NTNU – Trondheim
Norwegian University of
Science and Technology

Localization and Function of Factor XIII at the Fetal-Uterine Interface

Recurrent Abortion Management Issues

Line Syslak

Chemical Engineering and Biotechnology
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Supervisor: Per Bruheim, IBT

Norwegian University of Science and Technology
Department of Biotechnology

Preface

The work related to this master thesis was performed at "Unité de Pathologie Cellulaire et Génétique" at University of Versailles Saint-Quentin-en-Yvelines (UVSQ), located at the Poissy Hospital in France. The research facility focuses mainly on problems concerning placental pathology and pregnancy issues. The project started January 3rd 2012 and ended June 29th 2012, and the project was funded by the University of Versailles Saint-Quentin-en-Yvelines.

My gratitude goes to my external supervisor Professor Philippe de Mazancourt for accepting me as a student at his facility and allowing me to participate in this project. I have taken great interest in the project, and I have gained valuable knowledge for the years to come. I would also like to thank my internal supervisor Professor Per Bruheim at the Norwegian University of Science and Technology (NTNU) for his support throughout the stay abroad.

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I appreciate all the support and kindness I have been given from all the members of the laboratory team at UVSQ. I have been included from day one, even though language was to me a greater challenge than I had foreseen. You have all been patient and encouraging, and helped me in any matter, both project related and in personal matters. I would specially like to thank Marie-Noëlle Dieudonné, Mbarka Dakouane-Giudicelli, Linda Bellazi, Corinne Morvan and Dorothée Poidatz.

Performing the master thesis abroad has been a great experience; I have learned how to work in a different culture and also I have learned how to communicate with people of different ethnicity. Even though it has been challenging, I don't regret that I took the step and applied for a project abroad. I want to thank Maryse de Hauteur and Gerd Inger Sætrum for helping me with all the related paper work and making this experience possible for me.

Line Syslak, 24th July 2012, France.

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Abstract

The blood coagulation Factor XIII (FXIII) is a transglutaminase catalyzing γ-glytamyl ε-lysine crosslinks between various molecules. It is most known for its role in crosslinking fibrin and stabilizing the blood clot in the process of coagulation in wound healing. The Factor XIII is also essential in maintaining pregnancy, and recurrent spontaneous abortions are reported in FXIII deficient patients.

The localization and the role of FXIII in the development of the placenta were investigated in this study. Preliminary, FXIII was found to be located in macrophages, and in this study we verified by immunoblotting and mass spectrometry that FXIII is present in the decidual part of the placenta. The effect of FXIII on trophoblastic invasion during placental development was also examined. The invasion process was studied with the Boyden chamber assay using both an immortalized trophoblast cell line (HTR-8/SVneo), and primary culture of extravillous trophoblasts.

A significant effect of FXIII on inhibition of invasion using trophoblast cell line was found, but no effect was observed with the primary culture. The cause of the inhibition on invasion observed with the cell line was ruled out to be caused by other indirect effects of FXIII, such as cytotoxicity, effect on secretion of matrix metalloproteases and proliferation. The results showed unfortunately great variability; therefore better controls must be included in the assays in order to obtain more reliable results.

Future perspectives are to optimize the existing method or to use another technique to study the more *in vivo* approach on invasion using primary culture. Later, it would be interesting to examine the proteomics of FXIII; to identify the molecules involved in crosslinking by FXIII in placental development. This would allow us to understand more of the physiology of FXIII and its role in placental development which could aid in recurrent abortion managements.

Sammendrag

Blodkoaguleringsfaktoren Faktor XIII (FXIII) er en transglutaminase som katalyserer γ-glytamyl ε-lysin kryssbinder mellom ulike molekyler. Faktoren er mest kjent for sin rolle i kryssbinding av fibrin og stabilisering av blodlevring under koaguleringsprosessen i sårheling. Faktor XIII er også viktig for opprettholdelsen av svangerskap, og gjentatte spontanaborter er rapportert hos pasienter med FXIII mangel.

I dette studiet ble lokalisering av FXIII og faktorens rolle i utvikling av morkaken (lat.: placenta) under graviditet undersøkt. FXIII ble innledningsvis lokalisert i makrofager i morkaken, og i denne studien ble det bekreftet ved immunoblotting og massespektrometri at FXIII er tilstede i livmorshinnen (lat.: decidua) i morkaken. FXIIIs innvirkning på invasjon av trofoblastceller under graviditet ble også undersøkt. Invasjonsprosessen ble studert med Boyden kammer ved hjelp av både en trofoblast-cellelinje (HTR-8/SVneo), og primær kultur av ekstravilløse trofoblaster.

En signifikant effekt av FXIII på hemming av invasjonen ved bruk av trofoblast-cellelinje ble funnet, men ingen effekt ble observert med primær kultur. Årsaken til hemming på invasjonen observert med cellelinjen ble utelukket å være forårsaket av andre indirekte effekter av FXIII, for eksempel cytotoksisitet, effekt på utskillelsen av matrix metalloproteaser og proliferasjon. Resultatene viste dog store variasjoner, og for å oppnå mer pålitelige resultater må bedre kontroller inkluderes i analysene.

Fremtidige perspektiver er optimalisering av den brukte metoden eller utprøving av en annen teknikk for å studere en mer *in vivo* tilnærming på invasjonen med primær kultur. Senere vil det være interessant å undersøke FXIII proteomisk for å identifisere molekyler involvert i kryssbinding av FXIII i utvikling av morkaken. Dette ville tillate oss å forstå mer av fysiologien til FXIII og dens rolle under graviditet og igjen styrke behandlingen av gjentatt spontanabortering ved FXIII mangel.

Abbreviations

AP-FXIII	Activation peptide of Factor XIII
APS	Ammonium persulfate
BrdU	5-Bromo-2'-deoxy-Uridine
CT	Cytotrophoblast
ECM	Extracellular matrix
EVT	Extravillous trophoblasts
F13A1	Gene encoding human Factor XIII subunit A
FCS	Fetal calf serum
FXIII	Factor XIII
FXIIIa	Activated Factor XIII
FXIII-A	Factor XIII subunit A monomer
FXIII-A_2*	Activated Factor XIII subunit A dimer
FXIII-A_2'	Homodimer of FXIII-A without activation peptide
FXIII-B	Factor XIII subunit B monomer
FXIII-A_2B_2	Tetrameric form of FXIII
HBSS	HANKS' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kb	Kilo bases
kDa	Kilo Dalton
MMP	Matrix metalloproteinase
PCR	Polymerase chain reaction
rFXIII	Recombinant FXIII
SDS	Sodium dodecyl sulphate
ST	Syncytiotrophoblast
TBS-T	Tris buffered saline tween-20
TIMP	Tissue inhibitor of metalloproteases

1. Introduction

1.1 Factor XIII

Factor XIII was first mentioned as a "serum factor" in 1944, when Robbins studied the formation of fibrin from fibrinogen, a necessary step in coagulation of blood. Robbins found that in addition to the already known necessity of calcium ions, a serum factor was needed in order to render the clot insoluble in weak acid (Robbins, 1944). A few years later, this serum factor was discovered by Laki and Lorand and it was then called the "fibrin stabilizing factor" or the "Laki-Lorand factor" (Laki and Lorand, 1948). The fibrin stabilizing factor was first purified by Loewy et al. in 1957 and its enzymatic character and structure were studied thoroughly in the following years. In 1960, Duckert et al. discovered that its medical correlation was related to hemorrhagic diathesis[1] in a patient deficient in the fibrin stabilizing factor. Three years later, the International Committee on Blood Clotting Factors recognized the fibrin stabilizing factor as a blood clotting factor and was thereafter designated Factor XIII (FXIII).

1.1.1 Functions of FXIII

The Factor XIII is an important blood coagulation factor participating in the last step of the clotting cascade (Figure 1.1). The factor facilitates the formation of a stable blood clot after tissue damage, preventing excessive bleeding and aiding wound healing (Lauer et al., 2002). Activated FXIII (FXIIIa) acts as a transglutaminase[2] to form intermolecular γ-glutamyl ε-lysine crosslinks between fibrin fragments, the main protein in a blood clot (Pisano et al., 1968). The crosslinks stabilizes the clot network and increases its tensile strength (Shen and Lorand, 1983). Without the formation of the interlacing fibrous network, hemostasis[3] would be impaired by low resistance to degradation of the fibrin network.

[1] Hemorrhagic diathesis: An inherited predisposition to excessive bleeding.
[2] Transglutaminase: Enzyme that catalyze the linking between a free amine-group on proteins to the γ-carboxamide group of protein-bound glutamine.
[3] Hemostasis: The process to stop bleeding by coagulation and constriction of blood vessels.

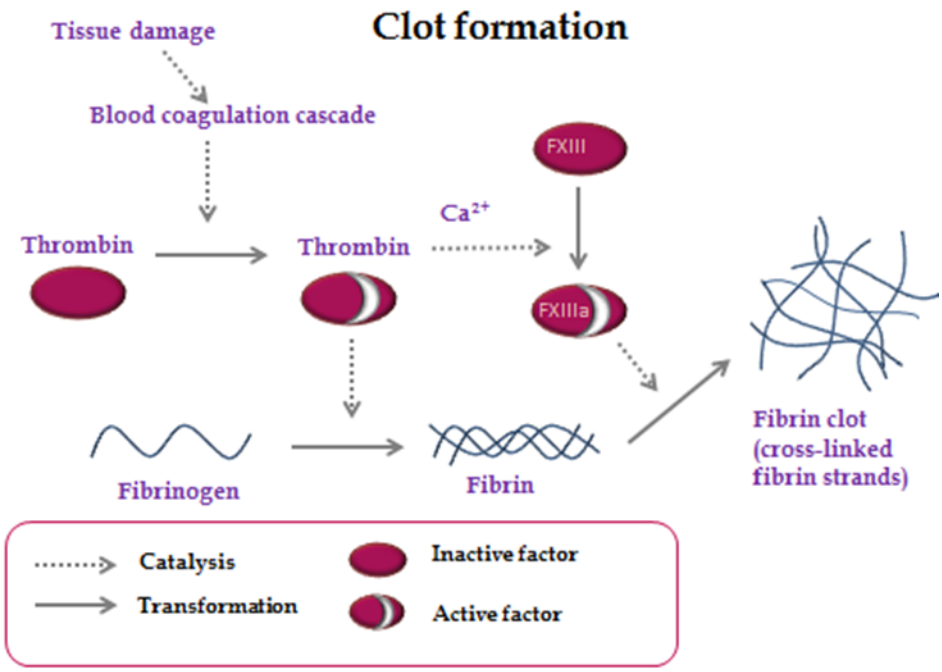

Figure 1.1: The last step of the blood coagulation cascade (After www.thrombosisadviser.com). *Tissue damage leads to activation of the blood clotting cascade where the enzyme thrombin is activated. Thrombin catalyzes the formation of fibrin from fibrinogen and also the activation of Factor XIII. The activated FXIII forms covalent bonds between the fibrin monomers and renders the clot into a more stable and strong protein network.*

FXIII can crosslink many proteins other than fibrin, such as α_2-Plasma Inhibitor which inactivates the enzyme plasmin, the main protein responsible for fibrinolysis (Sakata and Aoki, 1980). Not all substrates of FXIII are coagulation related, some are adhesive and extracellular matrix proteins, as well as intracellular cytoskeletal and contractile proteins (TRANSDAB database; http://genomics.dote.hu/wiki).

Since adhesive proteins are also substrates of FXIII, it can be said that the FXIII additionally has a role as an adhesive molecule, anchoring cells and proteins together. FXIII has been found to crosslink fibronectin, a structural component of the connective tissue, at matrix assembly sites and thus enhancing the formation of extracellular matrix (Barry and Mosher, 1988). Moreover, Dardik et al. discovered in 2002 that FXIII is involved in adhesion of platelets to endothelial cells via integrins[4] and postulated that this facilitation of cell attachment may be important in tissue remodeling and wound repair.

[4] Integrins: Cell receptors that mediates attachment between cells and tissue.

As cytoskeletal and contractile proteins such as actin and myosin are also substrates of FXIII, it has been proposed that FXIII may be involved in cytoskeletal remodeling by enhancing crosslinking between glutamic acid and lysine in actin polymerization (Cohen et al., 1980). FXIII has also been proven to have an effect in angiogenesis by promoting remodeling and sprouting of new capillaries (Haroon et al., 1999). Dardik et al. postulated in 2003 that the proangiogenic effect is due to down regulation of an antiangiogenic factor.

1.1.2 Structure of FXIII

FXIII circulates in plasma as a heterotetramer of two catalytic A-subunits (FXIII-A) and two carrier B-subunits (FXIII-B) (Schwartz et al., 1971). The FXIII-B subunits can be found in plasma both as the heterotetramer and in free form. FXIII-A is found in plasma bound in the heterotetramer, however, only homodimers of the A-subunit are present intracellularly. The enzymatic activity of FXIII is located on the FXIII-A subunits, and the B-subunits acts therefore only as transporter proteins in plasma to stabilize and protect the A-subunits (Saito et al., 1990).

The gene coding for human FXIII-A subunit (F13A1) is 160 kb long and is located at chromosome position 6p24-25 (Board et al., 1988), while the gene for human FXIII-B is shorter with 28 kb length and localized to chromosome 1q31-32.1 (Webb et al., 1989). The subunit FXIII-A consists of 730 amino acids and has a molecular weight of 83 kDa (Takahashi et al., 1986), whereas the FXIII-B consists of 641 amino acids and weighs 76.5 kDa (Ichinose et al., 1986b).

1.1.3 Cellular origin of FXIII

The FXIII-A circulating in the blood is derived from the bone marrow, which was determined by analyzes of phenotype change after bone marrow transplantation. Wölp et al. showed that the FXIII-A phenotype changed from origin phenotype to donor phenotype after the transplantation (Wölpl et al., 1987). In the same study, the authors presented also that FXIII-B phenotype did not change after bone marrow transplantation, but after liver transplantation, indicating therefore that FXIII-B is synthesized in the hepatocytes of the liver.

The homodimer of FXIII-A is mainly present in platelets (Luscher, 1957), megakaryocytes (Kiesselbach and Wagner, 1972), as well as monocytes and macrophages (Henriksson et al., 1985). FXIII-A has also been found in macrophages in the placenta and in the uterus (Adány et al., 1988). The secretion mechanism of FXIII-A from cells is not known as there has not been found a secretion peptide signal (Ichinose et al., 1986a, Ichinose and Davie, 1988). Some controversial theories suggested that release to plasma occurs during rupture of cells (Kaetsu et al., 1996). More recently it was demonstrated that a non-classical mechanism, requiring structures adjacent to the plasma membrane containing *trans*-Golgi network proteins, is involved in the secretion of FXIII (Cordell et al. 2010).

1.1.4 Activation of FXIII

FXIII is a zymogen[5] and needs activation by the concerted action of thrombin and calcium ions (Figure 1.2). FXIII is proteolytically cleaved between Arg-37 and Gly-38 by thrombin to release the activation peptide (AP-FXIII), yielding a peptide of 694 amino acids (FXII-A_2') (Takahashi et al., 1986). Calcium ions cause both the dissociation of B-subunits (Lorand et al., 1993) and exposure of the active site on the A-unit via conformational change (Hornyak and Shafer, 1991). FXIII is also inactivated again by thrombin due to a delayed cleavage at Lys-513, yielding two peptides of 56 kDa and 24 kDa (Takahashi et al., 1986).

