The role of IL-33 and ST2 in early pregnancy

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Abstract

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Regulation of the growth and differentiation of trophoblast cells is critical for successful embryo implantation and placentation. Cytokines are key players in these processes, as well as modulating the maternal immune response to prevent rejection of the conceptus. This thesis focused on the investigation of the cytokine interleukin (IL)s -33 and its receptor, ST2. ST2 has two isoforms, a functional cell surface receptor (ST2L) and a soluble decoy receptor (sST2). Previous work in this laboratory had shown that the human placenta expresses both IL-33 and sST2 at term. The aim of this thesis was to investigate IL-33 and ST2 in early pregnancy, the time when trophoblast is at its most active, with a view to better understanding their role. IL-33 and ST2 mRNA and protein were examined in 14 first trimester placentas from 6-12 weeks of gestation. IL-33 was localized to cells in the villous stroma, whereas ST2 was present in the syncytiotrophoblast, villous cytotrophoblast and the invasive extravillous cytotrophoblast of the cell columns. Secretion of sST2, but not IL-33, by the placenta was found. Investigation of pre-implantation embryos showed the presence of ST2, but not IL-33 protein. Decidualized endometrium was investigated as a potential source of IL-33 and sST2 at the maternal-fetal interface and, although mRNA for both was present, no protein could be found. The key finding was that sST2, rather than ST2L, was the predominant isoform in the placenta. This led us to reconsider the hypothesis that IL-33/ST2 interactions in the placenta are important for successful pregnancy and raised the possibility that they may have independent roles. Using trophoblast cell lines as a model, it was shown that sST2 binds to trophoblast cells, significantly inhibits their proliferation and stimulates their invasion *in vitro*. This is the first report of this novel role for sST2 in pregnancy. Thus these studies have shown that sST2 may play an important role in implantation and placentation through controlling trophoblast invasion.

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Contents

Abbreviations

8-Br-cAMP: 8-Bromoadenosine 3’,5’-cyclic monophosphate

APC: Allophycocyanin

ATP: Adinosine triphosphate

AV: Anchoring villi

BALF: Bronchoalveolar lavage fluid

BCA: Bicinchoninic acid

cAMP: Cyclic adenosine monophosphate

CAPG: Capping protein gelsolin-like

CD: Cluster of differentiation molecule

CK-7: Cytokeratin 7

cDNA: complementary deoxyribonucleic acid

C-section: Caesarean section

Ct: cycle threshold

CT: cytotrophoblast columns, CC

CTB: cytotrophoblast

DAMPs: damage associated molecular patterns

DAPI: Diamidino-2-phenylindole

dbcAMP: dibutyryladenosine 3’,5’ cyclic monophosphate

DC: Dendritic cells

dCTB: Distal cytotrophoblast

DMEM: Dulbecco’s modified Eagles culture medium

DSC: Decidualized stromal cells

ELISA: Enzyme linked immunosorbent assay

enEVT: Endovascular extravillous trophoblast

ErBb: Epidermal growth factor receptor

ERK: Extracellular signal regulated kinase

EVT: Extravillous trophoblast

FBXL: Skp-Cullin-F-box ubiquitin ligase

FCS: Fetal calf serum

FcεR: Fc epsilon receptor

FITC: Fluorescein isothiocyanate

FV: floating villi

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC: Giant cells

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GSK3B: Glycogen synthase kinase 3β

HSA: Human serum albumin

hCG: human chorionic gonadotrophin

HEV: High endothelial venules

His: polyHistidine

HLA: Human leukocyte antigen

hPL: Human placental lactogen

HRG: Heregulin

HRP: Horseradish peroxidase

HSS: Hank’s balanced salt solution

HUVEC: Human umbilical vein endothelial cells

ICM: Inner cell mass

iEVT: Interstitial extravillous trophoblast

IgG: Immunoglobulin

IHC: Immunohistochemistry

IL: Interleukin

IL-1R: IL-1 receptor

IL-1RA: IL-1 receptor antagonist

IL-1RAcP: IL-1 receptor accessory protein

IFNγ: Interferon gamma

IRAK: IL-1R-associate kinase

IVF: *In vitro* fertilization

JAK: Janus kinase

KI: Kallikrin Inhibitor

KIR: killer like immunoglobulin-like receptors

LGALS13: Placental protein 13, β-galactoside binding S-type galectin superfamily
LIF: Leukaemia inhibitory factor
LPS: Lipopolysaccharide
M: Macrophages
MAP: Mitogen activated protein
MGB: Minor groove binder
mRNA: messenger ribonucleic acid
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoluim bromide
MyD88: Myeloid differentiation primary response gene 88
NF: Nuclear factor
NFκB: nuclear factor kappa B
NK: Natural killer cell
NTC: No cDNA template control
PAGE: Polyacrylamide gel electrophoresis
PBS: Phosphate buffered saline
PBST: phosphate buffered saline with Tween
pCTB: Proximal cytotrophoblast
PFA: Paraformaldehyde
PR3: Proteinase 3
PVDF: Polyvinylidene difluoride
qRT-PCR: Quantitative real time polymerase chain reaction
rh: Recombinant human
RT: Reverse transcriptase
RT: Room temperature
SBB: Sudan black B
SC: Stromal cells
SDS: Sodium dodecyl sulphate
SEM: Standard error of the mean
SIGIRR: Single Ig IL-1-related receptor
sST2: Soluble ST2
ST2: growth factor stimulation 2

ST2L: Long membrane bound form of ST2

ST2V: ST2 variant

STB: syncytiotrophoblast

SVD: spontaneous vaginal delivery

TE: trophectoderm

Th: T helper cell

TLR: Toll like receptors

TNF: Tumour necrosis factor

TRAF: Tumour necrosis factor receptor-associated factor

Treg: T regulatory cell

TRITC: Tetramethylerhodamine-5-(and 6)-isothiocyanate

uNK: Uterine natural killer cells

VC: villous core

ZP: Zona pellucida

Chapter 1

Introduction

1.1 The human placenta

The placenta is essential for viviparity where fetal development takes place within the female reproductive tract. This form of reproductive strategy enables higher protection from environmental risks and controls the growth and development of the fetus in the uterus (1). The placenta is a specialized structure that develops simultaneously with the growing embryo. It is an indispensable human organ for fetal life. The placenta acts as an organ surrogate until the fetus's own organs develop, taking on the roles of the fetal lungs, gut and kidneys. It is also responsible for the transport of oxygen and nutrients and the elimination of waste from the fetal circulation (2). These roles are achieved by establishing an intimate vascular connection between maternal and fetal blood, which is critical for the survival of pregnancy (3). The second main function of the placenta is to provide an 'immunological camouflage', protecting the fetus from the maternal immune system that would otherwise attack the invading embryo (4). The placenta consequently acts as a physical barrier between the mother and fetus. It must establish a delicate equilibrium so that the maternal immune system can tolerate the necessary fetal requirements yet continue to protect the mother from excessive fetal intrusion (5). The placenta must perform these critical functions for a successful maternal-fetal relationship to be established and for the pregnancy to carry to term. Insufficient placental development or poor placentation is associated with pregnancy pathologies, such as pre-eclampsia, a syndrome that may develop from the late-second trimester onwards, characterised by hypertension and proteinuria (6).

1.1.1 Embryo implantation and the development of the placenta

Following fertilization, the zygote (fertilized egg) undergoes several rounds of divisions and morphogenesis to form the blastocyst, the embryonic stage where the first two distinct cell lineages arise: the outer specialized trophectoderm epithelium and the inner cell mass. The trophectoderm participates in the first physical and physiological interaction with the maternal endometrium to initiate implantation (7), beginning with the adherence of the blastocyst to the uterine epithelium (Figure 1). Then the blastocyst invades deep into the uterine wall via interactions between cells of the trophectoderm and the endometrium. The trophectoderm is the first cell lineage that exhibits a highly differentiated function during embryonic development, going on to form the placenta. The cells derived from the trophectoderm give rise to differentiated trophoblast cells through two general pathways (Figure 2). In the first differentiation pathway, the mononuclear cytotrophoblast (CTB) fuse into multinucleated syncytiotrophoblast (8) that cover the floating villi of the placenta, and are bathed in maternal blood. STB are primarily involved in the production of pregnancy-related hormones as well as the exchange of nutrients and waste at the maternal fetal interface. In the second differentiation pathway, the CTBs proliferate in the tips of the anchoring villi that attach to the uterine wall. Trophoblasts in the anchoring villi then acquire invasive characteristics and migrate from the placenta into the decidua; this subset of trophoblast is referred to as extravillous cytotrophoblasts (EVT) (7). The interactions between the different types of trophoblast cells and maternal cells take place in defined areas referred to as maternal-fetal interfaces (Figure 3). These interfaces contain different types of cells; the STB is in contact with maternal blood cells whereas the EVT are in contact with uterine stroma, macrophages and NK cells, whose interaction with trophoblast cells is essential for successful implantation and pregnancy.

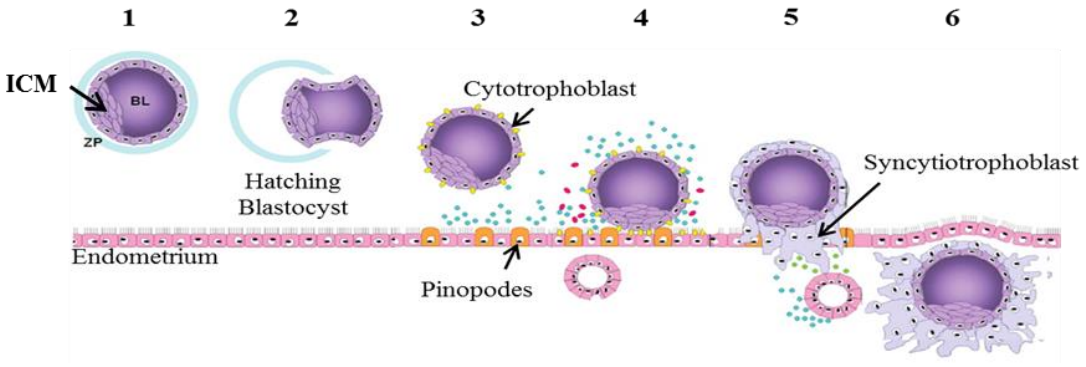


Figure 1. Human embryo implantation.
(1) Blastocyst with distinctive inner cell mass (ICM) and trophectoderm cell layer. (2) Once hatched out of the zona pellucida (ZP), cytokine (blue dots) and chemokine (pink dots) gradients attract the blastocyst (BL) to the implantation site (3). The blastocyst firmly attaches to the endometrium via adhesion molecules (yellow dots) on the endometrial pinopodes (4). For the invasion phase, cytotrophoblast cells fuse to form a syncytiotrophoblast layer to initiate the invasion by releasing matrix metalloproteinases (green dots) (5) and complete penetration of the endometrium (6). Figure, with modification, from (9).

1.1.2 Decidualization

1.1.2.1 Blastocyst-endometrial communication

Successful embryonic implantation requires the establishment of a two-way dialogue between blastocyst and maternal endometrium. After a well-defined period of uterine receptivity referred to as the ‘window of implantation’, the endometrium becomes refractive, and successful implantation can no longer occur. This window of implantation occurs from days 20-24 of the menstrual cycle or 6-10 days after the luteinizing hormone peak (10). In humans, decidualization can take place in the absence of an embryo and menstruation occurs in the absence of an implanting embryo. The process of decidualization is predominantly induced by progesterone which activates the second messenger cyclic adenosine monophosphate (cAMP), an important intracellular mediator of decidualization (11). Decidualization is maintained by the implanting embryo in the late secretory phase of the menstrual cycle, and continues after successful implantation to regulate trophoblast invasion and placentation (12). Full decidual transformation, with extensive stromal cell proliferation and differentiation, will only occur upon blastocyst invasion into the endometrium.

The differentiation of trophoblast cells during placental development is regulated by oxygen level, hormones and growth factors at the maternal-fetal interface. Survival of the fetus is dependent on the ability of the trophoblasts to differentiate into their respective subtypes, and for each subtype to undergo regulated phenotypic and functional changes. The behaviour of the trophoblast is controlled by many elements in the extracellular matrix as well as cell adhesion molecules, cytokines and growth factors (13).

1.1.3 Trophoblast cell fusion and formation of the syncytiotrophoblast

The mononucleated CTBs fuse into multinucleated STBs forming a syncytial layer that covers the placental villous tree. These cells form a protective layer that is involved in the exchange of gases, nutrients and waste across the maternal-fetal interface. The presence of microvilli on this layer increases the surface area which provide higher absorptive capabilities (14). The syncytial layer also plays a major role in the maintenance of pregnancy via the production of pregnancy-related hormones, such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL) (15).

1.1.4 Subtypes of extravillous cytotrophoblast

Proliferating CTBs participate in the generation of the cell column which forms a bridge between the placental villous tip and the maternal decidualized stroma. These cell columns can be found at the proximal ends of the anchoring villi. The sites of the anchoring villi are established during the second week following implantation (16). Human placental and decidual explant cultures suggest that decidual contact induces CTBs to break through the STB layer to form the cell columns (16).

CTBs located at the distal ends of the cell columns express high amounts of integrin, fibronectin and L-selectin molecules which are integral to the formation and maintenance of the anchoring villi in early pregnancy (17, 18). The population of extravillous trophoblasts in the cytotrophoblast columns is comprised of both proliferative and invasive cells, both of which have spatially and temporally regulated phenotypes (19). EVT within the cell column furthest from the maternal side are proliferative and non-invasive, whereas the extra-villous trophoblasts nearest to the maternal side stop proliferating and become invasive (20). Furthermore, CTBs at the distal ends of the columns lose their cell-cell contacts and detach from columns. As they come into contact with the decidual extracellular matrix, they differentiate into interstitial (iEVTs) and endovascular (enEVTs) and further migrate into the maternal decidua (21).

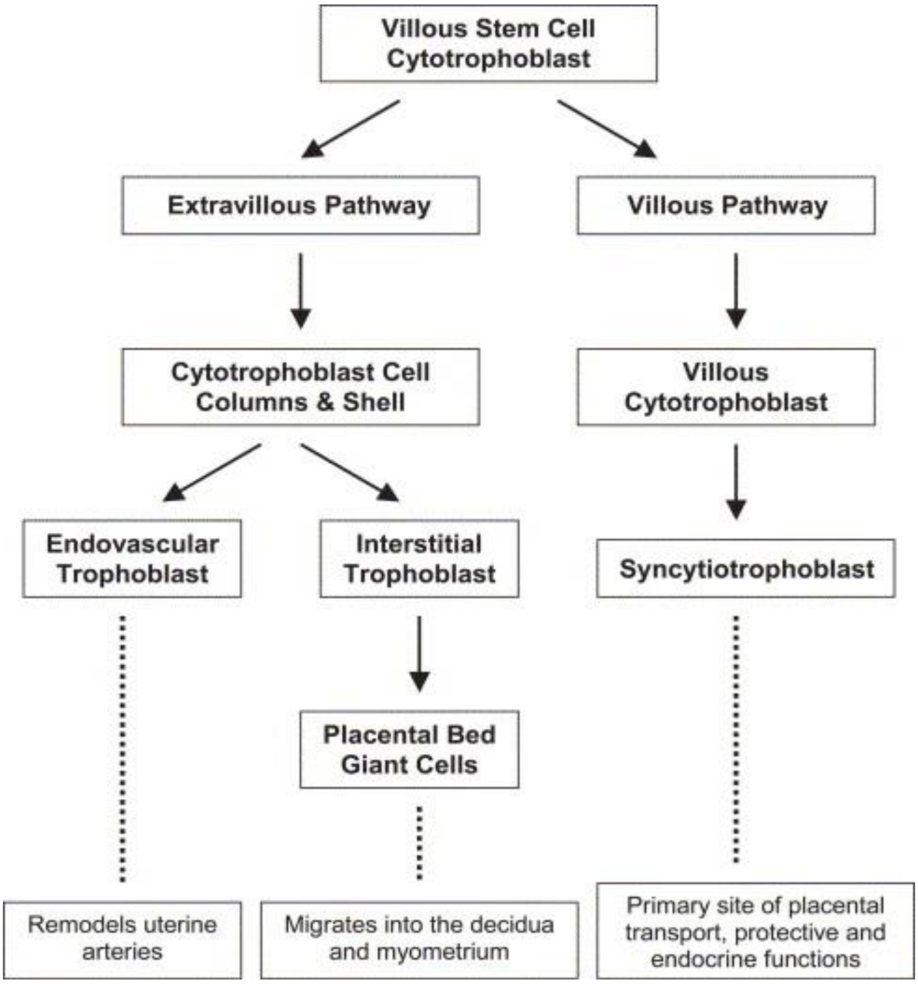


Figure 2. Trophoblast cell differentiation pathways.
The development of the various trophoblast cells that make up the placenta is through two main pathways: the villous pathway, which produces the syncytiotrophoblast, and the extravillous pathway that gives rise to endovascular and interstitial trophoblasts. The latter terminally differentiate into placental bed giant cells which may have a role in facilitating trophoblast interaction with maternal cells. Each trophoblast subtype has a unique role, as described in the diagram (22).

