The Expression of Coagulation Factors During Murine Development

by

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Abstract

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The interrelationship between hemostasis and vascular development is investigated in this study. Our current understanding of the regulation of blood coagulation is based primarily on postnatal mammalian physiology. In contrast, little is known about the regulation of blood coagulation during prenatal development, and in particular, during the formation of blood vessels.

Murine embryonic coagulation was examined by determining the temporal expression of coagulation factors during development using semi-quantitative RT-PCR. These results were then compared temporally to known events occurring in vascular development. In addition, the differentiation of embryonic stem cells was assessed as an *in vitro* model for embryonic coagulation.

We report, that while most coagulation-related factors are expressed coordinately, a number of factors are expressed in a temporally discordant fashion. In addition, several factors are expressed very early, prior to the onset of vascular development. These findings not only suggest that embryonic coagulation may be distinct from its adult counterpart, but that at least some factors have alternative developmental functions not necessarily associated with coagulation.

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Abbreviations

α_2AP	Alpha-2 antiplasmin
AGM region	Para-aortic splanchnopleure comprising the dorsal aorta, gonads and mesonephros
ATIII	Antithrombin III
BSLB4	*Bandeiraea simplicifolia* lectin
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic dcid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpc	days post coitum
DTT	dithiothreitol
EB	Embryoid body
EPCR	Endothelial protein C receptor
ES Cell	Embryonic stem cell
FCS	Fetal calf serum
FIBα	Fibrinogen alpha-chain
FII	Factor II/Prothrombin
FIX	Factor IX
FV	Factor V
FVII	Factor VII

FVIII	Factor VIII
FX	Factor X
FXII	Factor XII
FXIIIB	Factor XIII B subunit
GPIb	Glycoprotein Ib
HPRT	Hypoxanthine phosphoribosyl transferase
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	Isopropyl-thio-beta-D-galactoside
LB medium	Luria-Bertani medium
LIF	Leukemia inhibitory factor
MCM	Methylcellulose medium
MTG	monothioglycerol
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAR-1	Protease-activated receptor-1/Thrombin receptor
PAR-3	Protease-activated receptor-3/Thrombin receptor-2
PBS	Phosphate-buffered saline
PC	Protein C
PCR	Polymerase chain reaction
PLAS	Plasminogen
PS	Protein S
RNA	ribonucleic acid

RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
SDS	sodium dodecyl sulfate
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
t-PA	Tissue plasminogen activator
u-PA	Urokinase plasminogen activator
u-PAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

CHAPTER ONE: Introduction

1.1 Introduction

The purpose of this study is to investigate the developmental interrelationship that exists between the formation of blood vessels and hemostasis. We believe that these two processes must be coordinated during development as neither hemorrhagic nor thrombotic events are observed when developing blood vessels undergo extensive remodelling and reshaping, including the opening and closing of channels and redirection of blood circulation. Furthermore, our current understanding of the regulation of blood coagulation is founded exclusively on hemostatic reactions observed in adult mammalian physiology. In contrast, little is known about how the coagulation system is established and regulated during embryonic and fetal development.

To begin, an in-depth background of blood vessel development – including their embryonic origins and the mechanisms involved in their formation and stabilization – will be presented. Following this, the processes involved in maintaining hemostatic balance – thrombin generation, clot formation and fibrinolysis – will be explained. In addition, the effects of gene disruptions of specific coagulation factors will be discussed. Noticeably, from the past work presented in our introduction, vascular development and blood coagulation have, to date, only been studied independently. However, these prior observations will provide the initial framework from which we can investigate the developmental relationship between blood coagulation and the formation of blood vessels. Furthermore, an increasing body of evidence suggests that blood coagulation in the embryo may be distinct from that of the adult.

We begin our analysis of embryonic blood coagulation by investigating the temporal expression of a cohort of coagulation factors during murine development. We will examine by semi-quantitative RT-PCR, the expression patterns of these factors in mouse embryos ranging in age from 7.5-14.5 dpc and compare our findings with known developmental events occurring in vascular development. In addition, we will explore the usefulness of the *in vitro* differentiation of embryonic stem cells into embryoid bodies as a model for embryonic coagulation. While this model system has been shown to recapitulate *in vivo* conditions for processes such as hematopoiesis and vascular development, its potential as a model for developmental blood coagulation has not been evaluated. We will characterize the expression of coagulation factors in developing embryoid bodies, and compare these results with the expression of coagulation factors *in vivo*. The limitations of this system will be discussed in the third section of this thesis.

While coordinate developmental expression of coagulation factors is anticipated from our understanding that most coagulation factors must act in concert with each other to maintain hemostatic balance, we will show that there exists disparate temporal expression among some factors. Furthermore, we report the early expression of some coagulation factors, prior to vascular development. Our results show that our understanding of the coagulation process is incomplete, and illustrates particularly, our limited knowledge of how the coagulation system is established during development. Our observations not only suggest that embryonic coagulation is distinct from adult hemostasis, but imply that a number of coagulation factors have alternative roles in

development not necessarily associated with their coagulant functions. These claims will be discussed extensively in Chapter 3.

Documentation of the temporal expression patterns of coagulation factors in murine development has potentially far-reaching applications in the near future. A better understanding of the roles that coagulation proteins play during embryonic development may provide insights into pathophysiological processes that occur postnatally such as revascularization during ischemia, tumour growth, wound healing or inflammation. New targeted therapeutic strategies may thereby be devised to, for example, improve healing following surgery or possibly promote the growth of circulatory pathways around blocked blood vessels. Similarly, specific measures may be devised to interfere with the neovascularization associated with tumour growth.

1.2 Vascular Development

1.2.1 Embryonic Origins of Blood Vessels

Embryonic development at its early stages, including the development of organ systems, begins in the absence of vascularization.[1] However, the embryo is subsequently transformed rapidly from an avascular organism to one in which virtually every tissue is supported by functional blood vessels and primitive capillary plexuses.[2] Initial overt signs of blood vessel development appear outside the embryo, on the yolk sac, with the development of focal aggregations of mesenchymal cells within the splanchnic mesoderm adjacent to the extraembryonic endoderm.[1,3] These aggregations are known as blood islands largely because they are characterized by the early onset of hemoglobin synthesis[2]. Within each blood island, central cells segregate from those at the perimeter; the former constitute embryonic hematopoietic precursors and the latter endothelial cells.[1] Vascular channels are established as adjacent blood islands fuse, giving rise to a primary vascular plexus.[1,3] Although it was originally postulated that all blood vessels resulted from the elongation, branching and growth of extraembryonic precursors into the embryo proper[4], evidence of intraembryonic vascular origins has been supplied by Reagan[5] who showed that endothelial channels still formed in embryos that were surgically separated from the yolk sac. The cells giving rise to intraembryonic vessels are more commonly referred to as angioblasts, first described by His as individual mesenchymal cells committed to the endothelial lineage but not yet incorporated into endothelial tissue.[4] Angioblasts have also been defined by other early investigators to be endothelial precursor intermediates lying between the stage in which preendothelial cells

differentiate from the mesoderm and the stage in which they have aggregated into the basic endothelial monolayers of the blood vessel.[6,7]

While endothelial cells arise independently intra- and extraembryonically, these two systems of vessels subsequently fuse to form a contiguous circulatory system. After the onset of circulation, the primary plexuses are remodeled many times until a mature vascular system is formed.[8] The assembly processes are dynamic and include the formation of redundant channels and redirection and reversal of blood flow.[2,3] While some channels diminish and disappear, others enlarge to become principal vessels.[2] Thus, a vascular pathway, which at one point in development can play an integral role in circulation, may become obsolete and be replaced by other newly forming vessels.[2]

1.2.2 Mechanisms of Vascularization

Blood vessels can form and grow via two mechanisms, angiogenesis and vasculogenesis. Angiogenesis is a process by which new blood vessels arise from preexisting vessels through vascular sprouting and budding.[9,10] Vasculogenesis on the other hand, is used to describe the development of blood vessels by the differentiation *in situ* of endothelial cells.[9-11] While vasculogenesis seems to be restricted to embryonic development, angiogenesis can occur throughout the entire lifespan of the organism.[3] In the adult, neovascularization observed both in physiological conditions, such as the ovarian corpus luteum cycle and wound healing[3], and in pathological events such as tumour angiogenesis arises by an angiogenic mechanism.[12,13,14] Recently, studies using species-specific monoclonal antibodies (QH-1, MB-1) against quail endothelial cells

together with chick-quail transplantation chimeras have increased our understanding of the origin, assembly and migration of angioblasts, and confirmed the existence of intraembryonic origins of blood vessels.[11,15,16] Of particular interest is the migratory capacity of angioblasts, thought to be directed by local environmental cues.[16] Angioblasts are capable of migrating as individual cells, small groups, or sprouts. An understanding of the sites of angioblast migration as well as an understanding of the cues directing their migrations will help us to determine how the initial vascular pattern is established.[16] Studies using avian embryo chimeras have also shown effectively that vasculogenesis and angiogenesis are distinct mechanisms and that the type of vascularization occurring in developing organ rudiments largely depends on the germ layer from which they originated. Notably, organs derived from the splanchnopleura, that is, mesoderm associated with the endoderm, such as the lungs, pancreas and spleen, are vascularized by vasculogenesis[17], whereas organs of ectodermal or mesenchymal origins such as the brain and the kidney, seemed to be vascularized by an angiogenic mechanism.[17,18,19] The establishment of an experimental system to study murine vascular development, to determine whether the vasculogenesis and angiogenesis phenomena present in avian embryos also occurred in mouse embryos proved challenging because there were no specific histological markers for murine endothelium available, thus making the identification of murine angioblasts problematic. Coffin *et. al.* overcame this difficulty by performing double-labelling experiments on mouse embryos of varying ages using *Bandeiraea simplicifolia* lectin (BSLB4) and antisera to von Willebrand factor (vWF) as markers for angioblasts and endothelial cells.[20] vWF production is limited to

megakaryocytes and endothelial cells in several adult species[21] and BSLB4 was previously shown to label blood groups on endothelial cells.[22] Results from these double-labelling experiments not only demonstrated that the processes of vasculogenesis and angiogenesis were conserved between avian embryos and mammalian species but that angioblasts followed the predicted pattern for migration of mesodermal cells during gastrulation.[20,23]

1.2.3 Origins of Hematopoietic Cells During Development

The development of blood cells has been classically divided into two stages, namely primitive and definitive hematopoiesis. Primitive hematopoiesis occurs in the yolk sac blood islands (as mentioned above) and is characterized by erythropoiesis that is erythropoietin-insensitive, and produces short-lived red blood cells that are nucleated and contain only embryonic globin.[24] At mid-gestation, there is a switch to definitive hematopoiesis which occurs in the fetal liver, spleen, and finally in the bone marrow. Definitive hematopoiesis produces multiple hematopoietic lineages[25], and in particular, definitive erythropoiesis is characterized by the erythropoietin-sensitive production of longer surviving anucleated red blood cells containing both fetal and adult globins.[24]

Currently, it is not clear how embryonic and fetal/adult hematopoiesis are related.[26,27] Traditionally, primitive and definitive hematopoiesis in mammals were believed to be directly related, in that hematopoietic stem cells arising from extraembryonic mesoderm in yolk sac blood islands were thought to migrate into the embryo proper, seeding the fetal liver, where they quickly expanded to yield populations

of stem, progenitor and mature cells.[28] In the mouse, for example, yolk sac hematopoiesis is observed between 7.5 and 10.5 dpc, the onset of circulation begins at around day 9, and at 11.5 dpc when the liver has differentiated sufficiently, it becomes the principal site of definitive hematopoiesis. Furthermore, developing organs such as the spleen, bone marrow, thymus, and lymph nodes also become sequentially populated by cells similar in type to those found in adult organs.[29]

However, recent evidence suggests that hematopoietic development is more complex than that of the "yolk sac origin/migration" model initially proposed.[28] In fact, mammalian blood development appears to parallel a "dual origin" model first described in birds.[30,31,32] While primitive hematopoiesis in birds also arises extraembryonically in the yolk sac, an intraembryonic source of definitive hematopoiesis exists from the para-aortic/mesonephric region.[31] An analogous situation has since been proven to exist in the mouse[33,34,35,36] where hematopoietic stem cells are seen to arise from para-aortic splanchnopleure, also know as the AGM region (an area comprising dorsal aorta, gonads and mesonephros). The relationship between hematopoietic cells from the AGM region and yolk sac hematopoiesis is not yet fully understood and the origin of cells that ultimately colonize the fetal liver requires further clarification. What has been established is the presence of both yolk sac- and AGM-derived hematopoietic colonies in the fetal liver. This suggests that a two-wave model of fetal liver colonization is perhaps a more accurate way of describing the events observed in the previous experiments, whereby the liver is initially seeded transiently by hematopoietic cells from the yolk sac and subsequently more definitively by cells from the AGM region.[37,38,39]

1.2.4 Common Progenitor for Hematopoietic Cells and Endothelial Cells

Vascularization and hematopoiesis are closely coordinated and regulated to permit blood cells to enter circulation immediately after terminal differentiation is accomplished. As previously stated, blood islands/angioblasts contain both endothelial and hematopoietic precursors. The notion that these two lineages might share a common progenitor is an attractive one and one that is not new. Many investigators have hypothesized the existence of such a cell, termed the hemangioblast, to be localized in the blood islands.[4,40,41,42] More recent evidence put forth in support of the hemangioblast comes from cell surface markers that are expressed on both endothelial and hematopoietic stem cells such as CD34[43,44,45] and PECAM-1[46,47]. In addition, several receptor tyrosine kinases (RTKs) are found to be specific to the endothelium and they include Flk-1.[48,49,50,51,52] Of particular interest is the expression of the vascular growth factor receptor Flk-1, which is expressed in early mesodermal cells, before any evidence of vasculogenesis or angiogenesis, making it a very good candidate to label endothelial cell precursors, including possibly the hemangioblast[50,51,53] Unfortunately, definitive proof for the existence of this progenitor remains elusive.

1.2.5 Role of Receptor Tyrosine Kinases in Vascular Development and Integrity

Major insights into the development of the vascular (and hematopoietic) system have come from the discovery of the "endothelial" kinases Tek[54,55,56], Tie[57,58,59,60,61], Flt-1[62,63], and Flk-1. The roles of these RTKs and their respective ligands, in vascular development have been determined largely by investigations of the temporal and spatial

expression patterns of the respective genes in mouse embryos as well as through knockout studies. Identifying specific ligands to respective RTKs has also increased our understanding of the signalling pathways that ultimately shape and stabilize the vascular system.

Role of Flk-1 and VEGF in Vascular Development

The expression of *flk-1* was first detected at 7.0 dpc in the ectoplacental cone, extraembryonic mesoderm of the future yolk sac and in embryonic mesoderm that will develop into the heart. By 12.5 dpc, it is clearly localized to all parts of the embryonic vasculature while being downregulated in the yolk sac.[56] *flk-1* deficient mice do not form blood vessels and die *in utero* between 8.5 and 9.5 days post coitum.[50] In fact, yolk-sac blood islands are absent and a decrease in hematopoetic precursors is also observed.[50] These results point to a defect early in endothelial and hematopoietic cell development. It has been proposed that cells lacking Flk-1 are unable to reach the correct location to form blood islands, although *flk-1* -/- cells are able to differentiate into erythroid and myeloid cells under defined *in vitro* conditions.[51,64] Taken together, these observations suggest that one of the roles of Flk-1 in primitive hematopoiesis may be to ensure that mesodermal precursors are precisely placed to respond to environmental cues leading to hematopoiesis.[51]

VEGF (vascular endothelial growth factor), a mitogen for endothelial cells[65,66] has been shown to be a ligand for Flk-1 through *in vitro* affinity binding experiments and through a strong correlation between the temporal and spatial expression pattern of *VEGF*

and *flk-1*.[56,49] Since VEGF acts through this receptor, it is not surprising that a homozygous *VEGF* deficiency leads to embryonic lethality at mid-gestation from severe impairment of vascular development.[67] What is surprising, is the discovery by two independent laboratories,[67,68] that mice heterozygous for *VEGF*, also suffered embryonic lethality. Embryonic death occurred between day 11 and 12 and the deficiency impaired early stages of vascular development.[68] Factors that may have contributed to this severe phenotype include retarded differentiation or abnormal accumulation of endothelial cells, anomalies in fusion of angioblasts and aberrant capillary plexus formation or unwarranted regression of pre-existing vessels.[67] These results indicate a developmental requirement of VEGF in a dose-dependent manner.

Role of Flt-1 in Vascular Development

A RTK closely related to Flk-1, Flt-1 has been studied by gene inactivation in mice. Similar to what is observed in *flk-1* -/- mice, *flt-1* inactivation also results in embryonic lethality between 8.5 and 9.5 dpc.[63] However, whereas there is an absolute requirement for Flk-1 for the development of endothelial cells, *flt-1* -/- embryos form endothelial cells at both embryonic and extraembryonic sites.[63] In addition, the defect in these embryos lies more in the organization of endothelial cells in vascular channels.[63,69] Vascular structures are abnormally large with absence of progressive development of smaller and more defined branching vessels.[63] It would appear then that although Flk-1 and Flt-1 are highly homologous, they nevertheless transduce distinct signals in endothelial cells[53], even though they share the same ligand, VEGF.[62]

Role of Tie Receptor in Vascular Development

Through *in situ* hybridization data from the mouse, it was observed that *tie* is expressed early in endothelial precursors of the head mesenchyme, in the dorsal aorta, as well as in the blood islands.[59] However, in chimeric analysis, it appears that although *tie* is expressed early, it does not contribute significantly to the differentiation of angioblasts or early angiogenesis.[61] Rather, it is suggested that Tie supports later stages of angiogenesis during organogenesis at mid-gestation, and that it also has a role in ensuring endothelial cell survival and proliferation.[61] This notion is supported by results from a null mutation of the gene introduced by homologous recombination which showed that while *tie* -/- mouse embryos appeared normal prior to 13.5 dpc, all neonates died immediately after birth.[60] Upon closer inspection, it was observed that mutant embryos consistently had edema leading to localized hemorrhage.[60] The cause for this is believed to be a loss in vascular integrity and impaired survival of endothelial cells constituting vessels.[60,61] Since these defects occur later in development, Tie is thought not to play a significant role in early vascular development, but rather, plays an important later role in regulating vessel integrity and endothelial survival.

While *tie* expression has also been seen in some hematopoietic cells,[70] *tie* inactivation is without discernible hematopoietic effect.[61] Currently, the ligand(s) for the Tie receptor has/have not been identified.

Role of Tek and its ligands Angiopoietin-1 and -2 in Vascular Development

tek (also known as *tie-2*) expression has also been studied by *in situ* hybridization in mouse embryos varying from 6.0 through 12.5 dpc.[56] While its expression pattern is similar to that of *flk-1* and *tie* at early timepoints, like *flk-1*, it undergoes downregulation in the amnion suggesting that its main function lies in early stages of amnion development.[56] In addition, its expression is restricted mainly to endothelial cells and their precursors, indicating that it may have a role in the establishment of the vascular tree. Dominant-negative and targeted null mutations of this gene confirm this conclusion, as all embryos die between 9.5 and 10.5 dpc.[60,71] Vascular channels do form in such animals but are unable to organize themselves into proper branching networks. Furthermore, a lack of capillary sprouts to the neuroectoderm is observed in mutant embryos, suggesting a disruption of angiogenesis. Possibly, signalling through the Tek receptor allows endothelial cells to recruit stromal cells to encase endothelial channels, thereby regulating angiogenic growth and maintaining the structural integrity of blood vessels.[53]

The first ligand isolated for Tek, Angiopoietin-1, is an activating ligand that is initially expressed between 9 and 11 dpc, most prominently in the heart myocardium and in close proximity to endothelial cells of developing vessels.[72] Inactivation of the *angiopoietin-1* gene in mice, resulted in an embryonic lethal defect.[73] All embryos were dead by 12.5 dpc. although they appeared grossly normal up to 11 dpc. Of interest is the similarity of *angiopoietin* null animals to the *tek* -/- embryos. Both mutants demonstrate defects in vascular remodeling, and in particular, rudimentary blood vessels and

homogeneously sized channels are favoured over more complex vascular structures and networks. Thus, the activities that Angiopoietin-1 regulates may be late acting during development, and may include vessel maturation and stabilization in relation to their surrounding environment. Furthermore, the function of Angiopoietin-1 is believed to be linked cooperatively to another ligand acting during vascular development, namely VEGF. Such interactions may not be limited to embryonic vascular development, but also occur in angiogenic processes in the adult, including wound healing, the menstrual cycle, and tumour angiogenesis.

A second Tek/Tie-2 ligand, Angiopoietin-2, was recently isolated by screening cDNA libraries using an *angiopoietin-1* cDNA as a probe.[74] The finding that Angiopoietin-2 is an antagonist for the Tek receptor is surprising, considering its homology to Angiopoetin-1. *In situ* RNA hybridization studies show a different expression pattern for *angiopoietin-2* as well, as transcripts are not detected in the heart but rather in the dorsal aorta and aortic branches in early embryonic stages. Further observations of the expression patterns of *VEGF*, *angiopoietin-1* and *angiopoietin-2* in adult tissues undergoing vascular remodelling, namely the ovaries, uterus and placenta, suggest that Angiopoietin-2 may have an earlier role in angiogenesis by blocking the maturation and stabilization function of Angiopoietin-1, thereby reverting and maintaining vessels in a more plastic condition, where they may be more responsive to angiogenic cues produced by VEGF.[74] More definitive evidence of Angiopoietin-2 as an antagonist for the Tek receptor comes from transgenic overexpression of this factor in mice.[74] This condition leads to embryonic lethality between 9.5 and 10.5 dpc. As would

be expected, vascular defects are prominent features, and include vessel discontinuities and an absence of characteristic vessels. In fact, the phenotype is strikingly similar to that of mice homozygous for *angiopoietin-1* or *tek* gene disruptions, differing only by the increased severity of vascular defects.[74] Overall, therefore, Angiopoietin-1 and -2 appear to provide a natural system of positive and negative regulation of angiogenesis, each exerting opposing influences on vessel formation and overall vascular development.

1.2.6 An *in vitro* Model to Study Hematopoiesis and Vascular Development

As described above, mouse gene knockout studies have revealed the functional consequences of disrupting a number of genes involved in vascular development. The ES cells used to generate these "knockouts" have an additional potential use in that they can be induced to differentiate *in vitro* to form a variety of cell types in a sequence that recapitulates the first stages of murine embryogenesis.[75] The potential advantages that such an *in vitro* model system offers include: i) The ability to manipulate readily the genetic information in murine cells to dissect molecular processes that control development. ii) Some developmental processes are reproduced with high fidelity during the differentiation of ES cells although experiments *in vitro* cannot in all cases be substituted for *in vivo* analysis. Rather, *in vitro* approaches generally serve as an initial step to exploring developmental pathways. iii) *In vitro* studies are not limited by resources, nor are they as expensive as *in vivo* studies. To ensure that an *in vitro* model will produce valuable information, it must first be shown to express many of the genes of interest and furthermore to recapitulate closely the developmental sequence of events

occurring *in vivo*. Embryonic stem cells are derived from the inner cell mass of 3.5 day blastocysts and can be maintained in the undifferentiated state by growth on embryonic fibroblasts together with the addition of leukemia inhibitory factor (LIF). In suspension culture, ES cells will differentiate spontaneously into embryoid bodies of increasing complexity that contain blood islands[10,76], as well as express multiple hematopoietic lineages.[77] Studies using this model system have included the investigation of vessel formation and mechanisms of vascularization, that is, vasculogenesis and angiogenesis.[10,78] Other investigators have also used this model to study the onset of hematopoiesis[27,79] and found it to closely parallel events in the embryo. As such, embryoid bodies have provided a good experimental system to investigate hematopoiesis and blood vessel development and the connections between these two developmental processes. It is clear that a relationship exists between the hematopoiesis and vascular development, as illustrated by marked deficiencies of both hematopoietic and endothelial cells in embryos lacking the receptor tyrosine kinase Flk-1. In conclusion, while embryoid bodies have been used successfully to map out developmental events and mechanisms involved during blood vessel formation and hematopoiesis, the developmental potential of differentiating ES cells is not limited to only these processes and remains to be fully explored.

1.3 Blood Coagulation

1.3.1 Introduction and the Waterfall Hypothesis

When vascular damage occurs, physiological events are triggered to stop blood flow and restore vessel integrity. Early theories proposed that it is the exposure of plasma to injured tissues that initiated clotting.[80,81] Over several years, this concept has been expanded upon, as both chemical and laboratory data have elucidated several critical steps in coagulation.