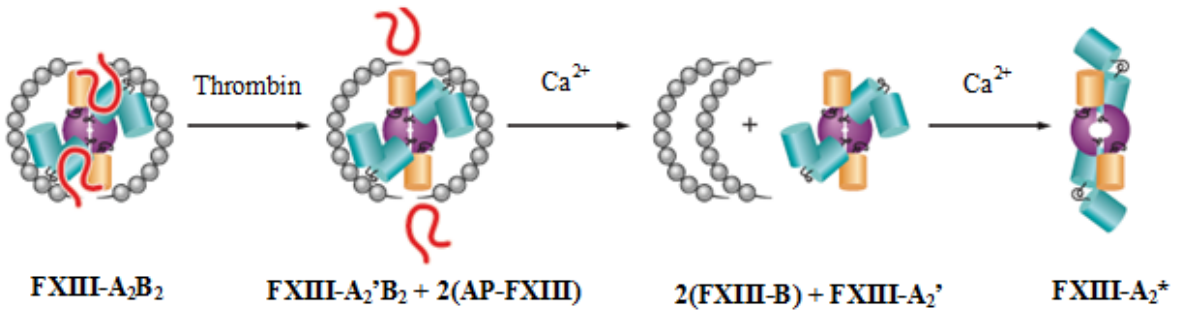

Figure 1.2: Proteolytic activation of plasma FXIII (Muszbek et al., 2011). *Thrombin cuts the activation peptide (AP-FXIII) from the FXIII-A_2B_2 tetramere. In presence of Ca^{2+} the FXIII-B subunits dissociate and the remaining protein without the activation peptide (FXIII-A_2') undergoes a conformational change to complete the activation of FXIII (FXIII-A_2^*).*

[5] Zymogen: Inactive precursor enzyme.

Human FXIII deficiency with less than 1% FXIII activity, although rare, occurs throughout the population with an incidence of 1 in 5 million people (Ichinose, 2001). Lack of functional FXIII may lead to poor wound healing, intracranial hemorrhage and recurrent miscarriages at early stages of pregnancy (Duckert et al., 1960, Fisher et al., 1966, Larsen et al., 1990). The roles of FXIII in wound healing and blood coagulation are well known, but the precise role of FXIII in maintaining pregnancy is still left to uncover.

1.2 Placental development

The placenta is the organ that forms the interface between the maternal tissue and the fetal tissue, and is developed from both fetal and maternal origin (Alsat et al., 1999). The placenta allows nutrient and gas exchange between the fetus and the mother, and it is also a producer of essentials pregnancy hormones such as progesterone and estrogens (Sadler, 2012). The placenta also provides a selective and immunological barrier, protecting the fetus against infectious agents and rejection from maternal tissue.

1.2.1 Implantation of the embryo

The development of the placenta starts at the implantation period of pregnancy, which is the attachment and embedding of the embryo in the uterus. Implantation begins around 6-7 days after conception, when the fertilized egg has migrated from the uterine tube to the uterine cavity and divided several times along its way (Sadler, 2012). At the stage of implantation the embryo is called a blastocyst, consisting of between 107 and 256 cells (Baergen and Benirschke, 2010). The blastocyst consists of an inner cell mass, which is the stem cells that will form the later embryo, and the outer cells, which are the trophoblasts that surround the blastocyst cavity and will form the embryonic part of the placenta (Figure 1.3). Before implantation the blastocyst is enveloped by a protective layer called the pellucid zone.

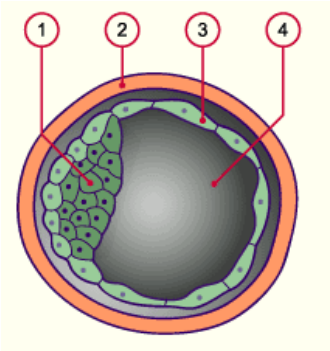

Figure 1.3: Blastocyst inside the pellucide zone (2) [www.embryology.ch]. *The blastocyst consists of the embryoblasts (1, inner stem cells) and the trophoblasts (3, outer cells) which are arranged so that a blastocyst cavity (4) appears.*

At the 5th day after conception, the blastocyst hatches from the pellucid zone by release of proteases from the trophoblast cells. The blastocyst orients its embryonic pole in a correct way to the endometrium, and the blastocysts and endometrium come in physical contact with each other, a process called apposition. To allow the following implantation, the maternal uterine mucosa must be in its implantation window in the secretory phase of the menstrual cycle where the endometrium is prepared for reception of the embryo (Sunder and Lenton, 2000). After the apposition of the embryo, the trophoblast cells interact with the epithelial cells of the endometrium via surface glycoproteins to adhere the embryo. Adhesion molecules, such as integrins and cadherins, bind cells to other cells and also to compounds of the extracellular matrix, and therefore play an important role in the anchoring of the embryo (Merviel et al., 2001).

When the embryo is adhered to the endometrium, the trophoblast cells proliferates and differentiates into an inner layer of cytotrophoblasts (CT) and outer layer of syncytiotrophoblast (ST), as shown in Figure 1.4. The cytotrophoblasts form the inner cell layer of mononucleate cells, while the syncytiotrophoblast becomes an external cell layer of multinucleate cells facing the maternal tissue. The layer of syncytiotrophoblast subsequently invades the endometrium through its production of lytic enzymes and secretion of necessary factors. From 9-12 days after conception the blastocyst is deeply implanted and the uterine epithelium closes over the implantation site (Baergen and Benirschke, 2010).

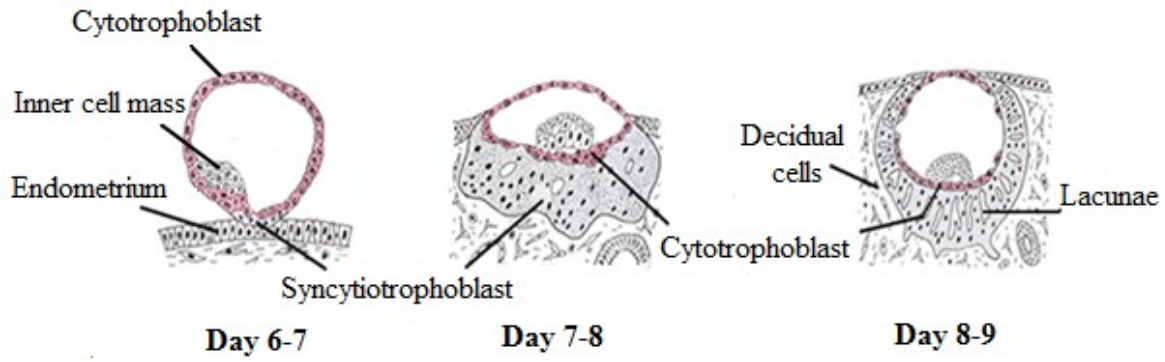

Figure 1.4: Implantation, day 6-9 (Kaufmann P, 1992). *This figure shows the implantation with the apposition and adhesion of the blastocyst (day 6-7), following the invasion of the uterine endometrium by the syncytiotrophoblast (day 7-8), and the final stage of implantation where the decidua closes around the embryo (day 8-9). Small vacuoles, called lacunae, start to form at day 7 to day 9, and provides later for the area where the villi are formed. Other cells shown in the figure are the inner embryonic cell mass, the outer cytotrophoblastic cells, and the decidual cells of the endometrium.*

Upon implantation, the endometrium is modified by a differentiation of maternal cells called decidualization. The objective of the decidual reaction is to form a favorable environment for the embryo, and the endometrium under pregnancy is now referred to as the decidua. The stromal cells[6] are transformed into decidual cells that produce a large amount of growth factors, such as Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF), as well as hormones (Prolactin, Renin), and proteins (collagen, laminins), (Healy and Hodgen, 1983). The decidua also secretes other factors that inhibit or stimulate the trophoblastic invasion in the endometrium to achieve a controlled invasion process.

The decidua throughout the pregnancy consists of various parts such as the Decidua basalis (BD), where the implantation takes place, the Decidua capsularis (CD), that envelopes the embryo; and the Decidua parietalis (PD), on the opposite uterus wall (Figure 1.5).

[6] Stromal cells: cells of the connective tissue.

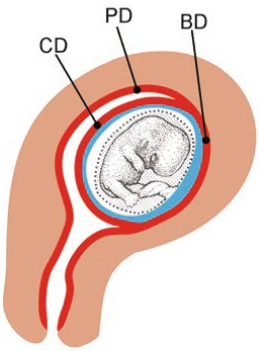

Figure 1.5: Decidual membranes (Baergen and Benirschke, 2010). *The blue lining constitutes the trophoblast of fetal origin, while the decidua of maternal origin is red. Here the three different parts of the decidua are shown; The Capsular Decidua (CD), the Basal Decidua (BD) and the Parietal Decidua (PD).*

1.2.2 Formation of the villi

During the implantation, small extra-cytoplasmic vacuoles are formed in the syncytiotrophoblastic mass to form a system of lacunae which is filled with tissue fluid and uterine secretions. The syncytiotrophoblast further invades the area between the lacunae and forms the start of the villi; the border between maternal and fetal blood. Subsequently, the cytotrophoblast penetrates and invades the syncytiotrophoblast layer forming cellular columns called the primary villi (Figure 1.6A).

At the end of the second week post conception, the implantation phase ends but the invading of maternal tissue continues with the forming of the villi. In the beginning of the third week, the cytotrophoblast reaches the trophoblastic shell, the end of the syncytiotrophoblastic layer, where it makes physical contact with the endometrium. The secondary villi are formed in the middle of the third week, by migration of the mesenchyme[7] into the primary trophoblast villi (Figure 1.6B). At the end of the third week, the tertiary villi are formed by differentiation of the mesodermal cells in the core of the villus into blood vessels (Figure 1.6C). This is the definitive placental villus and forms the capillary system that later supplies the embryo with nutrients and oxygen.

[7] Mesenchyme: Embryonic connective tissue regardless of origin.

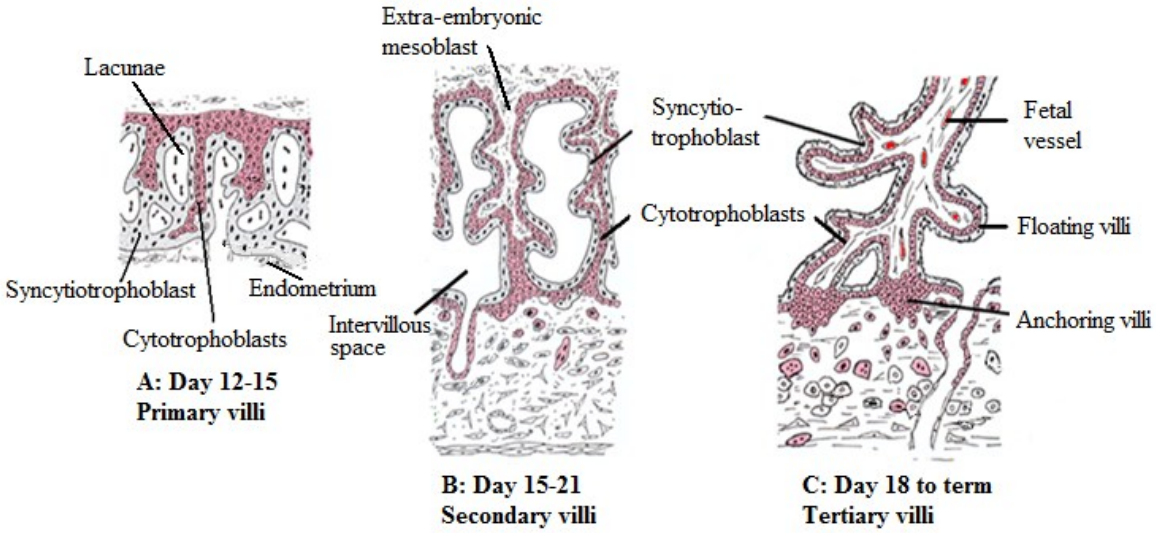

Figure 1.6: Formation of the villi (Kaufmann P, 1992). *Part A (Day 12-15) shows the formation of the primary villi between the vacuolar lacunae. The Primary villus consists of the outer layer of syncytiotrophoblast and inner layer of cytiotrophoblasts penetrating the outer layer. The secondary villi showed in part B (Day 15-21) are formed when the extra-embryonic mesoblast migrates into the interior layer of cytotrophoblast. In part C, the tertiary villus is presented with the differentiated fetal blood capillaries.*

1.2.3 Cytotrophoblast differentiation

The differentiation of the cytotrophoblast from the implantation stage throughout the development of the placenta follows two different roads; either into CT villi or CT extra-villi as shown schematic in Figure 1.7. The differentiation of the trophoblastic cells into villous phenotype or extravillous phenotype depends on the surrounding environment and contact with molecules in the extracellular matrix.

The CT villi are involved in growth support of the placenta as well as the exchange of nutrients and gas between the mother and the fetus, while the CT extra-villi are involved in remodeling of arteries and in the anchoring of the placenta (Evain-Brion, 2001). The extra-villi trophoblast (EVT) cells contain invasive properties and can therefore migrate through the decidua in the implantation process. The EVT also develops into giant cells and endovascular cells, while the CT-villi fuse into layers of syncytiotrophoblast.

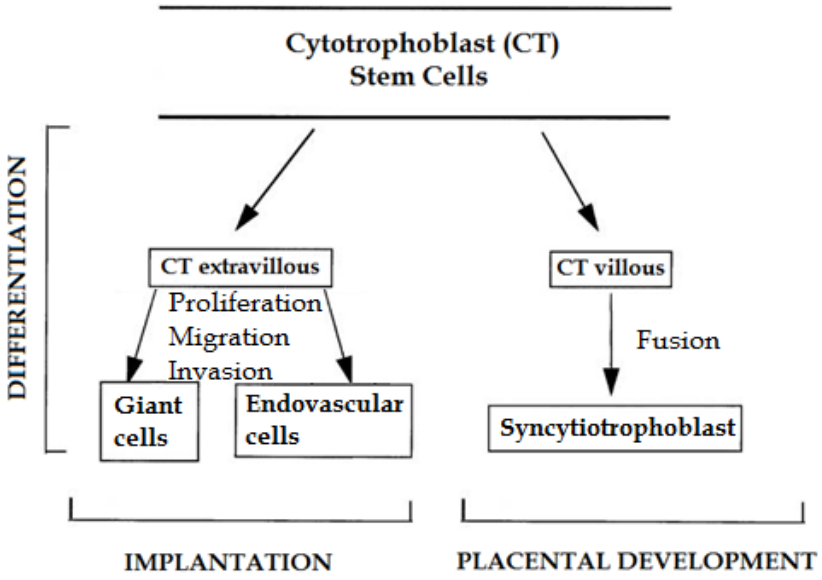

Figure 1.7: The two ways of differentiation of human trophoblast (Alsat et al., 1999). *Cytotrophoblastic stem cells differentiate into either CT extra-villous cells or to CT-villous cells. The CT extra-villi are involved in the implantation process, while the CT-villi are supporting the placental development. The CT extra-villi proliferate, migrate and invade, and differentiate into giant cells and endovascular cells. The CT villi cells fuse together into layers of syncytiotrophoblast.*

1.2.4 Invasion of the decidua

Both during and after the implantation, the cytotrophoblast migrates and invades the maternal tissue to form the villous structure. The invasion is a complex process and includes regulated degradation of the extracellular matrix (ECM). While invading the maternal tissue, the invasive cells encounter various components of the ECM, such as collagen, fibronectin, laminin and vitronectin. Cellular secretion of matrix metalloproteinases (MMPs) allows degradation of the ECM components so that the cells can migrate through. The matrix metalloproteinases MMP-9 and MMP-2, both involved in trophoblast invasion, are gelatinases that degrades collagen IV, a major ECM component (Staun-Ram et al., 2004). Other invasion mediators are the Tissue Inhibitors of Metalloproteases (TIMPs) that affects negatively the invasion.