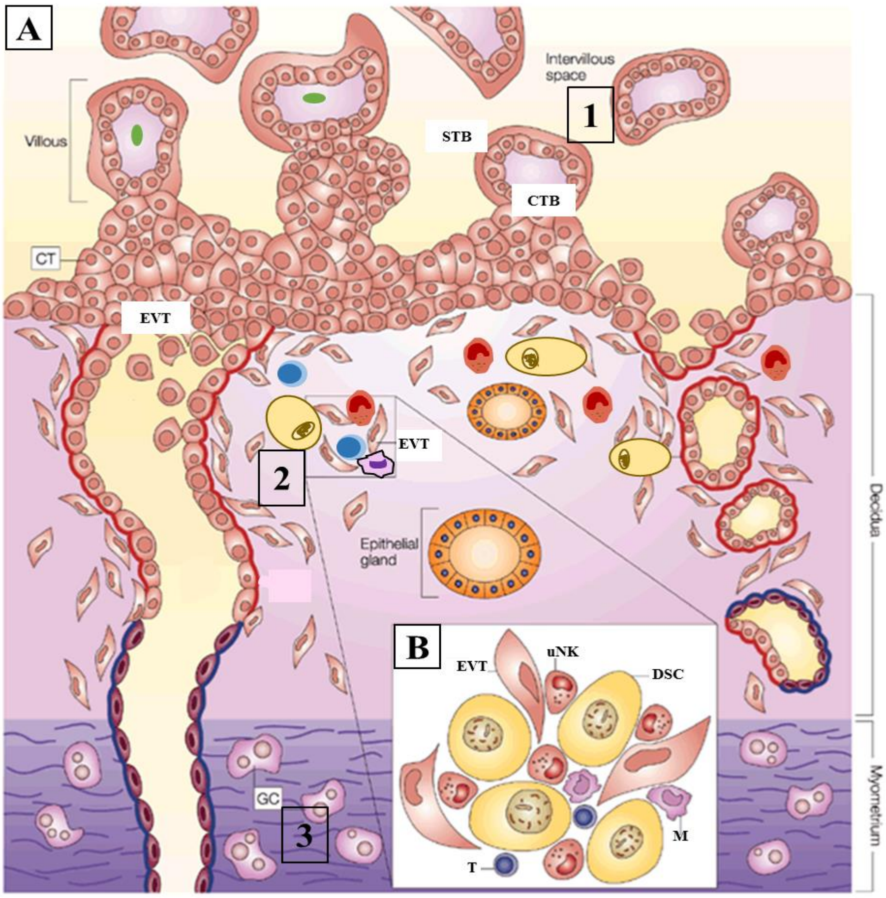


Figure 3. The two maternal-fetal interfaces of the placenta.

(A) Interface 1: syncytiotrophoblast (8) interaction with maternal blood. Placental villi are covered by villous trophoblast cells (an outer STB layer and an inner mononuclear CTB layer). Each villus core contains fetal macrophages, fibroblasts and blood vessels. Maternal blood reach the placenta through uterine spiral arteries and the exchange of molecules and gas takes place in the intervillous space where STB is the only fetal layer that is exposed to the maternal blood.

(B) Interface 2: extravillous cytotrophoblast (EVT) interaction with cells in the decidua. At the tips of the anchoring villi, the inner CTB cells grow out to form cell columns (CT) which are vital for placental attachment to maternal decidua. Extravillous trophoblast cells in the anchoring columns migrate toward the decidua and interact with different cell types in the maternal decidua, including decidual stromal cells (DSC), uterine Natural Killer (uNK), macrophages (M) and T cells (T).

EVT cells invade as far as the inner myometrium where they may fuse to form placental bed giant cells (GC). Figure adapted and modified from (23).

1.2 Immune paradox of pregnancy

1.2.1 An introduction to immune tolerance in pregnancy

The concept of immunological tolerance during pregnancy started almost a century ago from the work on fetus to fetus transfusion in dizygotic cattle twins (24, 25). Since then, scientists have been interested in the mechanism of maternal immune tolerance towards the semi-allogeneic fetus (26, 27). Medawar proposed three possible mechanisms by which the fetus is protected from being attacked by the maternal immune system. The first was that there was some kind of physical barrier that shields the fetus from maternal immune cells; the second was that the placental trophoblast expresses "immature antigens" which do not stimulate graft rejection responses in the mother, and thirdly that the mother's immune system is somehow suppressed during pregnancy. Detailed analysis of the structure of the placenta has shown that trophoblast cells and maternal immune cells are in intimate contact at both interfaces 1 and 2 (Figure 3), and therefore a physical barrier can be ruled out (28). However the "antigenic immaturity" and "maternal immunosuppression" hypotheses have turned out to be more interesting.

1.2.2 Human Leukocyte Antigen (HLA) Expression during Pregnancy

The immunological tolerance and symbiosis between the genetically distinct fetus and its mother is one of the most interesting paradoxes of life (29). It is now known that HLA molecules expressed by the trophoblast cells play a major role in the immune tolerance (30, 31), and they are tightly controlled during pregnancy (32).

In the human blastocyst, several HLA molecules are expressed with variation depending on the location. The fetus (derived from the ICM) presents the full range of

HLA molecules (33), while the trophoblast cells do not express HLA-A, HLA-B and HLA class II molecules, all of which are involved in rapid rejection of allografts in human (34, 35). Their lack of expression on trophoblast therefore prevents a typical T cell driven graft rejection response to the conceptus. However, trophoblast cells do express some HLA molecules, but ones with restricted polymorphism; namely HLA-C, HLA-E, HLA-F and, most importantly, HLA-G. (32, 36, 37).

HLA-G was the first of the HLA molecules found to be expressed almost uniquely by trophoblast cells and it remains an antigen of great interest and a focus of increased experimental investigations (36, 38). HLA-G is expressed by the pre-implantation embryo at the cleavage and blastocyst stage (39) and a soluble isoform of this protein can be detected in embryo culture supernatants, although conflicting results are reported between different groups (40). HLA-G has several important functions. It modulates the activity of T- and B- lymphocytes and macrophages by inducing apoptosis or reducing the cytotoxic activity of T cells (41). HLA-G expression by trophoblast cells also protects them from cell lysis by uNK cells (42), and plays an immunosuppressive role by controlling uNK cell cytokine production (43). Similar roles have been described for HLA-C in inhibiting the cytotoxic activity of uNK cells (37). These findings support the role of HLA molecules in maintaining maternal tolerance. STB does not express any HLA molecules and is therefore inert to the maternal immune system.

1.2.3 Immune cells in the decidua and the Th1/Th2 balance

Medawar’s third hypothesis, that there is suppression of the maternal immune response during pregnancy, relates to the different kinds of immune cells with which the trophoblast comes in contact. During early pregnancy, four major immune cell types are present in the decidua; uterine natural killer (uNK) cells, macrophages, dendritic cells (DC) and T cells (44). These cells, especially the uNK cells, are present in high numbers during the first trimester of pregnancy. uNK cell numbers are dramatically increased during decidualization and account for 65–70% of the decidual immune cells (45). uNK cells are known to be in intimate contact with extravillous trophoblast cells (46, 47).

A crucial phenotype of uNK cells is that they express highly diverse killer immunoglobulin-like receptors (KIRs) that recognize polymorphic HLA-C. Different maternal uNK KIR and paternal trophoblast HLA-C haplotype combinations can limit or foster uNK activation and the production of cytokines and angiogenic factors essential for uterine vasculature remodelling, and notably certain adverse combinations are associated with pre-eclampsia (48, 49).

In humans, differentiating stromal cells produce cytokines and chemokines that act as a chemo-attractants for immune cells, encouraging their infiltration into decidual tissue (50). Interleukin-1 (IL-1) stimulates decidual production of macrophage-attracting chemokines such as CCL2, CCL5, CXCL2, CXCL3, and CXCL8 (51). Macrophages play a major role in clearing apoptotic material that is released from trophoblasts during the different stages of pregnancy (52).

From work on mice, it was proposed that the maternal immune response in pregnancy is biased towards beneficial antibody-mediated T helper cell type 2 (Th2) responses and away from harmful T helper cell type 1 graft rejection responses that could damage the placenta (53). This concept of immune tolerance was later extended to NK cells (54) which showed equivalent cytokine secretion profiles to T cells (55). A model of Type-1 (T1)/Type-2 (T2) immune tolerance was proposed based on the ability of NK cells to provide an anti-inflammatory environment in normal pregnancy, dominated by T2 cytokines, but with a T1 cytokine predominance in pathological pregnancies such as implantation failure, recurrent miscarriage and pre-eclampsia (56).

There are several criticisms of the Th1/Th2 hypothesis, especially when considering human studies, as it assumed, at first, that immune responses in the circulation reflect those found locally in the uterus. It is therefore imperative to distinguish between events in the circulation and those occurring in the decidua (57). It was also noted that in humans, contrary to the hypothesis, Th1 activity is actually beneficial during the implantation period, as at this time, cytokines such IL-1 and TNF-α stimulate the production of LIF and increase angiogenesis. Furthermore, since Wegmann’s original hypothesis of 1993, many more cytokines have been identified, and their functions and expression profiles are now considered to be far more complex - they are no longer simply classified as ‘good’ or ‘bad’ based on their association with a Th1 or Th2 response (58).

Consequently, the Th1/Th2 pregnancy immunity model has evolved into a more complex theory, which extends to other types of immune cells. Naive $CD4^+$ T helper cells differentiate into four effector subsets, Th1, Th2, Th17 and T regulatory (Treg) cells, which secrete distinct combinations of cytokines (59). Also, it is recognized that many cell types (not just immune cells) are able to secrete different types of cytokines (60). The discovery of Treg cells, which supress rather than promote T cell responses, made it apparent that the immunology of pregnancy is not a simple model of Th1/Th2 balance only (61). Treg cells are detected in the human decidua at various stages of pregnancy, suggesting they play a role in establishing fetal-maternal immune tolerance, especially as their levels are highest in the peripheral blood of first trimester pregnancy women (62, 63). Recent evidence suggests that seminal fluid also contributes to maternal immune tolerance to paternal allo-antigens by promoting an increase in the Treg cell population during the early stages of pregnancy (64).

1.2.4 Cytokines and growth factors involved in implantation and early pregnancy

Numerous cytokines and growth factors are involved in the maintenance of successful embryo implantation and early pregnancy (65). The interleukin-6 (IL-6) family of cytokines which include leukaemia inhibitory factor (LIF), IL-6 and IL-11 play an important role in embryonic implantation. LIF is known to have a role in uterine preparation and embryo attachment (66). LIF deficient mice display implantation failure and when recombinant LIF is supplemented the failure is rescued (66). LIF is expressed in human endometrium with high levels detected in the secretory phase (67) and was reported to be vital for blastocyst implantation (68, 69), as an absence of LIF was associated with reduced fertility and unexplained recurrent pregnancy loss (70). LIF acts as a mediator of signals between immune cells in the decidua and the embryo's trophoblast cells, which are also a source of LIF (71). Furthermore, human chorionic gonadotropin (hCG) secreted by the blastocyst increases the expression of endometrial LIF (72).

IL-6 is another cytokine that is vital for successful pregnancy. In humans, IL-6 is produced by endometrial stromal and epithelial cells in a cyclical manner, with increased levels at the secretory phase and low levels at the proliferative phase indicating a supportive role of implantation (73, 74). Low levels of IL-6 were reported in with women with recurrent pregnancy loss and high levels (in comparison to normal levels) were reported in women with preterm delivery and pre-eclampsia (75). Blastocyst, trophoblast cells and endometrial stromal cells express the receptor for IL-6 (76). Hormones such as estrogen induce the expression of IL-6 (77) while hCG produced by the blastocyst inhibits its expression (78). Therefore it is important to note that factors released by the blastocyst (e.g. hCG) can act in 'two ways' by modulating cytokine release; hCG can increase inflammation by inducing LIF expression and decrease inflammation by inhibiting IL-6 (78) .

In addition to pregnancy promoting pro-inflammatory pathways, anti-inflammatory mediators such as IL-10 and adiponectin are able to prevent excessive inflammation (79). These mediators have the ability to reduce inflammation by inhibiting synthesis of pro-inflammatory cytokines and chemokines (79). Several other cytokines are being investigated for their role in fetal-maternal communication. Complex networks between cytokines and cells have been shown to be critical for promoting embryo implantation and successful pregnancy. This thesis focuses on one particular novel cytokine and its receptor, IL-33 and ST2L respectively, which are members of the IL-1 superfamily.

1.2.4.1 The IL-1 family of cytokines

Cytokines of the IL-1 family play important roles in immune regulation and inflammation in different types of tissues, and their dysregulation is involved with several diseases (80, 81). Members of this family include IL-1α, IL-1β, the IL-1 receptor antagonist (IL-1RA), IL-18, and interleukin-33 (IL-33). IL-1 family members bind specific IL-1 receptors, including IL-1 receptor types I and II (IL-1RI and IL-1RII) for IL-1, the IL-18 receptor (IL-18R) for IL-18 and ST2L for IL-33. Co-association with either the IL-1R accessory protein (IL-1RAcP) or IL-18 receptor accessory protein (IL-18RAcP) regulates functional responses of the cells to the cytokine stimuli (82, 83).

1.2.5 IL-33: a cytokine of the IL-1 family

IL-33 is a member of the Interleukin 1 family of cytokines. When first discovered, the IL-33 gene, *Dvs27*, was predicted to have a nuclear localization signal and was found to encode a nuclear protein expressed at high levels in vasospastic arteries in canines following subarachnoid haemorrhage (84). An orthologue of *Dvs27* was identified as a nuclear protein in human high endothelial venule (HEV) endothelial cells and was therefore given the name of nuclear factor from HEV (NF-HEV) (85). A computational database search of the IL-1R family identified a ligand for the orphan receptor T1/ST2 (ST2), also known as IL-1R-like 1. This ligand was a cytokine and was labelled IL-33 (83). The IL-33 sequence was mapped to human chromosome 9 (9p24.1) and the cDNA encodes 270 amino acids with a protein (full length IL-33) molecular weight of approximately 30 kDa (83).

IL-33 was identified to be an endothelium-derived, chromatin-associated nuclear factor identical to the NF-HEV, and because of its constitutive nuclear expression and IL-1-like cytokine domain, IL-33 was considered to be a dual function protein acting as both a cytokine and a nuclear factor (86, 87).

The identification of the receptor ST2 dates back to 1989 (88, 89). Its role in Th2 immune responses was first shown in 1998 (90). Since the identification of IL-33/ST2 as a ligand receptor pair, their involvement in the initiation of immune responses has been shown in a wide variety of human diseases (91, 92).

1.2.5.1 IL-33: gene and protein expression

Like most other IL-1 family members, IL-33 is synthesized as a full-length form making up a 30 kDa protein that is evolutionary conserved in mammals (91) (Figure 4). Human IL-33 is made out of 270 amino acids with a short chromatin-binding domain at its N terminus (amino acids 40-58); this domain mediates the nuclear localization of IL-33 by binding to the acidic groove of the histone H2A-H2B complex (93). On its C-terminal, human IL-33 contains an IL-1-like cytokine domain that is involved in the cytokine activity of IL-33 (83). The C-terminal domain is sufficient to bind and activate the ST2 receptor; the commercially available recombinant human IL-33 used widely is 18-kDa and represents the IL-33 C-terminal part (91). The structure of IL-33 cytokine domain was determined by using multidimensional heteronuclear nuclear magnetic resonance spectroscopy (94). The structure is characterized by a β-trefoil fold similar to that found in the IL-1α, IL-1β, IL-1R antagonist and IL-18. The N-terminal and C-terminal cytokine domains are evolutionary conserved in mammals; with 54% amino acid sequence homology between human and mouse (91). Splice variants of IL-33 mRNA lacking single or multiple exons (exons 3, 4, and 5) have been reported in human cells (95). Recently detected splice variant of IL-33 lacking exon 3 was found to be functionally active as a cytokine and inactivation of this form occurred via caspase dependant proteolytic cleavage (95, 96).

1.2.5.2 IL-33: posttranslational modification

It was first suggested that the full length IL-33 required caspase-1 cleavage for functional activity (83), but it is now widely accepted that the full length IL-33 is biologically active and caspase-1 cleaves the cytokine domain inactivating IL-33 (97). Apoptotic cells inactivate IL-33 by caspase-1,-3 and -7 cleavage (Figure 4) (97, 98). On the other hand, the neutrophil serine proteases elastase and cathepsin G enhanced by ten-fold the biological activity of IL-33, by producing three truncated IL-33 forms. These induced *in vivo* and *in vitro* pro-inflammatory actions in mice, which suggests that extracellular processing of IL-33 has a role in lung injury in mice (99). Furthermore, *in vitro* activation of a neutrophil protease, proteinase 3 (PR3), generated a truncated form of IL-33 which also possessed higher biological activity than the full length IL-33. However, longer incubation of IL-33 with PR3 resulted in the digestion of two sites in the cytokine domain which inactivated the cytokine (100).

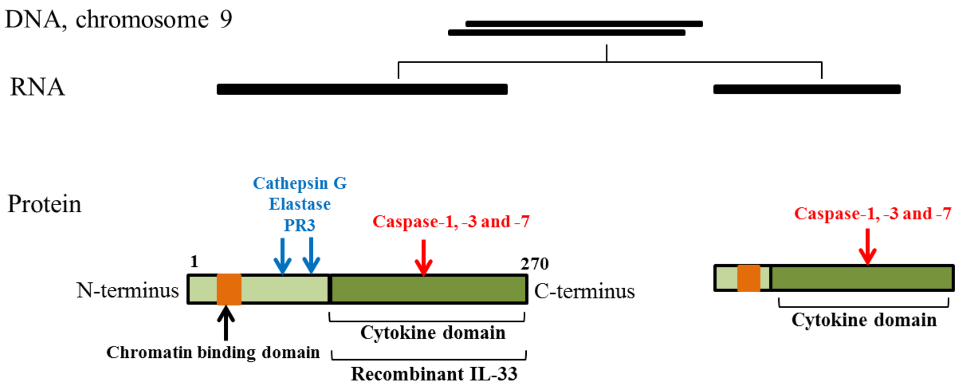


Figure 4. Production and processing of IL-33. The human IL-33 gene is found on chromosome 9 and two alternatively spliced forms of mRNA have been reported. Both contain the active cytokine domain found at the C-terminus and are processed by caspases-1,-3 and -7 into shorter inactive forms. The chromatim binding domain found at the N-terminus is responsible for translocating IL-33 into the nucleus. Cleavage by cathepsin G, elastase and PR3 increases IL-33 activity. The commercially available recombinant IL-33 represents the active cytokine domain. Figure adapted from (91, 101).

1.2.5.3 IL-33: expression, localization and secretion

1. **Expression**

IL-33 mRNA has been detected in a wide range of human and murine tissues and cells (83, 87, 102-109). IL-33 mRNA and protein are constitutively expressed by epithelial and endothelial cells (85, 86, 110, 111). *In vivo* and *in vitro* up-regulation of IL-33 mRNA and protein expression was detected following induction of IL-33 expression in different cell types in response to a wide range of molecules. These include pro-inflammatory cytokines such as IFN-γ and TNF-α, which induce the production of both the full length (30 kDa) and shorter cleaved (20 and 25 kDa) protein forms (112-114), ligands for Toll-like receptors such as IL-3 and IL-4 (115-118) and cross linking of the cell surface receptor FcεR in mast cells (119). Additionally, quiescent endothelial cells constitutively express IL-33 as a result of cell-cell contact that activated Notch signalling (120). A decrease in IL-33 protein levels has been reported following inflammation and angiogenesis in endothelial cells (110). However, inflammation up-regulated IL-33 protein expression in human tissues and murine disease models (121-125).