By 1964, the events of blood coagulation were ordered into sequential reactions referred to as the waterfall or cascade hypothesis (Fig. 1).[82,83] The main idea put forth by this model was that plasma proteins were activated by limited proteolysis and then served to activate other clotting factors further along the cascade. Since each activated factor or protease can serve to activate more than one coagulation factor, a cascade or amplifying effect ensues. This theory further divided the events of blood coagulation into two categories, the intrinsic and extrinsic pathways. The intrinsic pathway was so called because it involved only those factors circulating in the blood. Thought to be triggered by contact activation, factor XII was activated through interaction with a negatively charged surface. Factor XIIa then converted factor XI to XIa, which in turn activated factor IX to IXa, which proceeded to activate factor X to Xa, leading ultimately to the production of thrombin, and the cleavage of fibrinogen to fibrin. The extrinsic pathway is based on the observation that thrombin generation and consequent fibrin deposition may be initiated by exposure of subendothelial tissue to plasma following vessel injury. Specifically, vessel damage leads to exposure of the circulation to tissue factor, which

Figure 1: The original coagulation cascade hypothesis proposed in the 1960s. In this schematic, the cascade is divided into the extrinsic and intrinsic pathway. Factor XII was believed to be the initiator of coagulation when activated through its interaction with a negatively charged surface. Positive feedbacks are indicated by red lines. Activated coagulation factors are denoted by an "a".

then complexes with activated factor VII. In this complex, factor VIIa effectively activates factors IX and X, both of which lead to the generation of thrombin. Of the two, it appeared that the extrinsic pathway was the subordinate route to coagulation because natural human deficiencies of factors VIII and IX, components of the intrinsic pathway, produced severe bleeding disorders, hemophilia A and B respectively. However, from studies of patients with inherited deficiencies of coagulation proteins, it soon became clear that this cascade theory was not entirely accurate. For example, it was observed that patients deficient in factor XII showed no hemorrhagic symptoms. In contrast, patients severely deficient for factor VII had a high propensity for severe bleeding even though the intrinsic pathway was intact.[84] Furthermore, factor XII activation of clotting had only thus far been shown by *in vitro* studies. Together, these findings suggested that rather than having two independent coagulation pathways, the intrinsic and extrinsic pathways were coordinated and interrelated.

1.3.2 The "Revised" Coagulation Cascade

The current view of coagulation still uses the idea of a cascade as a central theme (Fig.2). However, importance has shifted to tissue factor as the initiating molecule of coagulation, and factor XII is now thought not to be involved in normal hemostasis.

Vessel disruption following injury causes tissue factor (TF), which is expressed ubiquitously throughout the vascular subendothelium, to be exposed to circulating blood. TF then serves as a high-affinity receptor for activated factor VII[85], and the resultant TF/VIIa complex in turn activates both factors IX and X. The mechanism by which

Figure 2: The current view of coagulation still maintains a cascade as its central theme. Initiation of coagulation begins with the complexing of factor VIIa to TF, which in turn activates factors IX and X, ultimately leading to the production of thrombin. Thrombin cleaves fibrinogen into fibrin generating a clot at the site of injury.

factor VII is activated is currently not well understood. The most common theory is that factor VII activation is accelerated by its interaction with tissue factor in the presence of factor Xa. Proponents of this idea believe that a small quantity of factor Xa is always present to activate factor VII.[86,87] Other sources suggest that factor VII undergoes autoactivation.[88] More recently, a novel membrane-associated serine protease called hepsin has been identified, which can convert factor VII to VIIa, thereby initiating coagulation.[89]

Amplification reactions are believed to take place predominantly on the surface of platelets but may also occur on endothelial cells, monocytes and neutrophils. Generation of small amounts of thrombin and factor Xa on specialized surfaces, results in activation of surrounding platelets, which in turn lead to the expression of specific receptors on their surface, including receptors for activated factors IX and X. Platelets thereby serve to localize the cofactors necessary for amplification (factors Va and VIIIa) along with coagulation factors at the site of vascular injury. Thrombin-activated factor XI consolidates the coagulation process by providing more factor IXa, thereby further perpetuating the amplification.

With the localization of essential coagulation factors on the platelet surface at the site of injury, clotting may effectively proceed. Factor IXa occupies a binding site in the vicinity of its cofactor VIIIa to form the *tenase complex* (IXa-VIIIa) which rapidly converts factor X to factor Xa. Coagulation continues with the formation of the *prothrombinase complex* (Va-Xa requiring Ca^{2+} and a phospholipid surface) whereby, factor Xa occupies a binding site near cofactor Va on the platelet surface. The central

event in coagulation occurs at this step, when prothrombin, an inactive zymogen, is transformed to the critically important serine protease, thrombin. Thrombin has multiple biological effects, one of which is to cleave fibrinogen, resulting in the formation of fibrin monomers which polymerize to form fibrin polymers. These are subsequently stabilized by factor XIIIa, a tetramer composed of two A chains and two B chains. While the catalytic activity of factor XIIIa resides in the A chains, B subunits stabilize the A subunits and regulate the rate at which factor XIII is activated by thrombin.[90] Finally, the maintenance and perpetuation of clotting is mediated by positive feedback mechanisms involving thrombin. Thrombin activates both cofactors V and VIII, and is also hypothesized to activate factor XI in a positive feedback reaction.[91] (Site of synthesis of coagulation factors is listed in Table 1.)

In order to achieve hemostatic balance, coagulation reactions must be carefully regulated and there are two main mechanisms to perform this task. Firstly, there exists the fibrinolytic system that counteracts coagulation by degrading the fibrin clot once healing is underway. Secondly, the actions of natural circulating anticoagulants serve to moderate and regulate amplification and propagation of coagulation. Procoagulants and anticoagulants work together in an equilibrium, such that when a stress such as vessel injury is introduced, each system shifts appropriately to regain hemostatic balance.

1.3.3 Role of Natural Anticoagulants in Regulating Procoagulant Pathways

The initiation phase of coagulation is controlled by an anticoagulant called tissue factor pathway inhibitor (TFPI). TFPI is a soluble plasma protein released by endothelial cells and platelets which inhibits both factor VIIa and factor Xa by forming a quaternary

TFPI/TF/VIIa/Xa complex.[92] Complex formation results in the indirect and direct inhibition of factor IX and factor X activation, respectively. Factor Xa that has escaped inhibition will continue the activation process to produce thrombin. However, in order to sustain clotting, additional factor Xa production must occur through the actions of factor IXa and its cofactor VIIIa. This amplification step is where the hypothesized positive feedback of thrombin to activated factor XI becomes important. Thrombin-mediated activation of factor XI may lead to the generation of factor IXa to partly supplement factor IXa production by the TF/factor VIIa complex prior to its inactivation by TFPI. A schematic representation of the effects of TFPI inhibition is shown in Fig. 3.

Other natural anticoagulants playing integral roles in regulating coagulation include antithrombin III and protein C (Fig. 4). ATIII, a plasma protein, binds to thrombin, forming a one-to-one stoichiometric complex, thereby resulting in the blockage of its active site.[93] This interaction is dramatically enhanced by heparin, an oligosaccharide synthesized by endothelial cells. The heparin-ATIII anticoagulant mechanism may similarly inactivate factors IXa, Xa, and XIa.

Protein C (PC) is also a plasma protein, but requires activation to a serine protease for its anticoagulant function. When complexed with thrombomodulin (TM), a transmembrane endothelial cell protein, thrombin cleaves and activates PC. In the presence of its cofactor protein S (PS), PCa degrades cofactors Va and VIIIa, thereby suppressing the generation of thrombin and effectively diminishing positive feedback mechanisms. In addition, TM also has natural anticoagulant properties, as it forms a one-to-one complex with thrombin, altering its conformation and modifying its procoagulant

Figure 3: Effect of tissue factor pathway inhibitor on the coagulation system. The direct inhibition of factors VIIa and Xa by TFPI results in a downregulation of cascade pathways (indicated by the dotted white lines). To compensate for this, thrombin generation is perpetuated by its positive feedback to activate factor XI. In this schematic, the pathways that play a more important role in response to TFPI are represented by thicker lines. Positive feedback mechanisms are shown in red.

Figure 4: The actions of the natural anticoagulants protein C and antithrombin III. In the presence of heparin, ATIII inactivates thrombin, factors IXa, Xa, and XIa by blocking their active sites. Protein C, when complexed with TM can be cleaved to generate activated protein C which in the presence of protein S, degrades cofactors Va and VIIIa.

properties. In this form, thrombin is unable to clot fibrinogen, activate factor V, or trigger platelet activation.[94,95] Recently, EPCR, another receptor for PC/PCa has been identified on endothelial cells.[96] However, at present, its role in coagulation remains obscure. (Refer to Table 1 for the site of synthesis of anticoagulant factors.)

1.3.4 Mechanism of TFPI Inhibition

The amino acid sequence of TFPI predicts the existence of three inhibitory domains called *Kunitz-type* domains. These domains are serine protease inhibitors which function by imitating substrate and binding the active site of enzymes. This type of inhibition is slow, competitive, and reversible. Factor VIIa of the TF/factor VIIa complex binds to the first Kunitz domain, but this reaction is aided by the binding of factor Xa to the second domain. The function of the third Kunitz domain is not yet fully understood. Although TFPI can interact independently with factor VIIa of the TF/factor VIIa complex, much higher concentrations of TFPI are required.[97] Thus, TFPI functions best as an inhibitor when it forms a quaternary complex with factor Xa and the TF/factor VIIa complex (Fig. 5). TFPI is unique among the inhibitors of coagulation in two ways. First, it is bivalent, i.e., it is one molecule having inhibitory sites for two proteases. Second, its requirement for factor Xa, which itself is a product of the interaction of factor VIIa and TF, for the inhibition of the TF/factor VIIa complex, constitutes a negative feedback mechanism.[97] Although there are other negative feedback pathways that regulate clotting, they involve the generation of an inactivating enzyme (such as PCa) rather than having the inhibitor itself perform dual roles.

Figure 5: Proposed mechanisms for the formation of a quaternary complex involving membrane-bound TF, factor VIIa, factor Xa and TFPI. Circulating factor Xa may combine with TFPI and these two may associate with the factor VIIa/TF complex. Alternatively, factor Xa, having been activated by the factor VIIa/TF complex, may remain associated with it and be inhibited along with factor VIIa, when circulating TFPI combines with this complex.

1.3.5 The Fibrinolytic System

Once hemostasis is established and healing is underway, the clot generated by procoagulant pathways must be removed. The most important enzyme for the dissolution of clots is plasmin. Plasmin lyses fibrin clots through proteolysis of an arginyl bond, producing fibrin fragments. The conversion of plasminogen to its active form – plasmin – requires the presence of activators. Two such activators have been identified, t-PA and u-PA. Both activators exist as single chains and must be converted to a two-chain molecule to be active. t-PA and u-PA activate plasminogen by cleaving an Arg-Val bond, thus exposing the active site of plasmin.[98,99] While both t-PA and u-PA activate plasminogen, they have different sites of activity. The function of u-PA is more relevant in cell-associated plasminogen activation, while t-PA is considered to be the physiologic activator of plasminogen within the circulation.

As with coagulation, fibrinolysis also requires tight regulation. The principal controlling factor for t-PA is PAI-1. PAI-2 is also an efficient inhibitor of u-PA and two-chain t-PA, although it acts at a slower rate than PAI-1. In addition, the regulation of plasmin is achieved by an antiprotease called α_2AP which forms a tight bond with plasmin to inhibit its actions. The reactions of the fibrinolytic pathway are depicted in Fig. 6 and the site of synthesis of the coagulation factors involved are listed in Table 1. Recently, it was found that fibrinolytic activity itself can be regulated by a protein called TAFI. When activated by thrombin, TAFI functions as a carboxypeptidase B enzyme and cleaves basic amino acid residues (i.e. lysines and arginines) from the ends of proteins.[100] TAFI removes C-terminal residues from partially degraded fibrin, thereby altering its structure and preventing plasminogen from binding. Since

Figure 6: The fibrinolytic system. The conversion of plasminogen to plasmin is mediated by two activators, t-PA and u-PA. Both these activators can be inhibited by PAI-1 and PAI-2 although the latter functions at slower rates than PAI-1. The major inhibitor of plasmin is and the antiprotease α_2AP.

fibrin is a cofactor for t-PA which converts plasminogen to plasmin when it is bound to fibrin clots, the actions of TAFI, therefore, significantly inhibit the process of fibrinolysis.[100]

Fibrin is a target of two opposing regulatory systems, coagulation and fibrinolysis. This duality suggests that a link might exist between procoagulant activity and fibrinolytic activity, although supporting *in vivo* evidence of such a direct link has been lacking. However, there are several lines of evidence *in vitro* to show that a connection between fibrin formation and fibrin dissolution exists. It is known for example, that factors XIIa and XIa can convert plasminogen to plasmin under certain circumstances.[101] Furthermore, under certain *in vitro* conditions, PCa can contribute to the initiation of fibrinolysis by disassociating t-PA from its inhibitor PAI-1.[102] Nonetheless, the physiologic relevance of putative links between coagulation and fibrinolysis remain to be established *in vivo*.

1.3.6 Contributions of Platelets in Coagulation

Platelets serve an important function in coagulation by providing cell surfaces on which clotting reactions can occur. They express receptors for a number of coagulation factors and thereby facilitate the amplification steps of coagulation. The contributions of platelets to hemostasis can be divided into three areas; 1) platelet adhesion to vessel wall, 2) platelet aggregation and 3) release reactions. Platelet adhesion to subendothelial surfaces – as a result of a damaged vessel wall – is assisted by a platelet glycoprotein Ib (GPIb) and von Willebrand factor (vWF). vWF supports both platelet adhesion and platelet-platelet interactions. Following platelet adhesion, activation occurs through the

TABLE 1. Site of synthesis of blood coagulant proteins in adult mouse

Coagulation Factor	Site of synthesis
α_2-Antiplasmin	Hepatocytes, endothelial cells
Antithrombin III	Hepatocytes
Factor V	Hepatocytes, megakaryocytes
Factor VII	Hepatocytes
Factor VIII	Hepatocytes, endothelial cells
Factor IX	Hepatocytes
Factor X	Hepatocytes
Factor XII	Hepatocytes
Factor XIII	Hepatocytes
Fibrinogen	Hepatocytes
PAI-1	Endothelial cells
PAI-2	Placenta, leukocytes, megakaryocytes
Plasminogen	Hepatocytes
Protein C	Hepatocytes
Protein C Receptor	Endothelial cells
Protein S	Hepatocytes
Prothrombin	Hepatocytes
TAFI	unknown
TFPI	Endothelial cells
Thrombin receptor	Platelets, endothelial cells, leukocytes, mesenchymal cells
Thrombin receptor-2	Splenocytes, megakaryocytes, bone marrow
Thrombomodulin	Endothelial cells
Tissue Factor	Many cell types
t-PA	Endothelial cells
u-PA	Kidney cells
u-PAR	Endothelial cells, monocytes, macrophages, keratinocytes, smooth muscle cells
von Willebrand factor	Megakaryocytes, endothelial cells

action of thrombin, resulting in the expression of thrombin receptor on platelet surfaces.[103] As well, sites for the tenase complex and the prothrombinase complex are also formed. Platelet aggregation occurs during this time through fibrinogen and other platelet membrane glycoproteins IIb/IIIa (GPIIb/IIIa), which expose binding sites for fibrinogen and vWF only after platelet activation. Fibrinogen is responsible for forming bridges between platelets during aggregation. Finally, the platelet-release reaction is a response to surrounding agonists to secrete platelet constituents stored in alpha granules and dense bodies. These constituents act to recruit additional platelets and also to promote vasoconstriction.

1.3.7 Natural Human Deficiencies of Coagulation Factors

Although many deficiency states of clotting factors lead to undesirable consequences, the symptomatic pathologies that they yield have provided valuable insights into blood coagulation. Intuitively, deficiencies in procoagulants should lead to hemorrhage, and deficiencies in anticoagulants and fibrinolytic agents should result in thrombotic events and impaired wound healing. In many cases, "deficiency" is a relative term, that is, for a specific factor, there exists a variation in the amount of deficiency among an affected population, generating asymptomatic to severe coagulopathies. The severity of a deficient condition is dependent on the nature of the specific genetic mutation involved, which may include frameshifts, point mutations, and terminations or deletions, resulting in the production of a dysfunctional or nonfunctional clotting protein.

One of the best characterized of the inherited coagulation deficiencies results in a pathological condition known as hemophilia. Hemophilia is characterized by frequent

spontaneous bleeding into joints, soft tissues, muscles and internal organs. Hemophilia is due to deficiencies of factor VIII and factor IX, known as hemophilia A and B respectively. In fact, because hemophilia A and B are clinically indistinguishable, they were not recognized as separate entities until it was demonstrated that a factor from one group of hemophiliac patients could correct the deficiency of another group in an *in vitro* clotting assay.[104,105] While approximately 60% of patients with hemophilia A have less than 1% factor VIII activity, only about 44% of hemophilia B patients exhibit a severe disease state. In 1953, a third type of hemophilia distinct from hemophilias A and B, was characterized. This third hemophilia was linked to a deficiency in factor XI, which produced a much milder phenotype and was first reported among members of a single family that experienced moderate hemorrhage, mostly bleeding subsequent to tooth extraction.[106] Parahemophilia, a disorder associated with factor V deficiency, is characterized by excessive bleeding following surgery, trauma, and dental extractions.[107] Clinical manifestations of factor V deficiency also include oral bleeding and menorrhagia. Of interest is a combined factor V and factor VIII deficiency which occurs at too high a frequency to be attributed to a coincidental concurrence of hemophilia A and factor V deficiency.[108] The molecular basis of this combined deficiency has recently been identified, and attributed to abnormal transport of both proteins from the endoplasmic reticulum to the Golgi apparatus.[109] The clinical symptoms of this combined deficiency are similar to those of isolated factor V deficiency.

Deficiencies in other factors also lead to bleeding. It was the severe bleeding tendency of patients significantly deficient in factor VII that changed the view that blood

clotting occurred primarily through the intrinsic pathway of the cascade hypothesis. Some factor VII deficiency homozygotes experience episodes of cerebral hemorrhage.[110] In addition, inherited fibrinogen deficiency or hypofibrinogenemia can be associated with a hemorrhagic diathesis related to trauma or surgery.[111]

Not suprisingly, deficiencies in anticoagulants produce thrombotic complications. For instance, antithrombin III deficiency is marked by an increase risk of thrombosis, primarily venous.[112] Hereditary, protein C deficiency is also associated with recurrent thromboembolic disease. Homozygotes or compound heterozygotes for protein C deficiency most often develop devastating purpura fulminans at birth, while heterozygotes may experience venous thromboses beginning at or soon after puberty.[113]

Although many deficiency states exist for different clotting factors, individuals with deficiencies in TF and TFPI have not been identified. This may be in part due to the fact that they have essential roles in normal hemostasis and if absent, may create a lethal condition *in utero*. Secondly, assay methodologies may not be adequate to measure and quantitate TF and TFPI reliably to confirm a deficiency state. For instance, measuring TF levels in a patient is problematic because the protein is normally not found in circulation. Furthermore, TF is expressed in varying levels in a variety of tissues. A similar difficulty exists when trying to measure TFPI levels in plasma, as approximately 80-85% on TFPI *in vivo* remains bound to the vessel wall while the rest is associated with lipoproteins in the plasma or is found in platelets.[114,115] Since most deficiency states are characterized using assays that detect the presence or absence of coagulation factors in

plasma, these conventional methods cannot be used to quantitate TF and TFPI, which are not found in the plasma.

Although our understanding of the mechanisms by which the coagulation system functions has been greatly increased by examining both clinical disorders, and the biochemistry of the critical components, much remains to be elucidated. For example, the roles that these proteins may play in development, in neovascularization and in coagulopathies, require further characterization. A molecular approach towards answering some of these questions involves the targeted disruption of the genes that encode coagulation proteins in mice, and the characterization of the phenotypic consequences of such mutations.

1.3.8 Gene Disruptions in Mice and Coagulation Deficiencies

Rapid advances in molecular biology techniques have made it possible to develop transgenic mouse models. Such animals have become valuable tools in a number of research disciplines including developmental biology and biomedical research. These models are used not only to study developmental events but have also been used to study the effect of treatment of many human pathological conditions such as cancer and cardiovascular disease. The mouse has quickly become the preferred experimental organism of choice, due to its accessibility and ease with which it can be manipulated, facilitated by improved biotechniques. In particular, the technology of gene disruption, which is used to introduce germ line mutations in a controlled fashion into the mouse, can be utilized to alter and understand the function of any gene of interest. The benefits of

"knocking out" a gene in a mouse include an understanding of a gene function *in vivo*, and in the context of the entire animal through its development. Furthermore, this technique has been used to produce animal models of human diseases. For example, the genes for factors VIII and IX have been disrupted in the mouse to produce models for hemophilias A and B, respectively.[116,117,118]

Briefly, the principle of gene disruption involves the construction of a targeting vector that includes a positive marker sequence (usually neomycin resistance) within the coding sequence of the test gene.[119] This vector is then introduced into mouse embryonic stem cells in culture in which homologous recombination – the exchange of genetic material between aligned identical sequences of the targeting vector and of the normal gene – occurs. Thus, the normal gene on the chromosome is replaced by an altered gene. Due to neomycin resistance, the positive marker, cells containing the targeting vector sequence can be selected for. ES cells containing the targeted mutation can then be injected into recipient mouse embryos at the blastocyst stage where they contribute to the developing organism. Resultant chimeric mice have patchy coat colouring and are screened using coat colour as a selection criterion, as the contribution of the ES cells results in a coat colour that is different from the wildtype. The extent of the ES cell contribution is roughly proportional to the amount or dominance of that coat colour over that of the wildtype. Chimeric males are mated and resulting heterozygote offspring containing the mutation are mated to each other such that a quarter of the F2 generation will have the mutation in both alleles, that is, they are homozygotes.

Deficiency states for coagulation factors have been created not only to further study their functions, but also to investigate their role in development. Especially interesting are those deficiency states that have not been found to occur naturally, such as TF and TFPI deficiencies. These deficiency studies have also provided new insights into requirements for normal hemostasis and revealed that, to some extent, factors having functional overlap allow coagulation processes to be somewhat versatile in compensating for the absence of one of these factors.

In recent years, the targeted disruption of a number of genes encoding coagulation factors has been accomplished in mice. In some cases, gene "knockouts" in mice produced phenotypes very similar to those in humans with corresponding deficiencies. Specifically, gene disruptions of *FVIII* and *FIX* have been created to reproduce the hemophilic phenotypes observed in humans deficient in these factors. Such animal models provide not only a means to study the pathophysiology of hemophilia, but also offer an opportunity to evaluate potential therapeutic strategies such as gene therapy, in this condition. Two independent laboratories have successfully disrupted *FVIII*[116,117], and FIX-deficient mice have also been generated.[118] While these "knockouts" have not yet been fully characterized, early data suggest that they produce phenotypes grossly similar to their human disease counterparts. One group observed spontaneous bleeding in the large joints of some mice deficient for FVIII.[117] Furthermore, these animals experienced subcutaneous hematomas even with the gentlest of handling. While the second group investigating FVIII disruption in mice did not make similar observations, the animals they generated suffered lethal bleeding subsequent to tail biopsy if the wounds were not

cauterized.[116] Tail biopsies were similarly lethal in FIX-deficient mice.[118] In addition, these animals exhibited hemorrhagic swelling of the footpads and experienced subcutaneous hemorrhages.[118]

Other "knockouts" with phenotypic consequences that correlate well with the corresponding human deficiencies include *FVII*, *FV*, and *fibrinogen* disruptions (discussed below). However, these deficiencies in mice produced additional severe consequences that are not observed in their human disease counterparts. For example, while the occurrence of intra-cranial hemorrhaging in most FVII-deficient mice[120] is not unlike the cerebral hemorrhage that some FVII-deficient patients experience[110] these animals also suffer fatal intra-abdominal hemorrhage.

Biological Consequences of Factor V Gene Disruption

In humans, a genetic deficiency of coagulation FV results in a bleeding disorder known as parahemophilia.[107] FV is an absolute requirement in coagulation, serving as a cofactor in the transformation of prothrombin to thrombin. Cui *et al.* have disrupted the *FV* gene by homologous recombination in mice and generated FV-deficient animals through mating of heterozygotes. Strikingly, not only were -/- mice born in half the expected numbers but neonates homozygous for this deficiency were unmistakable as they died within two hours due to massive intra-abdominal hemorrhage.[121] This phenotype is in contrast to the milder phenotype associated with parahemophilia and other human deficiencies of FVIII and FIX. It is unclear, however, whether absolute deficiency of FV in humans is compatible with life.

Embryonic loss of FV -/- animals generally occurred between 9.5 and 10.5 dpc. FV -/- embryos exhibited some degree of developmental delay and had a number of anomalies in yolk sac organization leading to fewer yolk sac blood islands and sluggish circulation. It was proposed by the authors that the lethality of FV deficiency may be due to a combination of interruption of fibrin formation and platelet activation due to decreased production of thrombin. This hypothesis is lacking in that platelets are not present in the circulation until the commencement of definitive hematopoiesis at 10.5-11.5 dpc. Also, since ~20% of FV is stored and released by platelets upon activation,[104,122] its local release at sites of injury aids in concentrating FVa to promote site specific hemostasis. Therefore, a deficiency in FV may reduce the effectiveness of clot formation at the site of vessel damage. The partial fetal loss of FV -/- mice is reminiscent of the 50% loss observed in mice homozygous for thrombin receptor gene disruption (discussed later). However, while functional redundancy exists for the latter, no such mechanism has yet to be observed in FV-deficient animals, as intra-abdominal blood obtained from these animals does not clot and is devoid of FV activity.[121] The possibility that FV may have physiological roles not related to coagulation cannot be ruled out since fetal loss does not appear to be due to hemorrhagic events.