In comparison to tumor invasion, the trophoblastic invasion is strictly regulated both in space and time. Several other factors than MMPs and TIMPs have been found to have an effect on invasion, such as hormones, cytokines and growth factors. The Tumor Necrosis Factor alpha (TNFα) was found by Meisser et al. to stimulate invasion by increasing MMP-9 activity (Meisser et al., 1999), while the Transforming Growth Factor β (TGFβ) has been showed by Graham and Lala to have an inhibiting effect on invasion (Graham and Lala, 1991). This controlled invasion is essential in order for the embryo to attach and develop in the uterus, as it is part of the stage of placental adhesion where the surfaces of mother and fetus bind to each other.

1.2.5 Placental structure at term

In the fourth month of pregnancy, the placenta takes its definite form. It consists of two components; the fetal side and the maternal side. The umbilical cord is located on the fetal portion, and the maternal portion is in contact with the endometrium (Figure 1.8). At the time of delivery, the placenta is discoid with a diameter of 15-25 cm, approximately 3 cm thick and weighs about 500 g (Sadler, 2012).

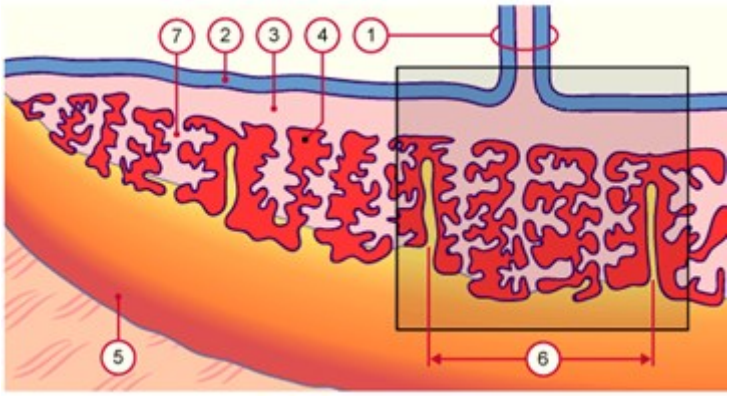

Figure 1.8: Anatomy of placenta at term [www.embryology.ch]. *The umbilical cord (1) is situated at the fetal side of the placenta, while the basal plate (5) is located at the maternal side. The villi (7) are the functional elements of the placenta, involved in exhanges between mother and fetus via the intervillous space filled with maternal blood (4). The villous structures are grouped into arrangements called cotelydons (6). The embryonic part are here shown as the amnion (2) and chronionic part (3).*

1.3 Factor XIII deficiency and pregnancy

1.3.1 Factor XIII deficiency

FXIII deficiency is an autosomal recessive disorder that is caused by homozygote or compound heterozygote defects in either the FXIII-A gene or the FXIII-B encoding genes. Heterozygote patients can synthesize both wild type and mutant protein, and carriers of the disease usually do not develop severe symptoms as a single allele is sufficient to drive the synthesis of chains above hampering levels (Hashiguchi and Ichinose, 1995).

The disorder is usually grouped in FXIII-A deficiency, FXIII-B deficiency and combined FXIII-A/FXIII-B deficiency (Ichinose et al., 2005). Most of the reported cases of FXIII deficiency are caused by mutations in the FXIII-A subunit where the active enzymatic site is located. At the current time, 89 mutations in the FXIII-A gene have been discovered; most of them are missense/nonsense or deletion mutations (Human Gene Mutation Database, Institute of Medical Genetics in Cardiff, www.hdmd.cf.ac.uk). The FXIII-B subunit is in excess in healthy individuals (Kohler et al., 2011), but some few cases of symptoms due to deficiency of FXIII-B subunit has been reported (Saito et al., 1990, Ichinose, 2001). Deficiency of subunit B usually has milder symptoms than deficiency of subunit A, but can lead to minor bleeding tendencies due to the absence of the stabilizing FXIII-B subunit, thus reducing the plasma level of FXIII-A.

The deficiency is not detected in the standard clotting test, since the fibrin fragments form blood clots even in the absence of FXIII, but without the crosslinked network which is not assessed by thrombin time[8]. A clot formed without FXIII is unstable in weak acids and bases and will readily dissolve. Testing of clot stability in 5 M urea have been used to diagnose FXIII deficiency (Robbins, 1944), however, this screening test detects only severe FXIII deficiency. Kohler et al. recommend a thorough laboratory diagnosis by first measuring the plasma FXIII activity, thereafter determining the FXIII-A_2B_2, FXIII-A and FXIII-B antigen concentration (Kohler et al., 2011). Several quantitative assay kits exist commercially, amongst other the FDA approved Berichrom FXIII (Dade Behring, Marburg, Germany) which measures photometrically the ammonia released in the first step of the transglutaminase reaction (Hsieh and Nugent, 2008).

[8] Thrombin time: A blood test that measures the time necessary for forming a clot in excess of thrombin.

The average plasma concentration of FXIII-A_2B_2 is 10-20 mg/L and the amount necessary in blood to prevent bleeding symptoms is 15% of the average plasma concentration (Bohn et al., 1973). Management of FXIII deficiency was traditionally treated with cryoprecipitate and fresh frozen plasma. Even though successful management of the disorder was obtained with this treatment, it included risk of infection by blood borne pathogens and allergic reactions and is therefore not recommended (Eshghi et al., 2010). Concentrates of pure plasma FXIII are now used instead and are now on the market. Also, a novel trial by Inbal et al. shows that recombinant FXIII (rFXIII) can be used safely and efficiently instead of plasma supplementation (Inbal et al., 2010).

1.3.2 Involvement of FXIII in pregnancy

FXIII has been known for several decades to have a role in maintaining pregnancy. The first report of a case of FXIII deficiency affecting pregnancy was made by Fisher et al. (1966) where a 29-year-old woman had experienced 12 recurrent spontaneous abortions with severe uterine bleedings. The woman was diagnosed with FXIII deficiency by investigating the clot stability in urea, and treatment was made with plasma transfusions allowing her to carry the 13^{th} pregnancy to birth to receive a healthy child.

A study performed by Koseki-Kuno et al. in 2003, showed that the occurring hemorrhage in pregnant FXIII-A knockout (KO) mice were of maternal origin by analyzing the location of blood pools. The uterine cavity of the KO-mice was filled with blood, but bleeding was not observed in the amniotic cavity and fetus region. The same study also detailed that FXIII deficient embryos do not contribute to bleeding or miscarriage, as FXIII deficient mice embryos developed normally. Thus spontaneous abortions occur only when the maternal FXIII is absent; when the mother is healthy, even with a FXIII deficient embryo, the pregnancy will be carried to term if no other unrelated complications occur.

Maternal FXIII is claimed not to be essential before 5-6 weeks after gestation, since abnormal decidual bleeding followed by first trimester spontaneous abortion will occur after this period if FXIII is absent (Asahina et al., 1998). Although Hsieh and Nugent states in 2008 that "massive bleeding associated with microvascular bleeds is no doubt one of the major causes of miscarriage and foetal loss in FXIII deficient women", other plausible theories have been considered. Due to the multifunctional nature of FXIII it is not certain that impaired

coagulation is the primary cause of abortions caused by nonfunctional maternal FXIII. Asahina et al. proposed that FXIII serves as an adhesive protein during the invasion of the endometrium by the cytotrophoblast, by promoting the binding between fibronectin receptors on cytotrophoblasts and fibronectin produced by stromal fibroblasts (Asahina et al., 1998). Fibronectin also binds to type IV collagen located at the surface of decidual cells, as well as fibrinogen. These bindings are assumed to strengthen the anchoring of the cytotrophoblasts. This theory was developed by showing that decidual Factor XIII is abundantly present at the decidual stroma around the invasive cytotrophoblasts at 5 weeks after gestation. No localization of FXIII-A was found at 7 weeks after gestation in the decidual stroma, however it was found in the mononuclear cells in the decidua. The same study showed that fetal FXIII-A was found in the Hofbauer cells[9], see Figure 1.9 for distribution of FXIII-A in the placenta.

In a later study by Asahina et al. in 2000 it was shown that when the concentration of FXIII-A is low, the cytotrophoblastic shell does not form adequately which results in an increased risk of abortion due to placental detachment at the Nitabuch's layer (Figure 1.9). The same authors found that FXIII-A was present in the extracellular space of the extravillous cytotrophoblast which forms the cytotrophoblastic shell adjacent to the Nitabuch's layer in normal pregnancy. Around poorly formed cytotrophoblastic shells the factor FXIII-A was not detected.

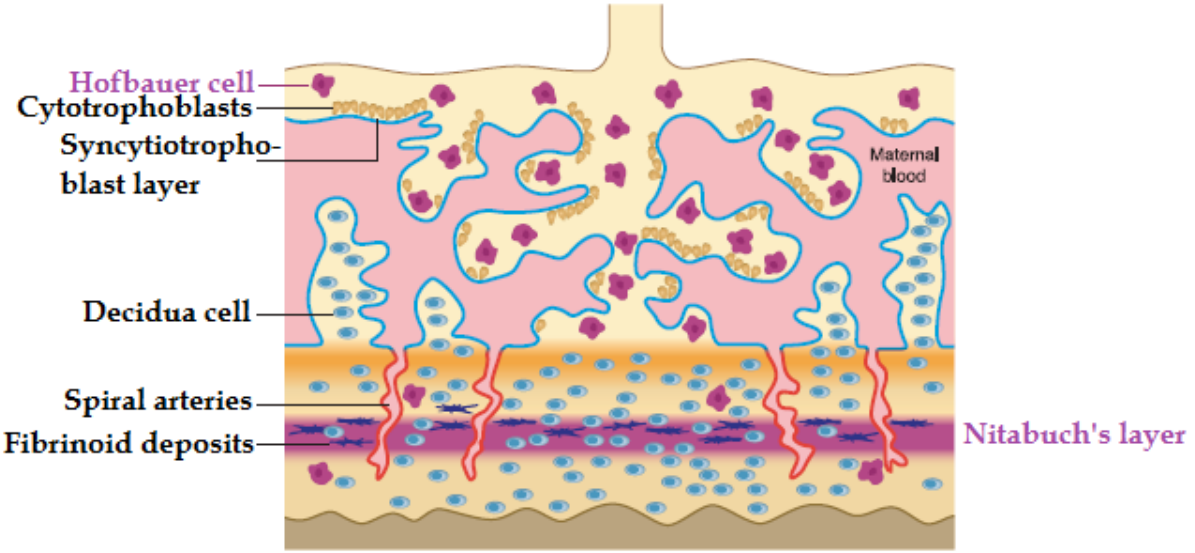

Figure 1.9: Distribution of FXIII-A in the definitive placenta (Muszbek et al., 2011). *FXIII-A containing cellular and structural elements are depicted in purple, including Hofbauer cells and the Nitabuch's fibrinoid layer.*

[9] Hofbauer cells: macrophages of embryonic origin.

1.3.3 Aim of study

In this study, two goals were sought to be achieved, both determination of the location of FXIII at the fetal-uterine interphase and also to examine the role of FXIII in maintaining pregnancy.

The complete role of plasma FXIII in maintaining pregnancy is debated, thus the aim of this study is to investigate and possibly clarify its' function in the development of the placenta. Our hypothesis is that FXIII could be involved in the control of embryonic invasion in maternal tissue by both inducing the crosslinking between the proteins located in the extracellular matrix and by forming a network binding the cells to the ECM via integrins and cadherins. In this case, the FXIII would impair the invasion, and complete development of the placenta will be altered, finally resulting in spontaneous abortion by placental detachment.

By determination of the origin and role of the FXIII involved in pregnancy we anticipate to understand better the physiology of FXIII, specifying the importance of FXIII of uterine, plasma or fetal origin. The current management of pregnancy in FXIII deficient women is based on a post hoc analysis described in literature; therefore the results would hopefully provide guidelines for treatment of FXIII more appropriate to management of pregnancy in FXIII deficient women.

2. Materials and methods

PART I: Materials

2.1 Chemicals and materials

The different chemicals and materials used in this project, as well as their producer and catalogue number are listed in Table 2.1.

Table 2.1: Chemicals used, their manufacturer and catalogue number

Chemical	Manufacturer	Catalog no.
Acetic acid 96%	CARLO ERBA	302002
Acrylamide 40%	Eurobio	018803
Albumin	SIGMA	A-7906
Ammonium persulfate (APS)	Fluka Biochemika	09917
Antimouse IgG antibody	Amersham Biosciences	NA931V
Bisacrylamide 2%	Eurobio	GEPB1200-1
Bromphenol blue	BIO-RAD	161-0404
Calcein AM	Interchim	FP895515
Collagen Type I	BD Biosciences	354236
Coomassie Brilliant Blue G	SIGMA	B-1131
Crystal violet	SIGMA	C-3886
Deoxyribonuclease (Dnase) type IV	SIGMA	D5025-375K
DMSO	SIGMA	D5879
F-12 medium	Sigma-aldrich	D2906
Fetal Calf Serum (FCS)	Gibco Invitrogen	10270-106
Gelatin Difco	BD Biosciences	214340
Glycerol	SIGMA	G-7757
Glycine	Eurobio	GEPGLY00-66
HANKS' Balanced salt solution (10x)	Gibco	14185-045
HEPES	Eurobio	GAUHEP00-64
Ki67	Dako	F078801
mAb anti-Factor XIIIa antibody	Novus Biologicals	NB100-696
Matrigel	BD Biosciences	354234
2-mercaptoethanol	SIGMA	M6250
Methanol	Carlo ERBA	309001
NaCl	Eurobio	GAUNAC01-66
Nonidet P40 substitue	Amresco	E109-50ML
NuPage MOPS SDS Running buffer (20x)	Invitrogen	NP001
NuPAGE 10% Bis-Tris Gel 1.5 mm, 10 well	Invitrogen	NP0315BOX
PBS (10x)	Cambrex	BE17-517Q
Penicillin G sodium salt	SIGMA	P3032
Percoll	GE Healthcare	17-5445-02
Precision Plus Protein All Blue Standard	BIO RAD	1610373
Protein Assay dye reagent concentrate	BIO-RAD	500-0006
Recombinant human Factor XIIIa (A-subunit)	Zedira	T070
Rb pAb tp Factor XIIIa	Abcam	ab97636

Table 2.1: Chemicals used, their manufacturer and catalogue number (continued)

Chemical	Manufacturer	Catalog no.
RPMI-1640 medium	Sigma-aldrich	R8755
SDS (10%)	SIGMA	L4522
Sodium azide	SIGMA	S2002
Sterile water for irrigation	AGUETTANT	600022
Streptomycin sulfate salt	SIGMA	S9135
Temed	Merck	1.10732
Thincert	Greiner bio-one	662638
Tris base	SIGMA	T1378
Triton-X-100	SIGMA	T-8787
Trypsin	SIGMA	T-4799
Tween-20	SIGMA	P1379
Protease inhibitor cocktail	SIGMA	118K412

2.2 Buffers and solutions

The following recipes were used to prepare various buffers and solutions. The chemicals were dissolved in deionized water, unless otherwise stated. Commercially available ready-to-use buffers are listed in Table 2.1.

Transfer buffer (1x)
12.5 mM Tris base
96 mM Glycine
0.005% SDS
10% Methanol
The buffer was stored at 4°C until use.

Tris Buffered Saline Tween-20 (1x)
10 mM Tris
75 mM NaCl
0.05% Tween-20
pH was adjusted to 7.6, and
the buffer was stored at 4°C until use.

Blocking buffer, milk
5% Dry milk in TBS-T (1x)
pH was adjusted to 7.6.

Blocking buffer, gelatin
2.5% Gelatin in TBS-T (1x)
pH was adjusted to 7.6.