2. Localization

The full length IL-33, similar to IL-1α, contains a nuclear localization sequence in its N-terminus which is responsible for the translocation of IL-33 protein into the nucleus (85, 86). Consistent with this, IL-33 protein has been detected in the nucleus of different cell types such as endothelial cells, fibroblasts and mast cells (84-86, 110, 111, 121-129). The function of IL-33 as a nuclear factor is not yet clear, as it can interact with the chromatin (93) activating gene transcription (130) or repressing it (131).

3. IL-33 secretion: a 'danger' signal

IL-33, like other members of the IL-1 cytokine family, lacks a specific signal peptide necessary for secretion via the endoplasmic reticulum-Golgi pathway and it is not fully defined how IL-33 is transported from the nucleus to the extracellular space (93). It has been proposed to be secreted by a mechanism involving compartmentalization in vesicles (132); these vesicles could be exocytotic vesicles (133), secretory lysosomes (134) or exosomes (135). However, the extracellular secretion of endogenous active IL-33 does not normally occur unless cells are induced to undergo death by necrosis (Figure 5) (97, 98, 128, 136). Therefore, the most recognised mode of IL-33 secretion is through necrotic cell death mediated release (101). The active full length IL-33 protein is released from cells following mechanical stress (137) or cell necrosis, but not during apoptosis where caspases-1,-3 and -7 cleave and therefore inactivate the cytokine domain (Figure 4) (97, 98). Therefore IL-33 acts as an alarmin or damage-associated molecular pattern (DAMP); endogenous pro-inflammatory factors released from damaged cells, such as IL-1α and high mobility group box 1 (HMGB1), responsible for activating innate and adaptive immune response (91, 111, 138, 139). Apoptosis on the other hand dampens inflammation, compared to necrosis, by inactivating IL-33 by caspase-1, -3, or -7 mediated cleavage (97, 98).

Despite being mainly released during cell necrosis, IL-33 was reported to be released from live cells following exposure to an aeroallergen, the fungus *Alternaria alternate*. In mice, airway exposure to an extract of this fungus induced rapid secretion of IL-33 into bronchoalveolar lavage fluid (BALF) and also induced IL-33 secretion from *in vitro* cultured epithelial cells. The induction of IL-33 secretion from live cells was mediated by an accumulation of ATP and subsequent elevation of intracellular Ca^{2+} level (140). Other allergens, such as ragweed pollen, have also been identified to induce *in vivo* secretion of IL-33 into nasal lavage fluid (129), as well as intratracheal administration of chitin, a biopolymer of *N*-acetylglucosamine found in insects and crustaceans, but the mechanism controlling IL-33 secretion by pollen or chitin is not yet determined (124).

Additionally, mechanical stress induced IL-33 secretion independent of necrosis from human primary skin fibroblast and fibroblast cell line and mice cardiac cells (Figure 5) (137). It was reported that mechanical strain induces ATP release and intracellular Ca^{2+} influx following cell damage (141, 142). The ATP-dependant release of IL-33 is further supported by the detection of secreted IL-33 in supernatant of glial cells and astrocytes (126), and from corneal epithelial cells (116) stimulated with Toll-like receptors (TLR) ligands associated with ATP.

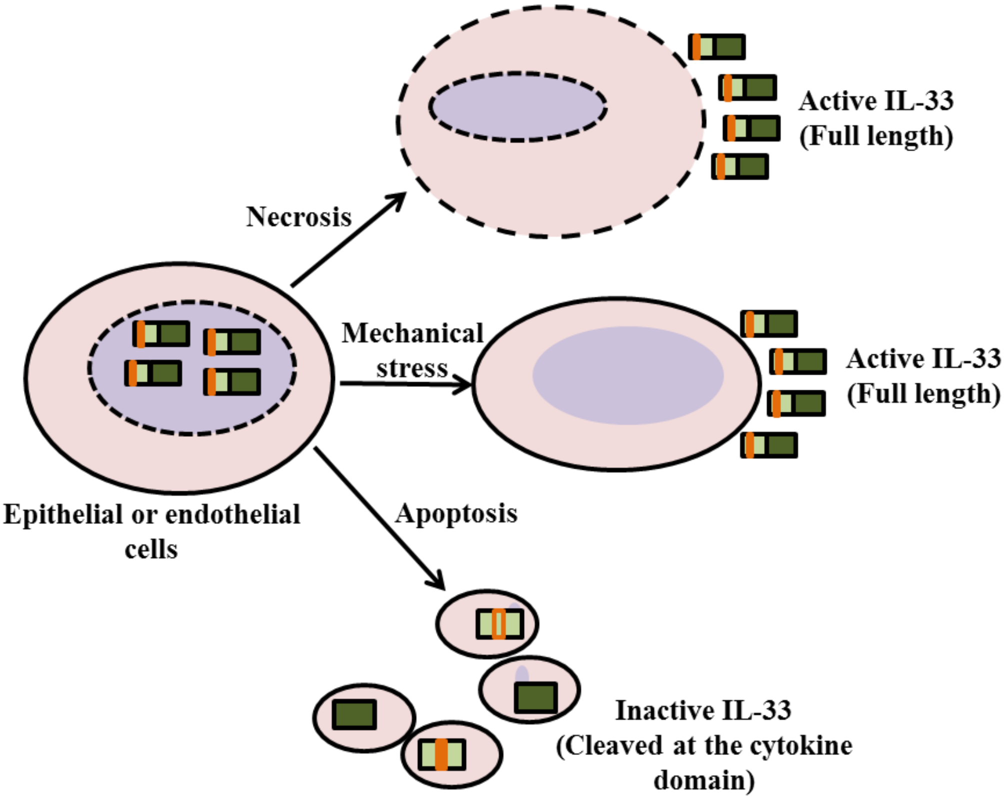


Figure 5. Schematic representation of cellular pathways responsible for IL-33 release. The full length IL-33 is constitutively expressed in the nucleus of endothelial and epithelial cells. It is actively released during cell necrosis and mechanical stress. However, IL-33 is inactivated by caspase-mediated cleavage during apoptosis.

1.2.5.4 IL-33 receptors and intracellular signalling

Direct binding between IL-33 and ST2 was first reported by Schmitz et al (83). The ST2, growth stimulation, gene was first identified in fibroblasts stimulated by serum and described as having a 2.7kb sequence with two initiation promoters coding for a protein with a predicted size of 37.7-38.5 kDa with multiple N-linked glycosylation sites (88, 89). Further analysis revealed that glycosylation resulted in a 50 kDa secreted protein (143-146). The amino acid sequence also predicted a protein of approximately 60 kDa (147). The resulting protein detected at 80 kDa was identified as ST2L and the shift in protein size was caused by glycosylation (109, 148). The ST2 gene is mapped to chromosome 2 (2q12-13) (149) and encodes four splice variants; a membrane-bound form (ST2L), a soluble secreted form (sST2) (109, 146) and two variants with as yet undetermined functions, ST2LV and sST2V (150, 151).

There are at least two splice forms of ST2 translated in humans and mice, sST2 (soluble form) and ST2L (long transmembrane form) (109, 152). ST2L is the functionally activating receptor for IL-33 (Figure 6A). ST2L is a type 1 transmembrane receptor composed of a transmembrane domain, an ectodomain with three linked immunoglobulin-like motifs and a cytoplasmic Toll-IL1R (TIR) domain. The soluble form, sST2, lacks the intracellular and transmembrane domains. sST2 is also able to bind IL-33 as a ‘decoy’ receptor which prevents the association of IL-33 and ST2L and as a result blocks the functional effect of IL-33 (Figure 6. B) (153). The binding of IL-33 to its receptor ST2 is mediated by the IL-1R accessory protein (IL-1RAcP) (Figure 6), also shared by members of the IL-1 family IL-1α and IL-1β. IL-1RAcP increases the binding affinity of IL-33 to ST2 (154-156). Mast cells isolated from bone marrow of IL-1RAcP deficient mice do not respond to IL-33 stimulation indicating the importance of this co-receptor for IL-33 binding to its receptor to exert downstream signalling effects (155). A soluble form of IL-1RAcP, sIL-1RAcP, has been shown to increase the binding affinity of IL-33 to sST2 (156).

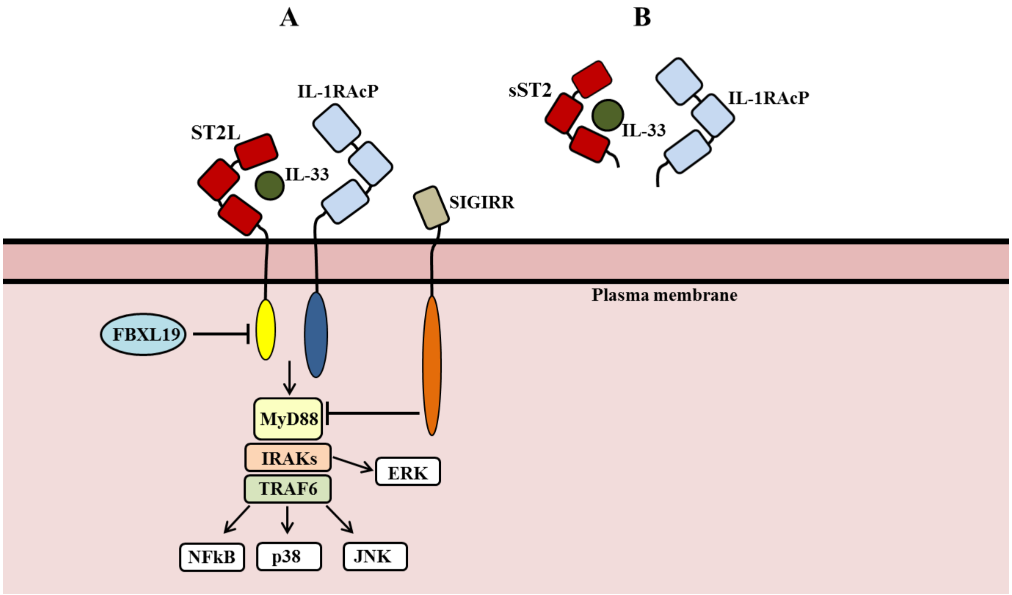


Figure 6. IL-33 and ST2 interaction and signalling. Secreted IL-33 released from endothelial, epithelial or necrotic cells binds to a heterodimeric receptor complex made up of ST2L and IL-1RAcP (A). This binding induces downstream signalling through myeloid differentiation primary response protein (MYD88), IL-1R-associated kinase 1 (IRAK-1) and IRAK-4, which induces the activation of the transcription factor nuclear factor $_k$B (NF$_k$B) and the activation of p38 with JNK to induce gene expression of proinflammatory cytokines and chemokines. The effect of the full length IL-33 can be neutralized with sST2 binding which acts as a decoy receptor for IL-33 (B). Figure adapted from (91).

Both ST2L and IL-1RAcP have a TIR domain which, once activated by IL-33 binding, initiates downstream signalling effects similar to those of other members of the IL-1 cytokine family (Figure 6) (83, 100, 154). Binding of IL-33 to ST2L induces the recruitment of the adaptor molecule myeloid differentiation primary response gene 88 (MyD88). MyD88 recruits IL-1R-associated kinase 1 (IRAK1), IRAK4 and tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) to the signalling complex.

This subsequently leads to the activation of NF$_k$B (nuclear factor kappa b) and mitogen-activated protein (157) kinases. TRAF6 activates NF$_k$B, c-Jun N-terminal kinase and MAP kinase p38, TRAF6 is not required for the activation of extracellular signal regulated kinase (ERK) (158). Fibroblasts derived from mice deficient for Janus Kinase 2 (JAK2) failed to activate NF$_k$B in response to IL-33 (159).

Inhibition of IL-33 signalling is regulated by the single Ig IL-1-related receptor (SIGIRR) by preventing the recruitment of IRAKs and TRAF6 to the MyD88-associated signalling complex, therefore inactivating the NF$_k$B and MAP kinases induced by IL-33/ST2L binding (Figure 6) (160). Mice deficient in SIGIRR showed enhanced Th2 response after stimulation with IL-33 (161) (indicating the importance of this molecule in regulating IL-33 function).

Binding of IL-33 to ST2L induces a negative feedback that regulates ST2L expression (162) so that a reduction in levels of ST2L are detected after IL-33 binding. Activation of ST2L by IL-33 is mediated by glycogen synthase kinase 3β (GSK3B) phosphorylation, which provides the binding site for an Skp-Cullin-F-box ubiquitin ligase 19 (FBXL19) (Figure 6). FBX19 catalyzes poly-ubiquitination of ST2L leading to proteosomal degradation of the protein. FBXL19 induces poly-ubiquitination targeting the cytoplasmic tail of ST2L and is therefore not able to target sST2 degradation. It has been suggested for inflammatory diseases that the IL-33 response could be therapeutically targeted by FBXL19 (162).

1.2.5.5 IL-33: Target cells and responses

The receptor for IL-33, ST2L is expressed on epithelial and endothelial cells of various tissues and on fibroblasts, monocytes and macrophages, T cells, mast cells, eosinophils, basophils, natural killer cells, neutrophils and dendritic cells (reviewed in (163)). These cells are therefore responsible for the functional activity of IL-33. ST2L is abundantly expressed on Th2, but not on Th1 cells (90) and binding of IL-33 to ST2L on Th2 cells induces the production of type 2 cytokines (83). IL-33 also induces the activation of innate type 2 cytokine producing cells; mast cells (164-166), basophils (167-169) and eosinophils (170).

Stimulation of eosinophils with IL-5 alone or in combination with GM-CSF up-regulates ST2L, which when bound to IL-33 induces IL-4 and chemokine production (170). IL-33 also stimulates innate lymphoid cells to produce the type 2 cytokines IL-5, IL-6 and IL-13 (171, 172). These cytokines play an important role in the development of IgE-independent innate-type allergy (reviewed (173)). IL-33 enhances the differentiation of M2 macrophages from bone marrow, characterized by the surface expression of mannose receptor and IL-4Rα, and inducing the production of CCL17 and CCL24 chemokines (174).

IL-33 also induces pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, production from basophils (167-169) and mast cells (166, 175, 176), as well as Th1 cytokines such as interferon-γ (IFN-γ) in human basophils (168, 177). IL-33 was reported to act as a chemoattractant for human Th2 cells (178). Furthermore, IL-33 facilitates the production of chemokines from eosinophils, neutrophils and basophils, recruiting them to sites of inflammation (83, 124, 167, 174). It is becoming clear now that IL-33 exerts a broad array of effector functions on various cell types indicating a complex involvement in wide range of diseases and is reported to be a promising therapeutic target for various human diseases such as asthma, rheumatoid arthritis, liver fibrosis and cardiovascular disease (179). However, there are very few reports on the presence of IL-33 and its receptor ST2 in human placenta or during pregnancy.

Our group was the first to detect IL-33 and ST2 protein expression in tissues of the term placenta (108) and in plasma samples of pregnant women throughout the duration of pregnancy. In another study, ST2 gene expression was reported to be higher in female than in male placentas (180), which was suggested to be a result of the higher immune response that is made by the female to immune stimulation such as infection or immunization (181).

1.2.6 IL-33 and ST2 in normal and pathological pregnancies

Normal pregnancy requires synchronization between the maternal adaptive and innate immune system to enable implantation of the immunologically foreign embryo and successful continuation of pregnancy. As previously discussed, maternal immune regulation in normal pregnancy was suggested to have a bias towards adaptive T-helper (Th)2 activity associated with inhibition of cytotoxic Th1 responses (53) . In pregnancy complications such as recurrent miscarriage and pre-eclampsia, Th1 responses dominate and the Th2 immune response is inhibited (182, 183). It was later found that pre-eclampsia, characterized by hypertension and proteinuria, induced an innate maternal immune response causing an increased systemic inflammatory reaction resulting in maternal endothelial cell dysfunction (57). Low level systemic inflammation was also detected in normal pregnancy characterized by the increase in pro-inflammatory cytokines such as TNF-α (184). Local inflammation has been reported to be important for the process of embryo implantation and the theory of Th1/Th2 balance was considered too simple to describe the maternal immune adaptation taking place during pregnancy (56). The Th2 shift in normal pregnancy was extended to the concept of ‘type-2’ shift that included cytotoxic T cells and NK cells in normal pregnancy (185) and as with Th1, a ‘type-1’ shift would dominate in pre-eclampsia. It then became clear that ST2 (ST2L) is a surface marker of Th2 and Type-2 NK cells (186, 187). Work in our laboratory then used flow cytometry to identify subsets of human ‘type-1’ and ‘type-2’ immune cells in the peripheral blood by their positive expression of surface markers such as IL-18 receptor for type-1 cells and ST2L for type-2 cells (188, 189). An increase in ST2L expression on NK cell population was detected in the blood circulation of normal (type-2) compared to pre-eclamptic (type-1) pregnancies (185).

The characterisation of ST2L as a marker of type-2 responses took place before the identification of its ligand, IL-33. As previously discussed, IL-33 is a potent inducer of type 2 cytokines (IL-4, IL-5 and IL-13) from both T and NK cells (83). But it can also activate type 1 responses in some pro-inflammatory environments leading to the production of cytokines such as IFN-γ (177).

The bias in type-2 response was detected in first trimester and continued to third trimester pregnancy and the dominance in type-2 response was detected as early as 10 days post embryo implantation in peripheral blood mononuclear cells of women undergoing IVF, this however was not detected when embryos failed to implant (190).

As a result of these findings, IL-33 and its receptor ST2 were suggested to play a major role in the immune regulation of pregnancy and pathologies associated with pregnancy (108). Circulating levels of IL-33 and sST2 were analysed in serum samples of normal and pre-eclamptic pregnancies (108). No significant difference in IL-33 levels was found between non-pregnant women compared to those who had normal and pre-eclamptic pregnancies (in all trimesters). The mean values of IL-33 were higher in women with pre-eclampsia than in non-pregnant and normal pregnant women, however the increase was not significant. Therefore IL-33 levels were not changing throughout pregnancy, either in women who had normal pregnancies or those who developed pre-eclampsia (108).