Hemostatic Responses in Mice Deficient in Fibrinogen

Fibrinogen is a symmetrical dimer composed of three different chains ($A\alpha_2B\beta_2\gamma_2$). Fibrinogen constitutes the building blocks of clots and serves as a bridging molecule linking activated platelets during platelet aggregation. Furthermore, fibrin may

have a broader physiological function aside from clot formation, since a variety of cell types including endothelial cells[123], macrophages[124,125], leukocytes[126,127], and fibroblasts[128] can bind to and migrate on fibrin matrices. These cells may infiltrate wound sites to initiate repair and/or may contribute to immune-inflammatory responses. Therefore, one might expect a deficiency in fibrinogen to result in compromised blood clotting as well as abnormal wound repair. To investigate these possibilities, fibrinogen-deficient mice were produced by homologous recombination. Chimeric males were used to produce heterozygous offspring which were then mated with each other to produce fibrinogen -/- animals.[129] The targeting vector was designed such that production of fibrinogen Aα-chain was eliminated. Although Bβ and γ chains mRNAs were transcribed (as shown by Northern blot analysis), the corresponding polypeptides were not found in the circulation (shown by Western blot analysis on whole blood samples) of -/- mice, suggesting a requirement of the Aα chain for protein secretion. In addition, platelets obtained from fibrinogen-deficient mice failed to aggregate in standard assays.[129]

Offspring obtained from heterozygous matings reveal that there is no fetal loss of -/- mice and that the pattern of transmission was consistent with a Mendelian pattern of inheritance. Although fibrinogen-deficient mice were born, ~30% of neonates suffered overt intra-abdominal hemorrhage and subcutaneous joint, and/or periumbilical bleeding 2 days after birth. Remarkably, two-thirds of these mice survived these spontaneous and severe bleeding events past the neonatal period. Closer inspection of fixed tissues obtained from -/- mice revealed that spontaneous bleeding occurred in a variety of tissues

and was generally the result of chance vascular breaks. Although fibrinogen-deficient mice had an increased risk of fatal bleeding, especially between 30 to 60 days, mice surviving past 90 days were likely to survive much longer.

A key issue in this study concerns how hemorrhages and spontaneous bleeding events were resolved in the absence of fibrin, such that they were compatible with life. Wound healing, although impaired, does occur in -/- mice. In particular, subcapsular hematomas seen in the liver and kidneys of deficient animals seem to initiate an unusual wound healing response.[129] These lesions consist primarily of blood pools, often surrounded by sites of necrosis. While some hematomas appear unconfined, others are enveloped in a fibrotic capsule. Presumably, the injury initiated the migration and proliferation of primarily fibroblasts to form a thick layer encapsulating but not infiltrating the hematomas.[129] The decrease of mortality in fibrinogen-deficient mice can be partly attributed to the formation of such fibrotic scars which may protect the liver from further injury or from rupture.[129] It is interesting to note that while it is expected that -/- female mice might have a higher risk for fatal intra-abdominal bleeding with each ovulation event, this is not observed. However, no litters could be generated from -/- females due to consistent overt vaginal bleeding that was often fatal. It is was found that embryo development was arrested between 9 and 10 days, notably when embryonic trophoblasts are invading maternal vasculature.

In conclusion, fibrinogen does not seem to be necessary for development as -/- embryos are carried to term without fetal loss. The lack of fibrinogen though, makes mice susceptible to severe spontaneous bleeding events, some of which are resolved with

the unconventional development of a fibrotic scar. It is striking that -/- mice do not bleed more often, as afibrinogenemic blood does not clot. In fact, individuals with inherited fibrinogen deficiency also only rarely experience spontaneous bleeding. One explanation for this is the fact that platelets are still activated and form a clot to control some bleeds. Presumably the ability to generate thrombin is not impaired in these individuals and animals.

In addition to creating mouse models of some coagulation factor deficiencies found in humans, genes encoding factors for which no corresponding human clinical diseases exist have been also disrupted in mice. The consequences of genetic disruptions of *PLAS*, *u-PA*, *t-PA*, *u-PAR*, *PAR-1*, *TM* and *TF* and their effects on coagulation, wound healing and development are discussed:

Consequences of Plasminogen Deficiency in Mice

Disruption of the fibrinogen gene, which encodes a coagulation protein believed to be critical for normal clotting, resulted in spontaneous bleeding. One might therefore expect that disruption of a key fibrinolytic agent would produce the opposite effect. In its active form, plasmin, acting in concert with other proteinases, is also thought to be involved in tissue remodelling and cell migration events, including trophoblast invasion[130], ovulation[131], wound healing[132,133], and tumour cell invasion.[134,135] Furthermore, there was additional interest in observing the effects of the disrupted plasminogen gene because potent biological activities such as angiogenesis- and

metastasis-suppressing activity have been associated with angiostatin, a plasminogen derivative consisting only of the kringle domains (triple loop disulfide-linked motifs) of plasminogen.[136] Plasminogen-deficient mice were generated through transgenic technology and homologous recombination. Crossing mice heterozygous for disrupted plasminogen allele produced +/+, +/- and -/- mice in the expected Mendelian ratios, indicating no fetal loss. However, plasminogen -/- mice were predisposed to severe thrombosis and developed thrombotic lesions in the liver, stomach, colon, rectum, lung, pancreas and other tissues early in life.[137] Reproductive systems did not appear to be affected by this deficiency as -/- females carried their litters to term and litter sizes were comparable to those of controls. It is interesting to note that the enzymatic activity of u-PA, a plasminogen activator well-documented to be activated by plasmin by positive feedback (see Fig. 6) was unaffected in -/- mice. Results from this study suggested that plasminogen and its proteolytic derivatives, plasmin and angiostatin are not essential for embryonic development or fertility and although there was a propensity for thrombosis, plasminogen deficiency is compatible with life.

Having established that mice deficient for plasminogen do survive, the next logical issue to address was their response to injury, and in particular how they resolve wounds if they are unable to clear fibrin clots. Incisional wounds were made in the skin of plasminogen +/+, +/- and -/- mice and while there were no differences in wound healing between +/+ and +/- mice, healing was severely impaired in plasminogen -/- animals.[138] These mice carried wound eschars for longer periods and even after eschar loss, a gaping red wound field remained, often having a scaly surface and lacking an

epidermal covering. It is important to note that healing activities in the wound field still occurred with the infiltration of inflammatory cells, formation of granulation tissue and evidence of neovascularization.[138] In contrast, keratinocyte migration required in reepithelialization appeared diminished, as epidermal cells were unable to dissect their way proteolytically past extracellular matrix underneath the wound crust.[138] However, the eventual resolution of these wounds suggested that there exists a functional overlap between plasminogen and other extracellular proteinases. Together, these two studies indicate that although the lack of plasminogen does not hinder developmental processes, it is essential in the normal progression of wound healing in adult mice.

Deficiencies of Plasminogen Activators and u-PA Receptor in Mice

The fibrinolytic system has been implicated in such biological processes as ovulation, embryo implantation, and embryogenesis, as well as in pathological conditions such as thrombosis and metastasis. Since no genetic deficiencies of t-PA and u-PA have been reported in man to date, their contribution to the fibrinolytic system *in vivo* has been determined through studies correlating fibrinolytic activity and physiological and pathological phenomena. Several studies have used homologous recombination to generate single deficiencies of t-PA, u-PA and u-PAR, as well as combined deficiencies, to investigate their roles in plasminogen activation in development, reproduction and thrombolysis.

Generally, single deficiencies produced milder effects than did combined deficiencies. Mice with single deficiencies of t-PA and u-PA were viable and were

fertile.[139] Both of these animals exhibited increased incidence of endotoxin-induced thrombosis, whereas only t-PA deficient mice showed decreased thrombolytic potential when plasma clots were injected through their jugular vein and embolized in the pulmonary arteries. u-PA -/- mice on the other hand, developed rectal prolapse and/or suffered extensive non-healing ulcerations in the facial area, and some also displayed fibrin deposits in ulcerated skin, prolapsed rectum, intestines and liver. A deficiency in the receptor for u-PA did not reveal any overt phenotypic abnormalities nor did it compromise fertility and hemostasis.[140] A combined deficiency of t-PA and u-PA resulted in viable mice with no gross abnormalities at birth.[139] However, these mice suffered postnatal growth retardation at ~17 weeks of age. They also had a shortened life span and reduced fertility. The latter might be explained by widespread and extensive multi-organ fibrin deposits in the gonads, liver, intestines and in the lungs. When challenged with skin incision wounds, t-PA -/-:u-PA-/- dual mutant mice similarly displayed delayed wound repair associated with impaired keratinocyte migration and re-epithelialization of the wound field as found in plasminogen-deficient animals.[138] In contrast, combined deficiency of t-PA and u-PAR produced no significant adverse consequences. While fibrin deposits were more localized to liver capsule with no other thrombotic lesions detected in other tissues, wound healing was not impaired in these double-deficient mice, suggesting that u-PA alone is sufficient to direct activation of plasminogen for the resolution of wound fields. The mildness of this phenotype is significant in light of the fact that u-PAR is thought to have a crucial role in u-PA-

mediated plasmin generation, and that t-PA is believed to be responsible for the removal of fibrin clots from the circulation.

Together, these findings show that t-PA, u-PA and u-PAR, are not required for normal embryonic development, and suggest that the mild phenotypes of single deficiencies of t-PA and u-PA may be partly explained by the ability of one plasminogen activator to complement the other. u-PAR deficiencies produced few effects in development and injury repair, raising the question of whether other receptors of plasminogen activators might exist to facilitate plasminogen activation.

Thrombin Receptor Deficiency and Evidence for a Second Receptor

To define the roles of the thrombin receptor (PAR-1) in coagulation, the gene was disrupted by homologous recombination. While thrombin receptor deficiency was expected to be lethal, deficient animals actually survived to birth in about half of the cases in two independent studies.[141,142] While at 8.5 dpc, PAR-1 -/- embryos were indistinguishable from their +/+ and +/- littermates, by 9.0 dpc, -/- embryos were uniformly smaller and at 9.5 dpc about half of these embryos had no beating heart. Those -/- embryos that survived to birth were grossly normal and did not experience spontaneous bleeding. In addition, their hemostatic ability was not compromised when challenged with tail bleeds. While this partial embryonic lethality has not yet been explained, this phenotype did suggest that the thrombin receptor has a physiological role in embryonic development.

Thrombin is the primary activator of platelets in coagulant function, activating platelets via the thrombin receptor. One might suspect that in "knocking out" this receptor, platelet activation would not be induced in the presence of thrombin. However, it was observed that platelets isolated from PAR-1 -/- and +/+ mice were activated equally by thrombin as assessed by secretion and aggregation responses. This result indicated that these responses may be due to thrombin signalling via a second thrombin receptor; this hypothesis having recently been proven.[143] This fact might also explain the partial lethality in PAR-1 -/- animals, since the presence of other thrombin receptors may provide functional redundancy and partly compensate for effects of thrombin receptor deficiency. In fact, the recently identified second thrombin receptor (PAR-3) has been found in mice to be highly expressed in megakaryocytes in the spleen and bone marrow of adult mice. The uncovering of another thrombin receptor may indicate different tissue-specific functions for different receptors.

The knock out of the thrombin receptor has not only shown a definite requirement for this receptor during development, but has revealed the existence of other receptors that can interact with thrombin in the coagulation process. Finally, the disruption of this gene may act as a model system to study its putative roles in pathological states such as tumor cell growth and metastasis.[144,145]

Disruption of the Thrombomodulin Gene

Thrombomodulin is an integral membrane protein involved in the regulation of coagulation. As previously mentioned, its interaction with thrombin serves to activate

protein C, which then proteolytically degrades activated cofactors Va and VIIIa. TM expression is initially localized to parietal endoderm at 7.5 dpc, but is produced in other organ systems at 9.5 dpc.[146] Investigators became interested in targeting this intronless gene for disruption when immunohistochemical studies revealed that the expression of TM, in addition to appearing in vascular tissues such as dorsal aorta, intersomitic arteries, endocardium and capillaries, was localized in tissues not in direct contact with body fluid.[147] Such widespread distribution of TM implies that this protein (or proteins with similar immunoreactivity) may have functions beyond its role as an anticoagulant.

Disruption of the TM gene was carried out by homologous recombination in D3 ES cells. As expected, mice heterozygous for the loss of TM function exhibited reduced levels of TM mRNA.[148,149] However, TM +/- mice were normal in size and fertility. Histologic analyses of organs including kidney, lung, heart, liver and spleen did not reveal any pathologic abnormalities. Furthermore, heterozygous offspring now exceeding 1 year in age, do not have adverse responses to experimental manipulations including tail biopsies.[149]

In contrast, the homozygous deletion of TM created an embryonic lethal phenotype as no TM -/- progeny were obtained from matings of heterozygous parents. Upon inspection of embryos at different times of gestation, TM -/- embryos were found to be present up to 9.5 dpc. While there were no gross differences between TM -/- embryos compared with TM +/- and TM +/+ littermates at 7.5 dpc, by 8.5 dpc, homozygotes were consistently smaller in size even though this stage is characterized by wide intralitter variation in maturity. By 9.5 dpc, TM -/- embryos were severely

abnormal with no discernable embryonic landmarks, and by 10.5 dpc, embryos were resorbed. Normally, extra- and intraembryonic vessels fuse to form a contiguous circulation at around 9.0 dpc. Consistent with this fusion, nucleated red blood cells derived from primitive hematopoiesis (in yolk sac blood islands) can normally be found in the embryonic heart after 9.0 dpc. In contrast however, 9.5 dpc TM-/- embryonic hearts have only one chamber, do not contain nucleated red blood cells and are deficient in endocardium development in comparison with +/+ embryos. These findings imply that extra- and intraembryonic vascular beds have not fused and that cardiac contractions have not been initiated. However, when 7.5 dpc TM -/- embryos were removed from maternal environment and cultured *in vitro*, embryos progressed to developmental stages not observed *in vivo*, including a well-developed contracting heart.[148] Thus, the maternal decidual environment may contribute significantly to the embryonic lethal phenotype in TM -/- offspring. The most plausible explanation offered is the observation by immunohistochemistry, using an anti-fibrinogen antibody, that increased levels of this protein surround extraembryonic membranes of TM -/- embryos as compared to TM +/- and +/+ littermates.[148] It is proposed that the deposition of fibrin could damage the parietal yolk sac (outermost of extraembryonic membranes) and prevent the exchange of macromolecules between mother and embryo that is essential for embryonic development. This notion also suggests that TM may be important in regulating hypercoagulable activities that may be present at the maternal and embryonic interface during placentation. However, antibodies specific to fibrin alone that do not cross react with fibrinogen must be developed to substantiate this hypothesis.

Studies into thrombomodulin gene disruption thus far, have not revealed an alternative function for this protein. However, the lethality of the homozygous state is indicative of the requirement of the anticoagulant properties of TM during embryonic development. It is interesting to note that thrombotic events are not present although they might be expected to contribute to the embryonic phenotype observed.

The Effects of Tissue Factor Gene Disruption

Since tissue factor was recognized as the primary initiator of hemostasis, the prospect of generating tissue factor deficient mice became exciting. Currently, humans with TF deficiency have not been identified. Furthermore, new lines of evidence suggest that TF function is not limited to initiating coagulation. In particular, tumor models have shown a requirement for TF to achieve an angiogenic and metastatic phenotype[150,151,152] and *in vivo*, TF may be useful as a marker for malignant breast cancer.[153] Tissue factor gene disruption was successfully performed by three independent laboratories with similar results.[154,155,156] Although these studies were in agreement that a homozygous deficiency for tissue factor leads to embryonic lethality with most -/- embryos lost between 8.5 and 10.5 dpc, the cause for this phenotype is still in debate. Carmeliet *et al.* observed vascular defects prior to wasting and necrosis in TF -/- embryos. In particular, they noticed that yolk sacs had vascular plexus anomalies, lacked vitelline vessels and lacked blood circulation.[156] From these findings, they inferred a role for TF in embryonic vessel development since at this stage of development, vessels begin to recruit muscle cells to stabilize their structure against increases in circulatory demands due to the

merging of intra- and extra-embryonic vasculature and the initiation of embryonic heart contractions. Bugge *et al.* on the other hand, attributed embryonic lethality to hemorrhagic events. They did not find abnormal vessel development but rather observed pools of nucleated red blood cells within yolk sac cavities and extreme pallor of both yolk sac and embryo.[154] They propose that TF has a physiological role in maintaining vessel integrity that would be crucial when vessels undergo remodelling. Therefore, TF -/- embryos are at risk for hemorrhage during vascular remodelling events such as the fusion of blood islands to form a vascular plexus in the yolk sac, and the development of vascular connection between embryo and placenta, which notably occur at around 10 dpc. Furthermore, the investigators suggested that TF is acting in a capacity unrelated to its role as a procoagulant since fibrinogen deficiency, although causing spontaneous bleeding, does not result in embryonic lethality.[129] A third investigation looking at TF deficiency performed by Toomey *et al.* is in general agreement with the two other studies in terms of TF deficiency inducing embryonic lethality, and cited possibly abnormal vessel development and/or hemorrhage to be the cause of death. However, unlike the other studies, these investigators found that approximately 15% of -/- embryos survived past the 10.5 dpc limit of embryonic life although no animals were born. Recently in a subsequent study, Toomey *et al.* found that different genetic backgrounds influenced the timing of embryonic lethality.[157] They cited the trauma of birth as a possible contributing factor to the absence of TF-/- mice and performed cesarean sections to deliver offspring from heterozygous matings at 18.5 dpc. One -/- offspring was found and lived precariously to 4 weeks of age until it suffered cerebral hemorrhage. These results favour

the idea that during early embryonic development, the role of tissue factor as a procoagulant may be secondary to one involved in maintaining vascular integrity.

Together, studies on tissue factor gene disruption suggest that TF may not be necessary during development, but is essential for surviving the major challenges to vascular integrity that occur at mid-gestation and at birth. Its role as the initiating molecule of coagulation remains undisputed, but its role in other physiological and/or pathological processes is still not clearly defined. The suggestion by one group[156] that TF deficiency leads to improper vascular development is consistent with the previous finding that TF is linked to angiogenesis as shown by Zhang *et al.*, who reported that Meth-A sarcoma cells transfected with and overexpressing TF, developed tumours with greater vessel density, while tumor cells transfected with a TF antisense construct displayed a lower degree of vascularity.[151] In another study using a different tumor model and methodology – teratomas/teratocarcinomas that were +/+, +/- or -/- for TF – failed to show a significant difference between tumors expressing or not expressing TF.[157] Although the disruption of the tissue factor gene has provided new evidence that TF possesses other functions besides a role in coagulation, these other functions require further definition and delineation.

In conclusion, while some human deficiencies such as hemophilia A and B are phenotypically recapitulated with high fidelity in their corresponding gene knockout model in mice, some "knockouts" produced more severe phenotypes than are found in human deficiency states. For example, gene disruptions of *FVII* and *fibrinogen* produced

much more severe hemorrhagic consequences than those found in humans with corresponding deficiencies. Furthermore, in contrast to the milder abnormal bleeding of FV-deficient humans, *FV* inactivation in mice led to both fetal loss and early post-natal mortality from intra-abdominal hemorrhage. It is important to note however, that most human deficiency states are characterized by a significant decrease of factor activity, and not a complete absence of the protein. In contrast, however, the disruption of coagulation factors genes in mice produces an "absolute" deficiency of these proteins. Therefore, it may not be surprising that the resultant murine phenotypes are more severe than those observed in humans. Nevertheless, these severe consequences suggest that the physiological role of these coagulation proteins requires further scrutiny at the developmental level. Consistent with this view, is a third group of "knockouts" comprising the inactivation of coagulation factor genes for which the corresponding human factor deficiencies have not been described. For example, embryos with *TF* and *TM* gene disruption did not survive to birth, indicating an absolute requirement for the corresponding factors in development. Thus, it is likely embryonic lethality that precludes the existence of the corresponding deficiency states in humans. Therefore, taken together, the unexpected hemorrhagic events and fetal loss seen in murine coagulation factors "knockouts" imply that the functions of these factors are likely not restricted to coagulation, and that their influence on development may be considerable.

1.4 Hemostasis and Vessel Formation During Murine Embryonic Development

So far, studies of blood vessel assembly and hemostasis have remained autonomous and mutually exclusive of one another. Our current understanding of the coagulation cascade and the fibrinolytic system are based on postnatal and primarily adult mammalian physiology. However, during development, vasculogenesis, angiogenesis, and hemostasis must be tightly regulated to ensure viability. We know that in development, the vascular system undergoes extensive remodelling with the emergence and disappearance of channels through vasculogenic and angiogenic processes.[8] Blood circulation begins at about 9.0 dpc, when the embryonic heart begins to contract and at this developmental stage, a contiguous vascular system is formed with the fusion of extra- and intra-embryonic vessels. Given the dynamic processes involved, it is remarkable that hemorrhagic and thrombotic events do not interfere with embryonic growth even though channels are opening and closing and direction of blood flow may change several times.[2,3] From the preceding, it can be inferred that a developmental interrelationship must exist between blood vessel formation and hemostasis, and that they must be coordinated during development such that the vascular architecture is not interrupted by clotting and bleeding events.

Recently, evidence has begun to accumulate indicating that mechanisms of coagulation in the embryo may be distinct from those of the adult. In particular, it has been the unexpected phenotypes resulting from knockouts of coagulation factors genes that have contributed to this idea. Specifically, deficiencies of procoagulants factor V,[121] PAR-1,[141-142] and TF[154-156] do not produce bleeding phenotypes but rather exhibit lethality

or fetal loss. Likewise, targeted disruption of the anticoagulant TM, also yields embryonic lethality with little evidence of thrombosis.[148-149] In addition, gene disruptions of factors believed to be essential for hemostasis and fibrinolysis – fibrinogen and plasminogen – are not only compatible with life, but mice with these deficiencies have developed unique methods to heal wounds. Other findings supporting distinct embryonic coagulation are derived from studies that show disparate temporal expression of factors that would be expected to be coordinately expressed during development by virtue of their functional relationship in the coagulation cascade. For example, one study found the thrombin receptor to be expressed during early organogenesis at 9.5 dpc, while its ligand, prothrombin, was not expressed until 12.5 dpc.[158] Also, it has been shown that some coagulation factors may have additional functions not related to coagulation at all. Protein S for example, is expressed in the nervous system and is a ligand for the Tyro 3/Axl family of receptor tyrosine kinases involved in remodelling developing neural networks.[159] These data are difficult to reconcile with our understanding of "adult" coagulation and suggest that developmental coagulation may be distinct from its adult counterpart.

The purpose of our study is to investigate further the developmental interrelationships between coagulation and blood vessel formation in the mouse. To attempt to characterize embryonic and fetal hemostasis, we have determined the temporal and developmental expression of twenty-seven different coagulation factors by semi-quantitative polymerase chain reaction (PCR) analysis. In addition, the *in vitro* model of differentiating ES cells has been used and characterized to determine its usefulness as a

model of coagulation during development. Our approach has identified a number of discrepancies between developmental and adult coagulation. We anticipate that studies such as this, that analyze the temporal patterns of coagulation factor expression, may provide further insights into the coagulation process and also into related areas such as wound repair and tumour angiogenesis.

CHAPTER TWO: Research Project

2.1 Materials and Methods

2.1.1 Cell Culture

R1-derived ES cells were grown in the presence of leukemia inhibitory factor (LIF) (Gibco Life Technologies, #13275-011) without feeder cells (fibroblasts). Undifferentiated ES cells were maintained on gelatinized dishes (Sardstet) in Dulbecco's modified Eagle's medium (Sigma, #D5796) supplemented with LIF, 0.1mM non-essential amino acids (Gibco Life Technologies, #11140-076), 2mM L-glutamine (Gibco Life Technologies, #25030-081), 1mM sodium pyruvate (Gibco Life Technologies, #11360-070), 50μg/ml penicillin-streptomycin (Gibco Life Technologies, #15140-122), 15% fetal calf serum (FCS) (Sigma, #F2442) and 1.5 X 10^{-4} M monothioglycerol (MTG) (Sigma, #M6145).[160] Under these conditions, more than 99% of cell population remained undifferentiated as determined by visual inspection by phase-contrast microscopy.

2.1.2 Differentiation of ES cells

The method used closely follows the protocol described by Wiles[160] and Keller *et al.*[79] For differentiation, ES cells grown on gelatinized dishes as described above were dissociated by trypsinization, centrifuged for 5 minutes at 1000rpm, and then cultured in 0.8-0.9% methylcellulose in Iscove's modified Dulbecco's medium (IMDM) (Gibco Life Technologies, #12200-036) with 15% FCS, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 0.2mM L-glutamine, 50μg/ml penicillin-streptomycin and a final concentration of 4.5 X 10^{-4}M MTG in the absence of LIF. Cultures were seeded at a density of 1000 cells per ml in a final volume of 1.5ml (except differentiation day 1-4

where the final volume was 10 ml) and grown in bacterial-grade dishes. Cultures were maintained in a humidified 5% CO2 atmosphere at 37°C for 15 days.

2.1.3 RNA Isolation and cDNA preparation from ES cells and mouse embryos

Total RNA was isolated from embryos of varying ages and from ES cells using TRIzol reagent and protocol (Gibco Life Technologies, #11596), a modification of the single-step isolation developed by Chomczynski and Sacchi.[161] The procedure was modified as follows: homogenized samples were incubated on ice, 20μg of RNAse- and DNAse-free glycogen (Boehinger Mannheim, #901393) were added to facilitate RNA precipitation, and the final RNA pellet was dissolved with DEPC-treated water (diethylpyrocarbonate, SIGMA #D5758). All RNA preparations were subjected to a DNAse digestion step prior to cDNA synthesis to remove any remaining genomic DNA. For each μg of RNA, 3 units of RQ1 RNAse-free DNAse (Promega # M6101) and 40-80 units of RNAse inhibitor (RNAguard, Pharmacia Biotech #27-0815-01) were incubated in a buffer containing 10mM Tris-Cl pH8.3, 50mM KCl and 1.5mM $MgCl_2$ in a 50μl reaction volume at 37°C for 2-5 minutes. The RNA was then purified using an equal volume phenol-chloroform extraction step followed by an equal volume chloroform extraction. 0.1 volume of 3M sodium acetate pH5.2 and 2.5-3 volumes of 100% ethanol were added to the top aqueous layer (which contains the RNA), and precipitated by at –80°C. 20μg of glycogen and 40 units of fresh RNAse inhibitor were also added. Finally, the RNA pellet was resuspended in RNAse-free water.