Running gel (Zymography)
10% Acrylamide
0.1% Bisacrylamide
0.38 M Tris pH 8,8
0.1% SDS
1 mg/mL Gelatin
The solution was heated to dissolve the
gelatin before adding the APS and TEMED.
0.1% APS
0.1% TEMED

Stacking gel
5% Acrylamide
0.1% Bisacrylamide
0.38 M Tris pH 8,8
0.1% SDS
0.1% APS
0.1% TEMED

Laemmli Buffer (2x)
4% SDS
20% Glycerol
10% β-mercaptoethanol
0.004% Bromphenol blue
0.125 M Tris-HCl

Substrate buffer
50 mM Tris pH=7.5
0.02% NaN_3
5 mM $CaCl_2$
150 mM NaCl

RPMI complete Medium
0.02 M HEPES
100 U/mL penicillin
0.1 mg/mL streptomycin
10% FCS
The reagents were mixed and dissolved in RPMI-1640 medium and sterile filtered (0.2 µm) before use.

RPMI-1640 medium (1 L)
RPMI 1640 SIGMA powder flask
2g $NaHCO_3$
pH was adjusted to 7.4 and the medium was filtrated (0.2 µm) before storing at 4°C.

F12 complete medium
100 U/mL penicillin
0.1 mg/mL streptomycin
10% FCS
The reagents were mixed and dissolved in F-12 medium and sterile filtered (0.2 µm) before use.

F-12 Medium (1L)
F-12 SIGMA powder flask
2.5 g $NaHCO_3$
pH was adjusted 7.4 and the medium was filtrated (0.2 µm) before storing at 4°C.

Solubilisation buffer
The following was added in the desired volume of ONC buffer:
0.1% SDS
1% Nonidet P40
0.5 mM Sodium deoxycholate
1 mM Sodium orthovanedate
30 mM B-glycerophosphate
10 mM NAF
5 µg/mL Aprotinin
12.5 µg/mL Leupeptin
0.1 mg/mL AEBSF

ONC Buffer
50 mM Tris
120 mM NaCl
1 mM EDTA
pH was adjusted to 8 and
the buffer was stored at 4°C.

Crystal Violet
20 mg Crystal violet was dissolved in 1 mL of 20% DMSO. The stock solution was stored at room temperature and diluted 1:40 with HANKS buffer (1x) before usage.

HANKS Buffer (1x)
136.7 mM NaCl
5.36 mM KCl
0.44 mM KH_2PO_4
0.42 mM Na_2HPO_4
4.16 mM $NaHCO_3$
The buffer was sterile filtered before used (0.2 µM).

Coomassie blue (250 mL)
62.5 mg Coomassie brilliant blue
100 mL Methanol
12.5 mL Acetic acid
12.5 mL Glycerol

Decoloration (1L)
200 mL Methanol
50 mL Acetic acid
25 mL Glycerol

Bradford
BIO-RAD Protein assay dye reagent concentrate was diluted 1:5 in ionized water and paper filtered before usage.

Trypsine
0.4% Trypsin was dissolved in HANKS buffer (1x), then sterile filtered (0.2 µm) and freezed until use.

Solution stock A
500 ml HBSS
25 mM HEPES
4.2 mM $MgSO_4$
To be stored at 4°C for 1 month

Digestion solution
100 mL Solution stock A
0.1% Trypsin
Dnase (50 U/mL)
pH was adjusted to 7.2-7.4
and the solution was sterile filtered (0.2 µm).

2.3 Commercial kits

Commercial kits utilized in the project and their producer is listed in Table 2.2.

Table 2.2: List over commercial kits

Name	Producer	Cat no.
5-Bromo-2'deoxy-Uridine labeling and detection kit III	Roche	11444611001
Supersignal West Pico Chemiluminescent substrate	Thermo Scientific	34080

2.4 Apparatus

The different instruments used and their producer are listed in Table 2.3.

Table 2.3: List over instruments used and their manufacturer

Name	Task	Producer
Fusion Fx7	Picturing gels	Vilber Lourmat
Infinite F200	Absorbance/fluorescence plate reader	Tecan
Ultrospec 3000	Absorbance reader	Pharmacia Biotech
Orbitrap LTQ Velos	Mass spectrometer	Thermoscientific

2.5 Tissue collection

This study was approved by the local ethical committee: "Comités de Protection des Personnes", and informed consent was obtained from each donor before clinical sampling. Human placental tissues from the first trimester (7-10 weeks of gestation) were obtained from healthy pregnant women from 18 to 43 years old when undergoing voluntary pregnancy terminations.

2.6 Cell culture

The Human Trophoblast cell line (HTR-8/SVneo) line was established by Graham et al. (1993) through introducing the gene encoding simian virus 40 large T antigen into first trimester human trophoblast HTR-8 cells. The cell line was kindly provided by Dr. Nadia Alfaidy (CEA Grenoble, France) in agreement with Dr. Charles Graham. This transfected cell line is a model of invasive extravillous trophoblast that shares a number of phenotypic properties with the non-transfected parental cells and therefore is an appropriate tool for the study of *in vitro* placental function (Graham et al., 1993).

In parallel, we preformed the same experiments with extravillous trophoblasts cells extracted from first trimester placentas. This primary culture represented a more *in vivo* approach to validate the results obtained with the HTR-8/SVneo cell line.

Part II: Methods

2.7 HTR-8/SVneo cell culture

The human HTR-8/SVneo cell line was cultured at 37°C in an atmosphere of 5% CO_2/95% air, plated in RPMI 1640 medium supplemented with fetal calf serum (FCS, 10%), HEPES (20 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The cells were grown in 60 cm^2 dishes, split with trypsin (0.2%) and not grown beyond 80% confluence. The cells were used in invasion assay (Section 2.10) between passage 5 and passage 13.

2.8 Human primary EVT cell isolation

Human placental tissue was rinsed in cold physiological water and sliced with scalpels. The sliced tissue was rinsed three times with physiological water and centrifuged at 200 g for 5 minutes. Digestion solution (0.1% trypsin in solution A, 50 mL) was added and incubated at 37°C for 30 minutes without agitation. To stop the tissue degradation, FCS (5 mL) was added. After tissue sedimentation, the supernatant was filtered through nylon screen (100 μm) and centrifuged at 200 g for 10 min. The cells were washed 4-6 times with warm HBSS (37°C) until the wash supernatant was free from extracted cells (controlled by microscopy). The supernatants from each wash was pooled, filtered and centrifuged as before. The cell pellets were dissolved and pooled in a final volume of 3 mL F12 medium containing FCS (10%) and filtered through a nylon screen (40 μm) and layered over a discontinuous Percoll gradient (20-55%). The gradient was centrifuged for 25 min at 1000 g. The cell layer corresponding to 40-55% Percoll that contains EVTs was aspired and washed with F12 medium containing FCS (10%), thereafter centrifuged at 10 minutes for 200 g. The pellet was dissolved in 100-200 μL F12 medium containing FCS (1%) according to size of pellet, and the cell concentration was determined with Crystal Violet Cell Counting. Finally, 10^5 of EVT were incubated per insert for the invasion assay (Section 2.10).

2.9 Explant cultures

The wells of a 24-well plate were coated with collagen I diluted in 0.02 M acetic acid (50 µg/mL, 200 µL). The plate was incubated for one hour at 37°C. The wells were then rinsed with sterile physiological water (150 mM NaCl) and air dried.

Pieces of villous placental tissue (1-2 mm in length and width) from first trimester were dissected and placed on the collagen I-coated 24-well plates. The explants were left for 2 hours at 37°C under 5% CO_2 and 95% air atmosphere to permit adhesion of explants to collagen, before addition of F12 medium containing FCS (1%), penicillin (100 U/mL) and streptomycin (100 µg/mL). After 24 hours the medium was changed to F12 medium containing only penicillin (100 U/mL) and streptomycin (100 µg/mL), and the effector was added as FXIIIa (50 µg/mL), or FCS (10%). After 24 hours of incubation the conditioned medium was collected for substrate gel zymography (Section 2.13) and the tissue explants were sent to the laboratory of anatomical pathology at the Poissy Hospital for histological proliferation studies (Section 2.12.2).

2.10 *In vitro* invasion assay

2.10.1 Principle of invasion assay

The Boyden chamber assay (Boyden, 1962) is the classical *in vitro* model used to study cell migration. In this project, Thincert culture insert (Greiner bio-one, Frickenhausen, Germany) was used, which consists of a porous membrane through which the cells can migrate from the upper chamber to the lower compartment (Figure 2.1). The size of the pores (0.8 µm) in the membrane is small enough to avoid the passive passage of epithelial and tumor cells, but large enough to allow migration. The cells capable of invasion will first degrade the ECM (invasive properties) and thereafter migrate through the membrane (migration properties).

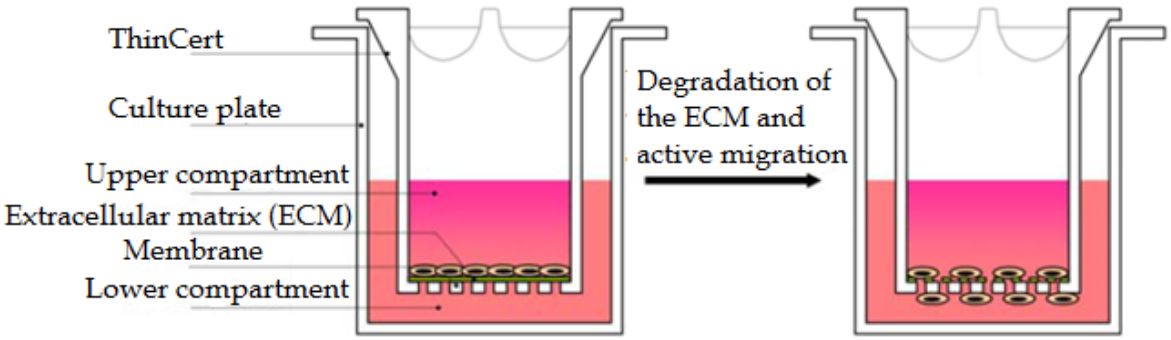

Figure 2.1: Schematic view of the Thincert principle [www.greinerbioone.com]. *The Thincert insert consists of a porous membrane (0.8 µm) which is placed in culture plates (24-well plates). Extracellular matrix is added on the membrane and allowed to polymerize. The cells are seeded in the upper compartment on the matrix together with the effector to study its influence on invasion. The system is incubated for a certain amount of time to allow the cells degrade the extracellular matrix in order to invade and migrate through the membrane from the upper to the lower chamber.*

The membrane is coated with an extracellular matrix for the cells to invade, e.g. matrigel (BD Biosciences, San Jose, CA) which contains extracellular matrix proteins such as laminin, collagen IV and heparan sulfate proteoglycans (Kleinman et al., 1982). The quantity and concentration of matrigel is critical for the assay. A large quantity of matrigel (150-200 $\mu L/cm^2$) allows the study of the ability to degrade and invade the ECM, and thereafter to migrate. However, an overly large quantity may not give the cells sufficient time to invade and migrate, and also an excessively high concentration of the matrigel can be too hard to invade.

A chemo-attracting effect is obtained by adding medium containing serum in the lower chamber. Since FXIII exists in serum, it is necessary to conduct the experiments at 0% FCS in the upper chamber. This is unfortunately an unavoidable stress factor for the cells since they must live without the necessary growth factors that the serum provides.

To detect the amount of cells that has invaded the ECM and migrated through the membrane, the fluorescent dye Calcein-AM (Interchim, Montluçon, France) was used. The dye is membrane-permeant and is retained in the cytoplasm of live cells. Inside the cells, the Calcein-AM is hydrolyzed by esterase into green fluorescent calcein that can be measured and therefore quantify the cell viability.

The invasion assay was used to study the effect of FXIII on both the ability of trophoblast cell line and of the primary cultures of extravillous trophoblasts to degrade ECM and both invade and migrate through extracellular matrix. Owing to the restricted availability of primary human EVTs, extended studies with controls were performed using immortalized extravillous trophoblast cell line (HTR-8/SVneo), followed by more limited studies using primary EVTs isolated from first trimester placenta to possibly validate the results.

2.10.2 Protocol of invasion assay

Thincert inserts (Greiner, Frickenhausen, Germany) were coated with BD matrigel (5.05 mg/mL, 20 µL) and allowed to gel at room temperature for 1.5 hours. Cells ($2.8x10^4$ cells/insert for HTR-8/SVneo, and 10^5 cells/insert for EVT primary culture) in medium (100 µL) containing 10% FCS were added to the top of the polymerized gel within the insert. Medium containing 10% FCS (600 µL) were added to the wells (in the compartment under the insert). The thincerts were incubated for 24 hours at 37°C before adding the effector. FXIIIa (50 µg/mL), or TNFα (50 ng/ml), dissolved in medium without FCS were added to the respective inserts, and the medium in the wells was changed in parallel when adding the effectors. The cells were incubated with the effectors for 24 hours. The matrigel and non-invading cells were removed by a moistened cotton swab, and the insert was washed with PBS (1x). Calcein (4 µM, 450 µL) was placed in the lower chamber with the insert, and was incubated for 30 min at 37°C. The inserts were washed again in PBS, and thereafter placed in wells containing trypsin (0,01%) and incubated at 37°C for 10 minutes with shaking. The fluorescence of the trypsin solution containing cells was read twice (2x150 µL) with Infinite F200 (Tecan , Männedorf, Switzerland) at $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=535 nm.

2.11 Cytotoxicity test

Lactate dehydrogenase enzyme activity released into the culture medium was quantified by an enzymatic method based on $NADH/H^+$ measurement performed by the biochemistry laboratory of the Poissy Hospital. The lactate dehydrogenase enzyme is localized in cells and its presence in culture medium reflects cellular lysis.

2.12 Proliferation

2.12.1 5-Bromo-2'-deoxy-Uridine labeling

HTR-8/SVneo cells were seeded in a 96-well culture plate ($7.7x10^3$ cells/well) in medium containing FCS (10%) and incubated for 24 hours at 37°C at 5% CO_2 and 95% air atmosphere. After 24 hours, the wells were washed with PBS and FXIIIa was added (50 µg/mL) in medium without FCS, and FCS (10%) was used as positive control. After 24 hours, the protocol of the 5-Bromo-2'-deoxy-Uridine labeling and detection kit III (Roche Applied Science, Indianapolis, IN) was followed. In brief, 5-Bromo-2'-deoxy-Uridine (BrdU) was added according to kit protocol to each well and incubated for 2 hours. The cells were then fixed with a fixing agent containing ethanol at -20°C for 30 minutes. After incubation with each reagent of the kit, the wells were washed with PBS. Nuclease was added and incubated for 20 min at 37°C without CO_2. Anti BrdU was added and incubated 30 minutes at 37°C. The substrate was then added and the fluorescence was read with Infinite F200 (Tecan, Männedorf, Switzerland) at 405 nm with a $\lambda_{reference}$=495 nm after 15 minutes.

2.12.2 Immunohistochemistry

Explants were cultured on collagen as explained in Section 2.9. The explants were fixed for 24 hours at room temperature in 4% (vol/vol) paraformaldehyde, and embedded in paraffin. Histological section (measuring 4 µm in thickness) were deparaffinized and prepared for immunohistochemistry by the laboratory of anatomical pathology at the Poissy Hospital. Thereafter, the tissue section was incubated with mouse monoclonal anti-Ki67 (Dako, Santa Barbara, CA) at 1:60 dilution. Proliferative extravillous trophoblast stained positive as Ki67 is a protein of the cellular cycle and therefore the anti-Ki67 functions as a proliferation marker.

2.13 Substrate gel zymography

Conditioned medium mixed with laemmli buffer without mercaptoethanol was deposited (5-20 µg total protein, quantified by Bradford absorbance measurement, see Section 2.14) on a acrylamide gel (10%) containing gelatin (1 mg/mL). Migration was performed at 50 mA/gel. The gel was then washed three times in Triton x100 (2,5%) to eliminate SDS, and afterwards three times in distilled water. The gel was incubated over night with substrate buffer at 37°C. The gel was colored in Coomassie blue for 1 hour, and decolored in decoloration buffer for 2 hours. The quantification of band intensity was conducted with Bio-1D computer software (Vilber Lourmat, France).