In contrast to IL-33, circulating sST2 levels were very high in pregnancy and comparable to those detected in heart disease (where sST2 is proposed as a biomarker for poor outcome in chronic heart failure and myocardial infarction (191). sST2 was detected in all pregnancy trimesters and its levels were increased as pregnancy progressed, reaching significantly higher levels in the third trimester. Furthermore, sST2 circulating levels were significantly increased in women with pre-eclampsia compared to the levels in normal pregnancy. This increase was detected before the onset of the clinical diagnosis of pre-eclampsia and a further increase was measured after diagnosis (108).

The examination of IL-33 and sST2 circulatory levels during pregnancy, and the findings of higher sST2 levels in pregnant women compared to non-pregnant ones suggested that the placenta is a possible source of the increased levels of sST2. IL-33 expression was also investigated despite the finding of no significant changes in circulating IL-33 throughout pregnancy. It was possible that it might still have a local effect that is not detected systemically. Therefore normal term and pre-eclamptic placentas were examined for IL-33 and ST2 expression and both proteins were detected in all samples examined (108). This is discussed in more detail in the next chapter.

1.3 Thesis aims

The findings described above suggested that ST2, and possibly IL-33, may play a role in maternal immune responses, and in particular inflammation, in pregnancy (108). However, most studies have been carried out at term. Early pregnancy is a crucial time for establishing maternal immune tolerance as that is when trophoblast invasion of the maternal decidua is at its height. The purpose of this thesis therefore was to investigate the expression of IL-33 and ST2 in early pregnancy, with a view to determining their roles in implantation and placentation. This was done by studying their expression in first trimester placental samples, human pre-implantation embryos and endometrial stromal cells, together with commonly used placental cell lines to explore their function. The aims of the thesis are summarized in Figure 7.

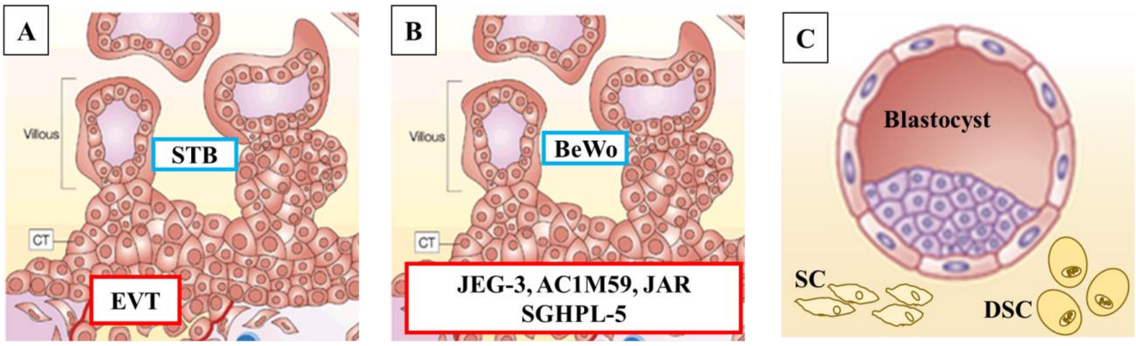


Figure 7. Aims of the thesis

(1) To investigate the expression of IL-33 and ST2 in first trimester placenta with a focus on the different subtypes of trophoblast cells present (Fig 7A).

(2) To use trophoblast cell lines to model the different trophoblast subtypes and further understand the possible role IL-33 and ST2 may play in trophoblast functions (Fig 7B).

(3) To search for IL-33 and ST2 expression before the establishment of pregnancy in human IVF blastocysts and endometrial stromal cells (undecidualized SC and decidualized: DSC) (Fig 7C). Figure, with modification, from (23).

Chapter 2

IL-33 and ST2 expression in the first trimester human placenta

2.1 Introduction

2.1.1 Placental cytokines

Cytokines are major modulators of placental development. They are mainly, but not exclusively, produced by cells of the immune system such as macrophages and NK cells. In the placenta, cytokines are also produced by syncytiotrophoblast and cytotrophoblast cells (192). The expression of cytokines by trophoblast cells in the placenta in relation to ligand and receptor expression is similar to that found in immune cells present at the maternal-fetal interface, such as macrophages (193). Cytokines at the maternal-fetal interface affect various functions of trophoblast cells, and defects in their expression can result in defective EVT invasion into the maternal decidua and subsequent failure of spiral artery remodelling during the early stages of placental development - a key mechanism underlying the development of pre-eclampsia (194). Therefore exploring mechanisms that regulate trophoblast differentiation into an invasive phenotype contributes towards our understanding of pregnancy pathologies and, with focus on pre-eclampsia, may identify key biological molecules that can potentially be used as biomarkers for this disorder (57).

As discussed in Chapter 1, our initial investigation of IL-33 and sST2 showing higher sST2 levels in the circulation of pregnant women compared to non-pregnant women revealed that the placenta is a possible source of the increased levels of sST2 (108) IL-33 expression in the placenta was also investigated, despite there being no significant changes in circulating IL-33 throughout pregnancy as it was possible that IL-33 might still have a local effect that is not detected systemically. Therefore normal term and pre-eclamptic placentas were examined for IL-33 and ST2 expression and both proteins were detected in all samples examined. The full length IL-33 form was detected in all normal and pre-eclamptic placentas, while the cleaved form of the protein (18-20 kDa) was only detected in some (108).

The ST2 protein detected in all normal and pre-eclamptic placentas was approximately 50 kDa in size and densitometric analysis revealed no difference in protein levels between normal and pre-eclamptic placentas. Immunohistochemical analysis revealed localization of IL-33 toward regions in the syncytiotrophoblast and fetal vessels found in the chorionic villi. ST2 localization was also detected in syncytiotrophoblast regions and on cells in the villous stromal core. This localization of IL-33 and ST2 was comparable in normal and pre-eclamptic placental sections.

Further investigation was performed to identify whether the placenta is a possible source of the secreted IL-33 and sST2 detected in pregnancy. Placental perfusion was carried out on normal and pre-eclampsia placentas. In this *in vitro* system, buffer is perfused over the maternal side of the placenta to mimic maternal blood flow. Therefore any molecules released by the syncytiotrophoblast will be found in the perfusate. High levels of sST2 were detected from the perfusates from both normal and pre-eclamptic placentas, while IL-33 was only detected in one perfusate sample from a normal placenta. Increased secretion of sST2 was detected in placental explants treated with IL-1β or TNF-α to model the pro-inflammatory environment of pre-eclampsia. Although hypoxic (1% O_2) conditions, similar to those seen in pre-eclampsia, induced lower sST2 secretion than that detected in normoxic conditions (8% O_2), higher levels of sST2 were secreted from explants that were cultured in hypoxic followed by normoxic conditions to mimic the ischemia/reperfusion seen in this disorder. IL-33 was not detected in any of the explant supernatants (108).

Elevated levels of sST2 were detected in women with very early onset pre-eclampsia (22 weeks) (108) which led us to speculate whether IL-33 and its receptor ST2 might play a role during early placental development.

2.1.2 Aims of this chapter

IL-33 and ST2 are expressed by term placentas, however little is known about these two proteins in the early stages of pregnancy. This chapter therefore aimed to investigate the following questions:

1) Are IL-33 and ST2 expressed at the gene and protein levels in first trimester placental tissues?
2) What are the cellular sources of IL-33 and ST2 in first trimester placenta?
3) Are IL-33 and ST2 secreted by the first trimester placenta?

2.2 Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma Aldrich.

2.2.1 Human placental tissue samples

First trimester placental tissue samples were obtained from women undergoing elective surgical pregnancy termination. The termination of pregnancy procedure was performed by surgical vacuum aspiration. Placental samples from single pregnancies with no known medical condition were used. Gestational age was calculated from the last menstrual period and was confirmed by ultrasound measurement of the fetal crown-rump length. These samples were kindly donated by Dr Judith Cartwright (St George's Hospital and Medical School, University of London). The gestational age of the samples ranged from 6+1 to 13+6 (weeks+days). Placental tissues were washed in Hank's balanced salt solution (HBSS) and were either stored in RNAlater (Qiagen), snap frozen in liquid nitrogen or fixed overnight in 4% paraformaldehyde (PFA) at 4°C and stored in 70% ethanol. Approval for this study was obtained from the Wandsworth Local Research Ethics Committee (Study number 02.6.8).

Term placental tissue samples were obtained from women (aged 23-35) with a normal pregnancy delivered at term either by spontaneous vaginal delivery or by an elective caesarean section. Term placental samples were kindly collected by the INTERBIO-21st research team and by research midwifes in the Nuffield Department of Obstetrics and Gynaecology. Approval for both studies was obtained from the National Research Ethics Service, South Central-Oxford Committee C (Study numbers 08/H0606/139, 09/H0606/10). Placentas were biopsied from the maternal side which is rich in different trophoblast subtypes (Figure 8).

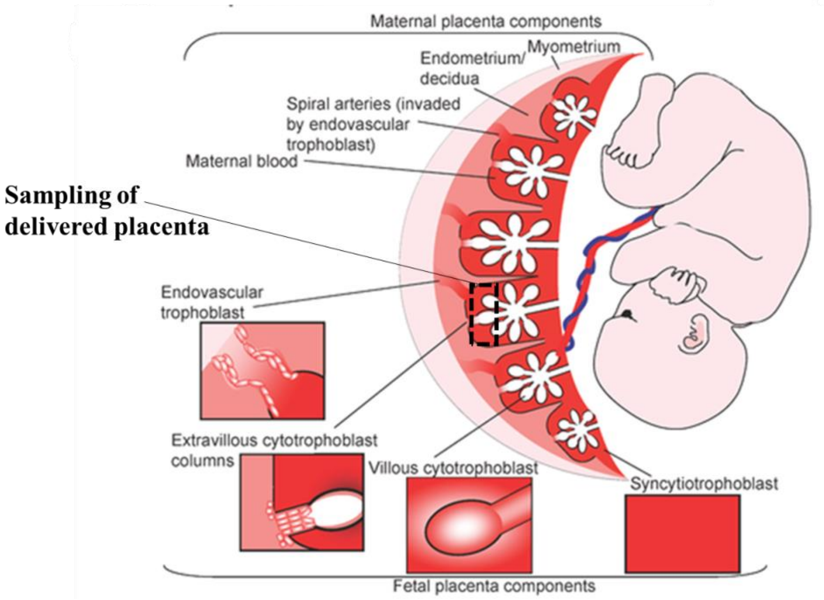


Figure 8. Sampling of placental tissues.
Term placentas were biopsied from the maternal side, rich in trophoblast cell populations (dotted black rectangle). Figure with modification adapted from (195).

2.2.2 First trimester chorionic villous explant culture media

First trimester chorionic villous explant culture media was collected by Dr Alison Wallace (St George's Hospital and Medical School, University of London) according to the protocol described previously (196) and were frozen at -80°C. The culture supernatants were obtained from the chorionic villous explants of placentas at 7+6, 8+0 and 9+3 (weeks+days) of gestational age. Chorionic villous tissue explants were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with no serum and grown on a thin layer of 10% Matrigel for 48 h outgrowth or invasion was assessed by measurement of the areas covered with migrated EVT cells as shown in (Figure 9).

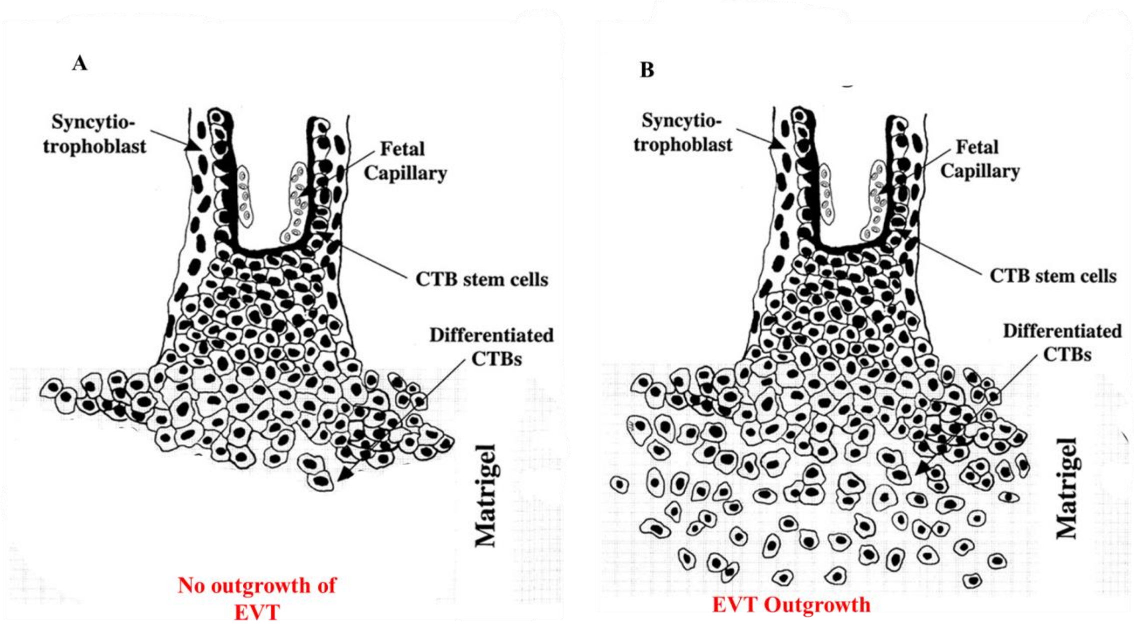


Figure 9. Placental explant culture.
In vitro cultured cytotrophoblast (CTB) cells from the anchoring villi behave as the *in vivo* CTBs when grown on Matrigel. Outgrowth of the villous explant is assessed by taking microscopic images at different time points and assessing the area of invasion commonly referred to as “outgrowth”. The Figure is adapted from (197).

2.2.3 Primary villous trophoblast isolation from term placenta

Human primary villous trophoblast cells were isolated by Miss Alexandra Webster (University of Oxford) according to the protocol previously described (198). The isolation method is based on the protocol originally developed by Kliman et al. (199). Isolation of trophoblast cells involved enzymatic digestion of villous tissues with trypsin and removal of cellular debris by density gradient centrifugation. Further purification was performed by immune-depletion of contaminating cells by passing them through a magnetic column with anti-human HLA class I conjugated magnetic beads, which removes all non-trophoblast cells resulting in a purified trophoblast cell population.

2.2.4 Placental tissue gene expression analysis

2.2.4.1 Purification of RNA from placental tissues

15-20 mg of RNAlater stabilized placental tissue was processed for RNA isolation and on-column DNase digestion by using an RNeasy Mini kit (Qiagen) according to the protocol provided by the manufacturer.

2.2.4.2 RNA quantification

A NanoDrop 1000 (Thermo Scientific) spectrophotometer with NanoDrop 1000 3.7 software was used to quantify the isolated RNA. The purity of the RNA isolated was assessed by the ratio of absorbance at 260 nm and 280 nm (260/280). A ratio of approximately 2.0 was accepted as a pure RNA. A lower ratio indicates the presence of contamination, such as from proteins. In addition, the 260/230 ratio was used as a secondary measure for RNA purity. Values within the range of 2.0-2.2 were deemed to be acceptable, lower values indicate contamination. RNA isolated from all placental samples was within the acceptable range of these two measurements.

2.2.4.3 Reverse transcription and cDNA preparation

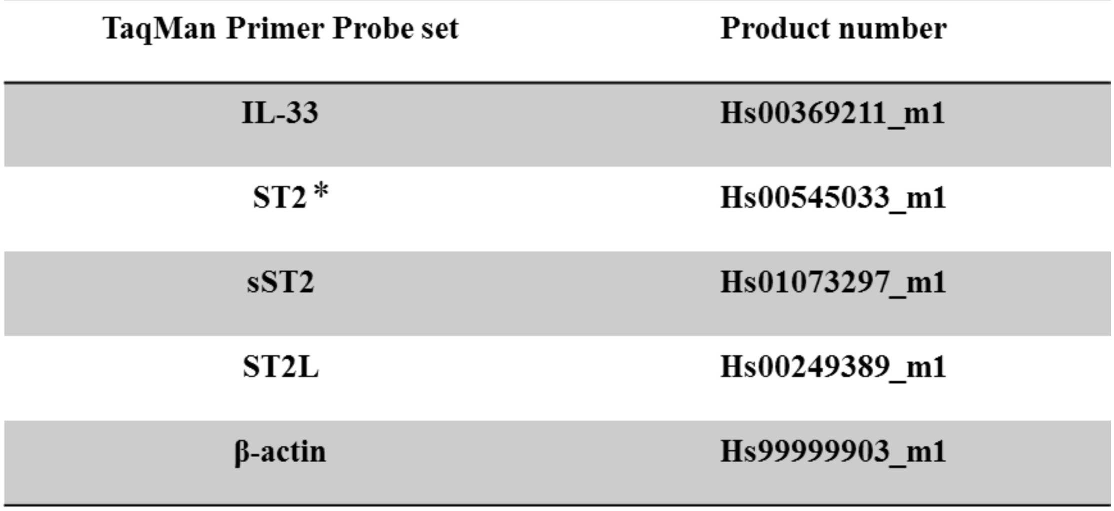
For the preparation of cDNA, the qScript cDNA SuperMix (Quanta Biosciences) was used. A 20 µl final volume reaction mixture was prepared with the following components: 1 µg of total RNA, 4 µl of qScript cDNA SuperMix (5X) suspended in RNase/DNase-free water. The reagents were mixed gently in 0.2 ml micro-tubes (Fisher Scientific) on ice. The thermal cycler (GStorm Cycler) was set for a reaction starting with 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and a final holding temperature at 4°C. Once the cDNA synthesis was complete, the cDNA product was diluted with 30 µl of 10 mM Tris-HCL (pH 8.0) (Ambion, Life Technologies) and stored at -20ºC. A no-RT (no reverse-transcriptase) control was run for each patient sample where the qScript cDNA supermix was replaced with RNase/DNase-free water.

2.2.4.4 Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR analyses were performed by using pre-designed TaqMan Gene Expression Assays (Applied Biosystems). These assays contained a TaqMan probe with a FAM dye on the 5' end and minor groove binder (MGB) non-fluorescenct quencher (NFQ) on the 3' end. The assays used in this section are shown in Table 1.

