Glu Ser Leu Phe Ile Arg Arg Glu Gln Ala Asn 20 25 30
Asn Ile Leu Ala Arg Val Thr Arg Ala Asn Ser Phe Leu Glu Glu Met 35 40 45
Lys Lys Gly His Leu Glu Arg Glu Cys Met Glu Glu Thr Cys Ser Tyr 50 55 60
Glu Glu Ala Arg Glu Val Phe Glu Asp Ser Asp Lys Thr Arg Glu Phe 65 70 75 80
Trp Asn Lys Tyr Lys Asp Gly Asp Gln Cys Glu Thr Ser Pro Cys Gln 85 90 95
Asn Glu Gly Lys Cys Lys Asp Gly Leu Gly Glu Tyr Thr Cys Thr Cys 100 105 110
Leu Glu Gly Phe Glu Gly Lys Asn Cys Glu Leu Phe Thr Arg Lys Leu 115 120 125
Cys Ser Leu Asp Asn Gly Asp Cys Asp Gln Phe Cys Asp His Glu Glu Gln 130 135 140
Asn Ser Val Val Cys Ser Cys Ala Arg Gly Tyr Thr Leu Ala Asp Asn 145 150 155 160
Gly Lys Ala Cys Ile Pro Thr Gly Pro Tyr Pro Cys Gly Lys Gin Thr 165 170 175
Leu Glu Arg Arg Arg Ser Val Ala Gin Ala Thr Ser Ser Ser Gly 180 185 190
Glu Ala Pro Asp Ser Ile Thr Thr Lys Pro Tyr Asp Ala Ala Asp Leu 195 200 205
Asp Pro Thr Glu Asn Pro Phe Asp Leu Asp Phe Asn Gin Thr Gin 210 215 220
Pro Glu Arg Gly Asp Ala Asn Leu Thr Arg Ile Val Gly Gly Gln Gin 225 230 235 240
Cys Lys Asp Gly Glu Cys Pro Trp Gin Ala Leu Leu Ile Asn Glu Glu 245 250 255
Asn Glu Gly Phe Cys Gly Gly Thr Ile Leu Ser Glu Phe Tyr Ile Leu 260 265 270
Thr Ala Ala His Cys Leu Tyr Gin Ala Lys Arg Phe Lys Val Arg Val 275 280 285
Gly Asp Arg Asn Thr Glu Gin Glu Glu Gly Gln Ala Val His Glu 290 295 300
Val Glu Val Val Ile Lys His Arg Phe Thr Lys Glu Thr Tyr Asp 305 310 315 320
Phe Asp Ile Ala Val Leu Arg Leu Lys Thr Pro Ile Thr Phe Arg Met 325 330 335
Asn Val Ala Pro Ala Cys Leu Pro Glu Arg Asp Trp Ala Glu Ser Thr 340 345 350
Leu Met Thr Gin Lys Thr Gly Ile Val Ser Gly Phe Gly Arg Thr His 355 360 365
Glu Lys Gly Arg Gin Ser Thr Arg Leu Lys Met Leu Glu Val Pro Tyr 370 375 380
Val Asp Arg Asn Ser Cys Lys Leu Ser Ser Ser Phe Ile Ile Thr Gin 385 390 395 400
Asn Met Phe Cys Ala Gly Tyr Asp Thr Lys Glu Gin Glu Asp Ala Cys Gin 405 410 415
Gly Asp Ser Gly Gly Pro His Val Thr Arg Phe Gly Asp Thr Tyr Phe 420 425 430
Val Thr Gly Ile Val Ser Trp Gly Gly Cys Ala Arg Lys Gly Lys
<210> SEQ ID NO: 23
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