2.14 Preparation of protein extracts from placental tissue

The placental tissue was washed three times with sterile physiological water (4°C), and thereafter solubilization buffer was added (300 µL buffer/100 mg tissue). The tissue was homogenized with Ultra Turrax (Janke & Kunkel KG, Staufen ik Breishau, Germany) in the solubilization buffer five short times to avoid overheating. The homogenized samples were left on ice for 1 hour under stirring to allow solubilization. The samples were centrifuged at 100 000 g for 10 min at 4°C, and the supernatant was recovered as the protein extract. Protein extract (1 µL) was mixed with Bradford (800 µL) and incubated for 30 minutes in the dark. The absorbance was read at 595 nm (Ultrospec 3000, Pharmacia Biotech, Cambridge, United Kingdom) against an albumin standard. After determination of protein concentration, the extracts were mixed with one part laemmli buffer and stored at -20°C.

2.15 SDS-PAGE gel electrophoresis and western blot

Precast gels of 10% polyacrylamide (Invitrogen, Karlsruhe, Germany) were used to separate proteins by size. The migration was conducted at 200 V for one hour in NuPAGE MOPS SDS running buffer. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting at 150 V for 1 hour and 30 minutes in 1x Transfer buffer (4°C). The membrane was washed with 1x Tris Buffered Saline Tween-20 (TBS-T) and thereafter blocked with either 5% milk in TBS-T (1 hour at room temperature with soft stirring) or 2,5% gelatin in TBS-T (2 hours at room temperature with soft stirring). Subsequently, an overnight incubation with one of two different primary antibodies was performed: "Antifactor XIIIa antibody Mouse" (Novus Biologicals, Littleton, CO) at 1:400 dilution, and "Rb pAb to Factor XIIIa" (Abcam, Cambridge, MA) at 1:200 dilution, in respective blocking buffer; milk at 4°C or gelatin in room temperature, both with soft stirring.

The membrane was washed with TBS-T (1x) before incubation with the secondary antibody; 1:5000 dilution of antimouse IgG (Amersham, Buckinghamshire, UK) diluted in 1x TBS-T, for 1 hour at room temperature with soft stirring. Afterwards, the membrane was washed again with TBS-T (1x) and detection of the signal was made with ECL supersignal kit (Thermo Scientific, Rockford, IL). Visualization of the chemiluminescence was made with the Fusion System Fx7 (Vilber Lourmat, France).

2.16 Mass Spectrometry analysis

A SDS-PAGE gel electrophoresis as described in Section 2.15 was performed, and coloration with coomassie blue followed (1 hour coloration, and decoloration overnight). We did the following at the proteomic platform of the Jacques Monod Institute in Paris:

The band with corresponding size was cut out of the gel with a scalpel, and added to a tube which contained bicarbonate ammonium solution (50 mM, 100 µL). The gel plugs were incubated with shaking for 15 minutes at room temperature. The supernatant was aspirated and the step with ammonium bicarbonate solution was repeated. Acetonitrile was added (100 µL) and incubated for 10 min with shaking at room temperature to dehydrate the plug. Then the plug was dried at 37°C for 10 minutes. Dithiothreitol (10 mM, 100 µL) was added to the

tube and incubated for 45 min at 56°C with soft shaking. The supernatant was aspired and idoacetamide (55 mM, 100 µL) was added, following incubation at 45 minutes at room temperature in the dark. The supernatant was aspired and ultra-pure water (100 µL) was added to the plug and incubated for 30 minutes with shaking. The water was removed and the plug was dehydrated in acetonitrile (100 µL) and incubated for 10 minutes with shaking. The plug was dried at 37°C for 10 minutes. Trypsin solution was added (10 ng/µL trypsin in 25 mM NH_4HCO_3, 20 µL) at 4°C and incubated for 20 minutes. Afterwards, NH_4HCO_3 (25 mM, 10 µL) was added and the plug was incubated overnight at 37°C. The next day, the supernatant was transferred to a new tube, and the remaining peptides in the plugs was extracted with a solution of acetonitrile (50%) with 0.1% formic acid (20 µL) and incubated with soft shaking for 15 minutes. The supernatant was pooled with the preceding supernatant and the liquid was allowed to evaporate. The pellet was reconstituted in water with formic acid (0.1%, 10 µL). The samples were then analyzed by mass spectrometry in Orbitrap LTQ Velos spectrometer (Thermoscientific, Bremen, Germany).

3. Results

3.1 Verification of the presence of FXIII in the decidua

3.1.1 Preliminary results

A preliminary project was performed at the research laboratory at the Poissy Hospital, with an aim to localize and quantitatively measure FXIII mRNA in decidual cells. The localization of FXIII was detected in cells that were labeled with a CD14 marker, showing its presence in monocytes and macrophages. However it was not detected in cells labeled with a CD56 marker, demonstrating that FXIII is absent in uterine Natural Killer cells. A genetic paternity test was also performed to prove the maternal origin of the FXIII positive cells.

The quantitative measurement of FXIII mRNA was done by extracting RNA from a biopsy sample of the decidua, with tissue verification by morphological analysis, and of a control of the placenta at term. The expression of FXIII-A was verified by Real-Time quantitative PCR in the decidual sample, and thus showing that FXIII-A is locally synthesized there.

3.1.2 Verification of FXIII localization using immunoblotting

To verify the presence of the Factor XIII in placental tissue (decidua) of maternal origin, protein extracts from placental tissue were separated on a SDS-PAGE gel and immunoblotted followed by visualization of bands specific to FXIII-A antibody (Section 2.15).

Biopsy samples from placental tissue at full term, which contained different types of cells from both maternal and fetal origin, were used as control. The decidua samples that were studied were protein extracted from decidua at first term, and its maternal origin was verified by the pathology service at the Poissy Hospital by morphological analysis.

The immunoblotting revealed a band between 75 and 100 kDa (Figure 3.1) that probably corresponded to the FXIII-A subunit (83 kDa), suggesting the presence of FXIII-A in the decidua. Moreover, this band at 83 kDa appeared more intensively in the placental control than in the decidual sample, as described in the literature (Fear et al., 1984). Two other bands at 37 kDa were present in the control and the decidual sample. These could correspond to isoforms of FXIII-A as a result of posttranscriptional proteolytic processing of the protein; this has previously been shown in osteoblast cultures (Al-Jallad et al., 2006) and in bone (Nakano et al., 2007). These results were verified by mass spectrometry, see Section 3.1.3. A probably non-specific band appeared also at approximately 60 kDa in the control but this was not verified by mass spectrometry as it did not appear in the decidual sample.

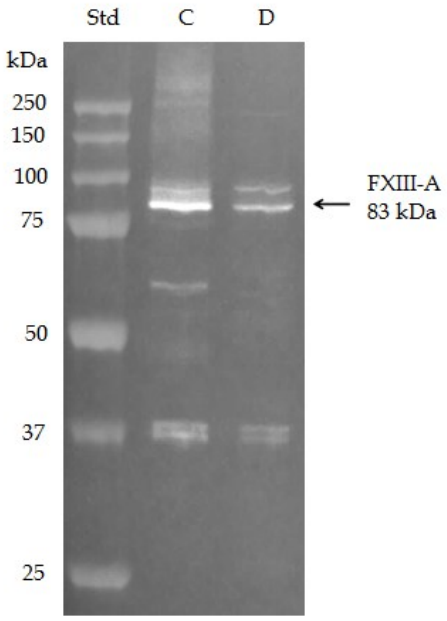

Figure 3.1: Verification of the presence of FXIII-A in placental tissue by immunoblotting. *The size of the standard (Std) is shown in kDa at left. The deposited samples are the placental tissue control from third trimester (C), and the decidual tissue sample from first trimester (D).*

Two different primary antibodies were tested; "Rb pAb to Factor XIIIa" (Abcam, Cambridge, MA) and "mAb anti-Factor XIIIa antibody" (Novus Biologicals, Littleton, CO). Both antibodies gave the same pattern of bands, but the antibody provided by Novus Biologicals was more specific, revealing less unspecific bands. Both blocking in dried milk and gelatin were tested, and it was found to give stronger signal and less unspecific bands using milk as blocking agent compared to gelatin.

3.1.3 Verification of antibody specificity by mass spectrometry

The same samples used in immunoblotting (Section 3.1.2) were separated by SDS-PAGE gel electrophoresis and the bands of interest (~80 kDa and 37 kDa) from Figure 3.1 were extracted and processed by mass spectrometry. Table 3.1 shows the identification of FXIII-A in the different samples. The score by which the protein was identified is the probability that the peptides identified belong to the actual protein. The probability is not expressed in percentage since 100% identification is not possible, but a higher score reflects a higher probability and also a higher quantity of the peptides in the sample which permits to identify the protein.

The table shows only Factor XIII, but several other proteins were detected, an extract of the list is shown in Appendix A. Since the bands at around 83 kDa and 37 kDa were cut out from the gel, and no selective immune-precipitation with antibody was conducted, a large amount of other proteins of the same molecular weight followed. Also proteins can co-migrate and this can explain the detection of proteins with different size, as well as contamination under handling can have occurred.

Table 3.1: Identity score of Human Blood coagulation Factor XIII-A with different databases

Database	**Score Placenta 37 kDa**	**Score Placenta 83 kDa**	**Score Decidua 37 kDa**	**Score Decidua 83 kDa**
NCBI	N.D.	718	N.D.	67
Swissprot	N.D.	756	N.D.	69

N.D: Not detected

The human coagulation Factor XIII subunit A was detected in both samples extracted from the 83 kDa band, but was not detected in the 37 kDa bands, verifying that only the band at approximately 83 kDa corresponded to the FXIII-A, and that the 37 kDa was due to unspecific binding of antibody.

3.2 Study of the role of FXIII in placental development

3.2.1 Effect of FXIII on invasion of trophoblast

3.2.1.1 Optimizing of invasion assay

To study the effect of FXIII on trophoblast invasion, an invasion assay using matrigel was performed as explained in Section 2.10. The invasion assay was performed with both cell line culture (HTR-8SV/neo) and primary cell culture (extravillous trophoblasts). Before examination of the effect of FXIII, the method was tested at different conditions explained in the literature (Kilburn et al., 2000, Busch et al., 2009, Kuang et al., 2009, Jovanovic et al., 2010), and optimized according to our requirements.

We tested different concentrations of matrigel (10 mg/mL, 5 mg/mL, 2 mg/mL, 0.5 mg/mL and 0.25 mg/mL), as well as the volume of matrigel to be used (20 µL, 30 µL and 100 µL). It was found that 5 mg/mL was optimal, corresponding to a 1:2 dilution of the matrigel. A volume of 20 µL seemed to be sufficient for testing the invasive properties and was thereafter used in the experiments with FXIIIa.

Following the protocol of the laboratory and different studies on invasion using HTR-8/SVneo cell line, we chose to work with an initial incubation of cells in the thincert insert, before adding effector, to allow attachment and initial proliferation, and thereafter 24 hours of incubation with the effector before studying the extent of invasion. We also tested if FXIIIa should be added directly in the matrigel when coating the thincert or in the medium containing cells added on top of the gelled matrigel. Since no difference was observed, we decided to add the effector in the medium containing cells and not in the matrigel.

Different concentrations of FXIIIa added were also tested in the invasion assay (20 µg/mL, 50 µg/mL and 100 µg/mL). No dose-dependent effect was observed in the concentration interval tested, so we chose to use 50 µg/mL FXIIIa as also used in another study of the effect of FXIII on angiogenesis (Dardik et al., 2003).

3.2.1.2 Invasion assay using trophoblastic cell line culture

Five experiments with the optimal conditions of the assay were completed with the HTR-8/SVneo cell line. In the experiments, FCS was used as a positive control but it showed great variability and was therefore excluded from the results. In the two last experiments, the TNFα was included as a positive control, but showed no effect on the invasion in our study. Raw data and calculations are given in Appendix B, and the results are shown in Figure 3.2, where a negative effect on invasion can be observed for FXIIIa compared to the control with no effector or serum added.

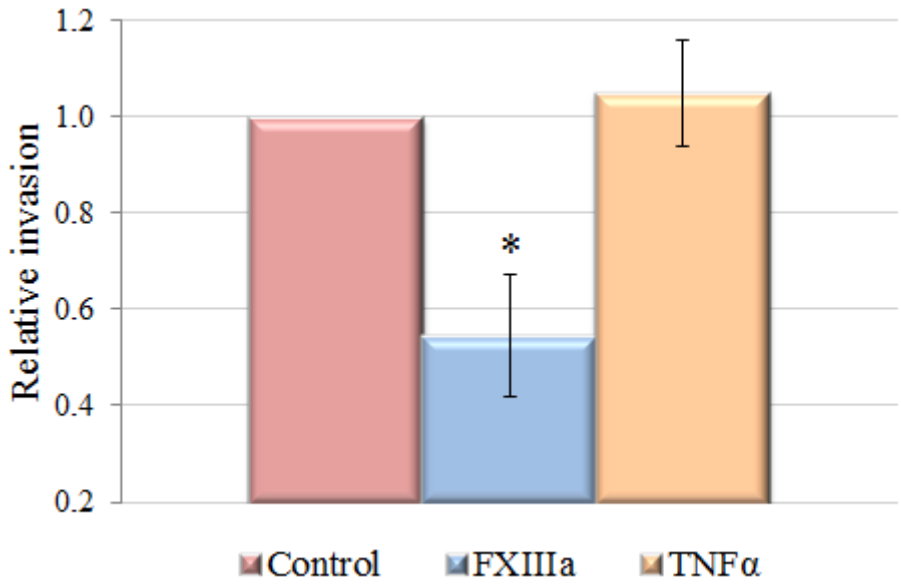

Figure 3.2 Effect of FXIIIa on trophoblast invasion relative to control using HTR-8/SVneo cell line. *Cells were incubated in presence of FXIIIa or TNFα, or without effector (control) during 24 hours in the invasion assay. The negative effect of FXIIIa was found to be significant (p=0.0313, *).*

The calculated effect of FXIIIa was found to be 45% ± 13% inhibition on the invasion of HTR-8/SVneo cells. Statistical analysis was performed using the software GraphPad Instat (Graphpad software Inc., San Diego, CA) and the effect was found to be significant by the one-tailed non-parametric Wilcoxon test (p=0.0313).

3.2.1.3 Invasion assay using trophoblastic primary culture

Ten experiments were conducted to study the effect of FXIIIa on trophoblast invasion using primary culture of EVT cells. Only control (without effector) and FXIIIa (50 µg/mL) was studied due to scarce access to EVT cells. The raw data and calculations are available in Appendix B. The results are shown in Figure 3.3 where no effect is observed compared to the control.