The qRT-PCR reaction mix contained the following: 10 µl of PerfeCTa qPCR FastMix-Uracil-N-glycosylase (UNG) (2X reaction mix), 1 µl of TaqMan gene expression assay, 5 µl of the cDNA template (containing 1 µg of RNA) and 4 µl of RNase/DNase-free water. To reduce pipetting error, a reaction cocktail for each TaqMan gene assay was prepared with all components except the cDNA template, which was added at the end to each tube. Two negative controls were included every time the qRT-PCR reaction was run. A no-RT negative control for each patient sample, which was prepared during the cDNA synthesis to check for any genomic DNA contamination (contained the no-RT reaction template and all components of the qRT-PCR reaction), and a no cDNA template control (NTC) (with all reaction components except the cDNA) for each gene assay to detect any reagent contamination. The qRT-PCR reaction was performed using a Rotor Gene Q (Qiagen) quantitative real-time PCR system with the following cycling conditions: initial UNG incubation at 50ºC for 2 minutes (the UNG removes uracil from the dUMP in contaminating molecules before the AccuFast Taq polymerase is activated at 95ºC, the AccuFast Taq polymerase is active and the UNG is inactivated – contaminating molecules are hydrolysed, therefore only the target cDNA is amplified), polymerase activation at 95°C for 30 seconds, and 40 cycles with these two steps; denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds.

Table 1. TaqMan Gene Expression Assays used in this study.



TaqMan Primer Probe set	Product number
IL-33	**Hs00369211_m1**
ST2 *	**Hs00545033_m1**
sST2	**Hs01073297_m1**
ST2L	**Hs00249389_m1**
β-actin	**Hs99999903_m1**

*Referred to as total ST2- recognizes the three ST2 isoforms (sST2, ST2L and ST2V).

The relative gene expression of IL-33 and ST2 normalized to the house-keeping gene β-actin was calculated by the formula $2^{\wedge -(\Delta Ct)}$, 2^ (-delta Ct (Ct gene of interest - Ct of housekeeping gene (β-actin))) (200).

2.2.5 Analysis of IL-33 and ST2 protein expression on first trimester placental samples by Western blot

Snap frozen placental tissue samples were lysed with 200-500 µl of the lysis buffer (50 mM Hepes (pH 7.5), 2% sodium dodecyl sulphate (SDS) and 10% glycerol) with Complete Mini-protease inhibitor cocktail tablet (Roche Diagnostics). Lysates were passed through a 21-gauge needle attached to a plastic syringe until a homogenous lysate was achieved and was then stored at -20˚C until required for analysis.

2.2.5.1 Determination of protein concentration using BCA assay

Placental lysate protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce, Thermo Scientific). The absorbance was read using a FLUOstar Optima (BMG Labtech) microplate reader with Optima Plus software.

2.2.5.2 SDS-PAGE and Western blot

15 µg of placental lysate samples was re-suspended in the same volume by adding the appropriate amount of lysis buffer and 3X reducing buffer (1 M Tris-Cl (pH 6.8), 20% SDS, 30% glycerol, 10% β-mercaptoethanol and 0.01% Bromophenol blue) to a 1X concentration. Samples were boiled on a heating block (100ºC) for 10 minutes, cooled to room temperature, and briefly centrifuged before loading onto the gel. Protein lysates were separated on 4-12% NuPAGE Noves Bis-Tris gel using 1X MOPS SDS running buffer (Invitrogen). Gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a semi-dry electroblotting system for 30 minutes set to 20V. Membranes were blocked with 5% Blotto non-fat dry milk (Alpha Diagnostics) in PBS with 0.1% Tween-20 (PBST) for 1 h at room temperature. Membranes were incubated overnight at 4˚C with the primary antibodies mouse IL-33 monoclonal antibody (clone Nessy-1) (1 µg/ml) (Enzo Life Sciences), goat ST2 polyclonal antibody able to identify both the soluble and the membrane bound forms of ST2 (AF523) (1 µg/ml) (R & D Systems) or mouse anti-β-actin specific antibody (0.155 µg/ml) (Abcam).

PVDF membranes were then washed three times (15 minutes each wash) with PBST and incubated for one and half hours with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Membranes were then washed three times (30 minutes each wash). Protein detection was carried out using the Enhanced Chemiluminescence Western blotting substrate detection system (Pierce, Thermo

Scientific). PVDF membranes were exposed to the imaging film (GE Healthcare) which was developed using an XOgraph imaging system. Densitometric analysis of detected bands was carried out using ImageJ software. The detected IL-33 and ST2 bands' intensity were normalized to β-actin levels.

2.2.6 Enzyme-linked immunosorbent assay (ELISA) of IL-33 and ST2 in first trimester chorionic villous explant cultures

First trimester chorionic villous explant culture supernatants were assessed for IL-33 and ST2 secretion using the commercially available IL-33 and ST2 Duoset ELISA kits (R & D Systems) and the Presage sST2 assay (gift from Critical Diagnostics) according to the manufacturer's protocol.

2.2.7 Fluorescence immunohistochemistry staining of formalin fixed and paraffin embedded first trimester placental sections

2.2.7.1 Deparaffinization and antigen retrieval of paraffin embedded placenta tissue slides

Placental tissue paraffin embedding and sectioning was performed by Mr Richard Stillion (University of Oxford). 5 µm-thick sections were deparaffinized using Histo-Clear solution (Fisher Scientific) and rehydrated in a series of alcohol washes (100%, 95%, 90%, 80%, 70%, 50% ethanol) then placed in H_2O, for 1 minute each. Slides were then boiled for 10 minutes in sodium citrate buffer (10 mM, pH 6.0) (VWR) and allowed to cool at room temperature for 30 minutes in the buffer. Slides were then washed in PBS and blocked for 1 h with 10% FCS/PBS.

2.2.7.2 Immunofluorescence staining of placental sections

Sections were stained overnight at 4ºC with 1 µg/ml the following primary antibodies; monoclonal mouse IL-33 antibody (clone Nessy, recognizes human full length and cleaved IL-33) (Enzo Life Sciences), polyclonal rabbit ST2 (HPA007406, recognizes the soluble and the membrane isoforms of ST2) (Sigma-Aldrich), monoclonal mouse cytokeratin-7, monoclonal mouse HLA-G (MEM-G/1 (ab7759), detects soluble and membrane bound HLA-G) (Abcam), polyclonal rabbit cofilin (Santa Cruz Biotechnology) and mouse IgG control isotype or rabbit IgG control isotype (Santa Cruz). Cytokerain-7 and HLA-G staining was performed to identify trophoblast populations in placental sections as reviewed in Figure 10. IgG negative controls were used at the same concentrations. Following overnight incubation with the primary antibodies, sections were washed three times for 10 minutes each and stained with 5 µg/ml of the appropriate secondary antibody; Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) or Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature. Sections were then washed three times for 5 minutes. All antibodies were diluted in 1% FCS/PBS and all washes were carried out with 0.01% Tween in PBS.

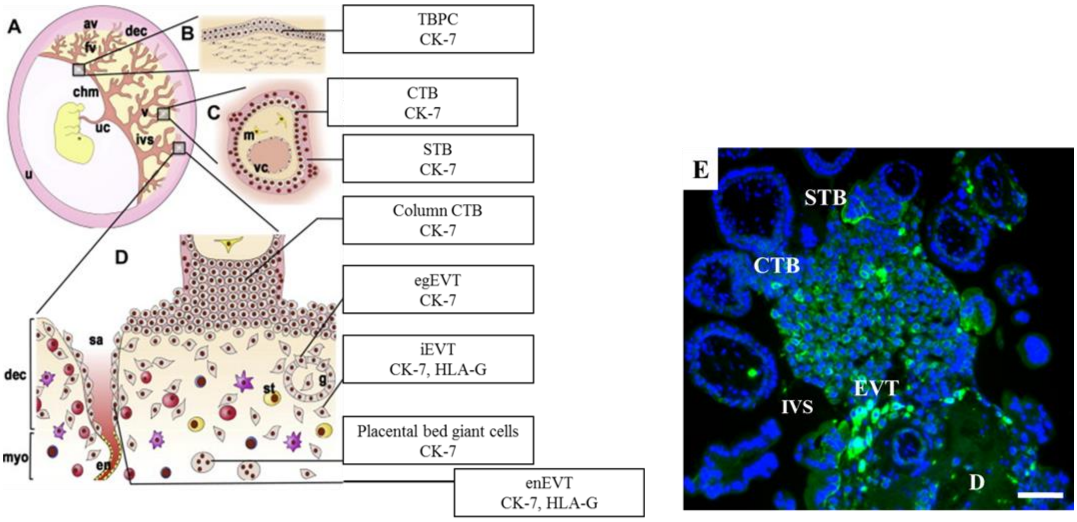


Figure 10. Illustration of human trophoblast differentiation pathways and their localization in the placenta.

(A) The placenta is anchored into the uterine wall (u). The umbilical cord (uc) branch into the placental villi (v) via its arteries and vein. The placental villi are surrounded by maternal blood in the intervillous space (ivs).

(B) The chorionic membrane (chm) is where the trophoblast progenitor cells (TBPC) or stem cells are located. These cells are the source of the different types of trophoblast.

(C) A floating villi (fv). Cytotrophoblast cells (CTB) are located underneath a layer of syncytiotrophoblasts (8). Mesenchymal cells (m) and villous capillaries (vc) are present in the villous core.

(D) Anchoring villous (av) proliferating cytotrophoblasts cells form column CTB. At the distal side of the column, CTBs detach from the villi and migrate towards the decidua (dec).These cells which acquire an invasive phenotype are called extravillous trophoblast (EVT) and they differentiate further to give rise to interstitial trophoblasts (iEVT) that invade the decidua and move deeper into the inner third of the myometrium (myo). Those reaching the myometrium are referred to as placental bed giant cells. Endoglandular trophoblasts (egEVT) invade the endometrial glands (g). Decidual NK cells (dNK) (round red cells), T lymphocytes (small blue cells) and macrophages (purple cells) are present in the decidua. Decidual stromal cells (st).

The primary characteristics and the differentiation relationship of different trophoblast subtypes are briefly presented. All trophoblasts express cytokeratin-7 (CK-7) while HLA-G is expressed in trophoblast subtypes as indicated.

(E) Histological section of a first trimester placenta at 9+3 weeks of gestation displaying the various types of trophoblast populations identified by positive staining with CK-7 (green). Nuclei stained with DAPI (blue). Scale bar =100 µm. sa: spiral artery. en: endothelial cells. IVS: intervillous space. Figure with modifications adapted from (201).

2.2.7.3 Auto-fluorescence minimization using Sudan Black B

A 0.1% (w/v) Sudan Black B (SBB) solution was dissolved in 70% ethanol for 1 h at RT before filtering with 0.2 µm pore size filter. Sections were exposed to SBB for 10 minutes following the secondary antibody staining and excess Sudan Black B was removed by washing the slides three times for 5 minutes with 0.01% Tween in PBS. Nuclei were counter stained with Vectashield mounting medium with DAPI (Vector laboratories). Sections were covered with size N 1º coverslips and the edges were sealed with nail varnish. Fluorescence signal was detected using a Leica DMIRE2 inverted fluorescence microscope and images were taken using a Hamamatsu Orca monochrome camera with Simple PCI software (C Imaging).

2.2.8 Statistical analysis

All data are presented as the mean +/- the standard error of the mean. Data are plotted with Graphpad software. A two sample *t*-test with Welch's correction was used to compare values between two groups. A p-value of less than 0.05 was considered statistically significant.

2.3 Results

Investigations of cytokine expression by the placenta have predominantly utilized techniques for gene expression such as qRT-PCR and protein analysis methods such as Western blotting, immunofluorescence staining and ELISA, all of which were used in this chapter to study the expression of the IL-33 cytokine and its receptor ST2 in first trimester placenta.

2.3.1 IL-33 and ST2 gene expression in placental tissues

Fluorescence-based quantitative real time PCR (qRT-PCR) using TaqMan probes was used to quantify IL-33 and total ST2, sST2 and ST2L mRNA expression in 9 first and 6 third trimester placentas. Placental tissue samples were preserved in RNAlater solution and processed in the same way for RNA isolation, cDNA preparation and the qRT-PCR reaction was carried out as described in the materials and methods section.

2.3.1.1 IL-33 gene expression is detected in first trimester placenta

IL-33 mRNA was detected as early as 6+2 (weeks+days) of gestation and in all of the first trimester placental sections analysed up to 12+3 weeks (Figure 11). The level of expression varied up to ten-fold between placentas.

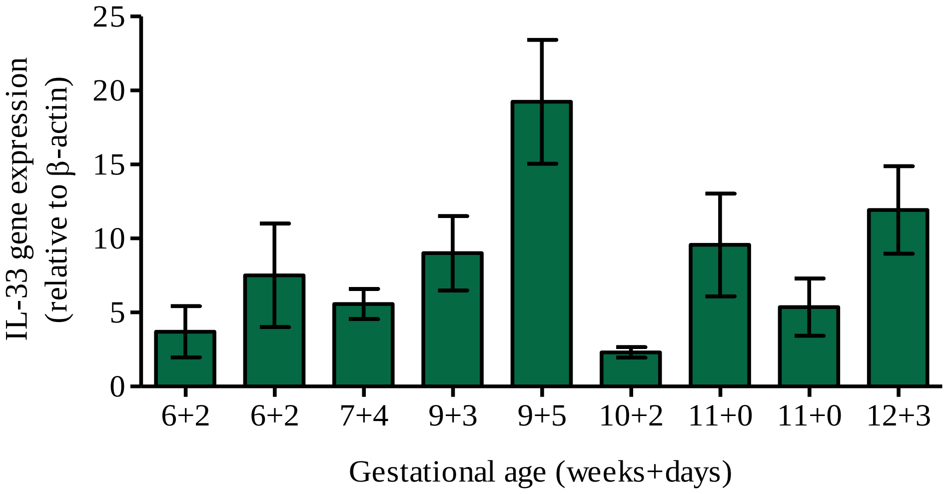


Figure 11. qRT-PCR analysis of IL-33 mRNA in normal first trimester placental tissues. IL-33 mRNA relative expression levels normalized to β-actin. Columns represent the mean of three independent experiments and error bars represent the standard error of the mean.

2.3.1.2 Detection of ST2 mRNA in first trimester placenta

ST2 mRNA expression was detected at all gestational ages in the first trimester placental tissues tested (Figure 12). Three sets of qRT-PCR primers were used which detect either all of the three ST2 isoforms (sST2, ST2L and ST2V) referred to as ‘total ST2’ (Figure 12A), sST2 alone (Figure 12B) and ST2L alone (Figure 12C). The levels of total ST2 mRNA expression were variable across the first trimester and relative levels of sST2 (soluble receptor) and ST2L (membrane receptor) followed a similar pattern from placenta to placenta. ST2L mRNA levels were very low in comparison to the sST2 mRNA levels and were almost undetectable in some samples. However, the sum of the sST2 and ST2L mRNA did not equal the total ST2 levels, indicating that placental tissues may also express the ST2V isoform.

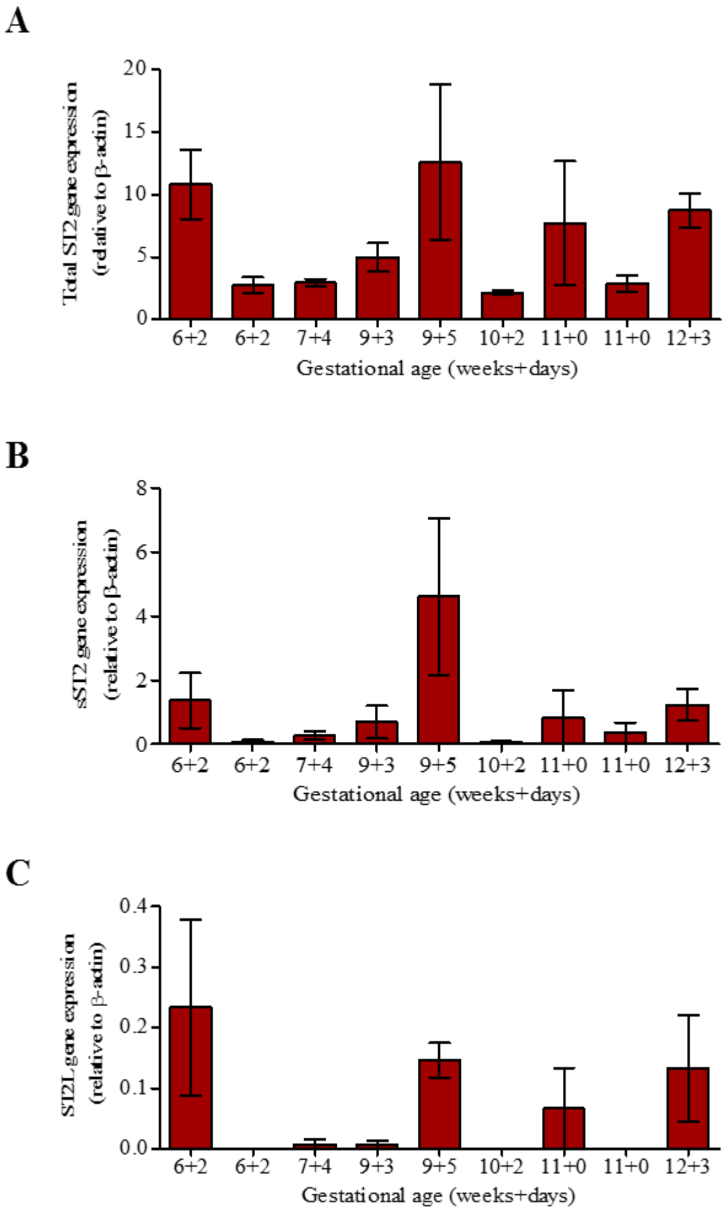


Figure 12. Total ST2 (A), sST2 (B) and ST2L (C) isoforms mRNA expression detected by qRT-PCR in first trimester placenta. mRNA levels are presented as relative expression to the endogenous control β-actin. Columns represent the mean values of three independent experiments and the error bars represent the standard error of the mean.