Embryos obtained from CD1 mice (Charles River) were dissected free of maternal decidua at various time points, and washed gently in a separate petri dish with chilled 1X PBS, (8g NaCl, 0.2g KCL, 1.44g Na_2HPO_4, 0.24 KH_2PO_4 in 1 liter of water) to prevent contamination by maternal matter. 0.5 dpc was designated as noon of the day the copulation plug was observed. Dissected embryos were examined on a gross morphological level and by somite counting to ensure that they conformed with published states for the times taken.[162] Polymerase chain reactions (PCRs) were conducted with cDNA derived from pooled embryos (3-5 embryos) for each time point ranging from 8.5 to 14.5 dpc. For a positive control in embryo PCRs, RNA was isolated from an adult mouse liver since many of the coagulation factors that we are investigating are synthesized by hepatocytes in the adult organism.

Embryoid bodies were collected from methylcellulose media (MCM) (StemCell Technologies Inc., #8CC-4100) by dilution with large quantities of Iscove's modified Dulbecco's media (IMDM). Washing of embryoid bodies with 1X PBS was avoided since it increases the possibility of mRNA degradation and it interferes with RNA isolation by forming a white precipitate in TRIzol. Embryoid bodies were pelleted by centrifugation at 1000rpm for 5 minutes and liquid media was removed by suction pipette. This wash was repeated once more to ensure complete removal of methylcellulose as it was shown to affect PCR reactions.

cDNA synthesis was carried out using 40-60 ng of random primers (Gibco Life Technologies, #48190-011, Pharmacia Biotech, #27-2166-01) and mouse-Moloney leukemia virus reverse transcriptase (RT) (Gibco Life Technologies, #18053-017) in a

TABLE 2. Oligonucleotide primers used for gene expression analysis by RT-PCR

Gene	Size (bp) cDNA	Left Primer	Right Primer	Reference
α_2AP	240	5'AGCCATTTCTACCAGAACCT	5'CACGGTGCTATCCGGCAGCT	170
ATIII	226	5'AACTGAACTGCCGACTCTAT	5'TCTTTGATGCGGCCTTCAGT	171
Factor V[a]		5'ACGATCAGACCAGTTCAACC	5'CCTTCTAACGTCCACATCAC	140
Factor VII	306	5'AAAGCAGAGGGGGAGGTAAA	5'AATGATGGACACACAGGCAG	172
Factor VIII	451	5'GAGGTGGCATACTGGCACAT	5'GTACTTTAAGCATCTCTGCT	173
Factor IX	341	5'TAACTGCTGCCCACTGTCTT	5'CAGTGGAACTCTAAGGTACT	174
Factor X	256	5'AGACTCCCATCACGTTCC	5'GCATCCTCTAACTTGGCC	175
Factor XII	984	5'GCCTCAGCTTCTCAAGTTCT	5'TGAACGTGAGGTGACAGGAT	176
Factor XIIIB	272	5'CGAAAGTGGGAAGCAAGAAG	5'GGGCCAAACATGTCTCTTGT	177
Fibrinogen α-chain	250	5'ACACAGGTAAAGCGGTCACT	5'TCAGAGAGTTCGTCGAGACT	178
HPRT[a]	**249**	**5'CACAGGACTAGAACACCTGC**	**5'GCTGGTGAAAAGGACCTCT**	**179**
PAI-1	390	5'AAGTCTGATGGCAGCACCGT	5'TGCCTGTGCTACAGAGAGCT	180
PAI-2	479	5'ACAATCAACACACCACAGGG	5'ATCTCATCGGGAAGCAACAG	181
Plasminogen	220	5'TTCGAAGACCCCAGAGAACT	5'CATGCAGTCTGTCTCAGAGT	182
Protein C	234	5'GGAGTTGCGCTTCCAGGACT	5'TGGATCTGGTTCCAGTTCAT	183
Protein C Receptor	380	5'AACTTCACCCTGAAGCAGCT	5'CAGACCTGGAGTTGTTGCTT	184
Protein S	230	5'CGGCTACCGATATGATCCCT	5'CAAACTGCTCGGCCAAGTAT	185
Prothrombin	330	5'GTGAACTACCTTGGGACTGT	5'TTGGTATAGTCGGCCACGCT	186
TAFI		5'AAGTCACTGTTGGGATGAAG CTTCATGGCC	5'GTACTTCTCGAATGATGATC CGATGTAGAT	
TFPI	435	5'TATGCCTTTTGGGCCACTGT	5'AGCCACCGTACACGAATCGT	
Thrombin Receptor	368	5'TGGCAGTTCGGGTCTGGAAT	5'TAGCAGACCGTGGAAACGAT	187
Thrombin Receptor-2	174	5'CTGCTGTTTGTGGTTGGTGT	5'TACCCAGTTGTTGCCATTGA	188
Thrombomodulin	230	5'CCAGGCTCTTACTCCTGTAT	5'TGGCACTGAAACTCGCAGTT	189
Tissue Factor	395	5'GACGGAGACCAACTTGTGAT	5'TGCTCGGTGCACACTGTACT	190
t-PA	160	5'AGCTGACGTGGGAATACTGT	5'TCTCCAGGAGACCTCTTGTT	191
u-PA	530	5'AGGGTGAGCGCCAATAGCAT	5'GATACATTCACGTGGAGCAT	192
u-PAR	385	5'TGAAGGATGAGGACTACACC	5'CAGGAGACAGAGACGTTGAG	193
VWF	201	5'GTAATCCAGCGCATGGACGT	5'GAAGCTGTGCTCAGAAAGGT	194

[a]Primers obtained from published oligonucleotide sequences.

Table 2: Oligonucleotide primers were specifically designed from published cDNA sequences indicated in the 'Reference' column with the exception of two sets of primers which were previously published and used in other studies. HPRT primers (highlighted) correspond to the housekeeping gene hypoxanthine phosphoribosyltransferase, the relative expression of which was used to calibrate the amount of cDNA synthesized by RT-PCR.

reaction volume of 20μl under conditions recommended by the manufacturer with the exception of several modifications: 40 units of RNAse inhibitor and 1μl of 0.1M DTT (Gibco Life Technologies, #Y00147) were added to the RNA priming step. Reactions were terminated by heat inactivation of the RT at 95°C for 5 minutes. We prepared cDNA from total RNA derived from 4 X 10^4 cells (at differentiation day 0) and from 1.5μg of mouse embryo RNA from each time point.

2.1.4 Semiquantitative RT-Mediated PCR (RT-PCR)

Semiquantitative RT-PCR was performed following the procedure developed by Keller *et al.*[79] To compensate for variable yields of RNA and cDNA, the amount of cDNA synthesized was calibrated using the relative expression levels of hypoxanthine phosphoribosyl transferase (HPRT), a housekeeping gene, as a standard. Using a series of dilutions of cDNA synthesized from a known number of ES cells (at day 0 of differentiation), PCR was performed with primers specific to HPRT (Table 2). Using HPRT as a standard, all cDNA samples were equalized to $\leq$ 60 cell equivalents of ES cDNA per PCR, of which 40% was subjected to gel electrophoresis. The amount of cDNA used from embryos per PCR was 30-40ng. To equilibrate cDNA between *in vitro* and *in vivo* studies, PCR using HPRT primers was performed on day 0 ES cells and on a serial dilution of cDNA from 14.5 dpc embryo sample. cDNA amounts were balanced usually by ethidium bromide staining. All PCRs were performed using oligonucleotide primer sets, specifically designed to coagulation related genes (Table 2).

In addition to careful inspection of embryos to confirm developmental stage, embryonic age and correct differentiation of ES cells into embryoid bodies were assessed by performing PCRs using primers corresponding to genes that have been established as endothelial and hematopoietic markers from previously published data[163,50]. These markers are listed in Table 3.

PCR reactions were carried out in 50μl reactions under standard Perkin Elmer-Cetus (Norwalk, Conn.) conditions; 50mM KCl, 10mM Tris-Cl pH9.0, 0.1% TritonX-100, 0.2mM dNTP's (Pharmacia Biotech, #27-2035-01, Boerhinger Mannheim, #1277049), 1 U Taq Polymerase (Gibco Life Technologies, #18038-018, Labquip, #) plus 50-60pmol of each primer. The PCR regimen of 94°C denaturation for 1 min, 55°C primer annealing for 1 min and 72°C extension for 1 min was run for 35 cycles preceded by a 5 minute 94°C hot start and followed by a 10 minute extension at 72°C using a Perkin Elmer-Cetus thermocycler (Perking Elmer thermocycler model#480). Approximately 40-50% of each product was size-separated electrophoretically on a 2% agarose gel containing ethidium bromide in 1X TAE buffer (40mM Tris-acetate, 1mM EDTA pH8.3).

PCR product specificity was confirmed by the following criteria: RT-PCR signal was present in the adult mouse liver known to express the transcript being investigated and the product was of predicted size. Furthermore, routine controls including cDNA reaction mix with no reverse transcriptase added (to check for genomic contamination) and a PCR control with no template added (to control for PCR artifacts caused by contamination) were performed. Because PCR efficiency can vary, even among

TABLE 3. Oligonucleotide primers of hematopoietic and endothelial genes used in RT-PCR

Transcript	Size (bp) cDNA	Left Primer	Right Primer	Reference
α-globin	331	5'CTCTCTGGGGAAGACAAAAGCAAC	5'GGTGGCTAGCCAAGGTCACCAGCA	179
βH1 globin	265	5'AGTCCCCATGGAGTCAAAGA	5'CTCCAAGGAGACCTTTGCTCA	179
β major globin	578	5'CTGACAGATGCTCTCTTGGG	5'CACAACCCCAGAAACAGACA	179
PECAM	260	5'GTCATGGCCATGGTCGAGTA	5'CTCCTCGGCATCTTGCTGAA	163
TEK	456	5'CCACTCCCTACTAGTGAAG	5'ATGCCCTTCTCCACCCTCT	195
TIE	228	5'CTCACTGCCCTCCTGACTGG	5'CGATGTACTTGGATATAGGC	163

reactions within the same experiment, all PCR reactions were performed at least twice (and usually 3 times) to ensure that consistent expression patterns were obtained. In addition, to ensure that 35 cycles of amplification did not produce a plateau effect[164], whereby the exponential rate of amplification is attenuated, resulting in an expression pattern that is not reflective of the actual condition, an excess of primers and dNTPs were included. Comparisons between 25 and 35 cycles using same templates were performed to confirm that patterns of expression were similar and do reflect events in development and exclude the possibility that results obtained are due to the plateau effect.

2.1.5 Touchdown PCR

In cases in which nonspecific PCR products were obtained, an alternative but equally effective and comparable touchdown PCR protocol[165,166] was utilized. We modified this method to use a range of annealing temperatures that is sequentially applied during the course of 20 cycles. The initial two cycles are well above the estimated annealing temperature and the annealing temperature of each subsequent two cycles is run at 1°C less than the preceding cycle. By the time the optimal Tm of the specific primer-template combination is reached through this range of temperatures, the specific product will have achieved a geometric advantage over nonspecific priming, thereby outcompeting nonspecific products for resources. The touchdown protocol follows a regimen of 94°C denaturation for 1min, a range of 65°C to 56°C annealing temperature for 1 min and a 1 min 72°C extension with two cycles per annealing temperature then

94°C for 1 min, 55°C for 1 min and 72°C for 1 min, for 20 additional cycles. As with standard PCR, a hot start and final extension were included.

2.1.6 Isolation of Murine TFPI cDNA clone

A C57BL/6 mouse liver cDNA library in λZAP (Stratagene, #93502) was screened for the cDNA encoding murine tissue factor pathway inhibitor (TFPI). The library was constructed with cDNA fragments greater than 1000 base pairs in length cloned into the *Eco*RI site of the λgt10 after addition of *Eco*RI linkers. Approximately 50000 phage were screened with entire rat TFPI cDNA probe (gift from Dr. K. Enjyoji and Dr. H. Kato, Japan). The phage library was plated on LB agar plates in 0.7% top agarose as described[167] with XL1-Blue host cells, OD_{600}=2.0 (grown in 0.2% maltose, 10mM $MgSO_4$ LB media). Duplicate filters of plaques were lifted from plates using supported nitrocellulose filters (Schleicher & Schuell, # 8320). Filters were processed sequentially through a denaturation solution, neutralization solutions, a 4X SSC (1X SSC: 150mM NaCl, 15mM Na_3citrate pH 7.8 in 1 liter of water) wash and water dip[167] before optimal UV crosslinking.

Probe was labelled with [α-^{32}P]dCTP and a random primer labelling kit following the protocol suggested by the supplier (Pharmacia Biotech, #XY-027-00-06) and purified using Nick Spin Columns (Pharmacia Biotech, #71-6033-00) equilibrated with STE buffer (50mM Tris-Cl pH 8.0, 100mM EDTA pH 8.0 and 0.5% sodium docedyl sulfate (SDS)). The probe reaction was loaded onto the column with equal volume of STE buffer and spun at 500g. Specific activity was determined by removing 1/100 of final

volume to the scintillation counter. Filters were prehybridized in 6X SSC, 0.5% SDS, 5X Denhardt's reagent (1X = 0.2% polyvinyl pyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) and 100mg/ml boiled sheared salmon sperm DNA at 68°C for 2 hours and hybridized at 60°C overnight.[167] 2 X 10^6 cpm/ml of high specific activity probe was added to the hybridization solution. Washes were carried out as follows: one rinse and two 20-minute washes in 2X SSC and 0.1% SDS at room temperature and one rinse, one 20-minute wash at 50°C and another 20-minute wash at 65°C in 0.1X SSC and 0.1% SDS.[167] Filters were exposed to autoradiographic film (Kodak, XAR-5) overnight at -80°C.

Clones hybridizing positively with the probe were picked and removed to 1ml SM buffer (5.8g NaCl, 2g $MgSO_4 \cdot 7H2O$, 50ml 1M Tris-Cl pH 7.5, 5ml 2% gelatin solution, water up to a litre) containing 40μl chloroform and vortexed to release phage into buffer. This eluate was replated and the plaques rescreened as above until a single plaque was purified. Usually, a secondary and tertiary screen is performed (using smaller plates and fewer plaques) to confirm plaque purification.

The lambda ZAP vector is unique in that pBluescript II SK– vector can be excised from the bacteriophage in a simple *in vivo* excision protocol using the ExAssist/SOLR kit (Stratagene, #200253). Positive plaques were stored in 0.5ml SM buffer plus 20μl chloroform at 4°C (phage stock). 200μl of XL1-Blue cells OD_{600}=1.0 were incubated with 100μl phage stock with the addition of 1μl of ExAssist helper phage (>1 X 10^6 pfu/ml) at 37°C for 15 minutes. 3ml of 2X YT medium (10g NaCl, 10g Yeast extract, 10g Bacto-tryptone in one litre of water) were then added and the mix was further

incubated for 2-2.5 hours at 37°C with shaking. The mixture was then heated at 70°C for 20 minutes and spun for 15 minutes at 4000g. The supernatant contains plasmid package as a filamentous phage particle. This phagemid was plated out by first incubating it with SOLR host cells, OD_{600}=1.0, (grown in 0.2% maltose, 10mM $MgSO_4$ LB media) for 15 minutes at 37°C and plating 100μl on LB-ampicillin plates treated with X-gal and IPTG for blue/white selection. Plates were incubated at overnight 37°C.

Colonies were analyzed by the alkali lysis miniprep preparation method[167] and isolated plasmid was digested with *Eco*RI (New England Biolabs, #101S) to release the insert. A band ~1.3 kilobases in length was observed when digestion reaction was electrophoresed on a 1.2% agarose gel containing ethidium bromide. This insert was characterized by DNA sequencing.

2.1.7 Nucleotide Sequencing

The cDNA insert obtained by library screening was sequenced by a combination of the Sanger dideoxynucleotide chain termination method[168] using ^{35}S-dCTP (NEN) and a T7 sequencing kit (Pharmacia Biotech, #XY-010-00-17) and automated sequencing. Separate A, G, C, T sequencing reactions were prepared using 1.5-2.0μg of plasmid DNA with insert as template and the T7 sequencing kit following manufacturer's guidelines. Manual sequencing reactions were resolved on a 6% polyacrylamide gel that is exposed to an autoradiographic film overnight. Cycle sequencing using the ABI prism dye-terminator sequencing kit (Perkin Elmer Applied Biosystems, #402079) was also performed to sequence the murine TFPI insert. Sequencing was done according to the

ABI standard protocol with 0.5μg of template. For all sequencing reactions, plasmid DNA prepared by alkali lysis was purified by polyethelene glycol (PEG) precipitation. Plasmid DNA was precipitated with an equal volume of 13% PEG in 1.6M NaCl on ice overnight. Samples were then centrifuged at 14000rpm for 15 minutes and pellet is washed with 70% ethanol. The final pellet was dissolved in 30-40μl sterile water. Alternatively, template used for sequencing was prepared using plasmid purified by centrifugation in a CsCl-ethidium bromide gradient with the subsequent removal of ethidium bromide by 1-butanol saturated in water[167], and desalting through a NAP-10 column (Pharmacia Biotech, #52-2075-00) equilibrated in 10mM sodium phosphate buffer pH 6.8. Sequencing was done using T3 and T7 primers and internal primers and was performed on both strands of cloned insert (refer to Table 4 for primer sequence). Nucleotide and protein homology searches and sequence comparisons were carried out with the BLAST[169] (Baylor College of Medicine, National Center for Biotechnology Information), GenBank, and SwissProt databases.

Table 4: Nucleotide Sequences of Primers used to Sequence Murine TFPI Clone

Primer Name	Antisense 5' to 3'	Sense 5' to 3'
T7	TAATACGACTCACTATAG	
T3		AATTAACCCTCACTAAAG
mTFPI	^{697}AGCCACCGTACACGAATCGT^{678}	^{162}TATGCCTTTTGGGCCACTGT^{181}
mTFPI-1	^{1163}GAAACAAGACACTGGTTTGT^{1144}	^{313}GCAAAGCAATGATAAGGAGT^{232}
mTFPI-2	^{887}GGCATTTCCCAGTGGCTGAAT^{867}	^{608}CCGCAACAACTTTGAAACTT^{627}
mTFPI-3	^{467}CTTCACAGCTGTCTTCTCAT^{448}	^{623}CCCTTCGTGAAAGTTGTATAT^{643}

Table 4: Nucleotide sequences of oligonucleotides used to determine cDNA sequence of murine TFPI. Sequencing locations are given in superscript at both ends of each nucleotide. mTFPI primer set was also used in our semi-quantitative RT-PCR analyses.

2.2 Results

2.2.1 Quantifying and Equilibrating HPRT Expression in Cells and Embryos

Our semi-quantitative PCR relies on the relative expression of a housekeeping gene, *HPRT*, as an internal control. All cDNA samples were carefully adjusted to yield equal amplification of an *HPRT*-specific band. RNA isolated from mouse embryos could initially be quantified by optical density and we could establish with some accuracy that after DNAse treatment and cDNA synthesis, 30-40ng of template be used per PCR. However, the amounts of RNA collected from embryoid bodies were too low to quantify by optical density measurements, and cDNAs were therefore equilibrated to ≤60 cells worth of cDNA per PCR. Because amounts of cDNA were equalized by relative band intensity of PCR products, it is estimated that balancing among samples is at least equal to or better than threefold and that the amount of cells and cDNA believed used in each PCR reaction (i.e. ≤60 cells worth of cDNA) is more likely to be an overestimation of the amount of template actually used per PCR.[79] Results from *HPRT* equilibration of ES cells and embryonic samples are shown in Fig. 1.

Having independently calibrated *HPRT* expression within *in vitro* and *in vivo* samples, it was then necessary to determine whether these samples were equalized to each other. As part of our study was to investigate the utility of the *in vitro* system to study *in vivo* developmental events, it was sensible to ascertain that results from these separate studies were comparable by ensuring that the initial expression of *HPRT* was similar. PCR was performed using cDNA from day 0 ES cells and 14.5 dpc mouse embryos (Fig. 1) using amounts of template as described above. Serial dilutions of the

Figure 1: (A) Relative expression of *HPRT* in differentiating ES cells and embryonic samples collected at various timepoints. Equivalent expression of *HPRT* is seen independently in differentiating ES cells and embryonic samples. (B) *HPRT* expression is shown to be equilibrated between *in vivo* and *in vitro* samples by comparison of *HPRT* expression in day 0 differentiating ES cells (D0 cells) and in 14.5 dpc embryonic sample. Dilutions of 14.5 dpc template are indicated by dilutions 1-4. Note that there was insufficient 6.5 dpc embryonic sample to detect *HPRT* expression and this timepoint was not used in our subsequent experiments. "-ve" represents the negative PCR control in which no template was added, to control for PCR artifacts caused by contamination.

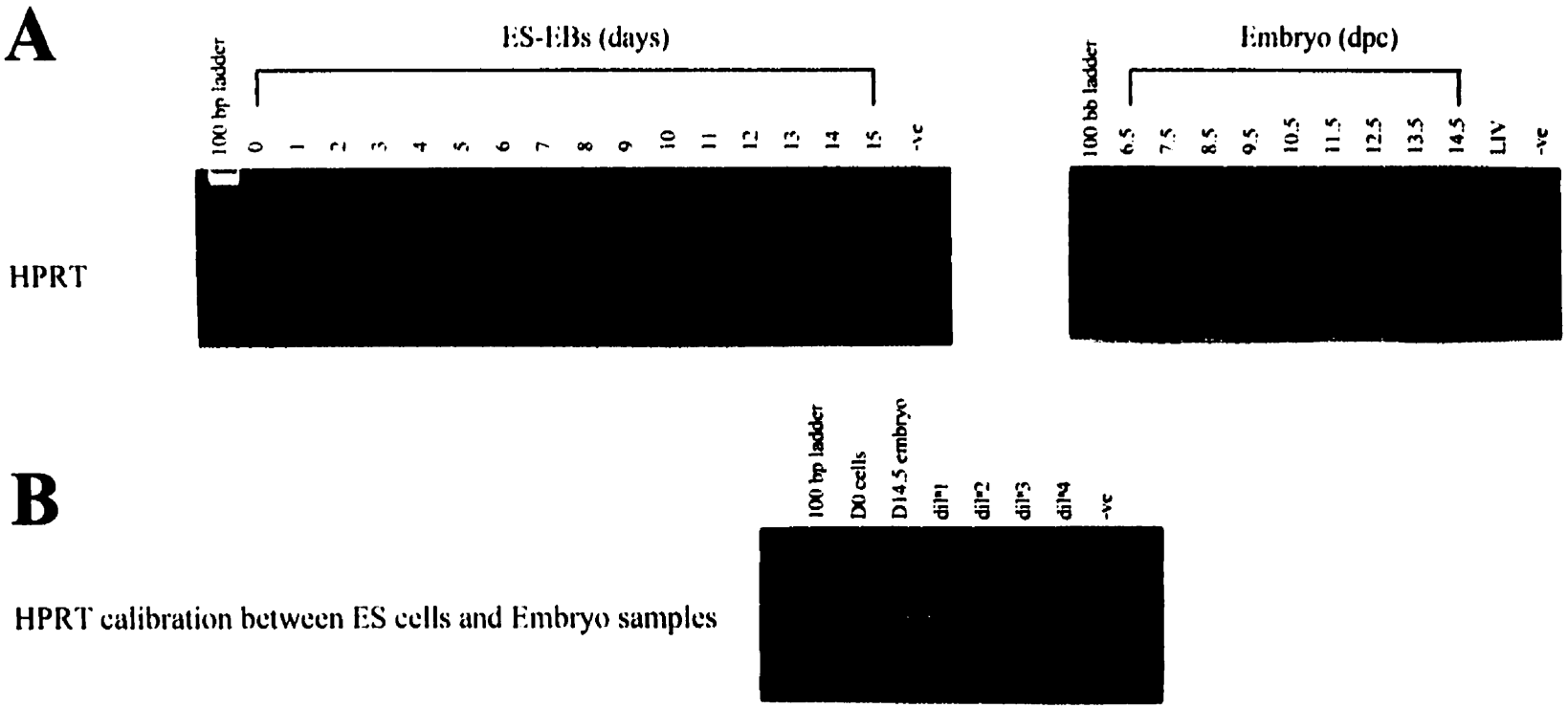

A
ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
Embryo (dpc)
100 bb ladder
6.5
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
LIV
-ve
HPRT
B
100 bp ladder
D0 cells
D14.5 embryo
dil^1
dil^2
dil^3
dil^4
-ve
HPRT calibration between ES cells and Embryo samples

Figure 1

14.5 dpc sample were performed as follows: the first dilution consisted of 1ul of template (30-40 ng) in 5ul water, and 1ul of this was removed into another 5ul of water (dilution 2). Subsequent serial dilutions were done similarly. Therefore, the initial dilution contained 5/6 the original amount of cDNA template and each following dilution is 1/6 of the previous. Dilution 1 produced an *HPRT* band closest to the band intensity seen for the day 0 ES cell sample. This suggested that results obtained from PCRs of *in vivo* and *in vitro* samples could be compared using the standards that we established. The amount of cDNA template for embryonic samples was not significantly more than template used in *in vitro* PCRs as seen by comparing band intensities of lane 1 and lane 2 of Fig. 1B.