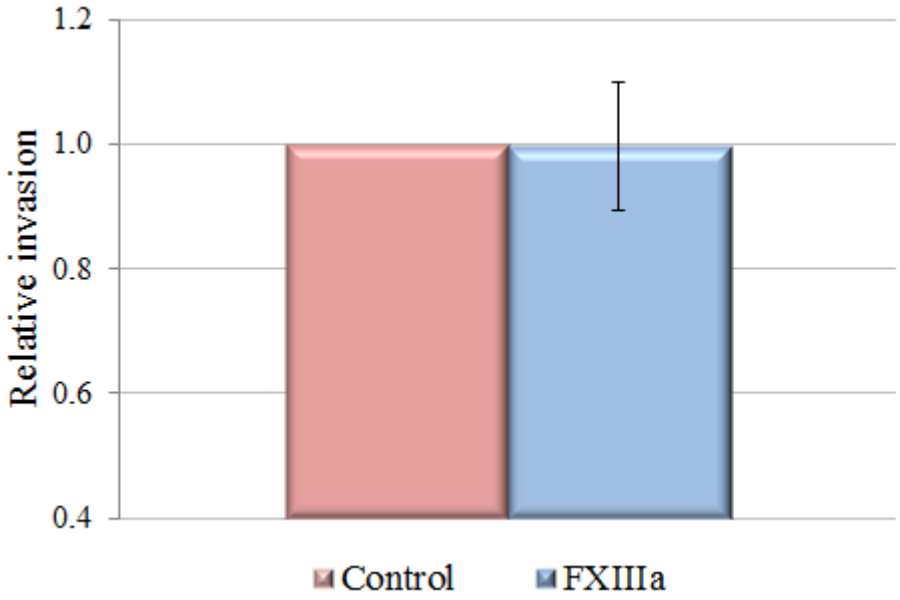

Figure 3.3 Effect of FXIIIa on trophoblast invasion relative to control using primary culture of extravillous trophoblast cells. *The primary culture cells were incubated without (control) or with effector (FXIIIa) during 24 hours in the invasion assay.*

3.2.2 Effect of FXIII on secretion of matrix metalloproteases

Substrate gel zymography was used to measure the gelatinase activity of secreted matrix metalloproteases MMP-9 and MMP-2, in conditioned medium of cell culture invasion assays and explants attached to collagen. Figure 3.4 shows a representative secretion pattern of MMP-9 and MMP-2 at 92 kDa and 72 kDa respectively.

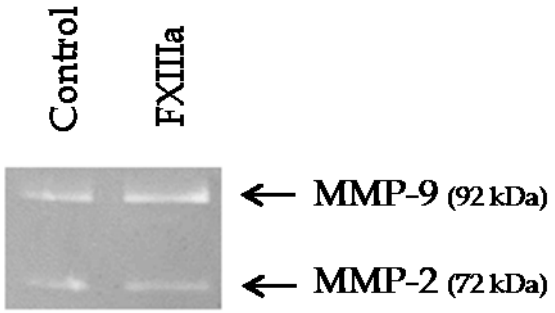

Figure 3.4: Representative secretion pattern of matrix metalloprotease activity. *The MMP-9 (92 kDa) and MMP-2 (72 kDa) secretion were examined with substrate gel zymography. The control was treated the same way as the FXIIIa sample, but without the addition of the effector.*

Each band was quantified with the Bio-1D software (Vilber Lourmat, France), for both MMP-9 and MMP-2. For raw data and calculations, see Appendix C. The result for each system; the HTR-8/SVneo cell line, EVT primary culture and the explants; regarding both MMP-9 and MMP-2, is shown respectively in Figure 3.5 and 3.6. The effect of each system studied on both MMP-9 and MMP-2 was found to be not significant with statistical analysis using a two-tailed non-parametric Wilcoxon test, with the software GraphPad Instat (data not shown).

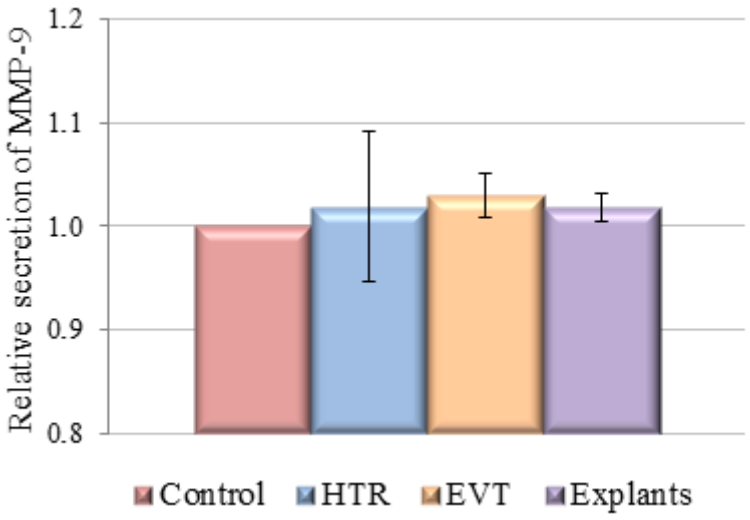

Figure 3.5 Effect of FXIIIa on MMP-9 secretion relative to control. *Substrate gel zymography was used to study the change in matrix metalloprotease activity in presence of FXIIIa in three different systems: HTR-8/SVneo cell line (HTR), extravillous trophoblast primary culture (EVT), and explants.*

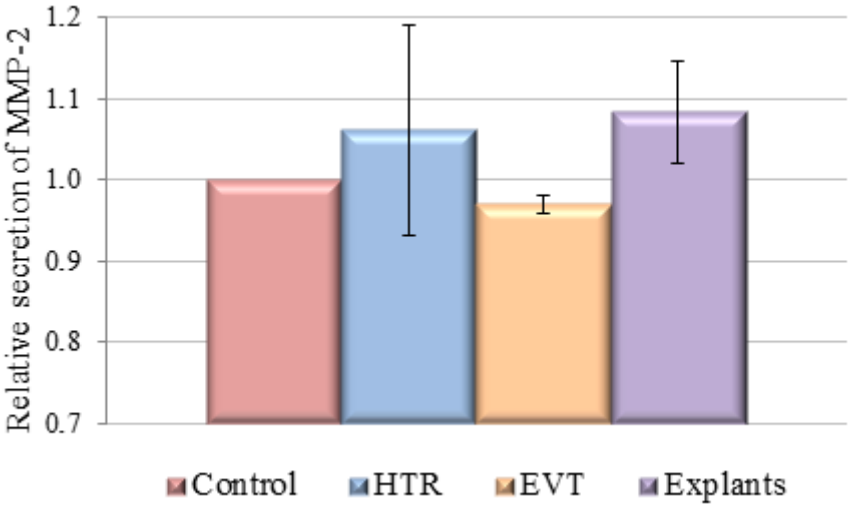

Figure 3.6 Effect of FXIIIa on MMP-2 secretion relative to control. *Substrate gel zymography was used to study the change in matrix metalloprotease activity in presence of FXIIIa in three different systems: HTR-8/SVneo cell line (HTR), extravillous trophoblast primary culture (EVT), and explants.*

3.2.3 Effect of FXIII on proliferation

3.2.3.1 Proliferation in HTR-8/SVneo cell line by BrdU incorporation

Proliferation studies of the effect of FXIIIa on the HTR-8/SVneo cell line were performed three times by using BrdU incorporation. A test of increasing cell amount (from $2.5x10^3$ to $1x10^5$ cells) was performed and it was found that a dose of $7.7x10^3$ cells/well was suitable as shown in Appendix D. Using this condition there was no observed effect of FXIII on proliferation (data not shown). However, no proliferation was observed in the cells with added serum either, which renders the results obtained for FXIIIa by this method unreliable.

3.2.3.2 Proliferation in explants by immunohistochemistry analysis

Another method for proliferation was performed with explants attached to collagen. After 24 hours incubation with/without effector, the explants were transferred for immunohistochemistry analysis as described in Section 2.9 and 2.12.2. The proliferative nuclei (marked with anti-Ki67) were counted by the software ImageJ (National institutes of Health, Bethesda, MD) and divided by the measured surface ratio of the tissue to obtain a comparable quantity between the samples. Three representative photographs are shown in Figure 3.7, where the proliferation in control tissue (A) and tissue treated with FXIIIa (B) is approximately the same, while the tissue treated with 10% FCS (C) shows an increased amount of nuclei in a state of proliferation. The non-treated control tissue shows approximately the same extent of proliferation as the tissue treated with FXIIIa, compared to the increased proliferation observed in tissue treated with serum (10% FCS).

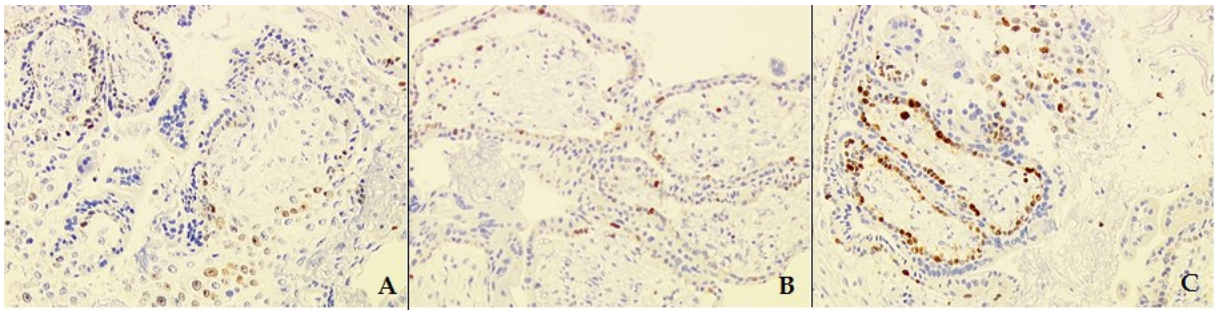

Figure 3.7: Proliferation study in explant using Ki67 marking. *Three representative microscopy photographs (100x magnification) of non-treated control tissue (A), tissue treated with FXIIIa (B), and tissue treated with 10% FCS (C) are shown.*

The effect of FXIIIa was compared to the control without effector and to the positive control with FCS (10%). The results are shown in Figure 3.8, where no effect of FXIII is observed. The effect of added serum shows a slight effect on proliferation as expected, however this effect was not found significant by a two tailed Wilcoxon test (p=0.1563).

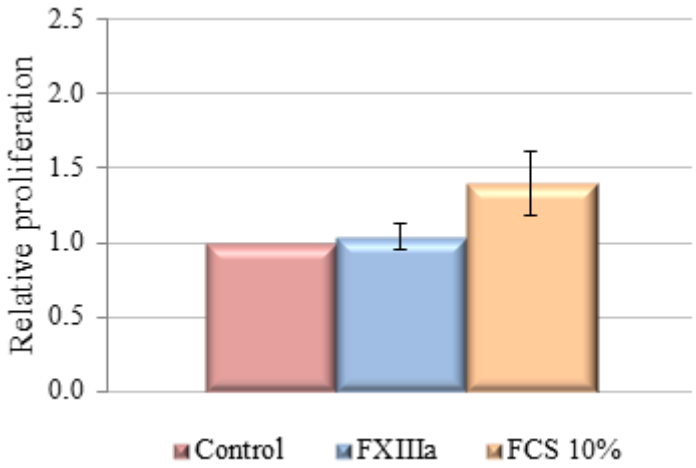

Figure 3.8: Proliferation effect of FXIII and serum on explants relative to control. *The effect of FXIIIa on proliferation was studied with anti-Ki67 marking. The control was treated the same way as the explants with added effectors (FXIIIa and FCS 10%) but without serum or effector added.*

3.2.5 Effect of FXIII on cytotoxicity

No cytotoxicity was observed with the various effectors tested as the lactate dehydrogenase level was not elevated in the conditioned medium (data not shown).

4. Discussion

4.1 Localization of FXIII

FXIII is already known to be located in monocyte-derived tissue macrophages in the placenta (Adány et al., 1988) and in the uterus (Adány and Muszbek, 1989). According to Adány and Muszbek, the presence of FXIII-A in the uterus is however not characteristic for the uterus, but for the monocyte/macrophage cell line including tissue macrophages in general.

Asahina et al. (2000) hypothesized that the level of maternal plasma FXIII-A is the most important for maintaining pregnancy, and not the cellular concentration, which would mean that the FXIII-A necessary for pregnancy is transported to the site of action by blood. This theory is well supported by the fact that blood supplementation throughout the pregnancy in a FXIII deficient woman is sufficient to carry the pregnancy to term.

In our study, FXIII-A was consistently found in the decidua from first term placenta by immunoblotting with antibody specific to FXIII-A, which supports the preliminary results obtained at the same laboratory where FXIII-A was found to be locally synthesized in the decidua. Therefore, we believe its presence and role in the placenta under development is not solely a result of transport from the blood circulation.

4.2 Role of FXIII in pregnancy

FXIII deficient patients experience recurrent pregnancy loss without replacement therapy. The fact that blood substitution of FXIII is sufficient to maintain the pregnancy proves the primary importance of plasma FXIII (Padmanabhan et al., 2004, Asahina et al., 2007, Ichinose et al., 2005). The role of cellular FXIII found in the macrophages of the decidua remains unclear.

Earlier it was believed that FXIII solely prevented severe decidual bleeding due to its role in blood clotting, as massive bleeding and following spontaneous abortion was the outcome in FXIII deficient pregnant patients. However, FXIII-A seems to have a non-coagulation related role by stabilizing of the fibrin layer at the fetal-uterine interface. According to Kobayashi et al. (1999), FXIII in macrophages may play a role in assisting the feto-maternal adhesion, attaching cells and extracellular matrix together. This can be achieved by crosslinking adhesive proteins, such as fibrinogen, fibrin and collagen.

We also believe that FXIII affects the trophoblast invasion during placental development, by crosslinking extracellular matrix proteins, and therefore also assisting in cell attachment to the extracellular matrix. Our hypothesis is that this attachment may hinder the cells from invading the maternal tissue. Thus, it is believed that FXIII controls invasion in an indirect way through cell adhesion. This may support the theory that FXIII is involved in anchoring of the placenta, but it remains to explore the mechanism of cell adhesion to a higher extent.

It was found in this study that FXIII asserts an inhibiting effect on the invasion using the HTR-8/SVneo trophoblastic cell line, but no effect was observed in the primary culture of extravillous trophoblastic cells. When using the EVT cells, we experienced problems with the blank samples (without cells) giving higher reading values than the samples with cells. The calculations were performed without retiring the blank values, thus the reliability of the results is in question. If the indecisive values obtained are due to problems with the invasion assay and the conditions used, either more optimizing of the technique or an another method needs to be considered. For the moment the laboratory is trying a new method, using DAPI-coloration of the cells and thereby counting the cells that have migrated through the membrane manually. It remains to be verified if this method is more reliable than the invasion assay using fluorescent calcein.

Explanations for the large variability observed in the results from the invasion studies with primary culture, could be due to that the primary culture cells were extracted from abortion products at different stages in pregnancy (7-10 weeks of gestation) and also that the termination of pregnancy was caused by both medicament treatment and suction abortion. Correlations between the results and the differences explained here were regarded, but no connection was found.

Also, the question of using EVT cells to study the effect of FXIII on invasion must be regarded. FXIII is found to be located in the Nitabuch's layer, and possibly the EVT cells are located further away from this layer, thus maybe not affecting the invasion to such a great extent. When studying an invasion model composed of explants instead, the whole range of cells involved in placental development can be examined.

Other factors that might affect the *in vivo* in contrast to the *in vitro* system could be the fact that oxygen is present during the experiments where cells come from 7-10 weeks placenta, while in fact under the placental development there is hypoxia until the 11^{th} week of gestation (Schneider, 2011).

Both BrdU incorporation and Ki67 marking were used to verify that the effect observed on invasion by the trophoblastic cell line was not caused by an effect on proliferation. The BrdU incorporation in the HTR-8/SVneo cell line showed no effect of FXIII on proliferation, but no effect of added serum either, which renders the results unreliable. It remains to verify the result by either optimizing the method or by using another technique, for instance a standard cell counting. The Ki67 marking showed no effect on proliferation when FXIII was added to explants, and an increase in proliferation, though non-significant by statistical analysis, was observed when serum was added. However, this verification of absence of effect on proliferation was made only on explants, and it remains to verify the same result using a trophoblastic cell line.