2.3.1.3 IL-33 mRNA expression in term placental tissue

IL-33 protein has previously been shown to be expressed by term placentas (108), however a comparison of mRNA levels between early and term placentas has not been performed. In this section, analysis of the expression of IL-33 mRNA in term placentas was carried out as described above on first trimester placental tissues.

IL-33 mRNA expression levels were higher in placental tissue samples obtained from Caesarean section (C-section) in comparison to samples obtained after spontaneous vaginal delivery (SVD) (Figure 13). However this increase was not statistically significant. The results presented are for samples from three patients only and may change with a higher number of analysed placentas.

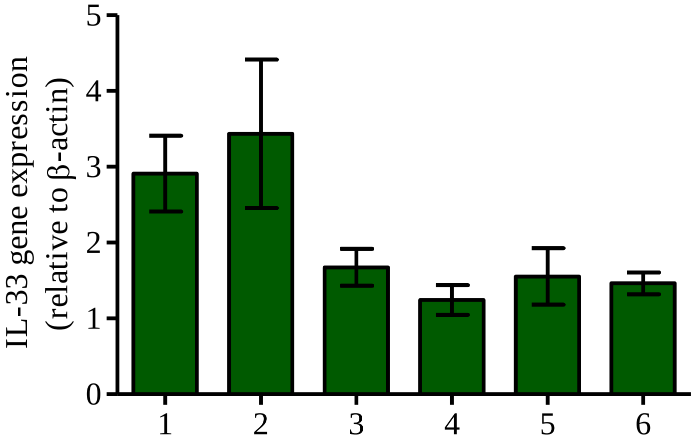


Figure 13. IL-33 mRNA expression in term placenta. IL-33 mRNA expression in placentas delivered either by C-section (1-3) or by SVD (4-6). The relative expression levels measured was normalized to the endogenous control gene β-actin. Columns represent the mean values of three independent experiments and the error bars represent the standard error of the mean.

2.3.1.4 ST2 gene expression in term placenta

Total ST2 (Figure 14A), sST2 (Figure 14B) and ST2L (Figure 14C) mRNA was detected in term placental samples for normal placenta that were delivered by C-section and by SVD. Similar to first trimester placental tissues, sST2 mRNA isoform levels are higher than ST2L therefore the total ST2 mRNA detected mainly consists of the soluble form of ST2. It is most likely that term placenta expresses the variable form of ST2 (i.e. ST2V) as the total ST2 expression was higher than the combined sST2 and ST2L mRNA levels.

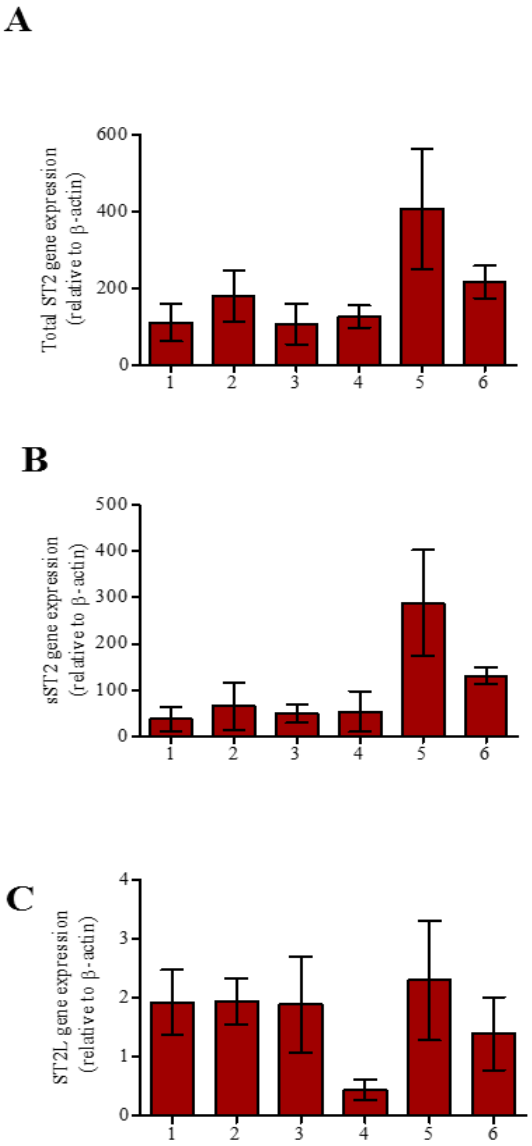


Figure 14. ST2 mRNA expression in term placenta.
Total ST2 (A), sST2 (B) and ST2L (C) mRNA expression measured by qRT-PCR for term placenta delivered by Caesarean section (1-3) and by spontaneous vaginal delivery (4-6). The relative expression measured was normalized to β-actin. Columns represent the mean values of three independent experiments and the error bars represent the standard error of the mean.

2.3.1.5 First trimester versus term placental IL-33 and ST2 gene expression

As stated earlier, there were no published reports on the levels of IL-33 and ST2 transcripts in first trimester placentas and how these levels compare to those found in term placenta. Therefore, we compared the mRNA transcript expression levels in all nine tissue samples from first trimester with the three term placentas delivered by C-section. Spontaneous vaginal delivered placentas were excluded as this mode of labour is associated with inflammation and may therefore affect the expression of various molecules present at the maternal-fetal interface (202) . IL-33 expression in first trimester placental tissues was twice that detected in term placentas (Figure 15A). In contrast, total ST2, sST2 and ST2L gene expression was significantly higher in term placental tissues than that in first trimester ones (Figure 15B, C and D).

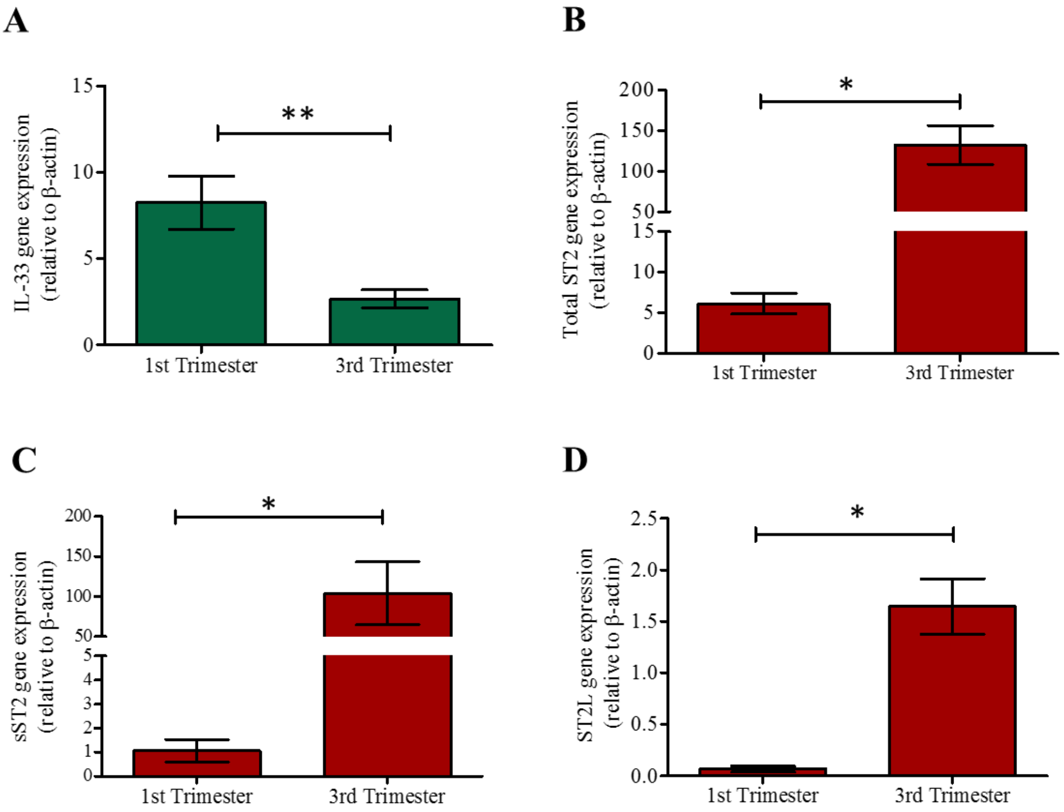


Figure 15. IL-33 and ST2 mRNA expression in first trimester and term placentas. mRNA expression of IL-33 (A), total ST2 (B), sST2 (C) and ST2L (D) measured by qRT-PCR in first and third trimester placental tissues. The expression represents relative mRNA quantities of nine first trimester placental samples and three term placentas. p-values of <0.05 were considered significant *, very significant (p <0.01) ** or highly significant (p<0.001) ***.

2.3.2 Analysis of IL-33 and ST2 protein expression in first trimester and term placentas by Western blot

2.3.2.1 Immunoblotting analysis of IL-33 and ST2

Western blotting was performed on fourteen first trimester placental tissue samples from women undergoing elective surgical pregnancy termination. The gestational age of the samples ranged from 6+2 to 13+6 (weeks+days). IL-33 and ST2 proteins had previously been detected by Western blotting in third trimester placental samples (108), therefore protein lysates from third trimester placenta were used as a positive control.

2.3.2.2 IL-33 expression in first trimester placental samples

The full length protein form of IL-33 was detected in all lysates from first trimester placental samples by the presence of a 30 kDa band which corresponds to the size of the uncleaved form of IL-33 (Figure 16A). Densitometric analysis of the full length IL-33 form of the protein in these samples revealed no changes in protein expression with increasing gestational age (Figure 17).

2.3.2.3 ST2 protein expression in first trimester placenta samples

ST2 expression was detected in all samples from first trimester placenta (Figure 16B). The bands identified at approximately 50 kDa correspond to the size of the soluble form of ST2. Only one sample (11+3) displayed a very faint band at approximately 80 kDa which is the expected size for the ST2L isoform. A band at approximately 100 kDa was detected in the control placenta; it is not known what it may correspond to.

Expression of soluble ST2 normalized to β-actin levels in all placental samples revealed no significant changes in expression with gestational age (Figure 18).

2.3.2.4 IL-33 and ST2 protein expression in first trimester placenta

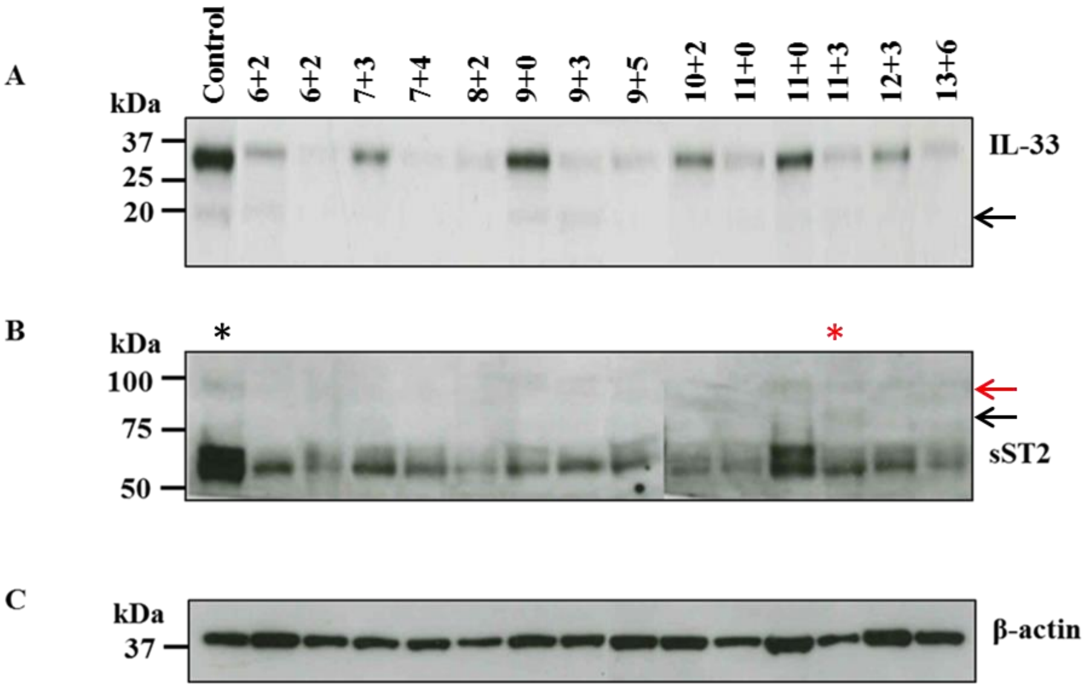


Figure 16. Western blotting analysis of IL-33 and ST2 in first trimester placental tissue lysates. The positive control used was a third trimester placental lysate. Gestational ages are indicated in weeks+days. β-actin (C) was used as a loading control. Black arrow in (A) points to the expected band size of the cleaved form of IL-33. Red Arrow in (B) points to a faint band at an approximately 100 kDa protein size in the control placenta (black asterisk) and the black arrow points to another faint band at approximately 80 kDa in the 11+3 placenta (red asterisk).

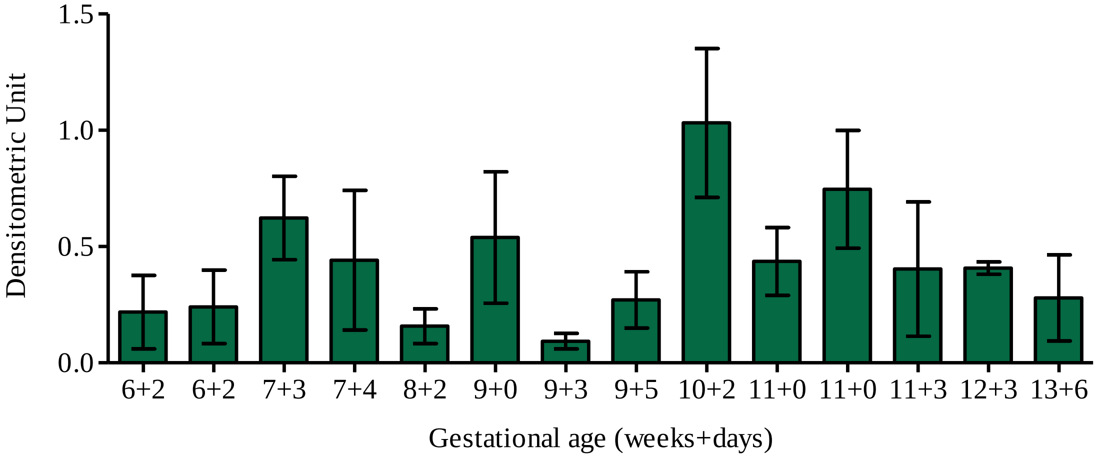


Figure 17. Densitometric analysis of IL-33 protein throughout the different gestational ages of first trimester placentas. Protein level was normalized to β-actin. Columns represent the mean of three independent immunoblots. Error bars represent the standard error of the mean.

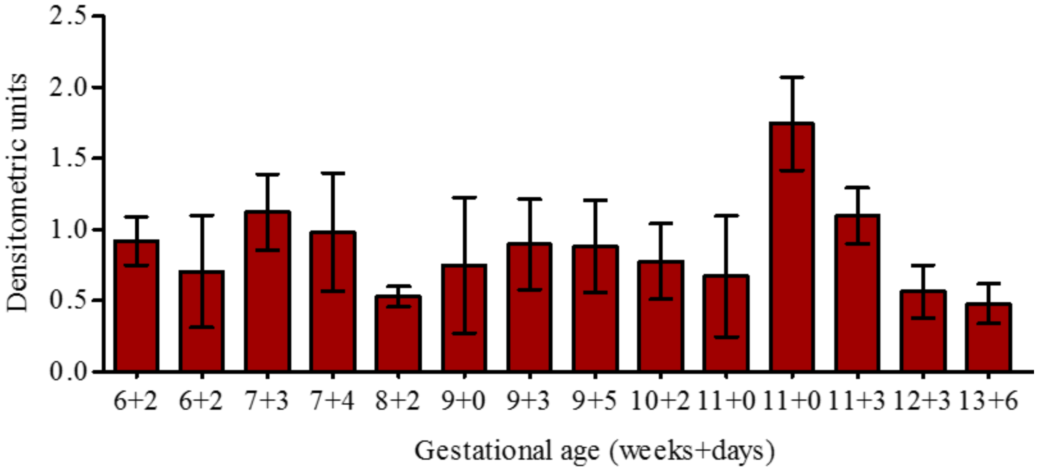


Figure 18. Densitometric analysis of sST2 protein expression in first trimester placental tissue lysates. Protein level was normalized to β-actin. Columns represent the mean of three independent immunoblots. Error bars represent the standard error of the mean.

2.3.2.5 IL-33 and ST2 protein expression in term placenta

IL-33 (Figure 19A) and sST2 (Figure 19B) were both detected in term placental tissue lysates. Densitometric analysis of protein levels (Figure 20) show higher IL-33 protein expression in two samples with lower ST2 protein. Thus samples expressing high ST2 protein levels appeared to have low IL-33 protein.

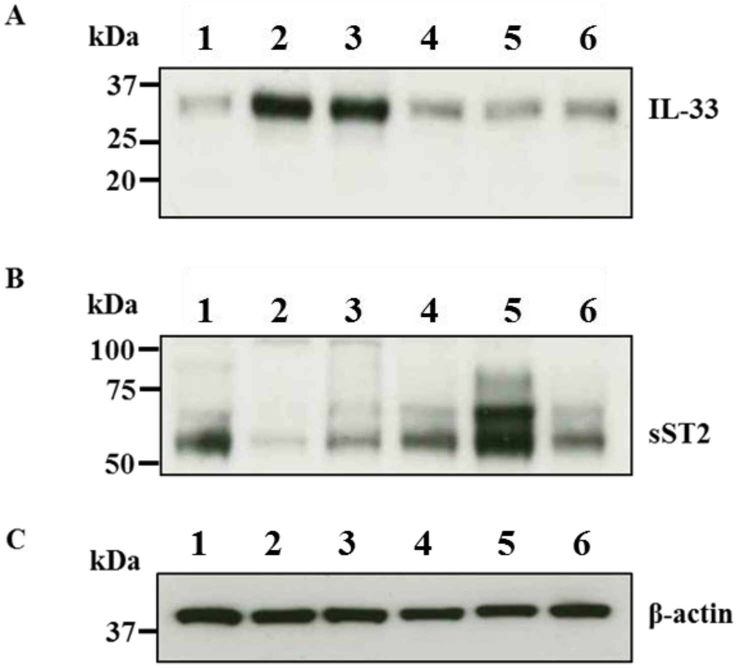


Figure 19. IL-33 (A) and ST2 (B) protein expression measured by Western blotting analysis in term placenta. The blot is representative of three independent experiments. β-actin (C) was used as a loading control. The blot shown is a representative example of three experiments performed. Samples 1-3 are of term placenta delivered by C-section. 4-6 samples are of placentas delivered after spontaneous vaginal delivery.