2.2.2 Generation and Characterization of Differentiating Embryoid Bodies

The *in vitro* system of differentiating ES cells to embryoid bodies has been used and well characterized in previous studies investigating hematopoiesis and vascular development, and has been shown to mirror *in vivo* events fairly closely.[10,27,78,79,163] In our study, we used this same system to study the expression of coagulation factors to determine whether it recapitulates the expression patterns observed in the developing mouse embryo.

We chose to differentiate R1 ES cells in a semisolid methylcellulose matrix not only to prevent cells from attaching to the plastic of culture dishes, thereby altering the desired differentiation pathway, but also to prevent cells from coalescing into heterogeneously-sized EBs, resulting in asynchronous differentiation. Growing cells in semisolid media reduces cell mobility and ensures that EBs are derived from single ES

cells. Upon visual inspection, ES cell differentiation appeared to be consistent with differentiation states published by Wiles.[160] However, to confirm that embryoid bodies were at correct differentiation stages, RT-PCR using primers corresponding to hematopoietic/endothelial markers was performed on days 0-7 ES cell samples, and results were compared with published findings.[79,163] Results of this analysis are shown in Fig. 2 and summarized in Table 5. In general, we observed that *PECAM*, *tek* and *tie* were expressed one day later than observed by Vittet *et al.*[163] However, it is important to note that Vittet *et al.* used different ES cell lines, and used Southern hybridization analysis of RT-PCR products to detect gene expression. This method is more sensitive in that although the PCR product might be below visual threshold by gel electrophoresis, its presence may still be detected by radioactive probes. Results of PCRs using globin primers were comparable to those presented by Keller *et al.*[79] and Weiss *et al.*[179] *β major globin* was not detected until day 6, two days later then published data. However, Keller *et al.* used at least 3 times more template and a slightly different PCR regimen.

In conclusion, the expression patterns of hematopoietic/endothelial markers in our embryoid bodies followed closely the trends observed by other investigators. We can therefore reasonably conclude that our *in vitro* system is correctly establishing embryoid bodies and expressing hematopoietic/endothelial genes in a predicted manner.

2.2.3 Assessment of Developmental Stages of Embryos

Gross inspection and somite counting of mouse embryos isolated at various timepoints confirmed that predicted developmental stages compared closely to published

Figure 2: Expression of hematopoietic/endothelial markers in differentiating ES cells, in embryonic samples from different timepoints, as well as in adult mouse liver. Semi-quantitative RT-PCR analysis using hematopoietic/endothelial markers were used to stage development of embryoid bodies and mouse embryos. In *in vitro* samples, expression of markers was detected reproducibly following 5-6 days of differentiation, with the exception of *βH1 globin* which was expressed at all time points. In *in vivo* samples, expression of markers was detected at 7.5 dpc. Expression of the endothelial markers, *PECAM*, *tek* and *tie* was not particularly strong in the liver sample. Analysis of embryos for the presence of *β major globin* was not included because of the high probability of contamination from maternal blood during their dissection from maternal decidua. "-ve" represents the negative PCR control in which no template was added to control for PCR artifacts caused by contamination.

Figure 2

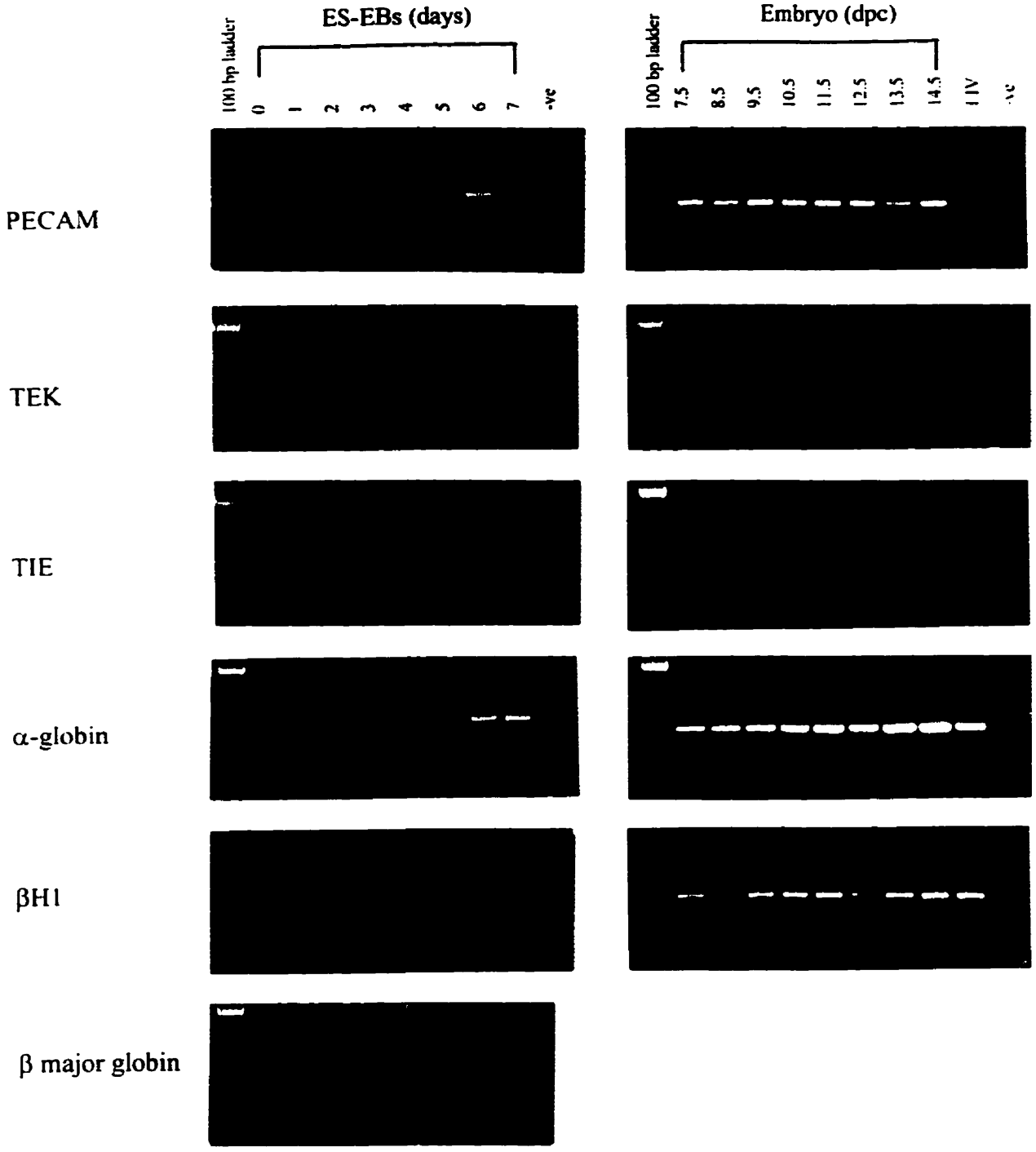

ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
-ve
Embryo (dpc)
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
PECAM
TEK
TIE
α-globin
βH1
β major globin

TABLE 5. Summary of PCR data on ES-EBs[b] using primers of endothelial/hematopoietic markers, days 0-7

Gene	Signal detected after indicated days of differentiation[a]							
	0	1	2	3	4	5	6	7
α-globin	–	–	–	–	–	±	+++	+++
βH1	++	+	+	+	+	+++	+++	+++
β major globin	–	–	–	–	–	–	+	+
PECAM	–	–	–	–	–	++	+++	+
Tek	–	–	–	–	–	–	+	±
Tie	–	–	–	–	–	+	+	+

[a]After 30-35 cycles of amplification, samples were gel electrophoresed and photographed. Scoring (done by eye) is from ± (trace signal) to +++, (very strong signal). –, no signal.

[b]ES cells (embryonic stem cells) are totipotent cells that have been previously established from the inner cell mass cells of mouse blastocysts. In suspension culture, they will spontaneously differentiate into blood-island containing cystic EBs (embryoid bodies).

TABLE 6. Summary of PCR data on embryos using primers of endothelial/hematopoietic markers

Gene	Signal detected at embryo age (days) of [a, b]								
	7.5	8.5	9.5	10.5	11.5	12.5	13.5	14.5	LIVER[c]
α-globin	+++	+++	+++	+++	+++	+++	+++	+++	+++
βH1	+++	++	+++	+++	+++	++	+++	+++	+++
β major globin	+	+	++	−	+++	+++	+++	+++	++
PECAM	+++	+++	+++	+++	+++	+++	++	+++	±
Tek	++	±	+	+	+	±	±	+	−
Tie	+	±	±	+	+	−	−	+	−

[a]After 30-35 cycles of amplification, samples were gel electrophoresed and photographed. Scoring (done by eye) is from ± (trace signal) to +++, (very strong signal). −, no signal.

[b]Noon the day the copulation plug was found was designated 0.5 days. Polymerase chain reactions (PCRs) were conducted with cDNA derived from pooled embryos.

[c]Adult mouse liver was used as a positive control. Definitive hematopoiesis first begins in the liver at about 12 days into gestation and later in the bone marrow and spleen.

timepoints. Mouse embryos of same time points were pooled to minimize the effect of intralitter developmental variation. As with embryoid bodies, developmental staging of embryos was further characterized by RT-PCR using oligonucleotide primers specific to hematopoietic/endothelial markers (Figure 2 and Table 6). Expression of *tie* and *tek* was seen slightly earlier than that observed by Dumont *et al.*[50] who determined expression by RNA *in situ* hybridization analysis. Temporal patterns of *tie* and *tek* expression in embryos correlated well with that of embryoid bodies. Furthermore, expression of *βH1 globin* was consistent with the pattern seen by Keller *et al.*[79] Thus, taken together, our findings indicate that mouse embryos collected at selected time points correspond closely to published embryonic ages, and express hematopoietic and endothelial markers in a temporal manner that is consistent with previous studies.

2.2.4 PCR Analysis: 25 vs. 35 cycles

To verify that our decision to use 35 cycles of PCR amplification did not result in a plateau effect, which may have masked the expression trends of the genes that we were investigating, we also examined by 25-cycle PCR the expression of a number of genes that had been found to be strongly expressed *in vivo* at early time points by 35-cycle PCR. The common concern in using a high cycle number is that amplification during later cycles might not remain geometric, but rather may become linear, as reagents are depleted. As a result, it is thought that PCR products corresponding to low abundance templates might appear to "catch up" during late reagent-depleted cycles to those of high abundance templates. As a consequence, therefore, differences in expression between the two might become obscured over time.

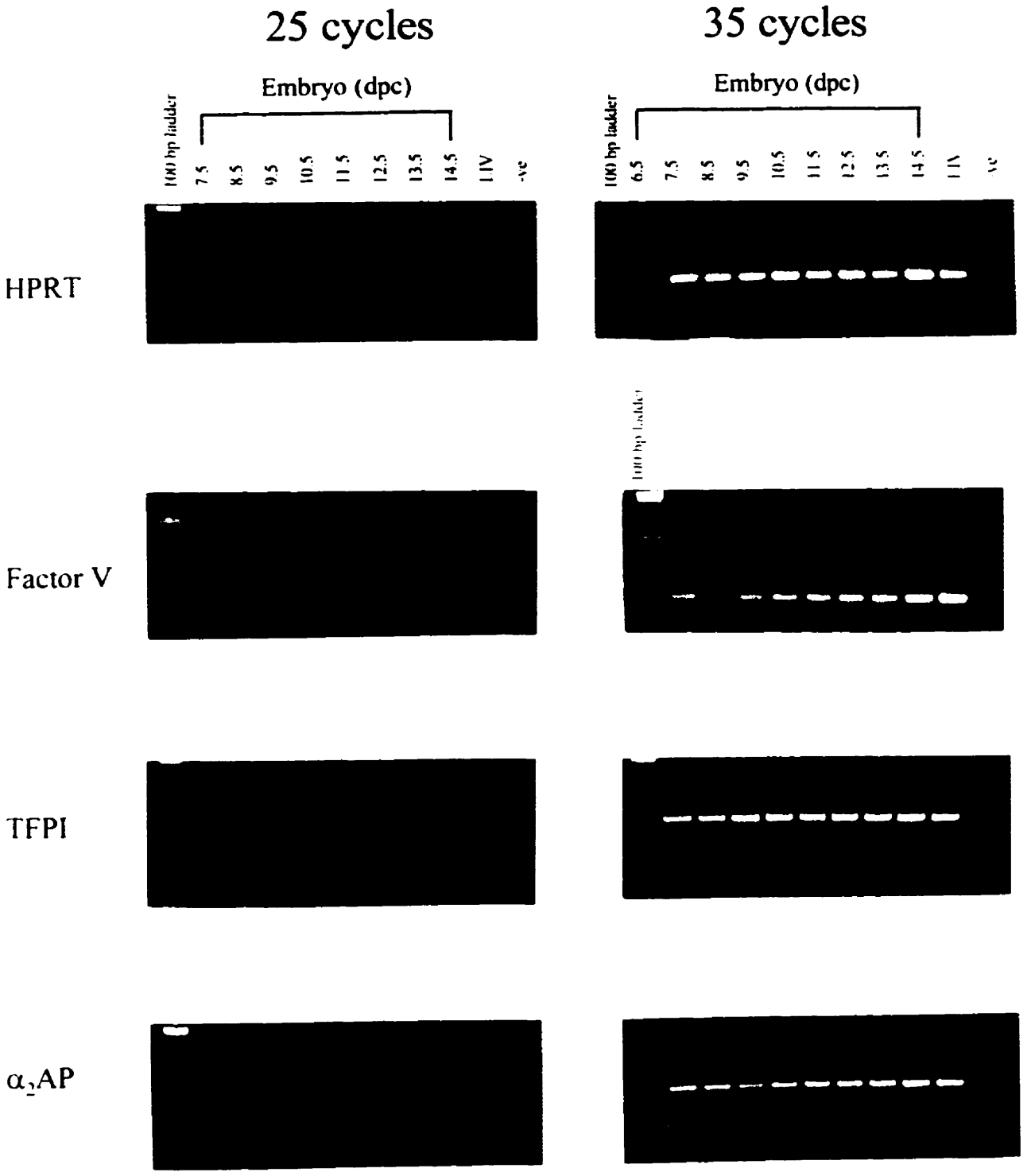
HPRT, *FV*, *TFPI* and *α_2AP* expression in embryonic samples was analyzed using 25 cycles of PCR (Fig. 3) and compared to results obtained after 35 cycles. As shown, *HPRT* bands are much fainter but still present in all samples. For *FV*, the products run are below the visual threshold and cannot be seen except for the signal in the liver sample. At 35 cycles, every time point expresses *FV* transcripts and the band in the positive control still shows a stronger signal. For *α2AP* expression, the trend seen for 25 cycles is similar to the one produced by 35 cycles of amplification. *TFPI* expression is also seen to be expressed at all time points with 25 cycles of amplification. 25-cycle PCR was similarly performed using *PAR-1*, *TM*, *vWF*, and *u-PAR* specific primers. In all cases, 25 cycles either did not yield enough product to be visualized by gel electrophoresis (*vWF, TM and u-PA*), or the expression pattern obtained followed the same pattern as seen with 35-cycle PCR (*PAR-1*). Thus, after 25 cycles of PCR, during which exponential amplification is still occurring, for some genes, there was not enough PCR product to produce visible bands. However, for those samples that yielded sufficient PCR product to be visible on ethidium bromide stained agarose gels, bands were faint and followed similar expression patterns to those after 35 cycles. Therefore, the expression patterns obtained from 35-cycle PCR remains representative of the developmental expression of genes being studied.

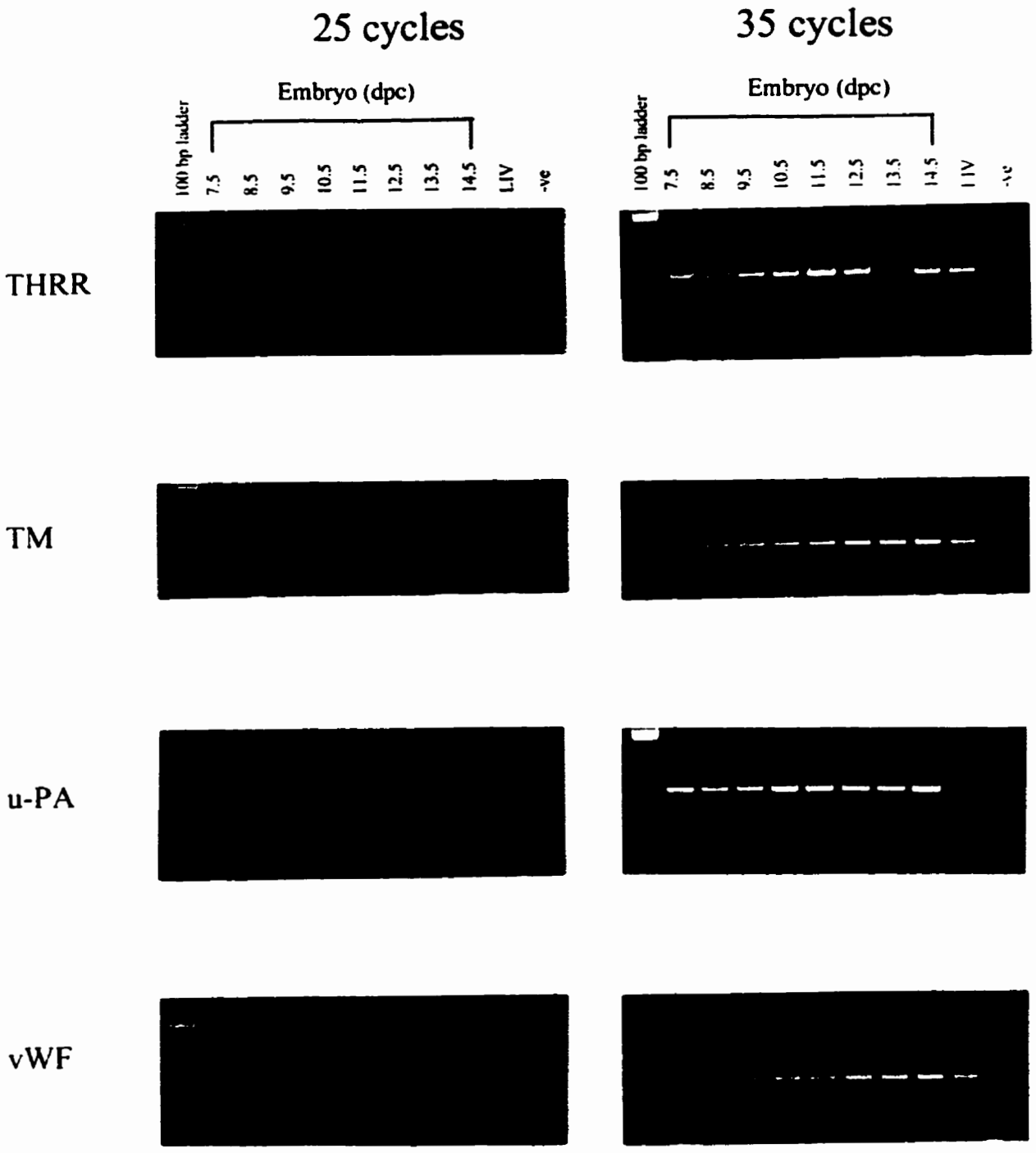
2.2.5 Expression of Coagulation Factors in Differentiating ES cells and Embryos

The expression of twenty-seven coagulation related factors was characterized using semi-quantitative RT-PCR analysis. As expected, most PCR reactions produced unique bands of expected sizes. In cases where multiple bands were observed, the

Figure 3: Comparisons of 25-cycle PCR vs. 35-cycle PCR in murine embryos. Expression of *HPRT*, *FV*, *TFPI*, α_2*AP*, *TM*, *PAR-1*, *vWF*, and *u-PA* was investigated using 25 cycles of amplification as well as 35 cycles of amplification. In general if signal was detected, it was weak although expression trends between 25-cycle and 35-cycle PCR were maintained as shown in *HPRT*, *TFPI*, α_2*AP* and *PAR-1* expression. In the other half of the cases, 25 cycles of amplification did not produce sufficient PCR products to be detected in agarose gels stained with ethidium bromide.

Figure 3


25 cycles
35 cycles
Embryo (dpc)
Embryo (dpc)
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
HPRT
Factor V
TFPI
α2AP


25 cycles
35 cycles
Embryo (dpc)
Embryo (dpc)
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
LIV
-ve
THRR
TM
u-PA
vWF

nonspecific bands were eliminated when PCR reactions were repeated using the touchdown PCR protocol. Of note, identical expression patterns were obtained with replicate PCR reactions performed in parallel, even with cDNA samples prepared from different stocks of RNA representing the same embryonic age or days of differentiation. The corresponding agarose gels are illustrated in figures 4 through 12 and these results have been summarized in tables 7 and 8. Several observations can be made from these results: First, *in vitro* expression patterns did not correlate well with their *in vivo* counterparts. Specifically, while the expression of factors *IX, XII, XIIIB, PAI-2* and *TAFI* was detected in embryonic samples, they were not detectably expressed in differentiating embryoid bodies. Furthermore, factors such as *ATIII*, α_2*AP*, *FVII*, *prothrombin*, and *plasminogen* produced very weak signals *in vitro* compared to embryonic samples. Thus, expression trends observed in embryoid bodies correlated very poorly with those seen in developing embryos. Second, analysis of *in vivo* samples indicates that with a few exceptions (see below), most factors are coordinately expressed by 7.5 dpc, well before the development of a functional circulatory system at around 9.5 dpc. Specifically, *TF*, *FV*, *FVII*, *FVIII*, *FX*, *prothrombin* and *fibrinogen* are all expressed by 7.5 dpc. Third, while most factors are coordinately expressed, the expression pattern of a number of factors is discordant with this general pattern: For example, the expression of *FIX* and *FXII* is not detected until 10.5 dpc, and *PAI-2* is not seen until 9.5 dpc. Finally, portions of our data are at variance with previously published reports. For example, we observed that prothrombin and thrombin receptor are not disparately expressed as observed by Soifer *et al.* using in situ hybridization. In fact, we found prothrombin and both thrombin receptors to be consistently expressed starting at our earliest time point of 7.5 dpc.

Figures 4-12: Expression of coagulation factors in ES-EBs and murine embryos. Temporal expression patterns observed in *in vitro* model of differentiating ES cells into embryoid bodies and in *in vivo* embryonic samples collected from 7.5 dpc to 14.5 dpc mouse embryos. *In vitro* expression results are shown on the left side of the page and *in vivo* findings on the right side of the page. Approximately 40% of each RT-PCR reaction was electrophoresed on a 2% agarose gel containing ethidium bromide. A 100 base pair ladder is located on the first lane of each gel unless otherwise indicated. In some cases, where a larger PCR product is anticipated, a 1 kilobase ladder was used. RT-PCR reactions were done in duplicates with similar results. The expression of some coagulation factors *in vitro* was consistent. For example, *TF* (Fig.4), *FV* (Fig.5), *PAR-1* and *PAR-3* (Fig.6), *TFPI* (Fig.9), *PC* and *PS* (Fig.10) and *u-PAR* (Fig.11) were expressed at all timepoints. In contrast, the *in vitro* expression of some coagulation factors fluctuated over time, particularly the expression trends of *FVII* (Fig.4), *FVIII* (Fig.5), *ATIII* (Fig.9) and α_2*AP* (Fig.12). Some factors were not expressed at all *in vitro* although they were detected in *in vivo* samples. These include, *FIX* (Fig.5), *TAFI* (Fig.6), *FXIIIB* (Fig.7), *FXII* (Fig.8), *PAI-2* (Fig.12). Almost all coagulation factors were expressed by 7.5 dpc in the embryonic samples with the exception of *FIX* (Fig.5), *FXII* (Fig.8) and *PAI-2* (Fig12). Interestingly, while *FIX* is expressed at 10.5 dpc, its only known cofactor, *FVIII* is expressed 3 days prior, at 7.5 dpc.