Met Trp Glu Leu Thr Ser Leu Leu Phe Val Ala Thr Trp Gly Ile 1 5 10 15
Ser Gly Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg 20 25 30
Ala His Glu Val Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu 35 40 45
Glu Leu Arg His Ser Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys 50 55 60
Asp Phe Glu Glu Ala Lys Glu Ile Phe Glu Asn Val Asp Thr Leu 65 70 75 80
Ala Phe Trp Ser Lys His Val Asp Gly Glu Cys Leu Val Leu Pro 85 90 95
Leu Glu His Pro Cys Ala Ser Leu Cys Gly His Gly Thr Cys Ile 100 105 110
Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp Glu Gly 115 120 125
Arg Phe Cys Glu Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn 130 135 140
Gly Gly Cys Thr His Tyr Cys Leu Glu Val Gly Trp Arg Arg Cys 145 150 155 160
Ser Cys Ala Pro Gly Tyr Lys Leu Asp Asp Leu Leu Glu Cys His 165 170 175
Pro Ala Val Lys Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys 180 185 190
Lys Arg Ser His Leu Lys Arg Thr Glu Asp Glu Glu Asp Glu Val 195 200 205
Asp Pro Arg Leu Ile Asp Gly Lys Met Thr Arg Arg Gly Asp Ser Pro 210 215 220
Trp Glu Val Val Leu Asp Ser Lys Lys Leu Ala Cys Gly Ala 225 230 235 240
Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala His Cys Met Asp 245 250 255
Glu Ser Lys Lys Leu Val Arg Leu Gly Glu Tyr Asp Leu Arg Arg 260 265 270
Trp Glu Lys Trp Glu Leu Asp Ile Lys Glu Val Phe Val His 275 280 285
Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Ile Ala Leu Leu His 290 295 300
Continued

Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu
305 310 315 320
Pro Asp Ser Gly Leu Ala Glu Ala Leu Asn Gln Ala Gln Glu Glu
325 330 335
Thr Leu Val Thr Gly Trp Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
340 345 350
Lys Arg Asn Arg Thr Phe Val Leu Asn Phe Ile Lys Ile Pro Val Val
355 360 365
Pro His Asn Glu Cys Ser Glu Val Met Ser Asn Met Val Ser Glu Asn
370 375 380
Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp Ala Cys Glu Gly
385 390 395 400
Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp Phe Leu
405 410 415
Val Gly Leu Val Ser Trp Gly Glu Gln Val Gly Leu Leu His Aen Tyr
420 425 430
Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gln His
435 440 445
Ile Arg Asp Lys Gln Ala Pro Gin Lys Ser Trp Ala Pro
450 455 460

<210> SEQ ID NO 24
<211> LENGTH: 721
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 24

Met Ala Pro Ser Leu Ser Pro Gly Pro Ala Ala Leu Arg Arg Ala Pro
1 5 10 15
Gln Leu Leu Leu Leu Leu Leu Ala Ala Glu Cys Ala Leu Ala Ala Leu
20 25 30
Leu Pro Ala Arg Glu Ala Thr Gln Phe Leu Arg Pro Arg Gln Arg Arg
35 40 45
Ala Phe Gln Val Phe Glu Ala Lys Gln Gly His Leu Glu Glu Glu
50 55 60
Cys Val Glu Glu Leu Cys Ser Arg Glu Glu Ala Arg Glu Ala Val Phe Glu
65 70 75 80
Asn Asp Pro Glu Thr Asp Tyr Phe Tyr Pro Arg Tyr Leu Asp Cys Ile
85 90 95
Asn Lys Tyr Gly Ser Pro Tyr Thr Lys Asn Ser Gly Phe Ala Thr Cys
100 105 110
Val Gln Asn Leu Pro Asp Gln Cys Thr Pro Asn Pro Cys Asp Arg Lys
115 120 125
Gly Thr Gln Ala Cys Gln Asp Thr Met Gln Asp Phe Phe Cys Leu Cys
130 135 140
Lys Ala Gly Trp Gly Gly Arg Leu Cys Asp Lys Asp Val Asn Glu Cys
145 150 155 160
Ser Gin Glu Asn Gly Lys Leu Ile Cys His Asn Lys Pro Gly
165 170 175
Ser Phe His Cys Ser Cys His Ser Gly Phe Glu Leu Ser Ser Asp Gly
180 185 190
Arg Thr Cys Gin Asp Ile Asp Glu Cys Ala Asp Ser Glu Ala Cys Gly
195 200 205
-continued