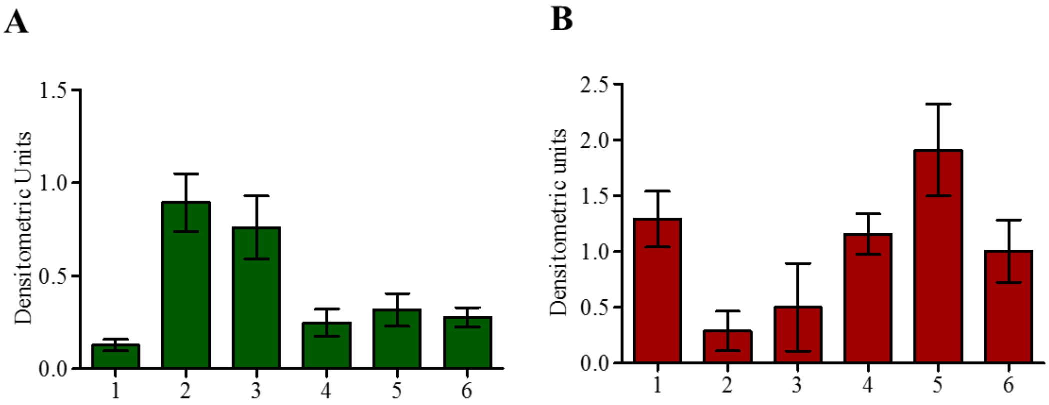


Figure 20. Densitometric analysis of IL-33 and ST2 proteins detected by Western blot in term placentas. IL-33 (A) and sST2 (B). The values are representative of three experiments. Columns represent the mean and error bars the standard error of the mean.

2.3.3 Immunofluorescence analysis of IL-33 and ST2 expression in first trimester and term placental sections

Having determined that IL-33 and ST2 were expressed by the first trimester placenta, the next step was to determine their cellular localization using immunofluorescence staining. Immunohistochemistry for IL-33 and ST2 was performed on formalin-fixed paraffin-embedded first trimester placental sections with gestational ages ranging from 6+1 to 12+1 weeks and on third trimester term placentas.

Trophoblast cells in first trimester and term placenta were identified by an antibody targeted towards isoform 7 of cytokeratin (cytokeratin-7) which is a component of the cell's cytoskeletal structure (203-205). Cytokeratin-7 has been reported to be expressed by all trophoblast subtypes and was therefore used to localize each subtype for the analysis of IL-33 and ST2 localization on placental sections. However, distinction between subtypes such as STBs and the underlying CTBs was not possible with the use of cytokeratin-7 alone. Therefore cofilin-1, a cytoskeletal protein that is essential for actin dynamics and polymerization (206), which has been demonstrated to be down-regulated in STB (Personal communication, Dr Gavin Collett, University of Oxford) was therefore used to distinguish between them.

The different types of trophoblast cells and the subtypes are demonstrated on placental sections from first trimester (Figure 21-23) and term placenta (Figure 24). In first trimester placenta, cytokeratin-7 strongly stains the STB, villous CTB, cell column (or anchoring column) CTB and EVT cells found on different placental sections in all gestations. STB and the underlying villous CTB cells make up the floating villi (Figure 21A) with STB cells in direct contact with the intervillous space filled with maternal blood. Trophoblast cell columns (CC) (Figure 21B) further develop and give rise to the anchoring column (Figure 21C, D and E). CTB cells making up the CC, start to dissociate and loose cell-cell contact, forming the EVT subtype characterised by its invasive capability, which can be seen clearly in Figure 21B. The CTB cells in the proximal region of the anchoring villi (Figure 21E) act as stem cells which continuously supply cells that can either move towards the syncytialization or the invasion pathway. Once out of the anchoring column, EVTs migrate further into the decidua (Figure 21F).

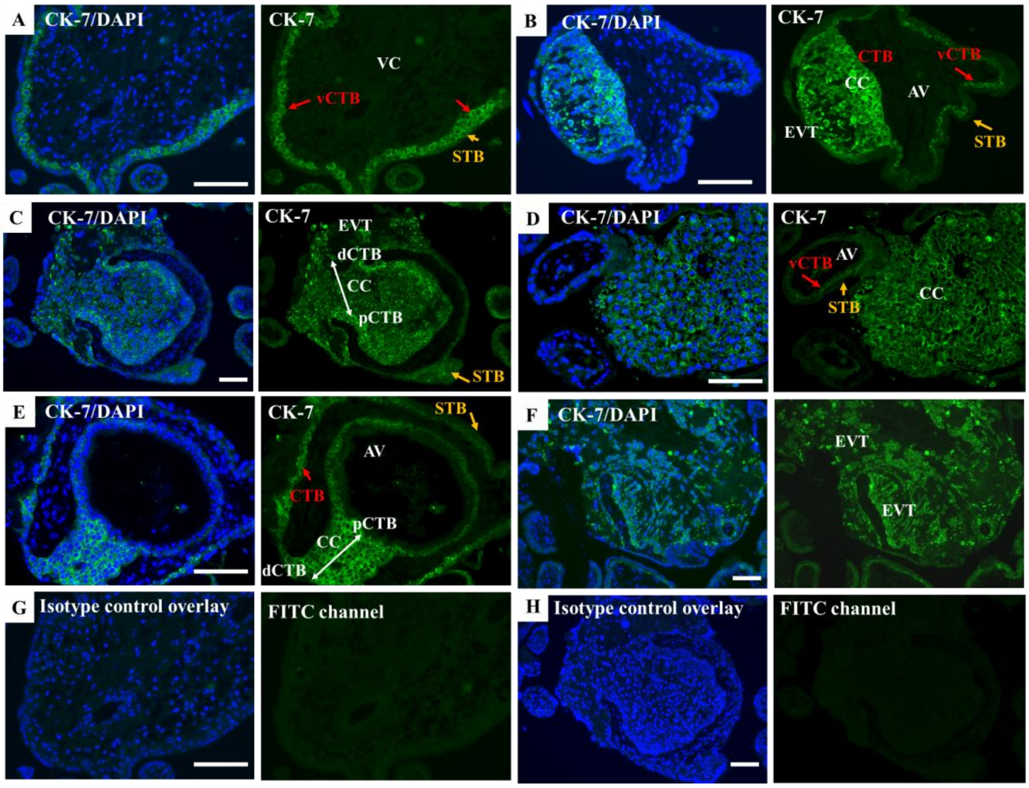


Figure 21. The use of cytokeratin-7 (CK-7) to localize the various types of trophoblast cells in first trimester placental sections. Each section is displayed as an overlay of CK-7 (green), or the isotype control and DAPI for nuclear staining (blue).

(A) Placental floating villi (6+1 weeks of gestation) showing CK-7 staining of the STB (yellow arrow) and the underlying villous CTBs (vCTB) (red arrow). No CK-7 is found in the villous core (VC) as it is largely stromal cells.

(B) A 10+5 week placenta showing CC in the anchoring villi, EVT are seen at the distal end of the CC. These cells are clearly distinct from the CC.

(C) Trophoblast CC from an 11+1 weeks placenta showing proximal (p) and distal (d) CTB.

(D) A placental anchoring villus (AV) at 7+3 weeks of gestation with the vCTB, STB cell layers and trophoblast cell columns (CC) that are attached to the anchoring villi.

(E) A 9+0 weeks anchoring villus with both STB and CTB cells. The arrow on the trophoblast cell column points to the proximal CTB (pCTB) (Stem cells, proliferative) and the distal (dCTB) (EVT, invasive).

(F) EVT in 11+1 weeks placenta are dispersed at different sites in the decidua. (G) and (H) are isotype controls which were taken at the same exposure. Scale bars =100 µm.

The villous core of the floating villi, as explained in the introduction, contains different cell types. The focus of this thesis was aimed at the identification of trophoblast subpopulation expressing either IL-33 or ST2. Therefore, in addition to cytokeratin-7 (CK-7), cofilin-1 was used to stain placental sections and distinguish the STB cells (Figure 22) which are negative for cofilin-1. Also, CK-7 and cofilin-1 were used to distinguish STB from the underlying CTB layer (Figure 23).

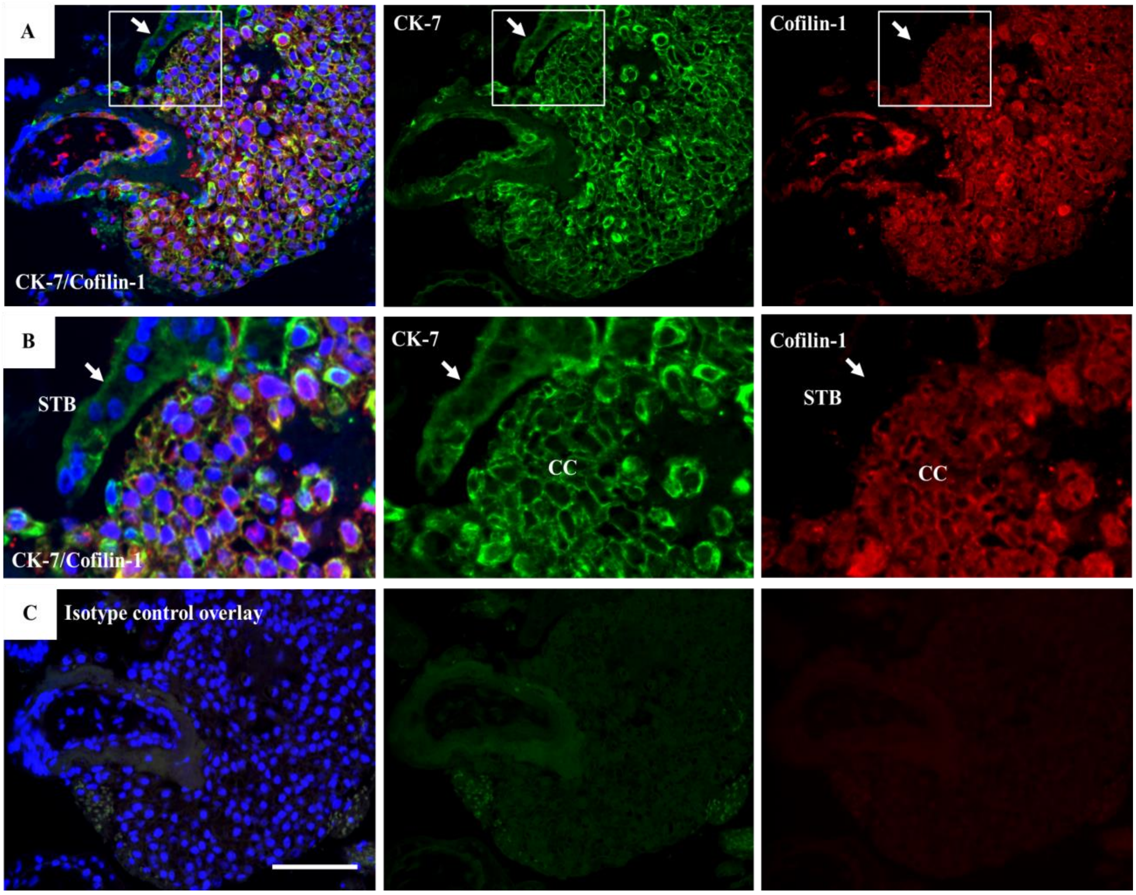


Figure 22. Identification of the different subtypes of trophoblast cells by cytokeratin-7 and cofilin-1 fluorescence staining.
(A) Overlay of CK-7 (green) and cofilin-1 (red) counterstained with DAPI (blue). White square insert is enlarged in (B) for demonstration. White arrow points to STB layer formed with the fusion of approximately 13 cells labelled with CK-7, this region is negative for cofilin-1. (C) Isotype control overlay stained with red and green image channels taken at the same parameters as those in CK-7 and cofilin-1. Gestational age = 7+3. Scale bar =100 µm.

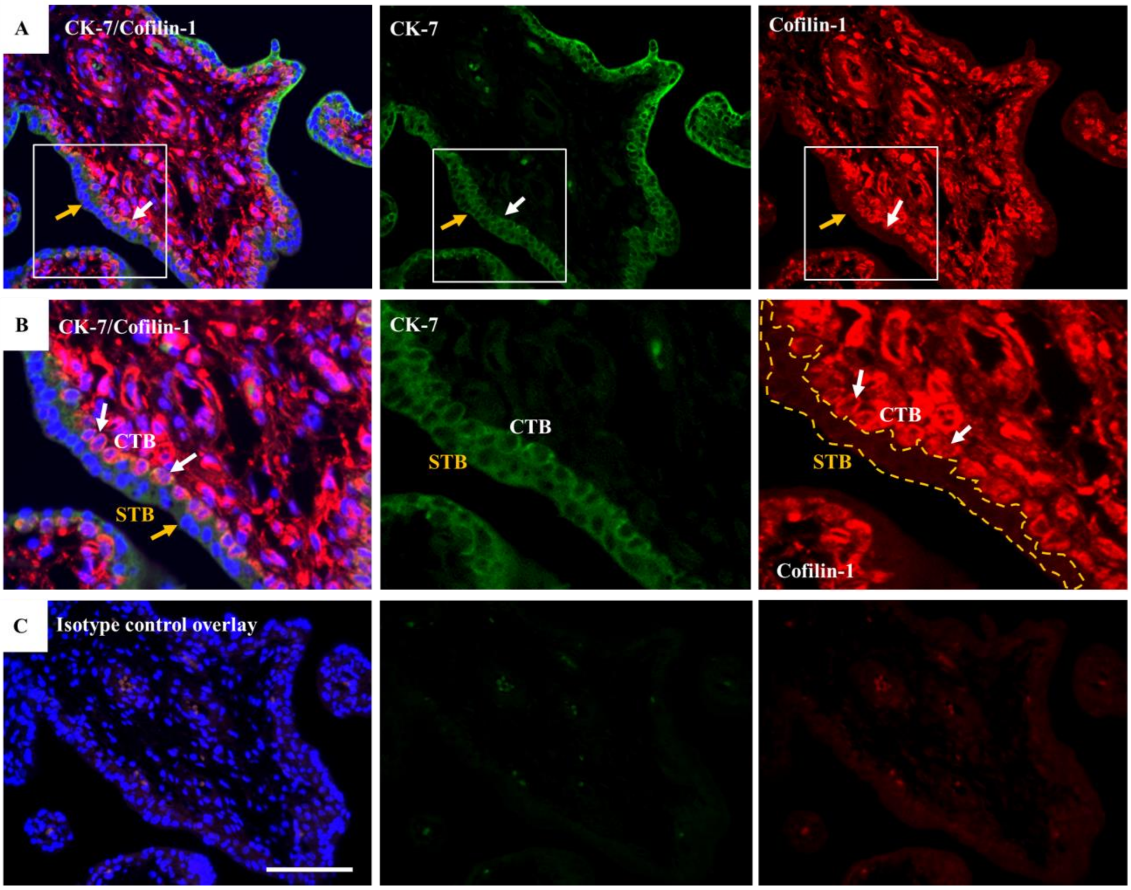


Figure 23. Immunofluorescence localization of the different trophoblast subtypes using cytokerain-7 and cofilin-1. (A) Overlay of CK-7 (green), cofilin-1 (red) and DAPI (blue). (B) Enlarged insert from the white square in A. CTB (while arrow) and STB (yellow arrow and dashed line). (C) Isotype control overlay with images of green and red channels at parameters as those in CK-7 and cofilin-1 overlay. Gestational age = 10+5. Scale bar =100 µm.

Trophoblast cells in term placenta were also identified by using CK-7 (Figure 24). As in first trimester placenta, CK-7 stained different trophoblast subtypes, such as STB and the underlying CTB cells (Figure 24A). Also, EVT cells invading the maternal decidua were strongly positive for CK-7 (Figure 24B).

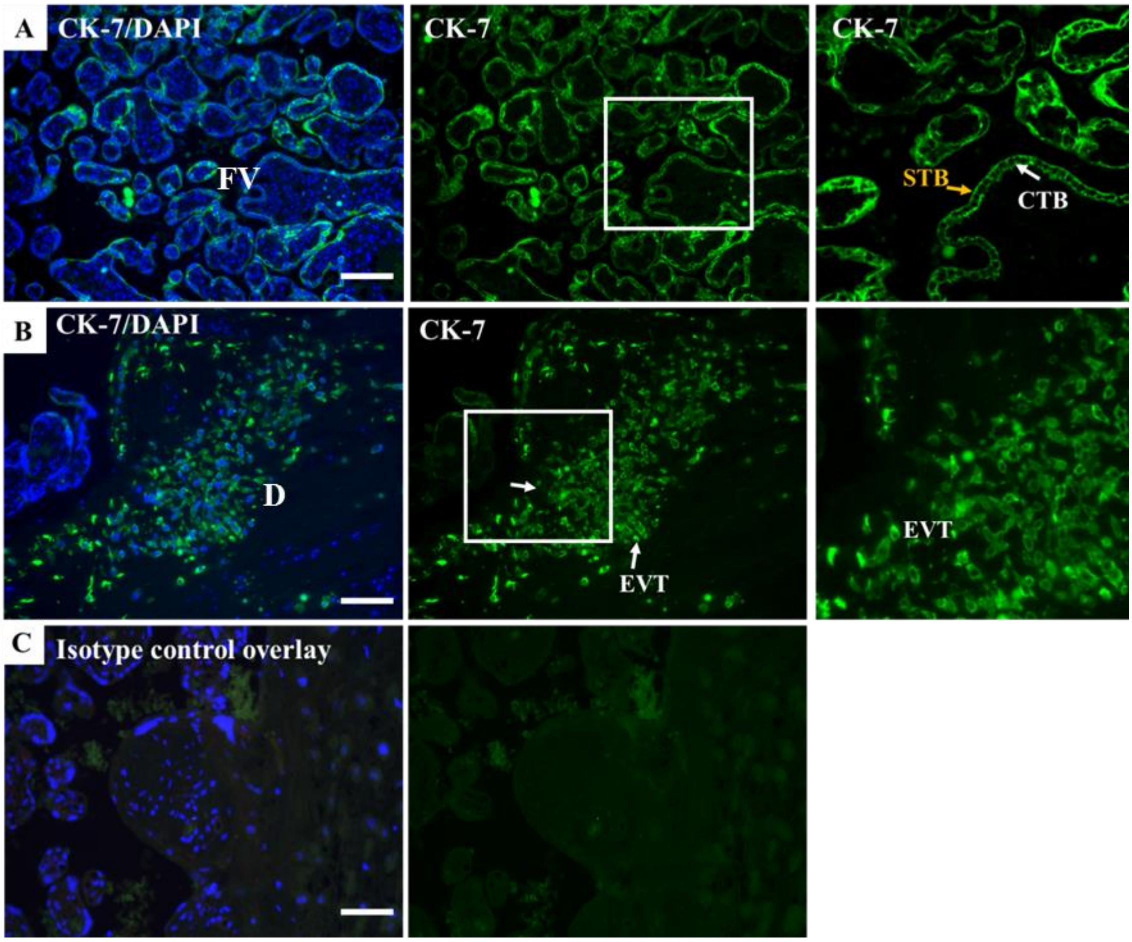


Figure 24. Term placental trophoblast staining with CK-7. (A) Dense population of floating villi stained with CK-7 (green) and DAPI (blue nuclear stain). White square insert is enlarged for demonstration to the right of each panel. STB cells (yellow arrow) and the underlying CTB (white arrow). (B) EVT cells invading maternal decidua -D.
(C) Overlay of the isotype control with the green channel to the right. Scale bars =100 µm.