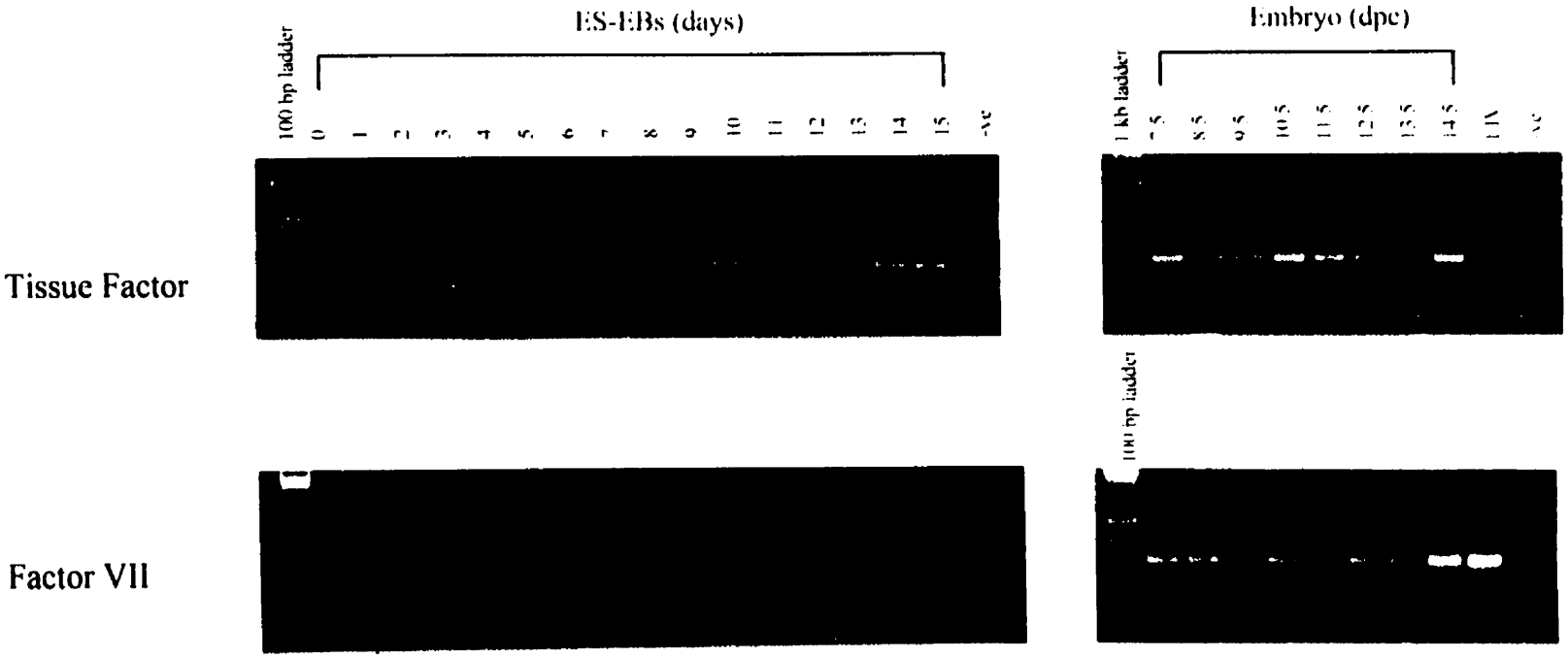

ES-EBs (days)
Embryo (dpc)
100 bp ladder
1 kb ladder
Tissue Factor
Factor VII

Figure 4

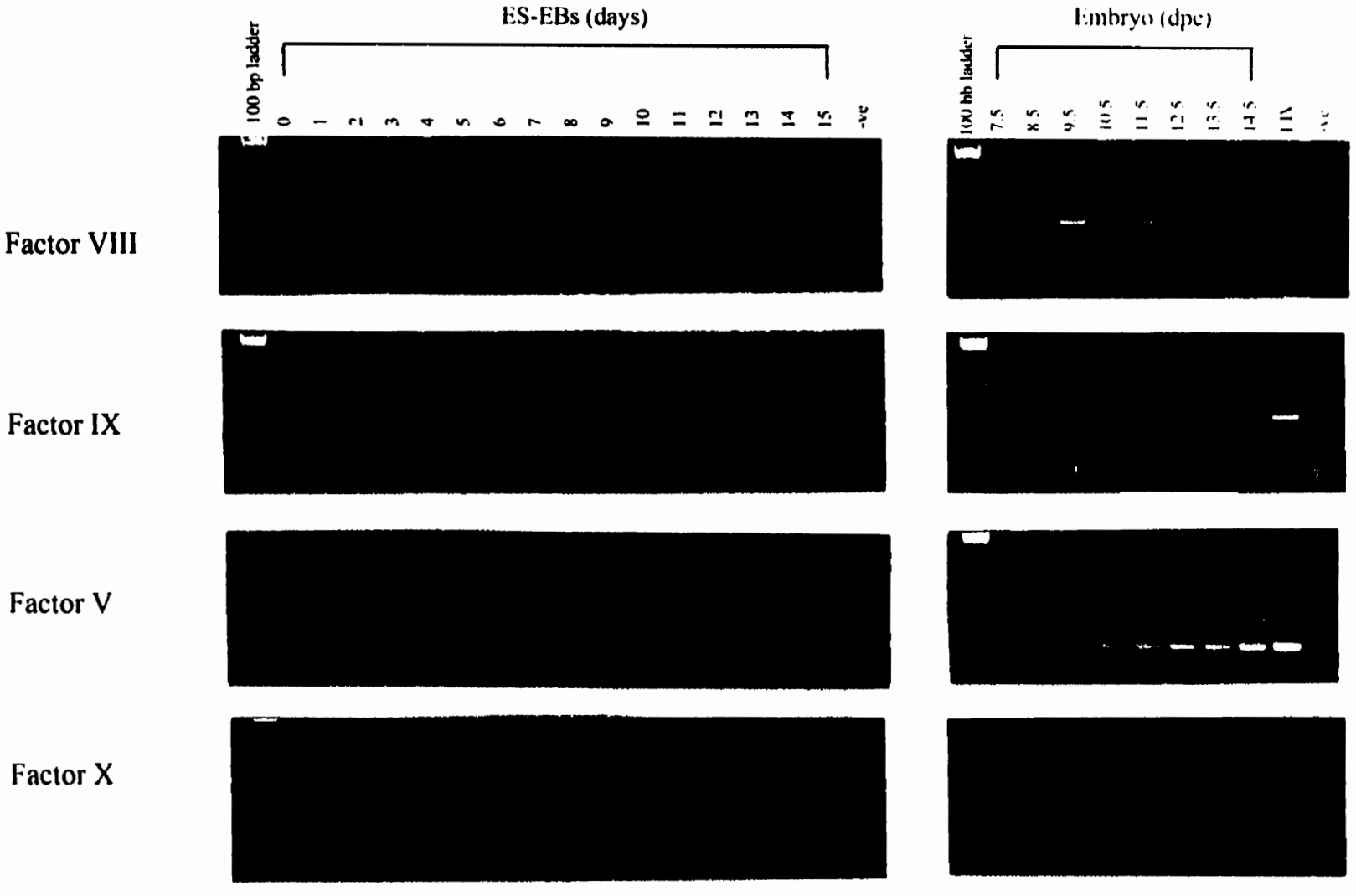

ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
Embryo (dpc)
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
Factor VIII
Factor IX
Factor V
Factor X

Figure 5

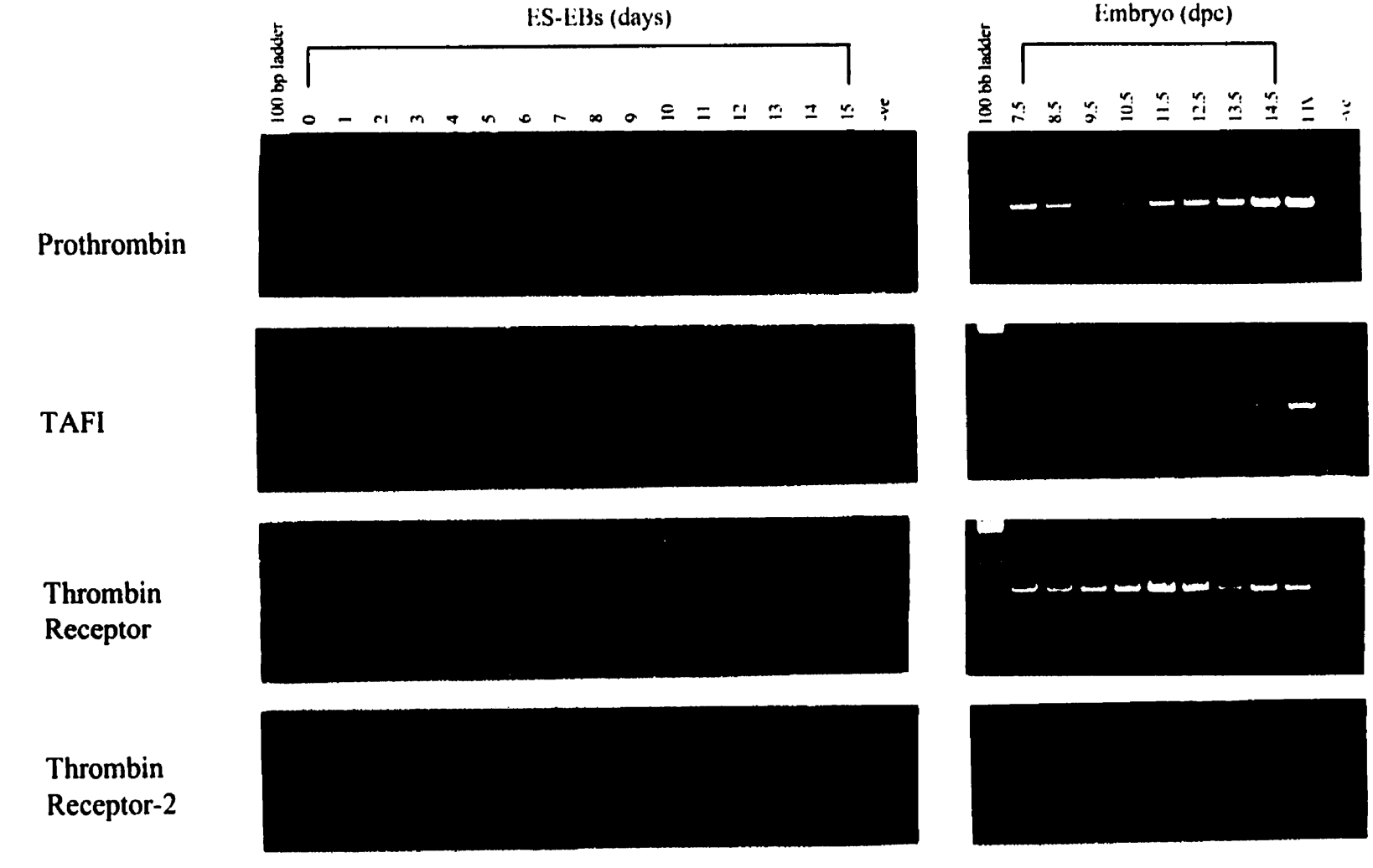

ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
Embryo (dpc)
100 bb ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
-ve
Prothrombin
TAFI
Thrombin Receptor
Thrombin Receptor-2

Figure 6

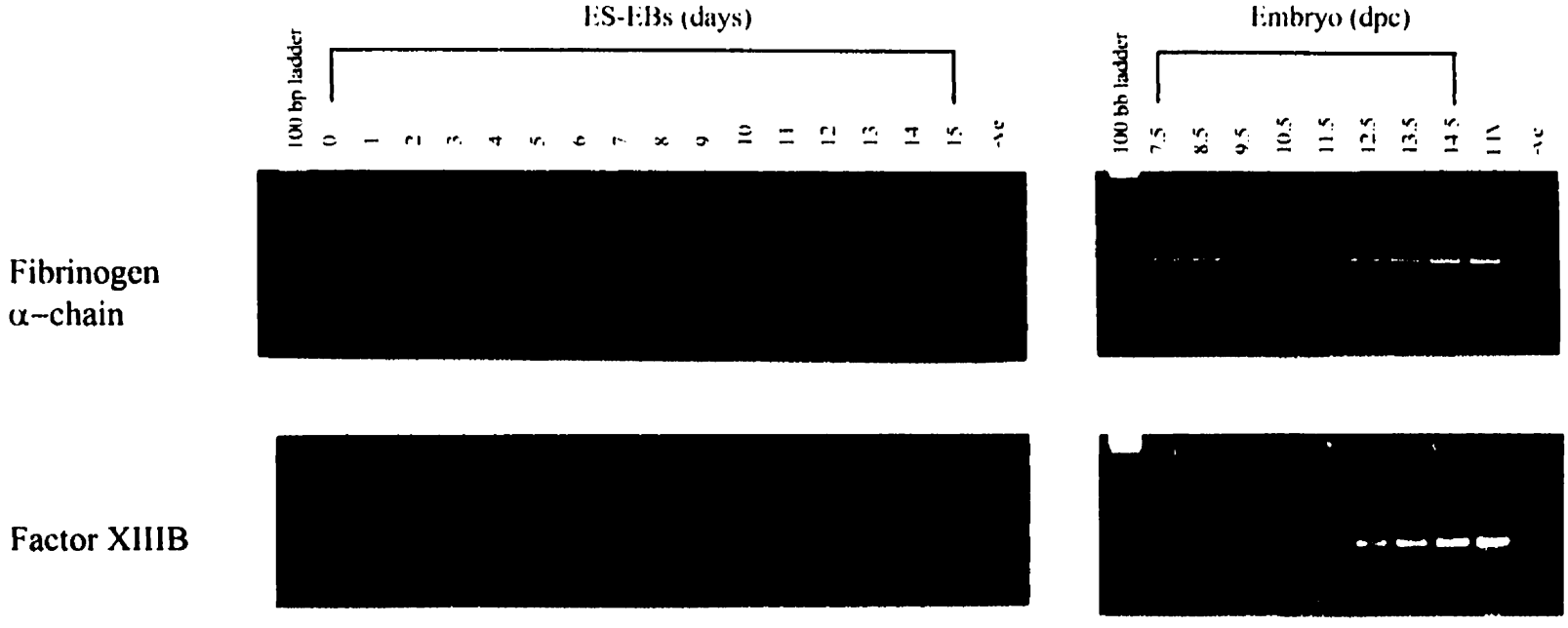

ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
Embryo (dpc)
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
-ve
Fibrinogen
α–chain
Factor XIIIB

Figure 7

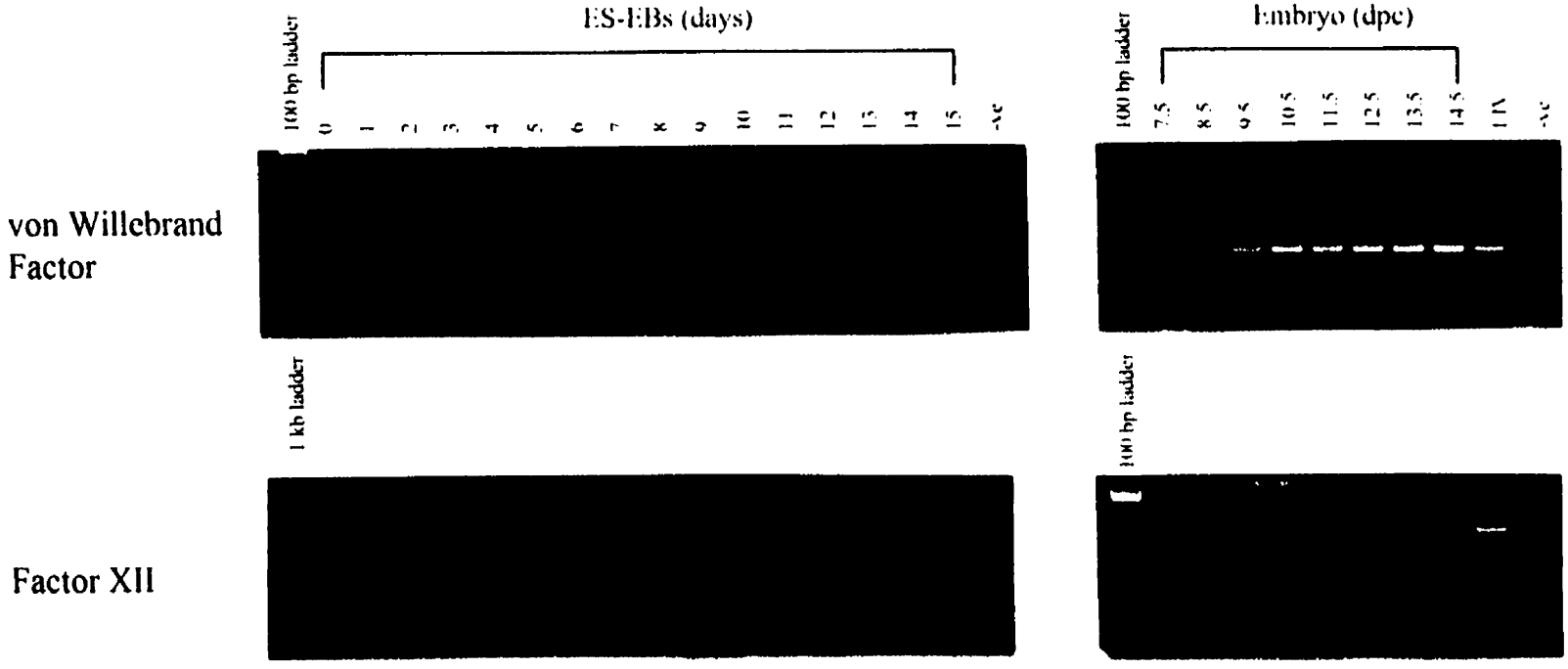

ES-EBs (days)
Embryo (dpc)
100 bp ladder
1 kb ladder
von Willebrand Factor
Factor XII

Figure 8

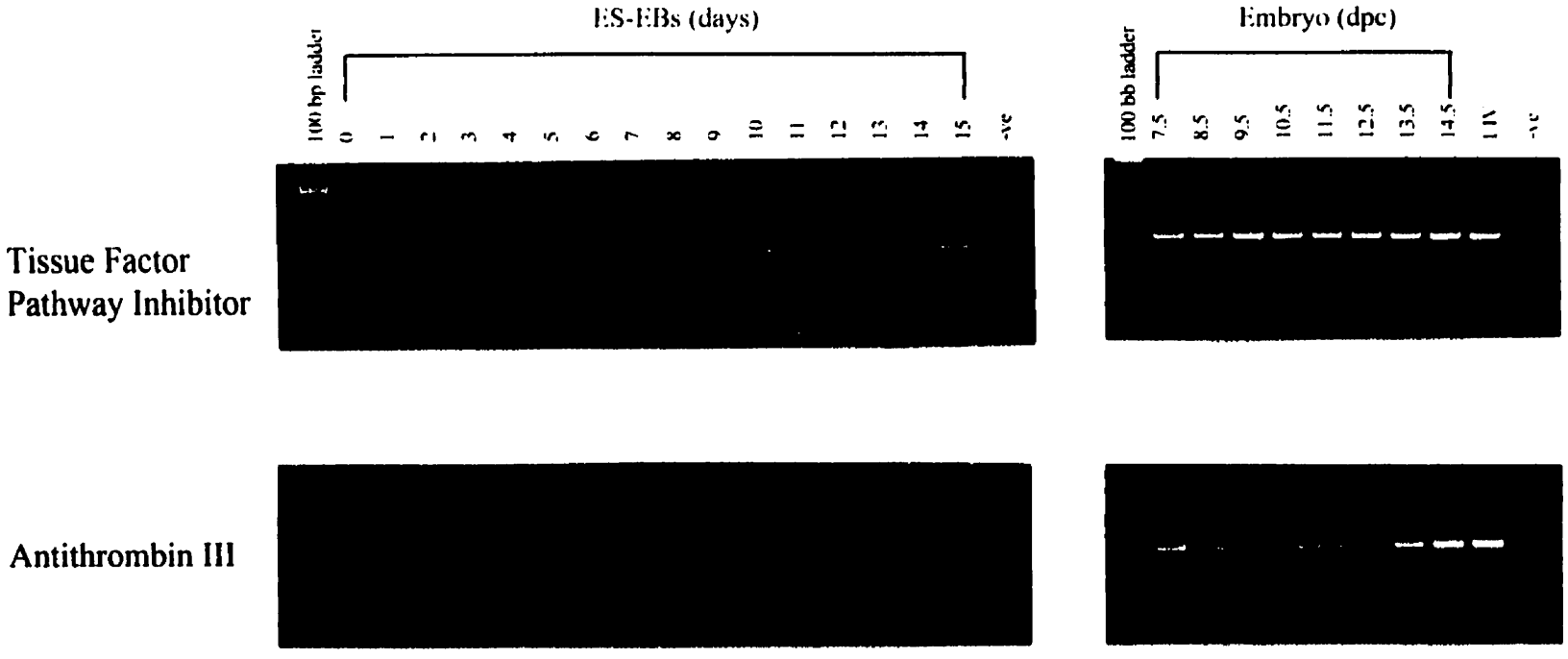

ES-EBs (days)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
Embryo (dpc)
100 bb ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
-ve
Tissue Factor Pathway Inhibitor
Antithrombin III

Figure 9

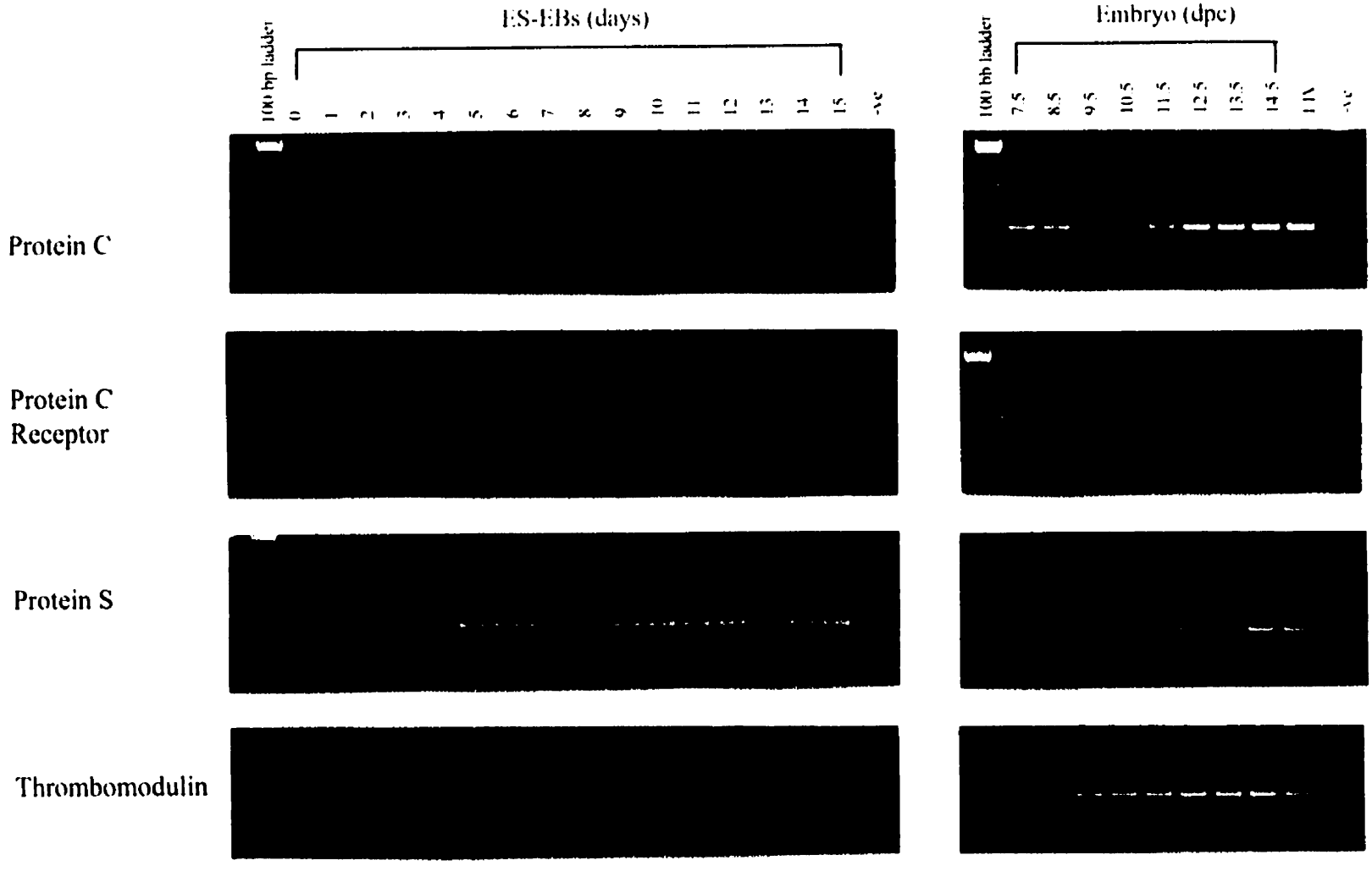

ES-EBs (days)
Embryo (dpc)
100 bp ladder
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
-ve
100 bp ladder
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
-ve
Protein C
Protein C Receptor
Protein S
Thrombomodulin