Regarding the secretion of matrix metalloproteases, no effect was observed in the trophoblastic cell line, primary cultures or explants. This shows that the possible effect on cell invasion by FXIII is not due to altered secretion of matrix metalloproteases, and thus may imply that the effect is solely caused by cell and protein adhesion. Even though no effect was observed in the pilot studies, it remains to include controls known for their effect on secretion of MMPs to be more certain of our conclusions.

4.3 Controls

Serum (10% FCS) was tested as a control in this project, both for invasion, proliferation and examination of secretion of MMPs. Due to large variability in the results obtained, it was concluded that the serum is not an optimal control. The large variability and low reproducibility might be due to differences in content of different batches of serum, as well as the fact that serum contains a vast amount of different molecules. A more reliable control would be a single molecule that is used in the same way as FXIII, proven to have an effect on invasion and secretion of MMPs, e.g. TNFα. In the two last experiments of invasion using the HTR-8/SVneo cell line, TNFα was included. Unfortunately, it was discovered later that the reported effect of TNFα is somewhat ambiguous. Meisser et al. found a proinvasive outcome by presence of TNFα (Meisser et al., 1999), but lately there has been reported an inhibiting effect of TNFα on invasion (Xu et al., 2011, Otun et al., 2011). This makes TNFα not an optimal control either. Thus, it remains to find a better control to validate the results obtained.

Regarding a negative control, it was found in the literature that FXIIIa could be inactivated by treatment with iodoacetamide, an inhibitor of transglutamination. It could therefore be a promising negative control to compare the values obtained of inhibited FXIII versus active FXIII (Dardik et al., 2003).

5. Conclusion and perspectives

Exploring the effect of FXIII on invasion needs further work, both concerning excluding the possibility that the effect observed with the *in vitro* system is due to proliferation or apoptosis events, and finding a system approaching *in vivo* conditions supporting these results. In order to verify the results obtained, functional controls need to be included, and also it would be interesting to block the function of FXIII and compare the results obtained from the inhibition.

It is also interesting to study the effect of FXIIIa on gene expression by microarray, and RNA extraction of explants used in the experiments in this study is ongoing. FXIIIa is likely to require adaptive changes in the cell that needs modifications of gene expression. Altering of gene expression might give indirect information about the processes involving FXIII.

Further on, it is desirable to explore the molecules involved in crosslinking that FXIII may induce during placental development. A method for studying this might be to extract proteins from the matrigel containing cells, after addition of FXIIIa. An immunoprecipitation of the protein extract with an antibody directed against N-epsilon (γ-L glutamyl)-L-lysine isopeptide can be used to select the proteins crosslinked by FXIII. Hopefully, this could identify the molecules involved in the crosslinking and thereby increase our knowledge of the role of FXIII in placental development.

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Appendix A: Mass spectrometry results

Two different protein extracts (placental and decidual extract) were separated by size with gel electrophoresis. Two bands were cut out from each sample: the 37 kDa band (A) and the 83 kDa band (B) from the placental sample, and the 37 kDa band (C) and the 83 kDa band (D) from the decidual sample. An extract of the list obtained from mass spectrometry analysis of some of the proteins identified from the NCBI database and the Swissprot database are shown respectively in Table A.1 and Table A.2. The factor XIII-A identified is marked in yellow. The score by which the protein was identified is the probability that the peptides identified belong to the actual protein. The probability is not expressed in percentage since the 100% identification is not possible, but a higher score reflects a higher probability and also a higher quantity of the protein in the sample.

Table A.1: List of proteins identified with the NCBI database

Accession	Description	Score A	Score B	Score C	Score D	MW [kDa]	calc. pI
154146191	heat shock protein HSP 90-alpha isoform 2 [Homo sapiens]	N.D.	2883.6	49.7	2125.5	84.6	5.0
20149594	heat shock protein HSP 90-beta [Homo sapiens]	N.D.	2770.2	N.D.	2560.8	83.2	5.0
190026	plasminogen [Homo sapiens]	N.D.	1277.0	N.D.	196.7	90.5	7.2
20072188	Aconitase 2, mitochondrial [Homo sapiens]	N.D.	1238.7	N.D.	1336.7	85.5	7.7
221045102	unnamed protein product [Homo sapiens]	N.D.	1235.5	N.D.	1924.6	78.8	6.1
62020522	Glucosidase I [Homo sapiens]	N.D.	933.9	N.D.	180.9	91.8	9.0
154354966	mitochondrial inner membrane protein isoform 3 [Homo sapiens]	N.D.	866.1	N.D.	257.9	82.6	6.6
12053275	hypothetical protein [Homo sapiens]	N.D.	844.7	N.D.	773.0	91.7	5.5
168988718	Chain A, Structure Of Human Serum Albumin With S-Naproxen And The Ga Module	612.0	828.0	944.5	1578.6	65.8	5.9
119620058	glucosidase I, isoform CRA_a [Homo sapiens]	N.D.	820.0	N.D.	180.9	74.3	6.9
154354962	mitochondrial inner membrane protein isoform 2 [Homo sapiens]	N.D.	806.3	N.D.	230.2	83.5	6.6
1127268	Chain A, Three-Dimensional Structure Of A Transglutaminase: Human Blood Coagulation Factor Xiii	N.D.	717.9	N.D.	67.5	83.1	6.0
11935049	keratin 1 [Homo sapiens]	581.6	695.5	899.1	63.8	66.0	8.1
153217289	Complement component 4A (Rodgers blood group) [Homo sapiens]	N.D.	671.3	N.D.	607.2	192.7	7.1

A: Placental sample 37 kDa, B: Placental sample 83 kDa, C: Decidual sample 37 kDa,
D: Decidual sample 83 kDa, MW: molecular weight, calc. pI: calculated pI, N.D.: Not detected.

Tabel A.2: List of proteins identified with the Swissprot database

Accession	Description	Score A	Score B	Score C	Score D	MW [kDa]	calc. pI
P07900	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN]	N.D.	3153.4	49.7	2267.1	84.6	5.0
P08238	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4 - [HS90B_HUMAN]	N.D.	2969.6	N.D.	2704.0	83.2	5.0
P00747	Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2 - [PLMN_HUMAN]	N.D.	1335.4	N.D.	185.9	90.5	7.2
Q99798	Aconitate hydratase, mitochondrial OS=Homo sapiens GN=ACO2 PE=1 SV=2 - [ACON_HUMAN]	N.D.	1287.2	N.D.	1341.4	85.4	7.6
P06396	Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1 - [GELS_HUMAN]	N.D.	1279.4	N.D.	1949.1	85.6	6.3
Q13724	Mannosyl-oligosaccharide glucosidase OS=Homo sapiens GN=MOGS PE=1 SV=5 - [MOGS_HUMAN]	N.D.	1011.8	N.D.	196.6	91.9	8.9
P02786	Transferrin receptor protein 1 OS=Homo sapiens GN=TFRC PE=1 SV=2 - [TFR1_HUMAN]	N.D.	918.3	N.D.	211.0	84.8	6.6
Q16891	Mitochondrial inner membrane protein OS=Homo sapiens GN=IMMT PE=1 SV=1 - [IMMT_HUMAN]	N.D.	890.9	N.D.	278.0	83.6	6.5
Q96QK1	Vacuolar protein sorting-associated protein 35 OS=Homo sapiens GN=VPS35 PE=1 SV=2 - [VPS35_HUMAN]	N.D.	880.6	N.D.	798.6	91.6	5.5
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	654.3	863.8	996.3	1641.6	69.3	6.3
P00488	Coagulation factor XIII A chain OS=Homo sapiens GN=F13A1 PE=1 SV=4 - [F13A_HUMAN]	N.D.	756.4	N.D.	69.4	83.2	6.1
P35222	Catenin beta-1 OS=Homo sapiens GN=CTNNB1 PE=1 SV=1 - [CTNB1_HUMAN]	N.D.	731.1	N.D.	150.2	85.4	5.9
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	617.1	722.9	952.1	63.8	66.0	8.1

A: Placental sample 37 kDa, B: Placental sample 83 kDa, C: Decidual sample 37 kDa,
D: Decidual sample 83 kDa, MW: molecular weight, calc. pI: calculated pI, N.D.: Not detected.

List of peptides detected corresponding to the FXIII-A from the NCBI database and the Swissprot database are shown in Table A.3 and A.4, respectively.

Table A.3: Detection of peptides belonging to FXIII-A, identified with the NCBI database

Sequence	Protein Group Accessions	A	B	C	D
QIGGDGMMDITDTYK	1127268	N.D.	High	N.D.	N.D.
QIGGDGMMDITDTYK	1127268	N.D.	High	N.D.	N.D.
SNVDMDFEVENAVLGK	1127268	N.D.	High	N.D.	N.D.
GTYIPVPIVSELQSGK	1127268;194385606	N.D.	High	N.D.	N.D.
LALETALMYGAK	1127268;194385606	N.D.	High	N.D.	N.D.
STVLTIPEIIIK	1127268;194385606	N.D.	High	N.D.	N.D.
MYVAVWTPYGVLR	1127268;194385606	N.D.	High	N.D.	N.D.
QIGGDGMMDITDTYK	1127268	N.D.	High	N.D.	N.D.
KPLNTEGVMK	1127268;194385606	N.D.	High	N.D.	N.D.
LIASMSSDSLR	1127268;194385606	N.D.	High	N.D.	N.D.
FQEGQEEER	1127268;194385606	N.D.	High	N.D.	Medium
MYVAVWTPYGVLR	1127268;194385606	N.D.	High	N.D.	N.D.
DGTHVVENVDATHIGK	1127268;194385606	N.D.	Medium	N.D.	N.D.
EIRPNSTVQWEEVCRPWVSGHR	1127268	N.D.	Medium	N.D.	N.D.
VGSAMVNAK	1127268;194385606	N.D.	Medium	N.D.	High
KPLNTEGVMK	1127268;194385606	N.D.	Low	N.D.	Low
LSIQSSPK	1127268;194385606	N.D.	Low	N.D.	Medium
KDGTHVVENVDATHIGK	1127268;194385606	N.D.	Low	N.D.	N.D.
WDTNKVDHHTDKYENNK	1127268;194385606	N.D.	Low	N.D.	N.D.
VDHHTDKYENNK	1127268;194385606	N.D.	Low	N.D.	N.D.

A: Placental sample 37 kDa, B: Placental sample 83 kDa, C: Decidual sample 37 kDa,
D: Decidual sample 83 kDa, N.D.: Not detected.

Table A.4: Detection of peptides belonging to FXIII-A, identified with Swissprot database

Sequence	Protein Group Accessions	A	B	C	D
QIGGDGMMDITDTYK	P00488	N.D.	High	N.D.	N.D.
QIGGDGMMDITDTYK	P00488	N.D.	High	N.D.	N.D.
SNVDMDFEVENAVLGK	P00488	N.D.	High	N.D.	N.D.
GTYIPVPIVSELQSGK	P00488	N.D.	High	N.D.	N.D.
LALETALMYGAK	P00488	N.D.	High	N.D.	N.D.
STVLTIPEIIIK	P00488	N.D.	High	N.D.	N.D.
MYVAVWTPYGVLR	P00488	N.D.	High	N.D.	N.D.
QIGGDGMMDITDTYK	P00488	N.D.	High	N.D.	N.D.
KPLNTEGVMK	P00488	N.D.	High	N.D.	N.D.
LIASMSSDSLR	P00488	N.D.	High	N.D.	N.D.
FQEGQEEER	P00488	N.D.	High	N.D.	High
MYVAVWTPYGVLR	P00488	N.D.	High	N.D.	N.D.
DGTHVVENVDATHIGK	P00488	N.D.	High	N.D.	N.D.
EIRPNSTVQWEEVCRPWVSGHR	P00488	N.D.	High	N.D.	N.D.
VGSAMVNAK	P00488	N.D.	High	N.D.	High
KPLNTEGVMK	P00488	N.D.	Medium	N.D.	Low
LSIQSSPK	P00488	N.D.	Medium	N.D.	High
KDGTHVVENVDATHIGK	P00488	N.D.	Medium	N.D.	N.D.
WDTNKVDHHTDKYENNK	P00488	N.D.	Medium	N.D.	N.D.
VDHHTDKYENNK	P00488	N.D.	Low	N.D.	N.D.

A: Placental sample 37 kDa, B: Placental sample 83 kDa, C: Decidual sample 37 kDa,
D: Decidual sample 83 kDa, N.D.: Not detected.

Appendix B: Raw data from invasion studies

B.1 Cell line Invasion Assay

The raw data obtained from the invasion assay using the trophoblastic cell line is given in Table B.1-B.5 respectively for each of the five different experiments performed. The parallel 1 and 2 are fluorescent measurements done twice of the same sample. In experiment 2-5 triplicates of each sample were performed (a-b-c).

The calculation of the effect was conducted using equations shown in equation B.1, standard deviation in equation B.2 and the standard error of mean (S.E.M) in B.3, where σ is standard deviation, N is number of experiments, x_i is value of sample i, and $\bar{x}$ is the average value. The net value was calculated by subtracting the blank value (without cells) from the measured fluorescent value of each sample.

$$\frac{Sample-Blank}{Control-Blank} = Effect \quad \text{(B.1)}$$

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \bar{x})^2} \quad \text{(B.2)}$$

$$S.E.M = \frac{\sigma}{N} \quad \text{(B.3)}$$

Table B.1: Fluorescent readings of samples from invasion assay no. 1

Conditions	Parallel 1	Parallel 2	Average	Net value	Effect
Blank	11359	12250	11805	-	-
FCS (10%)	16186	19869	18028	6223	0.25
Control (FCS 0%)	36899	37096	36998	25193	1
FXIIIa (50 µg/mL)	20922	23212	22067	10263	0.41

Table B.2: Fluorescent readings of samples from invasion assay no. 2

Conditions	Parallel 1	Parallel 2	Average	Net value	Effect
Blank – a	19996	20413	21123	-	-
Blank – b	22364	21076			
Blank – c	21180	21708			
FCS (10%) – a	39087	38794	32456	11333	1.72
FCS (10%) – b	33532	33285			
FCS (10%) – c	25140	24898			
Control (FCS 0%) – a	26986	26819	27702	6580	1
Control (FCS 0%) – b	27996	27691			
Control (FCS 0%) – c	28102	28620			
F13a (50 µg/mL) – a	23589	24185	22558	1435	0.22
F13a (50 µg/mL) – b	19841	20248			
F13a (50 µg/mL) – c	23058	24426			

Table B.3: Fluorescent readings of samples from invasion assay no. 3

Conditions	Parallel 1	Parallel 2	Average	Net value	Effect
Blank – a	334	328	348	-	-
Blank – b	317	307			
Blank – c	411	391			
FCS (10%) – a	819	888	693	345	0.55
FCS (10%) – b	538	526			
FCS (10%) – c	-	-			
Control (FCS 0%) – a	1477	1126	973	625	1
Control (FCS 0%) – b	850	817			
Control (FCS 0%) – c	758	811			
FXIIIa (50 µg/mL) – a	674	634	869	387	0.62
FXIIIa (50 µg/mL) – b	771	859			
FXIIIa (50 µg/mL) – c	1037	1242			
TNFα (50 ng/mL) – a	1086	1136	1086	738	1.18
TNFα (50 ng/mL) – b	1035	-			
TNFα (50 ng/mL) – c	-	-			

Table B.4: Fluorescent readings of samples from invasion assay no. 4

Conditions	Parallel 1	Parallel 2	Average	Net value	Effect
Blank – a	20686	22141	24174	-	-
Blank – b	23463	24278			
Blank – c	27430	27048			
FCS (10%) – a	18710	18826	26278	2104	0.47
FCS (10%) – b	32118	32731			
FCS (10%) – c	27319	27964			
Control (FCS 0%) – a	28203	28386	28696	4522	1
Control (FCS 0%) – b	21340	21688			
Control (FCS 0%) – c	43017	44204			
FXIIIa (50 µg/mL) – a	21072	21657	28298	4124	0.91
FXIIIa (50 µg/mL) – b	30689	30493			
FXIIIa (50 µg/mL) – c	24099	25089			
TNFα (50 ng/mL) – a	33231	33304	26733	2559	0.57
TNFα (50 ng/mL) – b	24414	25064			
TNFα (50 ng/mL) – c	29612	29977			

Table B.5: Fluorescent readings of samples from invasion assay no. 5

Conditions	Parallel 1	Parallel 2	Average	Net value	Effect
Blank – a	14953	15127	14532		
Blank – b	13447	13498			
Blank – c	15083	15082			
FCS (10%) – a	18411	18380	17387	2855	0.16
FCS (10%) – b	14413	14659			
FCS (10%) – c	19292	19165			
Control (FCS 0%) – a	40947	37545	32663	18132	1
Control (FCS 0%) – b	30013	30122			
Control (FCS 0%) – c	36957	37344			
FXIIIa (50 µg/mL) – a	24059	24319	24866	10335	0.57
FXIIIa (50 µg/mL) – b	31171	30216			
FXIIIa (50 µg/mL) – c	20711	21515			
TNFα (50 ng/mL) – a	29444	28786	24280	9749	0.54
TNFα (50 ng/mL) – b	18039	19048			
TNFα (50 ng/mL) – c	16229	16081			

Summary of effects and calculated average, standard deviation and standard error of mean of the invasion assays performed using the trophoblastic cell line are shown in Table B.6.