Glu Ala Arg Cys Lys Asn Leu Pro Gly Ser Tyr Ser Cys Leu Cys Asp
210  215  220
Glu Gly Phe Ala Tyr Ser Ser Gln Glu Lys Ala Cys Arg Asp Val Asp
225  230  235  240
Glu Cys Leu Gln Gly Arg Cys Gln Glu Val Cys Val Asn Ser Pro Gly
245  250  255
Ser Tyr Thr Cys His Cys Asp Gly Arg Gly Gln Leu Lys Leu Ser Gln
260  265  270
Asp Met Asp Thr Cys Glu Leu Glu Ala Gly Trp Pro Cys Pro Arg His
275  280  285
Arg Arg Asp Gly Ser Pro Ala Ala Ala Arg Pro Gly Arg Gly Ala Gln Gly
290  295  300
Ser Arg Ser Glu His His Pro Arg Arg Arg Gly Pro Arg Pro Trp
305  310  315  320
Gln Asp Ile Leu Pro Cys Val Pro Phe Ser Val Ala Lys Ser Val Lys
325  330  335
Ser Leu Tyr Leu Gly Arg Met Phe Ser Gly Thr Pro Val Ile Arg Leu
340  345  350
Arg Phe Lys Arg Leu Gln Pro Thr Arg Leu Val Ala Glu Phe Asp Phe
355  360  365
Arg Thr Phe Asp Pro Glu Gly Ile Leu Leu Phe Ala Gly Gly His Gln
370  375  380
Asp Ser Thr Trp Ile Val Leu Ala Ala Ala Gly Arg Leu Glu Leu
385  390  395  400
Gln Leu Arg Tyr Asp Gly Val Gly Arg Val Thr Ser Ser Gly Pro Val
405  410  415
Ile Asn His Gly Met Trp Gln Thr Ile Ser Val Glu Glu Leu Ala Arg
420  425  430
Asn Leu Val Ile Lys Val Asn Arg Asp Ala Ala Val Met Lys Ile Ala Val
435  440  445
Ala Gly Asp Leu Phe Gln Pro Glu Arg Gly Leu Tyr His Leu Asn Leu
450  455  460
Thr Val Gly Ile Pro Phe His Glu Lys Asp Leu Val Gln Pro Ile
465  470  475  480
Asn Pro Arg Leu Asp Gly Cys Met Arg Ser Thr Pro Cys Trp Leu Asn Gly
485  490  495
Glu Asp Thr Thr Ile Gln Glu Thr Val Lys Val Asn Thr Arg Met Gln
500  505  510
Cys Phe Ser Val Thr Glu Arg Gly Ser Phe Tyr Pro Gly Ser Gly Phe
515  520  525
Ala Phe Tyr Ser Leu Asp Tyr Met Arg Thr Pro Leu Asp Val Gly Thr
530  535  540
Glu Ser Thr Trp Glu Val Val Ala Ala His Ile Arg Pro Ala Ala
545  550  555  560
Asp Thr Gly Val Leu Phe Ala Leu Trp Ala Pro Asp Leu Arg Ala Val
565  570  575
Pro Leu Ser Val Ala Leu Val Asp Tyr His Ser Thr Lys Lys Leu Lys
580  585  590
Lys Glu Leu Val Val Leu Ala Val Glu His Thr Ala Leu Ala Leu Met
595  600  605
What is claimed is:

1. A method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a chimeric Factor VIIa polypeptide with a prolonged functional lifespan in vivo or prolonged functional half life in vivo as compared with a non-chimeric Factor VIIa polypeptide.

2. The method of claim 1, wherein the chimeric Factor VIIa polypeptide further comprises a domain that mediates binding of a protein to a structural molecule of the extravascular tissue such as a basement membrane component.

3. The method of claim 2, wherein the domain is selected from the group consisting of:
   a) a single chain antibody Fab fragment or single chain variable fragment (scFv) that specifically binds a structural molecule of the extravascular tissue such as a basement membrane component;
   b) a laminin binding domain of a laminin binding receptor;
   c) a laminin binding domain of a proteoglycan;
   d) a laminin binding domain of a bacterial adhesin protein;
   e) a nidogen/entactin binding domain;
   f) a collagen type 4 binding domain; and
   g) any combination of (a)-(f) above.

4. The method of claim 1, wherein the chimeric Factor VIIa polypeptide further comprises a GLA domain of a vitamin K dependent coagulation protein.

5. The method of claim 4, wherein the GLA domain is a Factor IX GLA domain or a Protein S GLA domain.

6. The method of claim 1, wherein the chimeric Factor VIIa polypeptide further comprises an EGF-1 domain of a vitamin K dependent coagulation protein.