Figure 10

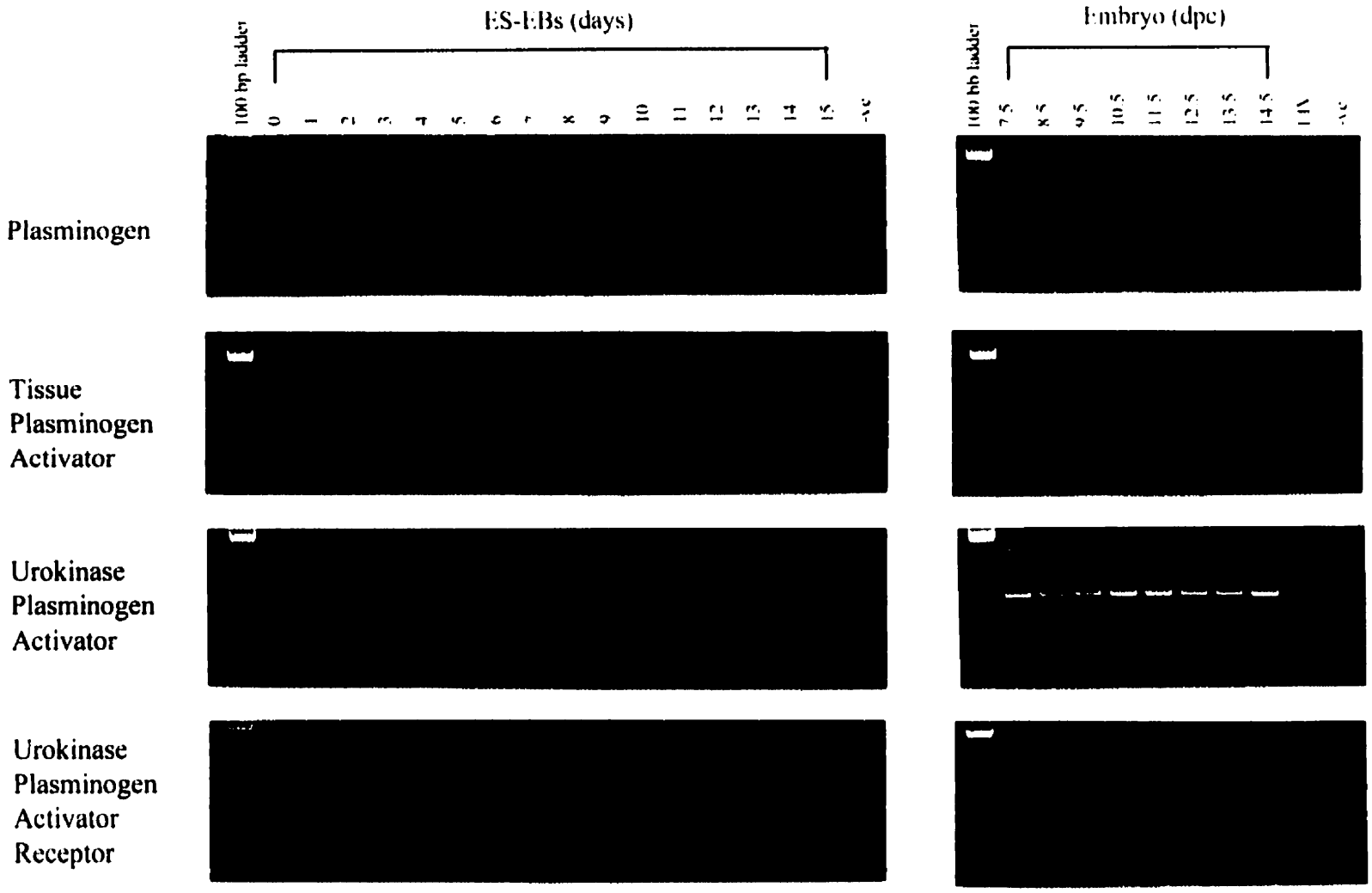

ES-EBs (days)
Embryo (dpc)
100 bp ladder
Plasminogen
Tissue Plasminogen Activator
Urokinase Plasminogen Activator
Urokinase Plasminogen Activator Receptor

Figure 11

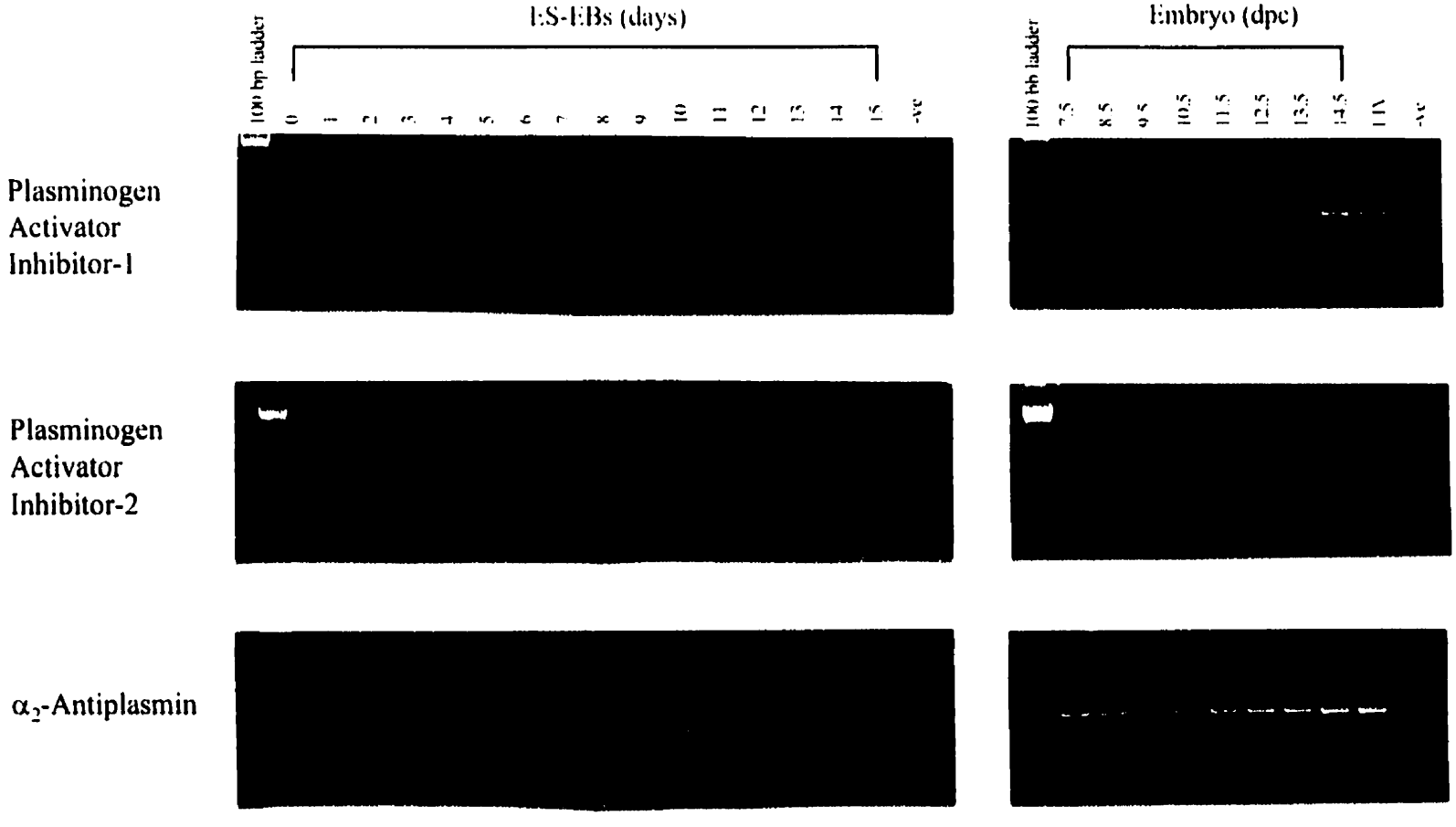

ES-EBs (days)
Embryo (dpc)
100 bp ladder
Plasminogen Activator Inhibitor-1
Plasminogen Activator Inhibitor-2
α_2-Antiplasmin

Figure 12

TABLE 7. Complete summary of PCR data on ES-EBs time course, days 0 - 15

Gene	Signal detected after indicated days of differentiation[a]															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
HPRT	++	++	++	++	++	++	++	++	+	++	++	++	++	++	++	++
α_2AP	±	–	+	–	–	±	+	+	+	–	++	+	±	+	+	+
ATIII	–	+	++	+	–	+	++	+	+	+	++	+	±	–	–	++
FV	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+
FVII	–	–	–	±	±	±	+	–	±	–	±	–	–	±	+	–
FVIII	–	±	–	+++	–	+	–	++	±	±	++	±	±	–	++	±
FIX	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FX	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FXII	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FXIIIB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FIBα	±	+	++	+	–	–	–	–	+	±	+	±	±	±	+	++
PAI-1	++	–	+	–	–	+	++	+	+++	++	+++	++	+	+++	++	+++
PAI-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PLAS	–	–	–	–	–	–	±	–	±	–	–	–	–	–	–	–
PC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EPCR	±	±	±	±	–	+	+	–	++	+	++	+	+	++	++	+++
PS	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
FII	++	–	–	–	–	–	–	–	+	–	+	±	±	±	±	+
TAFI	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TFPI	+	+	+	+	+	+	++	++	++	++	++	+	++	+	+	++
PAR-1	++	+	++	++	++	++	+++	+++	++	++	+++	++	++	++	++	++
PAR-3	±	–	±	+	+	+	+	+	++	+	++	++	+	+	+	++
TF	++	+	+	+	+	+	+++	+++	++	++	+++	++	++	++	+++	+++
TM	+	–	±	±	+	+	+	++	+++	±	++	+	+	+	++	++
t-PA	+	–	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
u-PA	–	–	–	±	±	+	+	+	+	+	+	+	+	+	+	+
u-PAR	+	+++	++	++	++	++	+++	+++	+	+	+++	+	++	+++	++	+++
vWF	+++	–	±	±	±	+	++	++	+++	+++	+++	+	++	±	+	++

[a]After 35 cycles of amplification, samples were gel electrophoresed and photographed. Scoring (done by eye) is from ± (trace signal) to +++, (very strong signal). –, no signal.

TABLE 8. Complete Summary of PCR data on embryos, days 7.5 to 14.5

Gene	Signal detected at embryo age (days) of [a, b]								
	7.5	8.5	9.5	10.5	11.5	12.5	13.5	14.5	LIVER[c]
HPRT	+++	+++	+++	+++	+++	+++	+++	+++	+++
α_2AP	+++	+++	+++	+++	+++	+++	+++	+++	+++
ATIII	+++	++	+	+	++	+	++	+++	+++
FV	++	++	++	++	++	++	++	++	+++
FVII	++	++	+	++	+	++	+	++	+++
FVIII	+	–	+++	+	++	+	+	+	++
FIX	–	–	–	±	±	±	+	+	++
FX	++	++	+	++	++	++	++	+	+
FXII	–	–	–	±	+	–	±	++	+++
FXIIIB	±	+	–	±	+	++	++	+++	+++
FIBα	+++	+++	+	++	++	++	++	+++	+++
PAI-1	++	+++	++	++	++	++	±	+++	++
PAI-2	–	–	+	++	++	++	±	++	±
PLAS	++	++	+	+	++	++	++	++	++
PC	+++	+++	++	++	++	+++	+++	+++	+++
EPCR	+++	+++	++	++	++	++	++	+++	±
PS	++	++	+++	+++	+++	+++	++	+++	+++
FII	+++	++	+	+	++	++	++	+++	+++
TAFI	++	+	±	±	+	±	+	++	+++
TFPI	+++	+++	+++	+++	+++	+++	+++	+++	+++
PAR-1	+++	+++	+++	+++	+++	+++	+++	+++	++
PAR-3	++	+	+	++	++	++	+	++	+
TF	+++	++	++	+++	++	++	+	+++	+
TM	+++	+++	+++	+++	+++	+++	+++	+++	+++
t-PA	+	+	+	+	+	+	+	+	±
u-PA	+++	++	++	+++	+++	+++	++	+++	+
u-PAR	+	+	–	–	–	–	–	+	–[d]
vWF	+++	++	+++	+++	+++	+++	+++	+++	+++

[a]After 35 cycles of amplification, samples were gel electrophoresed and photographed. Scoring (done by eye) is from ± (trace signal) to +++, (very strong signal). –. no signal.

[b]Noon the day the copulation plug was found was designated 0.5 days. Polymerase chain reactions (PCRs) were conducted with cDNA derived from pooled embryos.

[c]Adult mouse liver was used as a positive control.

[d]Although adult liver did not express u-PAR, adult mouse spleen did produce a strong signal for u-PAR when used in RT-PCR (result not indicated in table).

2.2.6 Isolation and characterization of the cDNA clone of mouse TFPI

As the murine TFPI cDNA had not been previously isolated, we were initially unable to synthesize murine TFPI-specific oligonucleotide primers. We therefore proceeded to isolate the murine TFPI cDNA, and determine its nucleotide sequence. A λZAP library containing cDNAs prepared from mouse liver mRNA was screened for the mouse TFPI cDNA by employing the entire rat TFPI cDNA (1228bp), as a probe. One plaque giving a strong positive signal was obtained, purified and found to contain a cDNA insert of ~1.3kb in length. Since the size of the insert was fairly close to the size of the rat TFPI cDNA, it was thought that this insert might contain the entire cDNA for mouse TFPI. The DNA sequence was determined on both strands.

The nucleotide sequence and predicted amino acid sequence obtained from the positive clone are shown in Fig. 13. The mouse cDNA is 1297 bp in length, excluding the poly(A) tail. A 137-bp 5' untranslated region precedes an open reading frame of 918 nucleotides which encodes 306 amino acid residues, followed by a TAG stop codon, 239 nucleotides of 3' non-coding sequence, and a poly(A) tail of 18 nucleotides. The polyadenylation sequence, AATAAA, is 16bp upstream of the poly(A) tail.

Comparisons among the mouse cDNA sequence and TFPI cDNA from 5 different species – rat[196], human[197], monkey[198], and rabbit[199] – were performed. Fig. 14 shows the sequence alignment of mouse, rabbit, human, monkey and rat TFPI. Notably, mouse TFPI showed 68.3% (88%) and 54.7% (75%) amino acid (nucleic acid) sequence similarity to rat and human TFPI, respectively. Furthermore, cysteine residues are conserved among all species (important in forming disulfide bridges during protein

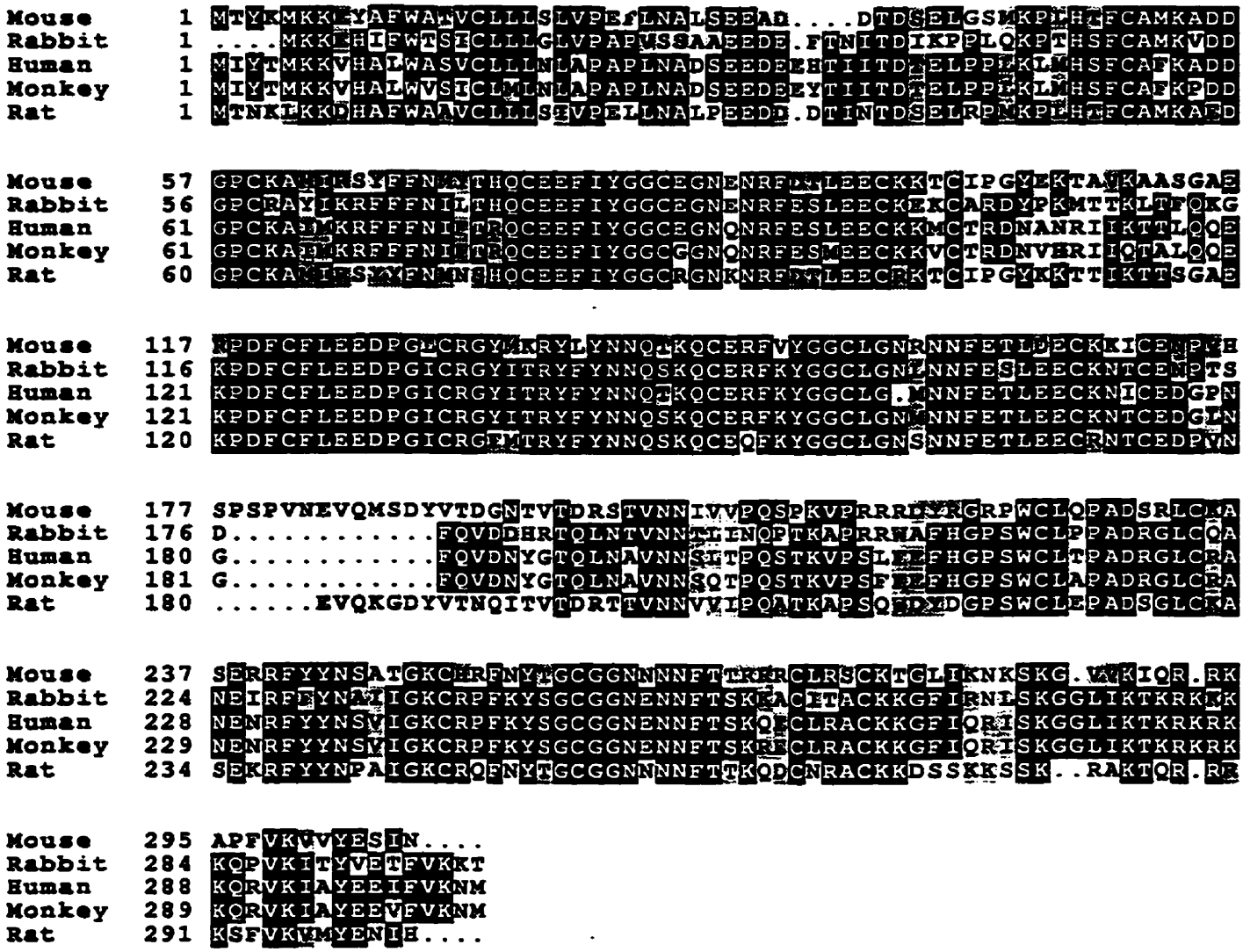
folding). In addition, the Kunitz-type inhibitory domains are also highly conserved. The amino acid sequences of the three mouse TFPI Kunitz domains are: ^{78}FIYGGCEGNENRFDTLEEC^{96}, ^{149}FVYGGCLGNRNNFETLDEC^{167}, and ^{252}FNYTGCGGNNNNFTTRRRC^{270}, respectively.

Figure 13: Nucleotide sequence and deduced amino acid sequence of murine TFPI. The nucleotides are numbered positively from the first coding residue of the cDNA sequence. Numbers in bold represent the amino acids, beginning with the initiation codon methionine.

Murine TFPI cDNA 1315 base pairs

```
-137  TGCCTCGCTGTGAATAAACATGGTCCCTCCTAAGACTGTATCACTTTCGGGACCTATCTCCGGATTTC  -70
 -69  TTCTGAAGACGGTATAATTTTGGGACCTCATCTCCAGATCTCATTTAAGGACTACTGACTCATCAAGAA  -1

   1  ATGACTTACAAAATGAAGAAAGAATATGCCTTTTGGGCCACTGTGTGTCTGTTGCTTAGCCTTGTTCCC   69
   1   M  T  Y  K  M  K  K  E  Y  A  F  W  A  T  V  C  L  L  L  S  L  C  P   23

  70  GAGTTTCTTAATGCTCTGTCTGAGGAAGCTGATGACACAGATTCTGAGCTGGGGTCAATGAAACCGCTG  138
  24   E  F  L  N  A  L  S  E  E  A  D  D  T  D  S  E  L  G  S  M  K  P  L   46

 138  CATACATTTTGTGCAATGAAGGCAGATGATGGTCCATGCAAAGCAATGATAAGGAGTTATTTTTTCAAT  207
  47   H  T  F  C  A  M  K  A  D  D  G  P  C  K  A  M  I  R  S  Y  F  F  N   69

 208  ATGTATACTCATCAATGTGAAGAATTTATATACGGGGGATGTGAAGGGAACGAGAACCGATTTGATACC  276
  70   M  Y  T  H  Q  C  E  E  F  I  Y  G  G  C  E  G  N  E  N  R  F  D  T   92

 277  CTGGAAGAGTGTAAGAAAACATGCATACCAGGTTATGAGAAGACAGCTGTGAAGGCAGCATCTGGAGCA  345
  93   L  E  E  C  K  K  T  C  I  P  G  Y  E  K  T  A  C  K  A  A  S  G  A  115

 346  GAAAGGCCAGATTTCTGCTTCTTGGAAGAGGACCCTGGACTCTGCCGAGGTTACATGAAGAGGTATCTT  414
 116   E  R  P  D  F  C  F  L  E  E  D  P  G  L  C  R  G  Y  M  K  R  Y  L  138

 415  TATAACAACCAGACAAAGCAGTGTGAACGATTCGTGTACGGTGGCTGCCTGGGCAACCGCAACAACTTT  483
 139   Y  N  N  Q  T  K  Q  C  E  R  F  V  Y  G  G  C  L  G  N  R  N  N  F  161

 484  GAAACTTTGGATGAGTGCAAGAAGATCTGTGAGAATCCAGTCCACTCCCCTTCCCCAGTGAATGAGGTA  552
 162   E  T  L  D  E  C  K  K  I  C  E  N  P  V  H  S  P  S  P  V  N  E  V  184

 553  CAGATGAGTGACTACGTAACTGATGGAAATACTGTAACTGATCGCAGTACTGTAAATAACATCGTGGTT  621
 185   Q  M  S  D  Y  V  T  D  G  N  T  V  T  D  R  S  T  V  N  N  I  V  V  207

 622  CCCCAGTCTCCCAAAGTGCCCAGGCGTCGGGATTATCGTGGCCGTCCCTGGTGTCTTCAACCAGCAGAC  690
 208   P  Q  S  P  K  V  P  R  R  R  D  Y  R  G  R  P  W  C  L  Q  P  A  D  230

 691  AGCAGATTATGTAAAGCCAGTGAGAGAAGATTCTACTACAATTCAGCCACTGGGAAATGCCACCGATTT  759
 231   S  R  L  C  K  A  S  E  R  R  F  Y  Y  N  S  A  T  G  K  C  H  R  F  253

 760  AACTACACTGGATGTGGGGGAAATAATAATAATTTTACTACCAGAAGGAGATGTCTGAGATCGTGTAAA  828
 254   N  Y  T  G  C  G  G  N  N  N  N  F  T  T  R  R  R  C  L  R  S  C  K  276

 829  ACAGGTCTCATCAAAAACAAGTCAAAAGGAGTAGTTAAGATTCAGAGAAGAAAGGCGCCCTTCGTGAAA  897
 277   T  G  L  I  K  N  K  S  K  G  V  V  K  I  Q  R  R  K  A  P  F  V  K  299

 898  GTTGTATATGAAAGCATTAATTAGTGCTAAGATATAAGCACATTAAATTTTTACATTAGTTTCACTAAA  966
 300   V  V  Y  E  S  I  N                                                  306

 967  TATTCCTTATACGGTGCTCTACTGTGATTTCTATTCTTCTACAAACCAGTGTCTTGTTTCCATTGGTGA 1035
1036  CCATCTATTAGAAGATATATTTTCGCCAGTTATGATCACAAACATTACATACCCTTGACAGTCTAAACC 1104
1105  TTTTCGAATGATTTTAATTTTTAACAATGTGAGAAATAAAATCTTTCATCCTGAGCAAAAAAAAAAAAA 1173
1174  AAAAA                                                                 1178
```

Figure 14: Alignment of the complete amino acid sequences for mouse, rabbit, human, monkey and rat TFPI. The amino acid numbers are shown at the beginning of each sequence for every line. Identical residues boxed in black. Residues with similar properties are shaded in gray. At least half the residues being compared must be similar or identical in order for shading to take place. Dots denote gaps inserted to optimize the homology of TFPI among different species. Sequence alignments was done using ClustW[200] (version 1.6) and homology presentation was prepared using BOXSHADE (version 3.21), written by K. Hofmann and M. Baron.


Mouse 1 MTYKMKKKEYAFWATVCLLLSLVPEFLNALSEEAD....DTDSELGSMKPLHEFCAMKADD
Rabbit 1 ....MKKKHIFWTSICLLLGLVPAPVSSAAEEDE.FTNITDIKPPLQKPTHSFCAMKVDD
Human 1 MIYTMKKVHALWASVCLLLNLAPAPLNADSEEDEEHTIITDTELPPLKLMHSFCAFKADD
Monkey 1 MIYTMKKVHALWVSICLMLNLAPAPLNADSEEDEEYTIITDTELPPLKLMHSFCAFKPDD
Rat 1 MTNKLKKDHAFWAAVCLLLSGVPELLNALPEEDD.DTINTDSELRPNKPLHEFCAMKADD
Mouse 57 GPCKAMIRSYFFNIITHQCEEFIYGGCEGNENRFDSLEECKKTCIPGVEKTAMKAASGAE
Rabbit 56 GPCRAYIKRFFFNILTHQCEEFIYGGCEGNENRFESLEECKEKCARDYPKMTTKLTFQKG
Human 61 GPCKAMMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTRDNANRIIKTTLQQE
Monkey 61 GPCKAMMKRFFFNIFTRQCEEFIYGGCGGNQNRFESMEECKKVCTRDNVHRIIQTALQQE
Rat 60 GPCKAMIRSYYFNMNSHQCEEFIYGGCRGNKNRFDTLEECRKTCIPGVKKTTIKTTSGAE
Mouse 117 RPDFCFLEEDPGLCRGYMKRYLYNNQTKQCERFVYGGCLGNRNNFETLDECKKICEDPYH
Rabbit 116 KPDFCFLEEDPGICRGYITRYFYNNQSKQCERFKYGGCLGNLNNFESLEECKNTCEDPTS
Human 121 KPDFCFLEEDPGICRGYITRYFYNNQTKQCERFKYGGCLG.MNNFETLEECKNICEDGPN
Monkey 121 KPDFCFLEEDPGICRGYITRYFYNNQSKQCERFKYGGCLGNMNNFETLEECKNTCEDGIN
Rat 120 KPDFCFLEEDPGICRGEMTRYFYNNQSKQCEQFKYGGCLGNSNNFETLEECRNTCEDPVN
Mouse 177 SPSPVNEVQMSDYVTDGNTVTDRSTVNNIVVPQSPKVPRRRDYRGRPWCLQPADSRLCKA
Rabbit 176 D.....FQVDDHRTQLNTVNNTLINQPTKAPRRWAFHGPSWCLPPADRGLCQA
Human 180 G.....FQVDNYGTQLNAVNNSLTPQSTKVPSLELFHGPSWCLTPADRGLCRA
Monkey 181 G.....FQVDNYGTQLNAVNNSQTPQSTKVPSFELFHGPSWCLAPADRGLCRA
Rat 180 ......EVQKGDYVTNQITVTDRTTVNNVVIPQATKAPSQSDYEDGPSWCLEPADSGLCKA
Mouse 237 SERRFYYNSATGKCERFNYTGCGGNNNNFTTKERCLRSCKTGLIKNKSKG.VKKIQR.RK
Rabbit 224 NEIRFEYNAIIGKCRPFKYSGCGGNENNFTSKEACITACKKGFIRNLSKGGLIKTKRKKK
Human 228 NENRFYYNSVIGKCRPFKYSGCGGNENNFTSKQECLRACKKGFIQRISKGGLIKTKRKRK
Monkey 229 NENRFYYNSVIGKCRPFKYSGCGGNENNFTSKRECLRACKKGFIQRISKGGLIKTKRKRK
Rat 234 SEKRFYYNPAIGKCRQFNYTGCGGNNNNFTTKQDCNRACKKDSSKKSSK..RAKTQR.RR
Mouse 295 APFVKVVYESIN....
Rabbit 284 KQPVKITYVETFVKKT
Human 288 KQRVKIAYEEIFVKNM
Monkey 289 KQRVKIAYEEVFVKNM
Rat 291 KSFVKVMYENIH....