Table B.6: Summary of invasion assay using trophoblastic cell line

Experiment	**Control (0% FCS)**	**FXIIIa (50 µg/mL)**	**FCS (10%)**	**TNFα (50 ng/mL)**
1	1	0.41	0.25	-
2	1	0.22	1.72	-
3	1	0.62	0.55	1.18
4	1	0.57	0.15	1.40
5	1	0.91	0.47	0.57
Average	1	0.55	0.63	1.05
Standard deviation	-	0.26	0.63	0.16
SEM	-	0.13	0.32	0.11

B.2 Primary culture invasion assay

The raw data obtained for the ten times we did the invasion assay using extravillous trophoblast primary culture are given in Table B.7. The parallels 1 and 2 are fluorescent measurements done twice of the same sample. In experiment no. 2, duplicates of samples were conducted (a-b), however, due to scarce access of primary culture cells only singular samples were made in the other experiments. The calculation of the effect was conducted as the same way explained in Section B.1.

Table B.7: Fluorescent readings of invasion assay 1-10 with primary culture cells

Experiment	Conditions	Parallel 1	Parallel 2	Average
1	Control (0% FCS)	41131	42412	41772
	FXIIIa (50 µg/mL)	34456	34779	34618
2	Control (0% FCS) – a	20159	20622	21301
	Control (0% FCS) – b	22075	22347	
	FXIIIa (50 µg/mL) – a	18504	18683	17783
	FXIIIa (50 µg/mL) – b	16756	17189	
3	Control (0% FCS)	41378	43942	42660
	FXIIIa (50 µg/mL)	32010	32934	32472
4	Control (0% FCS)	40990	42045	41518
	FXIIIa (50 µg/mL)	33511	34424	33968
5	Control (0% FCS)	16585	16838	15914
	Control (0% FCS)	14985	15247	
	FXIIIa (50 µg/mL)	23585	23344	20203
	FXIIIa (50 µg/mL)	16807	17077	
6	Control (0% FCS)	32859	32800	32830
	FXIIIa (50 µg/mL)	35245	35482	35364
7	Control (0% FCS)	33039	33213	33126
	FXIIIa (50 µg/mL)	42016	41898	41957
8	Control (0% FCS)	14991	15034	15013
	FXIIIa (50 µg/mL)	16025	16445	16235
9	Control (0% FCS)	30008	29835	29922
	FXIIIa (50 µg/mL)	42483	42786	42635
10	Control (0% FCS)	33196	36625	34911
	FXIIIa (50 µg/mL)	39926	45723	42825

Summary of effects and calculated average, standard deviation and standard error of mean of the invasion assays performed using primary culture cells are shown in Table B.8.

Table B8: Summary of invasion assay using primary culture

Experiment	Control (0% FCS)	Effect of FXIIIa (50 µg/mL)
1	1	0.59
2	1	0.83
3	1	0.76
4	1	0.45
5	1	1.27
6	1	1.08
7	1	1.27
8	1	1.08
9	1	1.42
10	1	1.23
Average	-	1.00
Standard Deviation	-	0.32
SEM	-	0.10

Appendix C: Raw data from zymography studies

C.1 Substrate gel zymography using trophoblast cell line

Conditioned medium from invasion assays using the trophoblastic cell line was studied with substrate gel zymography, and the intensity of the bands of the matrix metalloproteases MMP-9 and MMP-2 was quantified with Bio-1D software (Vilber Lourmat, France). The obtained values of volume intensity calculated by the software are given in Table C.1.

Table C.1: Volume intensity of MMP-9 and MMP-2 secretion using trophoblast cell line

Experiment	Conditions	Volume intensity of MMP-9	Volume intensity of MMP-2
1	Control (0% FCS)	214862	229367
	FXIIIa (50 µg/mL)	215857	190586
2	Control (0% FCS)	192961	181473
	FXIIIa (50 µg/mL)	175876	178494
3	Control (0% FCS)	144034	165034
	FXIIIa (50 µg/mL)	172675	225263
4	Control (0% FCS)	179647	195562
	FXIIIa (50 µg/mL)	172520	208699

Summary of effects and calculated average, standard deviation and standard error of mean of effect on matrix metalloproteases secretion using trophoblastic cell line are shown in Table C.2, using formula B.1, B.2 and B.3 given in Appendix B.

Table C.2: Summary of effects of FXIIIa on MMP secretion using trophoblast cell line

Experiment	Control (0% FCS)	Effect of FXIIIa on MMP-9 secretion	Effect of FXIIIa on MMP-2 secretion
1	1	1.00	0.83
2	1	0.91	0.93
3	1	1.20	1.36
4	1	0.96	1.07
Average	1	1.02	1.05
St dev	-	0.13	0.23
SEM	-	0.064	0.12

C.2 Substrate gel zymography using primary culture

Conditioned medium from invasion assays using primary culture was studied with substrate gel zymography, and the intensity of the bands of the matrix metalloproteases MMP-9 and MMP-2 was quantified with Bio-1D software (Vilber Lourmat, France). The obtained values of volume intensity calculated by the software are given in Table C.3.

Table C.3: Volume intensity of MMP-9 and MMP-2 secretion using primary culture

Experiment	Conditions	Volume intensity of MMP-9	Volume intensity of MMP-2
1	Control (0% FCS)	104793	109301
	FXIIIa (50 µg/mL)	105253	107199
2	Control (0% FCS)	85302	N.D.
	FXIIIa (50 µg/mL)	91342	N.D.
3	Control (0% FCS)	91306	124880
	FXIIIa (50 µg/mL)	92590	119811

N.D.: Not detectable

Summary of effects and calculated average, standard deviation and standard error of mean of effect on matrix metalloproteases secretion using primary culture are shown in Table C.4, using formula B.1, B.2 and B.3 given in Appendix B.

Table C.4: Summary of effects of FXIIIa on MMP secretion using primary culture

Experiment	Control (0% FCS)	Effect of FXIIIa on MMP-9 secretion	Effect of FXIIIa on MMP-2 secretion
1	1	1.00	0.98
2	1	1.07	-
3	1	1.01	0.96
Average	-	1.03	0.97
St dev	-	0.04	0.015
SEM	-	0.02	0.011

C.3 Substrate gel zymography using explants

Conditioned medium from explants culture was studied with substrate gel zymography, and the intensity of the bands of the matrix metalloproteases MMP-9 and MMP-2 was quantified with Bio-1D software (Vilber Lourmat, France). The obtained values of volume intensity calculated by the software are given in Table C.5.

Table C.5: Volume intensity of MMP-9 and MMP-2 secretion using explants

Experiment	Conditions	Volume intensity of MMP-9	Volume intensity of MMP-2
1	Control (0% FCS)	242703	203328
	FXIIIa (50 µg/mL)	245264	244962
2	Control (0% FCS)	268027	215148
	FXIIIa (50 µg/mL)	280145	225095
3	Control (0% FCS)	356128	415315
	FXIIIa (50 µg/mL)	355794	414981

Summary of effects and calculated average, standard deviation and standard error of mean of effect on matrix metalloproteases secretion using primary culture are shown in Table C.6, using formula B.1, B.2 and B.3 given in Appendix B.

Table C.6: Summary of effects of FXIIIa on MMP secretion using explants

Experiment	Control (0% FCS)	Effect of FXIIIa on MMP-9 secretion	Effect of FXIIIa on MMP-2 secretion
1	1	1.01	1.20
2	1	1.05	1.05
3	1	1.00	1.00
Average	-	1.02	1.08
St dev	-	0.02	0.11
SEM	-	0.014	0.06

Appendix D: Raw data from proliferation studies

D.1 Proliferation studies on trophoblast cell line using BrdU

The effect of FXIII-A on proliferation was studied with BrdU incorporation. The initial experiment was conducted with 3000 cells/well in a 96-well plate after the existing technique in the laboratory. The measured absorbance was lower than expected (Table D.2), therefore a test of increasing cell dosage was performed and the result is shown in Table D.1. The net value of absorbance (blank absorbance measurement subtracted from sample absorbance) was plotted against cell quantity in Figure D.1. From the graph it was chosen to use 7.5*10^3 cells/well since a higher quantity of cells would reach the plateau phase immediately. Absorbance measurements should be read after 10-15 minutes to reach the optimal absorbance interval.

Table D.1: Increasing cell dosage test for proliferation studies using BrdU incorporation

Cell quantity	Absorbance after 5 min	Absorbance after 10 min	Absorbance after 15 min	Absorbance after 20 min	Absorbance after 30 min
2.5*10^3	0.208	0.283	0.318	0.460	0.634
5.0*10^3	0.275	0.387	0.523	0.610	0.610
1.0*10^4	0.283	0.442	0.516	0.600	0.600
5.0*10^4	0.384	0.519	0.639	0.740	0.890
1.0*10^5	0.462	0.609	0.700	0.830	1.040
Blank	0.0867	0.0875	0.0853	0.0881	0.0878

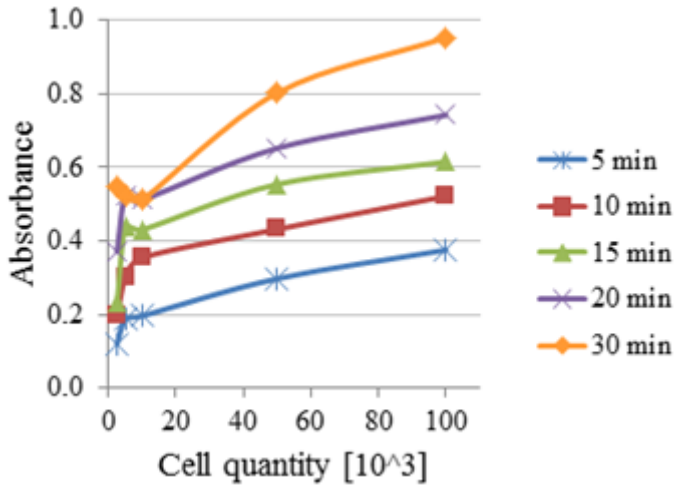

Figure D.1: Absorbance plotted against cell quantity for different time measurements. *Absorbance was read after 5, 10, 15, 20 and 30 minutes for the different cell quantities.*

The absorbance measurements with and without effectors are given in Table D.2. Quadruplicates of each sample were conducted and the blank (substrate from BrdU kit only) was measured once. The average of the quadruplicates was calculated and used to find the effect of FXIIIa compared to the control (0% FCS). Formulas given in Appendix B were used in the calculation and the results are listed in Table D.3.

Table D.2: Proliferation study on trophoblast cell line using BrdU incorporation

Experi-iment	Conditions	Absobance Parallell 1	Absorbance Parallell 2	Absorbance Parallell 3	Absorbance Parallell 4
1	FCS (10%)	0.2042	0.2164	0.2695	0.2529
	FXIIIa (50 µg/mL)	0.2316	0.2030	0.2321	0.3123
	Control (0% FCS)	0.2087	0.2647	0.2368	0.2052
	Blank	0.1379	-	-	-
2	FCS (10%)	N.A.	0.6212	0.7815	0.9089
	FXIIIa (50 µg/mL)	0.7893	0.7957	0.8352	0.6818
	Control	0.6256	0.6358	0.9556	0.6965
	Blank	0.0751	-	-	-
3	FCS (10%)	0.6407	0.5584	0.5128	0.6308
	FXIIIa (50 µg/mL)	0.6357	0.8410	0.7373	0.7975
	Control	0.5643	0.9360	0.9740	0.5279
	Blank	0.0876	-	-	-

N.A: No answer

Table D.3: Summary of effects of FXIIIa and serum on proliferation of trophoblast cell line

Experiment	Control (0% FCS)	FXIIIa (50 µg/mL)	FCS (10%)
1	1	1.17	1.08
2	1	1.07	1.06
3	1	1.00	0.75
Average	1	1.08	0.96
St dev	-	0.85	0.19
SEM	-	0.049	0.11

D.2 Proliferation studies on explants using immunohistochemistry

The effect of FXIII-A on proliferation was also studied by marking sections of explants with a proliferation marker (Ki67). The amount of nuclei in a proliferation state was counted with ImageJ computer software (National institutes of Health, Bethesda, MD). The surface ratio of the tissue was measured with the same software, and the proliferation ratio was calculated as shown in equation D.1. The counted nuclei and measured surface is listed in Table D.4. The effect of FXIII and serum (10% FCS) compared to the control (0% FCS) was calculated, as well as the average, standard deviation and standard error of the mean (using formulas given in Appendix B), which is listed in Table D.5.

$$Proliferation\ ratio = \frac{Nucleus\ marked}{Surface} \qquad \text{(D.1)}$$

Table D.4: Proliferation study on explants using immunohistochemistry

Experiment	Conditions	Nucleus marked	Surface	Nucleus marked	Surface	Nucleus marked	Surface
1	Control	82	329861	48	254620	68	334716
	FXIIIa	61	242532	62	239433	86	338931
	10% FCS	103	281383	95	325327	130	359629
2	Control	56	383725	68	381874	54	371568
	FXIIIa	63	344950	85	277742	32	291117
	10% FCS	163	415234	124	377876	145	390901
3	Control	49	379013	50	346856	178	370334
	FXIIIa	78	317553	93	357139	81	271596
	10% FCS	133	351170	113	362209	128	354222
4	Control	80	395816	76	415219	51	367832
	FXIIIa	49	342599	55	369433	96	347926
	10% FCS	81	320697	128	378992	57	392400
5	Control	169	335307	113	394613	117	390233
	FXIIIa	60	323784	60	238218	51	308152
	10% FCS	-	-	-	-	-	-
6	Control	82	329861	48	254620	68	334716
	FXIIIa	61	242532	62	239433	86	338931
	10% FCS	103	281383	95	325327	130	359629
7	Control	49	379013	50	346856	178	370334
	FXIIIa	78	317553	93	357139	81	271596
	10% FCS	133	351170	113	362209	128	354222

Table D.5: Summary of effects of FXIIIa and serum on proliferation study using explants

Experiment	Control (0% FCS)	FXIIIa (50 µg/mL)	FCS (10%)
1	1	1.19	1.59
2	1	1.27	2.33
3	1	1.07	1.40
4	1	1.08	1.40
5	1	0.55	-
6	1	1.19	1.59
7	1	1.07	1.40
Average	-	1.04	1.40
St dev	-	0.24	0.55
SEM	-	0.090	0.21