7. The method of claim 6, wherein the EGF-1 domain is a Factor IX EGF-1 domain, a Protein S EGF-1 domain, a Factor X EGF-1 domain, a Factor VII EGF-1 domain, a Protein C EGF-1 domain or a Gas 6 EGF-1 domain.

8. The method of claim 5, wherein the chimeric Factor VIIa polypeptide further comprises an EGF-1 domain of Factor IX or an EGF-1 domain of Protein S or an EGF-1 domain of Factor X.

9. The method of claim 5, wherein the GLA domain is a Factor IX GLA domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by arginine.

10. The method of claim 8, wherein the GLA domain is a Factor IX GLA domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by arginine.

11. The method of claim 5, wherein the GLA domain is a Factor IX GLA domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by alanine and/or a substitution of valine at residue 56 in the amino acid sequence of SEQ ID NO:19 by another amino acid.

12. The method of claim 8, wherein the GLA domain is a Factor IX GLA domain comprising a substitution of lysine at residue 51 in the amino acid sequence of SEQ ID NO:19 by alanine and/or a substitution of valine at residue 56 in the amino acid sequence of SEQ ID NO:19 by another amino acid.

13. The method of claim 7, wherein the EGF-1 domain is a Factor VII EGF-1 domain comprising a substitution of isoleucine at residue 129 in the amino acid sequence of SEQ ID NO:18 by alanine.

14. The method of claim 7, wherein the EGF-1 domain is a Factor VII EGF-1 domain comprising a substitution of arginine at residue 139 in the amino acid sequence of SEQ ID NO:18 by alanine.

15. The method of claim 8, wherein the catalytic domain of Factor VII comprises a substitution of methionine at residue 366 of the amino acid sequence of SEQ ID NO:18 with alanine, valine or isoleucine.

16. The method of claim 8, wherein the catalytic domain of Factor VII comprises a substitution of valine at residue 218 of the amino acid sequence of SEQ ID NO:18 with aspartate.

17. The method of claim 8, wherein the catalytic domain of Factor VII comprises a substitution of glutamate at residue 356 of the amino acid sequence of SEQ ID NO:18 with valine.

18. The method of claim 8, wherein the catalytic domain of Factor VII comprises a substitution of methionine at residue 358 of the amino acid sequence of SEQ ID NO:18 with glutamine.
19. The method of claim 8, wherein the chimeric Factor VIIa polypeptide further comprises a domain that mediates binding of a protein to a structural molecule of the extravascular tissue such as a basement membrane component.

20. The method of claim 19, wherein the domain is selected from the group consisting of:
   a) a single chain antibody Fab fragment or single chain variable fragment (scFv) that specifically binds a structural molecule of the extravascular tissue such as a basement membrane component;
   b) a laminin binding domain of a laminin binding receptor;
   c) a laminin binding domain of a proteoglycan;
   d) a laminin binding domain of a bacterial adhesin protein;
   e) a nidogen/entactin binding domain;
   f) a collagen type 4 binding domain; and
   g) any combination of (a)-(f) above.

21. The method of claim 1, wherein the bleeding disorder is selected from the group consisting of: a clotting factor deficiency; defective platelet function; thrombocytopenia; von Willebrand’s disease; inhibition of clotting factors; bleeding induced by surgery; bleeding induced by trauma and any combination thereof.

22. The method of claim 21, wherein the bleeding disorder is a clotting factor deficiency.

23. The method of claim 22, wherein the clotting factor deficiency is hemophilia.

24. The method of claim 1, wherein treating the bleeding disorder comprises administering to the subject a nucleic acid molecule comprising a nucleotide sequence encoding the chimeric Factor VIIa polypeptide.

25. A method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has an increased affinity for tissue factor as compared with a non-modified Factor VIIa polypeptide.

26. A method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has an increased binding affinity for a basement membrane component as compared with a non-modified Factor VIIa polypeptide.

27. A method of treating a bleeding disorder in a subject in need thereof, comprising administering to the subject an effective amount of a modified Factor VIIa polypeptide that has a reduced clearance rate from the body of the subject or reduced binding to Factor VIIa protease inhibitors that inactivate FVIIa protease activity or facilitate the clearance of bound FVIIa in the body of the subject as compared with a non-modified Factor VIIa polypeptide.

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