CHAPTER THREE: Discussion

3.1 Discussion

3.1.1 Differentiating Embryoid Bodies as an *in vitro* model of coagulation

Several groups have used the differentiation of ES cells into embryoid bodies to study developmental hematopoiesis and vascular development, and found it a valid representative model of actual *in vivo* events.[10,27,78,79,163] We have briefly repeated some of these same expression studies and confirmed their findings. Because embryoid bodies not only express hematopoietic and endothelial genes but also contain blood islands and develop vascular channels, we believed that this *in vitro* system might also be useful to investigate the expression of coagulation factors during development.

The limitation of this *in vitro* model system however, is seen with the finding that some coagulation factor genes are expressed *in vivo* but not *in vitro*. These include *FIX*, *FX*, *FXII*, *FXIIIB*, *PAI-2*, and *TAFI*. In addition, *plasminogen* and *fibrinogen* exhibit trace expression in comparison to embryonic samples. While the lack of *FXII* expression in EBs may be of little consequence since it is no longer thought to have a direct role in hemostasis, critical factors necessary for clot formation, such as FIX, FX and FXIIIB, are not expressed. FX, when activated by FIXa and the FVIIa/TF complex, is the serine protease responsible for cleaving prothrombin to thrombin during the initiation and the propagation of clot formation. An absence of this factor would effectively prevent coagulation reactions from proceeding to fibrin generation and result in bleeding events. In addition, FIX, integral in amplification reactions of blood coagulation, was also not expressed in EBs. FIX deficiency results in the bleeding disorder, hemophilia B. Furthermore, FXIIIB, required for crosslinking fibrin, is not detected in differentiating

EBs. Taken together, these data suggest that a functional coagulation system is not present in EBs. Since we predict that EBs cannot sustain clot formation due to the absence of key factors, it is not surprising that a fibrinolytic system is not established in EBs as well. Since only trace amounts of *fibrinogen* are detected, and these have no way to produce a clot, the presence of plasminogen, TAFI and PAI-2, all of which are involved in fibrin clot dissolution, would be pointless. While the effects of TAFI and PAI-2 deficiencies are not known, a deficiency in plasminogen predisposes homozygous -/- mice to thromboses.[137] In contrast, the absence of plasminogen would have no effect on EBs if we accept that clotting itself cannot occur in EBs. Thus, while Wang *et al.* have shown that cystic embryoid bodies not only develop vascular channels, but that blood cells found within these channels do circulate[78], as we have shown, several key factors required for clotting are absent in this system, suggesting that it may not be a very useful model of developmental coagulation. Indeed, it has not been determined whether EBs would trigger any hemostatic mechanisms if the vessels were disrupted.

In conclusion, although embryoid bodies do express many coagulation factors under investigation, we have found little evidence to suggest that these factors are expressed in any coordinate fashion or that a reasonable semblance of a coagulation system is being established. Some factors are not expressed at all *in vitro*, making it difficult to postulate the existence of a coherent hemostatic system in embryoid bodies. Furthermore, the expression of some factors including *FVII*, *FVIII*, α_2*AP* and *ATIII* appeared to fluctuate over time. It is not clear whether this variable pattern of expression can be attributed to the limited accuracy of semi-quantitative RT-PCR analysis or

whether embryoid bodies really exhibit drastic changes in gene expression over time. Nevertheless, the latter possibility further underscores the inadequacy of embryoid bodies as a model of coagulation during development.

It may be argued that the absence of some factors *in vitro* can be explained by the inability of ES cells to differentiate appropriately into functional liver rudiments, thereby precluding the synthesis of liver-derived factors. This argument cannot account entirely for the pattern of factor expression observed *in vitro* however, as *FVII*- and *PS*-specific transcripts (which are believed to be produced by hepatocytes, at least in the adult, see Table 1) are readily detected in EBs. Nevertheless, whatever the explanation, the disparity between the *in vitro* expression of factors and their corresponding expression patterns observed in embryonic samples, underscores that EBs are unsuitable as a model for developmental coagulation. We have therefore restricted our discussion of coagulation during development to observations generated from *in vivo* samples.

3.1.2 Early Expression of Coagulation Factors in Embryonic Development

Presently, little is known of the temporal expression, during development, of most coagulation factors. In the literature, there exist only a handful of experiments that investigate the expression of coagulation factors during murine development and they have been restricted to a limited number of factors – PTHR[158], PAR-1[158], TF[158,204] and TM[146]. The developmental roles that coagulation factors may have, have been examined primarily through gene inactivation studies. Therefore, our study of the expression of twenty-seven coagulation factors during murine development will not only clarify our

understanding of coagulation in terms of its assembly in early development, but may provide insights into their interrelationship with vascular development as well.

The majority of coagulation factors investigated were expressed coordinately during development with expression first detected at 7.5 dpc, well before the formation of a functional circulatory system. This finding suggests strongly that at least some coagulation factors have additional non-hemostatic functions and physiological roles in development. This interpretation is supported by several coagulation factor gene "knockouts" that resulted in early embryonic lethality, thereby suggesting that the corresponding factors had important early developmental roles, in addition to their later roles in hemostasis. For example, *TF*, *FV*, and *TM* are all expressed by 7.5 dpc, and in all cases, inactivation of the corresponding genes resulted in embryonic lethality.[154-157,121,148,149]

Similarly, other factors such as FV and TF, which are also expressed early, have been postulated to be involved in vessel formation and maintenance processes prior to their commitment to the coagulation mechanism.[121,154,157] Fetal loss in *FV* knockout mice was not due to hemorrhagic events, and in fact fetal loss was not expected since a FV deficiency exists in humans.[106] In addition, there is support that TF is involved in vascular integrity. Carmeliet *et al.* observed vascular defects prior to wasting of TF -/- embryos[157] and Bugge *et al.* proposed a role for TF in vessel remodelling during blood island fusion and establishment of vascular connections between embryo and placenta.[154] Furthermore, TF has been linked to angiogenic processes in promoting tumorigenicity.[151-153]

In contrast to previous work that showed disparate expression of *prothrombin* and *PAR-1* in developing embryos[158], our findings clearly show that *prothrombin* is expressed as early as 7.5 dpc, as are both thrombin receptors suggesting that these proteins have additional or alternative functions unrelated to coagulation. Consistent with this idea, Soifer *et al.* found *PAR-1* to be expressed in mesenchymal cells within the endocardial cushions and possibly in smooth muscle cells.[158] This suggested that PAR-1 might have a role in septation and valve formation, as well as in controlling smooth muscle proliferation and/or differentiation.

Having cloned and sequenced the mouse TFPI cDNA, we found it also to be expressed early, before the establishment of the vascular tree. This observation suggested the possibility that it may also have an alternative role in development. Recently, it has been reported that disruption of the first Kunitz domain (which binds factor VIIa of the VIIa/TF complex) of TFPI resulted in intrauterine lethality.[201,202] With the loss of the TFPI regulatory mechanism, one might expect an increased risk of thrombosis. This was not observed. Rather, when -/- embryos were examined at various gestation periods, yolk sac hemorrhage was commonly observed early, while older -/- embryos showed evidence for hemorrhages in the spine, head and tail. In addition, structural abnormalities in yolk sac vasculature were observed. The authors proposed that a functional deletion of the first Kunitz domain of TFPI resulted in a loss of vascular integrity leading to embryonic lethality. While the role of unregulated factor VIIa/TF in contributing to fetal loss has not been ruled out, bleeding events would be unexpected as both of these factors initiate and promote clotting. The expression of *TFPI* early in the embryo supports the

contention that TFPI likely has biological roles distinct from its anticoagulant properties. This notion is further supported by the recent discovery of an alternatively spliced *TFPI*, *TFPIβ*, which contains only the first two Kunitz domains.[203] Preliminary results suggest that the anticoagulant functions of TFPIβ are not compromised. Since to date, the role of the third domain has not been determined, it is conceivable that full length TFPI may have additional functions not necessarily linked to the coagulation system.

Our data are also consistent with gene "knockout" studies that suggested that some coagulation factors have overlapping functions. Putatively overlapping factor pairs include PAR-1 and PAR-3, and t-PA and u-PA, respectively. In the "knockout" studies, it was postulated that if the function of one of the genes in each pair was removed, the remaining factor could complement the deficiency, although coordinate early expression of these specific factors was not proven. In fact, the second thrombin receptor was postulated to exist because platelets of mice deficient in the thrombin receptor still responded to thrombin.[141] Our study reinforces this interpretation and confirms that the "functionally overlapping" genes are indeed coordinately expressed early in development.

Several other lines of evidence support the idea that coagulation factors may have alternative functions unrelated to hemostasis: i) PS, a potent natural anticoagulant, is now known to be a ligand for a family of receptor tyrosine kinases expressed largely in the nervous system.[159] The notion that PS can trigger intracellular signalling cascades via the Tyro 3 receptor suggests several non-coagulant roles for protein S both as a neurotrophic factor and as a regulatory molecule functioning in a neuronal protease cascade system

aimed at synaptic remodelling and shaping. ii) An investigation into tumour suppression unearthed a potent inhibitor, which specifically blocked the growth of endothelial cells. This inhibitor, angiostatin, was revealed to be an internal fragment of plasminogen.[136] In this case, it is not so much that plasminogen itself had an alternative role, but rather, that it contains a separate molecule that is not involved in maintaining hemostatic balance, but rather acts strictly as an endogenous angiogenesis inhibitor. iii) RNA *in situ* hybridization studies have localized the expression of some coagulation factors to tissues not involved in vascular development or hemostasis. Notably, TF is expressed widely in epithelia, nervous system, epidermis and hepatocytes[204], and PAR-1 is expressed in the neurons of the hindbrain.[158] Our findings are consistent with these observations, as we have shown that protein S, plasminogen, TF and PAR-1 are all expressed prior to the formation of a functional vascular system, supporting the idea that they have an alternative function unrelated to hemostasis.

Our evidence for the expression of coagulation factors prior to a functional vascular system is based exclusively on RNA analyses. That the expression of some coagulation factors is detected before the development of structures to sustain their circulation lends support to our notion that these coagulation factors have non-hemostatic functions. However, as we have not demonstrated that actual functional proteins for these coagulation factors exist at early timepoints in our studies, it is conceivable that the factor-specific mRNAs we have detected are not translated into protein. We think that this possibility is unlikely, however, for the following reasons: i) Superfluous expression of genes that do not contribute to the developmental program is not only wasteful in

terms of energy but also in terms of depleting resources. ii) Furthermore, RNA has a limited half-life in the cytoplasm and excessive mRNAs are targeted for degradation. Therefore, the continuous expression of mRNAs for days, without their translation into proteins and their subsequent degradation, is also an unnecessary expenditure of cellular energy. iii) Finally, although there is evidence that specific mRNAs that encode for structural and/or housekeeping proteins can be stored in unfertilized eggs, long-term storage of non-maternally-derived RNA is unusual. Thus, while we cannot exclude the possibility that the early expression of some coagulation factors might be stored and translated later in development, we feel that the points presented above, along with our data, imply the existence of as yet, uncharacterized non-coagulation-related functions for these factors.

It may be argued that our findings of early expressing coagulation factors might have resulted from the contamination of our RNA samples by maternal components since maternal contamination is a common problem in embryo RT-PCR. We believe that maternal contamination is not a concern in our study. Our embryonic samples were prepared by careful dissection, and were washed repeatedly in 1X PBS, to remove maternal material. Indeed, the fact that our early-stage embryos did not express *FIX*, *FXII*, and *PAI-2* – genes that are normally expressed in adults – provides strong evidence that our approach eliminated maternal contamination and that the expression patterns observed were embryonically-derived.

While our study examines the temporal expression of coagulation factors, it does not localize the sites of their expression. We know that most coagulation factors (at least

in the adult) are produced by endothelial cells, the liver and megakaryocytes. However, our results detect their expression prior to the establishment of vascular networks, liver organogenesis, and definitive hematopoiesis. This observation indicates that these factors must have alternative sites of synthesis, at least during early development. Furthermore, the fact that most of these factors exhibit coordinate expression in the absence of a circulatory system may be significant. While the coagulation cascade cannot be assembled due to the absence of a vascular system and platelets, this does not preclude other protein cascades from existing. As shown, by 7.5 dpc, most receptor/ligand pairs such as *TF* and *FVII*, *FV* and *FX*, *EPCR* and *PC*, and *PAR-1* and *FII* are coordinately expressed. While these factors may not contribute to hemostasis during early development, they define potential mechanisms for regulating a variety of developmental processes. Indeed, some of these receptor/ligand pairs are believed to comprise signal transduction systems that lie at least partly outside of blood coagulation. For example, the FV and FX, and EPCR and PC receptor/ligand pairs have been implicated in the regulation of immune-inflammatory responses.[96,205]

3.1.3 Disparate Temporal Expression of Coagulation Factors

Intuitively, one would expect the expression of coagulation factors to be coordinated since they all act in concert with each other to maintain hemostatic balance. A failure to observe coordinate expression of coagulation factors in the developing embryos would imply that our understanding of the coagulation system (based primarily on post-natal and adult physiology) is inadequate or incomplete, or that the embryonic

coagulation system itself is distinct from that of the adult. Notably in this regard, we have found the discordant expression of a number of coagulation factors:

First, *FXII* is not expressed until 10.5-11.5 dpc. This observation may not be surprising, and is consistent with the idea that FXII is synthesized by the liver, which does not form until this time.[162] In addition, the role of FXII in hemostasis and its interaction with other coagulation proteins *in vivo* have not been characterized.

Second, the discordant expression of *PAI-1* and *PAI-2* was observed. *PAI-1* was expressed by 7.5 dpc (as was *plasminogen*), but *PAI-2* signal was not detected until 9.5 dpc. While PAI-1 and PAI-2 share homologous structures and similar mechanisms of action, their discordant expression suggests that they may have non-overlapping functions. In addition, while PAI-2 is an effective inhibitor of u-PA *in vitro*, its actions may not be limited to blood coagulation. Indeed, consistent with this view and with our data, its expression in placenta[206] and by macrophages[207] may imply a coagulation-independent role for PAI-2 in maternal-fetal immune reactions.

Third, *u-PA* and its receptor, *u-PAR*, also showed disparate temporal expression as well as contrasting levels of expression. While *u-PA* expression was fairly consistent at all embryonic timepoints, expression of *u-PAR* was variable and weak throughout development and not present in the adult liver. *u-PAR* was detected in the adult spleen (not shown), however, an organ not fully formed in the embryo until 16 dpc.[162] This pattern of expression suggests that u-PAR is not crucial for normal development, and knockout studies confirm this, as u-PAR-deficient mice did not experience any major defects in development, fertility or wound repair.[140] Our data are also consistent with the

notion that u-PAR is not the primary receptor for u-PA, and that an alternative receptor may perform this function in early development.

And fourth, discordant expression between *FIX*, and its only known cofactor *FVIII* was also observed. While *FIX* expression was not detected prior to 10.5 dpc, that of *FVIII* was apparent by 7.5 dpc. These results (together with the discordant expression of *u-PA* and *u-PAR* above) raise several questions. First, are there other interacting proteins that exist for these factors which have not yet been identified and which are expressed earlier during development? Secondly, if such proteins exist, do they elicit similar hemostatic reactions (i.e. functional redundancy) or do they promote reactions of a different physiologic pathway altogether? Both questions recognize that our understanding of coagulation during development is not complete. While one suggests that other as yet uncharacterized coagulation factors exist, the latter proposes that embryonic coagulation might be distinct and differently assembled compared to adult hemostasis.

The idea of a distinct coagulation system in the embryo is complicated by the possibility that soluble coagulation factors might be transplacentally transferred from mother to fetus. However, this transfer can only occur after chorio-allantoic fusion at around 9.0-9.5 dpc. Furthermore, it has not been determined whether significant levels of these proteins can be transferred such that they interact with embryonically-derived coagulation factors and/or compensate for deficiencies. Some evidence suggests that only minimal maternal-fetal transfer occurs, if at all. For example, in the factor VII knockout, plasma from FVII -/- embryos at 11.5 dpc, 14.5 dpc and 18.5 dpc did not

exhibit any procoagulant activity.[120] Also, when recombinant human FVIIa was injected into pregnant mice, it was not detectable in embryos 9.0-14.5 dpc of age.[120] Our study overcomes this difficulty, as we are investigating the expression of coagulation factors in embryonic samples as early as 7.5 dpc, before chorio-allantoic fusion. In addition, presumably only proteins are transferred and not mRNAs. We can therefore be reasonably satisfied that the expression of all the coagulation factors under our investigation is embryonically-derived. Thus, while the potential effects of maternally-derived coagulation factors cannot be entirely ruled out, our experiments are designed such that our findings can be construed independently of this issue.

Presently, whether clotting occurs at all in embryos is debatable. Coagulation in adult physiology depends greatly upon the expression of receptors for coagulation factors on platelets, which also provide surfaces on which the coagulation cascade can be assembled. However, as we know, the presence of platelets is not detected until 11.5 dpc. Furthermore, while fibrinogen was expressed coordinately with other factors, factor XIIIB, crucial in crosslinking fibrin polymers into a visible clot, was not appreciably detected until 11.5-12.5 dpc. Thus, it is not clear whether early embryos are capable of generating cross-linked fibrin. Consistent with this notion, the presence of fibrin in early mouse embryos has never been proven. First, when searching for fibrin clots in mouse embryos, it can not be determined whether the clots are a result of the experimental study itself (e.g. knocking out an anticoagulant to induce hypercoagulability) or whether it is due to the trauma and/or handling that embryos undergo when they are dissected from the maternal decidua. Second, antibodies that detect only murine fibrin and that do not cross

react with fibrinogen have not been generated. Thus, immunohistochemical detection using these antibodies does not necessarily mean that the protein identified was ever activated or cross-linked. In addition, it is also possible that cross-linked fibrin is not required until later in development and/or may be detrimental to the mouse embryo before 11.5 dpc. This could be determined by either overexpressing FXIIIB or expressing the protein earlier in development and observing whether this produces negative consequences.

In conclusion, analysis of the temporal expression of coagulation factors during development provides some insights into embryonic coagulation. That some factors are not coordinately expressed, as expected, illustrates that our knowledge of how the coagulation system is established during development is limited. Possibly, other undetermined coagulants or anticoagulants expressed early during development may compensate for those factors that are detected only later in embryogenesis. In contrast, it may be, that the ability to clot is not a requirement at all in early development, given that some factors likely have other biological functions, and factor XIIIB, responsible for crosslinking fibrin, exhibits strong expression only later in development. The surprising finding from knockout studies that the inactivation of supposedly "essential" genes such as *fibrinogen* is compatible with embryonic survival is consistent with this view. At the minimum, these data suggest that an understanding of coagulation based on adult physiology cannot remain a fixed concept and should be modified to take into account the discrepancies observed at the embryonic level, and argue strongly that the embryonic hemostatic system is distinct from its adult counterpart.

3.2 Future Directions

Clearly, more data are required to complement the findings of other investigators and substantiate our own conclusions regarding a distinct coagulation system during embryonic development. To follow up our results, it is logical to undertake RNA *in situ* hybridization studies. Localization of factor expression will further characterize possible alternative functions for coagulation factors, especially if it can be shown that they are also expressed in tissues not involved in vascularization and/or hematopoiesis.

In a clinical setting, new therapeutic strategies may be developed if an understanding of how coagulation functions during development is considered. It is conceivable, for example, that functionally redundant factors may be upregulated to supplement a deficiency, and coagulation or fibrinolytic mechanisms may be directly augmented to alleviate such blood disorders as hemophilia and thrombosis, or used to promote healing after surgery. Furthermore, neovascularization during tumour angiogenesis may be specifically targeted if factors contributing to this process are recognized.

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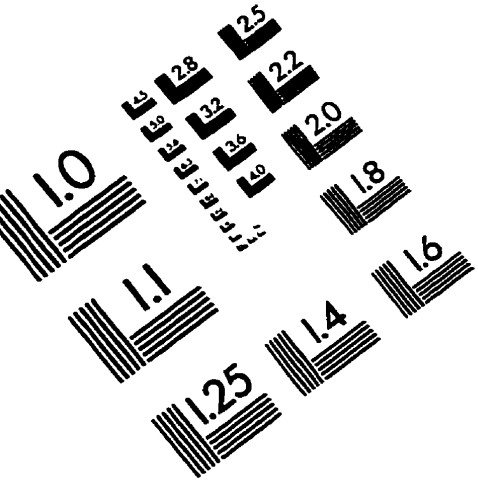

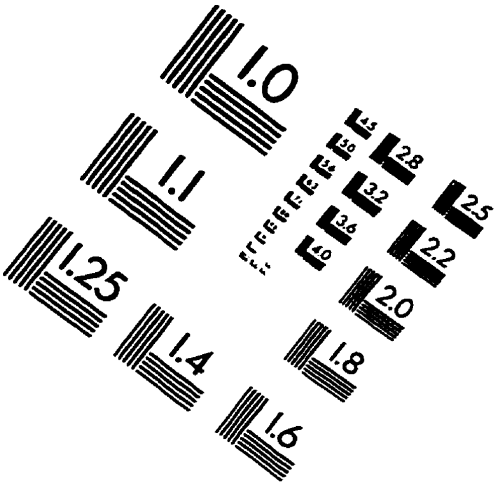

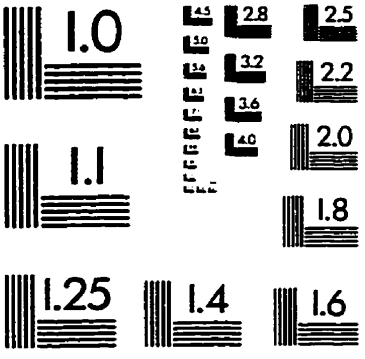

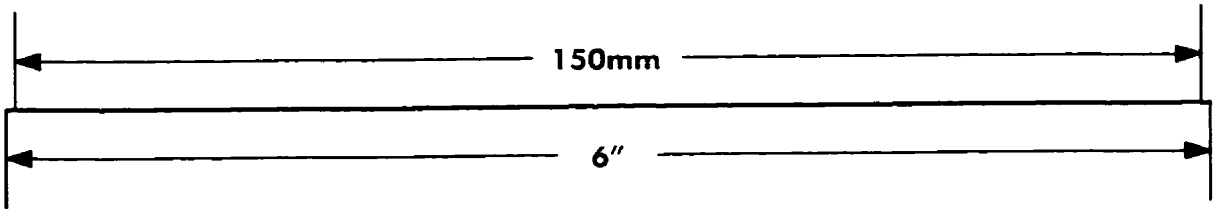

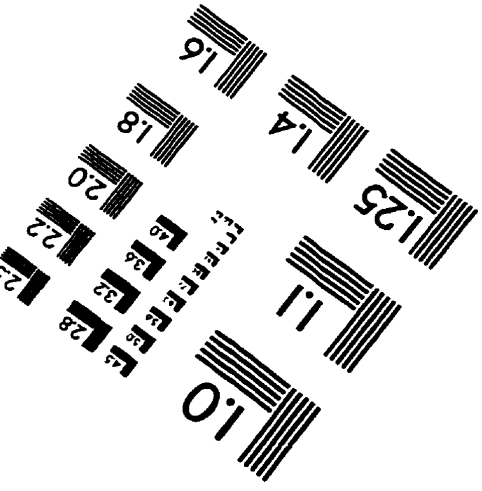

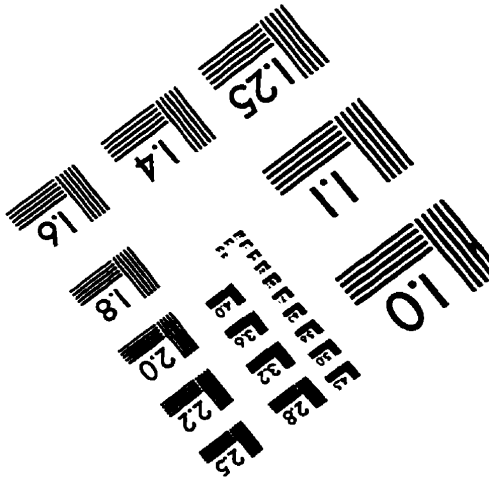

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