Cofilin-1 was also used to distinguish STB from the underlying CTB cell population in term placenta (Figure 25). The STB in term placenta is thinner than in the first trimester; they are however negative for cofilin-1 as those in first trimester sections. Figure 24 and Figure 25 displayed a denser population of villous trophoblast in term placenta than that found in first trimester placental sections.

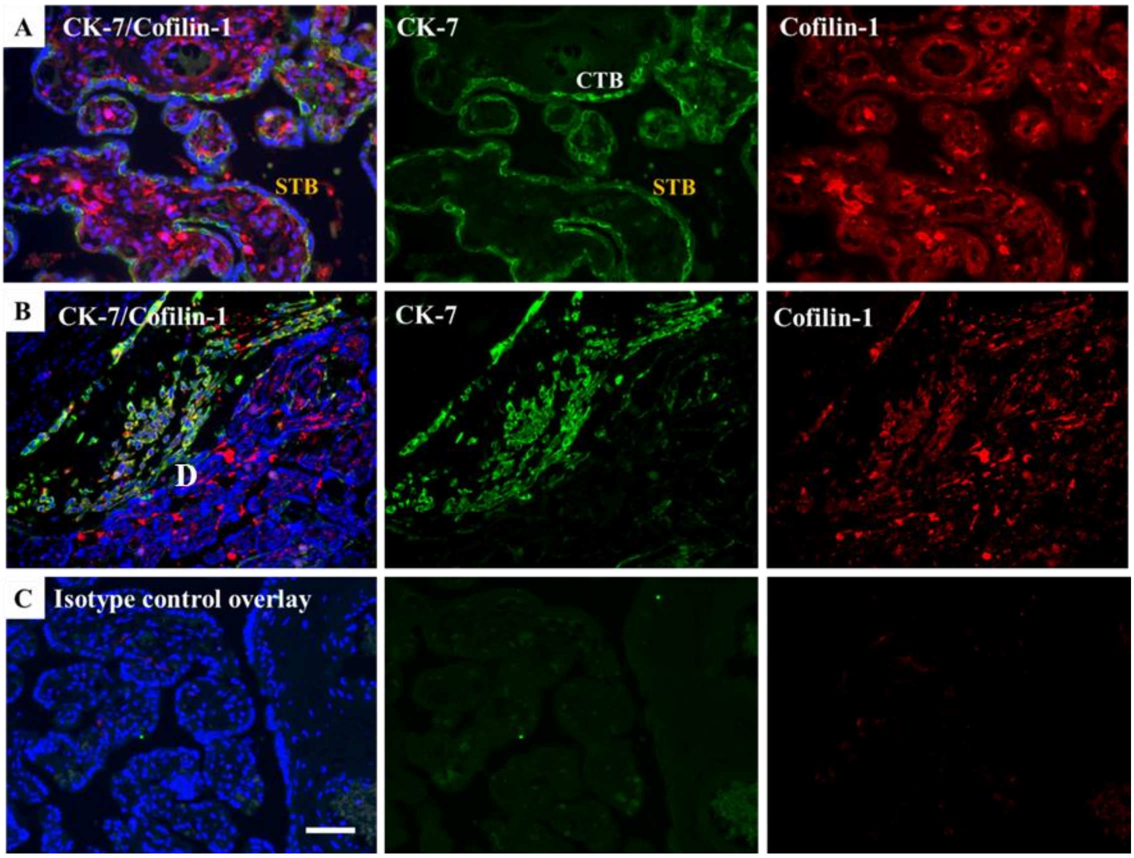


Figure 25. CK-7 and cofilin-1 immunofluorescence staining on term placenta. (A) Overlay of CK-7 (green) and cofilin-1 (red) counterstained with DAPI (blue). (B) EVT cells labelled with CK-7 coming into close contact with decidual cells D.
(C) Isotype control overlay with images of green and red channels at parameters as those in CK-7 and cofilin-1 overlay. Scale bar =100 µm.

2.3.3.1 IL-33 localization in placental sections

The previous section described the normal distribution of trophoblast cells on placental sections. STB, underlying CTB and EVT cells can all be identified with CK-7 antibodies. The positive identification of IL-33 protein expression by Western blotting in some first trimester placentas and all term placentas led to the assumption that trophoblast cells in the placenta were a possible source, as the sampling of tissues was from areas rich in trophoblast cells. Therefore, immunofluorescence staining was carried out to investigate the localization of IL-33 in first trimester and term placentas. These experiments were performed three times and representative images from one experiment are displayed in Figure 26 and Figure 27 for first trimester placenta and in Figure 28 for term placenta.

2.3.3.2 IL-33 localization in first trimester placenta

IL-33 protein localization in first trimester placental sections was detected in cells of the villous stromal core (Figure 26 and Figure 27). These cells are clearly negative for CK-7 indicating that they are of non-trophoblast origin. The localization of IL-33 in these cells was detected in the cytoplasmic and nuclear regions. The sections with positive IL-33 staining corresponded to those positive with Western blot detection at gestational ages of 7+3 and 9+0. Other sections examined were either negative or displayed a high background staining when compared with their isotype control (data not shown).

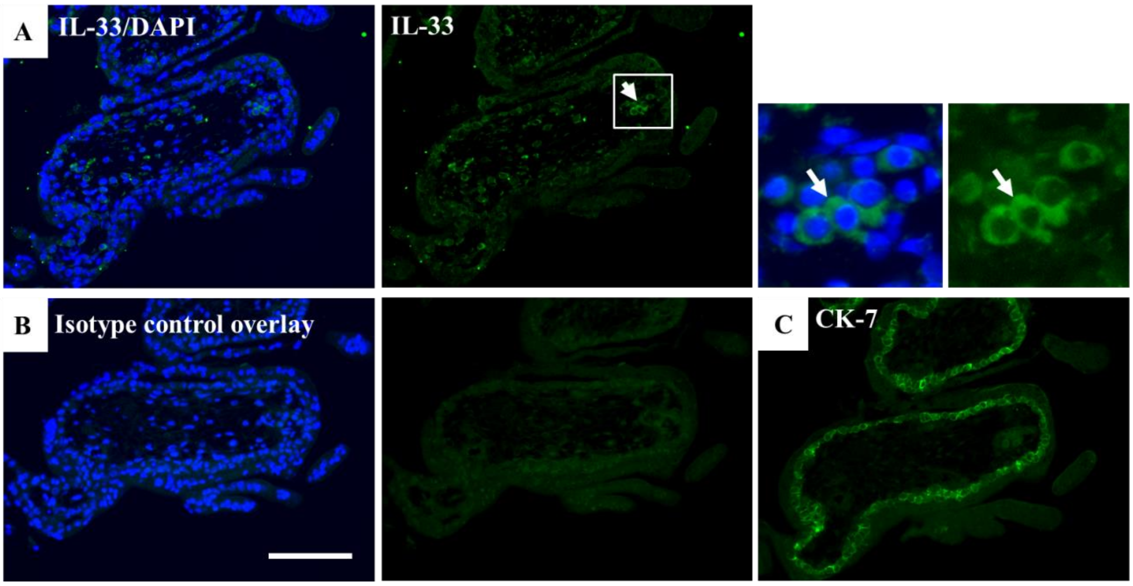


Figure 26. IL-33 localization in first trimester placenta detected by immunofluorescence staining. Placenta at 7+3 weeks of gestation stained for (A) IL-33 (green) and DAPI (blue) placental floating villi. White square insert in the green channel is enlarged in the right panel. (B) Isotype control with the green channel image taken at the same parameters. (C) CK-7 (green) staining was used to locate trophoblast cells. White arrows point to cells positive for IL-33. Scale bar =100 µm.

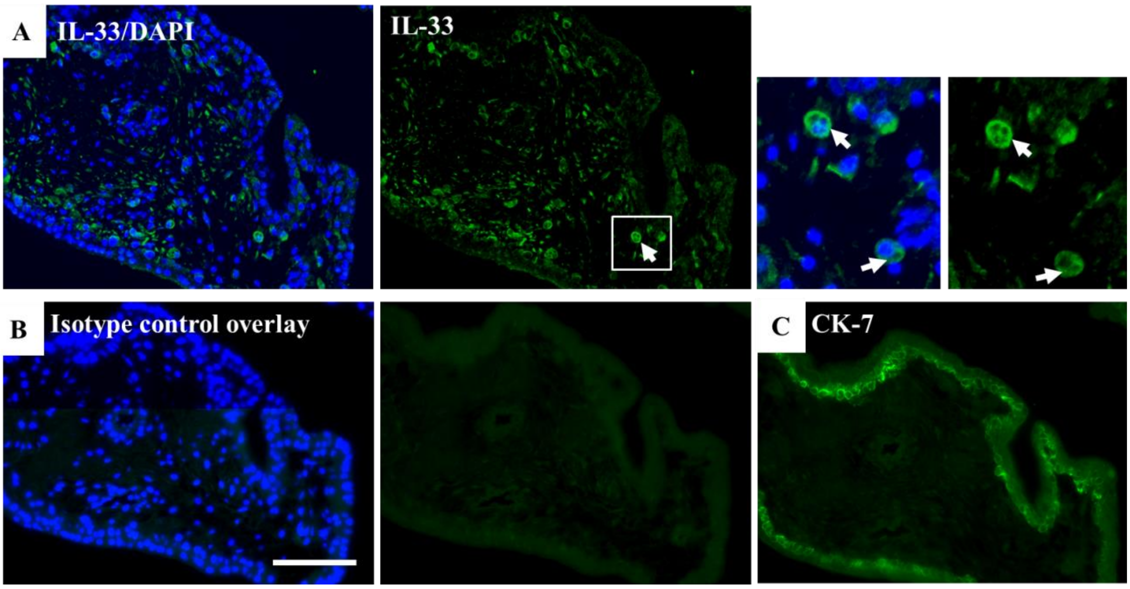


Figure 27. Immunofluorescence staining of IL-33 on first trimester placenta. Placenta at 9 weeks of gestation stained for (A) IL-33 (green), (B) isotype control and (C) CK-7 (green). Nuclei were counter stained with DAPI (blue). Upper panel white square insert is enlarged to the right. White arrows point to cells positive for IL-33.
Scale bars = 100 µm.

2.3.3.3 IL-33 localization in term placenta

IL-33 protein signal was detected near the fetal capillaries of term placenta (Figure 28). There was a weaker signal distributed throughout the stromal core of the floating villi. The signal was stronger at the fetal capillaries than anywhere else in term placentas.

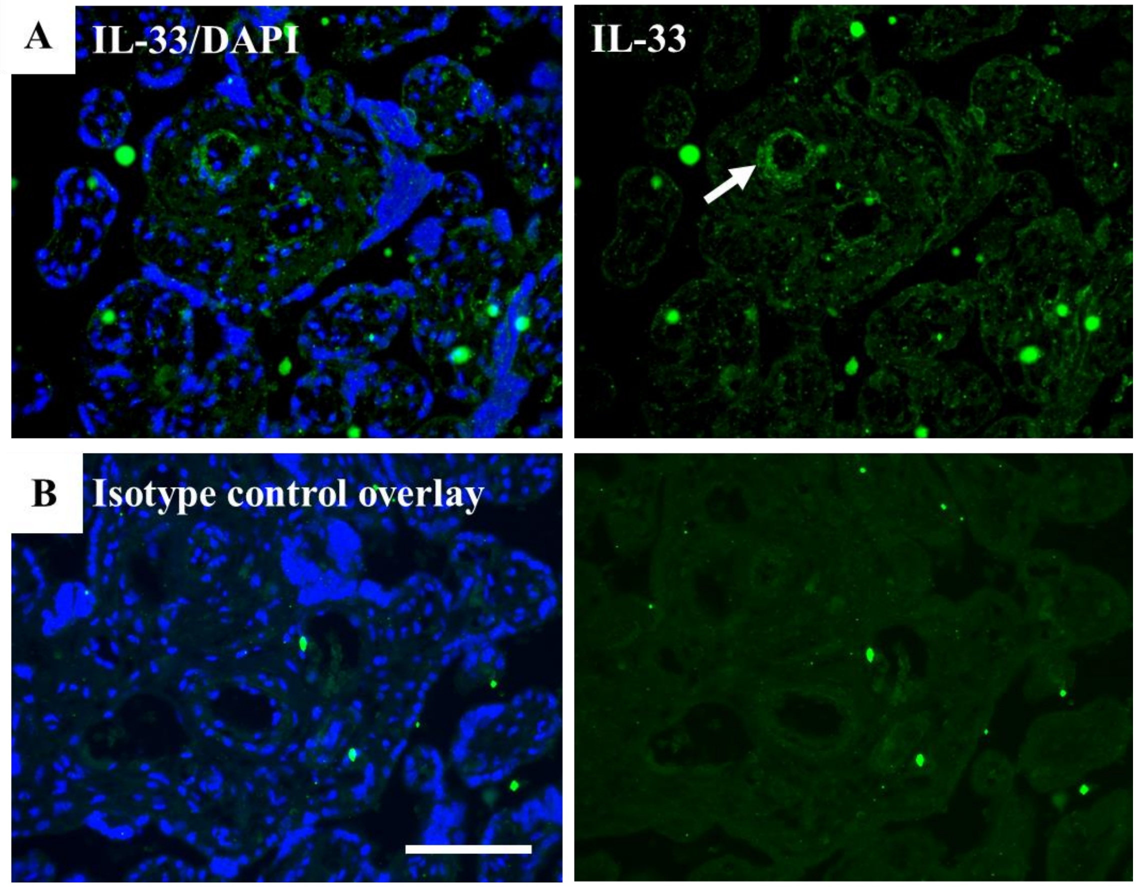


Figure 28. IL-33 is found in fetal capillaries and stromal core of term placenta. (A) Overlay of IL-33 (green) and DAPI (blue). White arrow points to the fetal capillary (B) Isotype control overlay with the green image channel to the right taken at the same parameters as that for IL-33. Scale bar =100 µm.

2.3.3.4 ST2 localization in placental sections

Previous results showed that first trimester placenta expressed ST2 at the gene and protein levels. In this section, immunofluorescence staining was performed to analyse the cellular localization of this molecule in first trimester placenta and compare it to that in term placenta. ST2 was analysed in eleven first trimester placentas of varying gestational age and three term placentas obtained after elective C-section of normal pregnancies.

The immunofluorescence staining was carried out three times and representative images from one experiment are displayed in the following section. During initial investigations, it was noticed that ST2 was present on both the apical and the basolateral surface of the syncytiotrophoblast. Cytokeratin-7 was used as a marker for all trophoblast cells present in first trimester and term placentas. It was however of interest to distinguish between the STB and the underlying CTBs to investigate further the localization of ST2 around these cells. Therefore, cofilin-1 staining was used to further distinguish the localization pattern of ST2 at the STB and underlying CTB barrier. The following section reports the localization of ST2 at the apical and basolateral surface of STB and the underlying CTB cells in first trimester placental sections.

2.3.3.5 ST2 localization in STB and CTB across different gestational ages in first trimester pregnancy

ST2 was detected both at the apical surface of the syncytiotrophoblast and the basolateral side where it comes into contact with the underlying cytotrophoblast cells (Figure 29). This pattern of localization was seen on floating villi of different gestations of first trimester placentas (Figure 29A-F). However, this localization was not uniform as there were some areas where ST2 was expressed only on the apical side but not on the underlying CTB side and other areas with the opposite localization where ST2 was found on CTB that were in contact with STB, but not on the apical surface of the STB layer. Furthermore, the use of cofilin-1 staining allowed further characterization of ST2 localization in first trimester placental sections. In addition to the localization on apical and basolateral surface of STB layer, ST2 was also found, albeit with a weaker signal than that on trophoblast cells, in stromal core cells of floating villi (Figure 29A and C). Most of these cells were of non-trophoblast origins as they were not positive for CK-7 staining (data not shown). Also a weaker ST2 signal was evenly distributed in stromal core in some, but not all regions, of the same placenta (Figure 29C and Figure 29E).

Further investigation of ST2 localization patterns in first trimester placental sections detected ST2 in trophoblast cells forming anchoring columns. The distribution of ST2 across the different gestational ages (six to 12 weeks of gestation) is shown in Figure 30-Figure 35. ST2 was detected as early as six weeks of gestation (Figure 30). It was found on the apical (facing the intervillous space) and the basolateral (in contact with the underlying CTBs), and this is clearly shown in Figure 30A, Figure 31A and Figure 33B. In Figure 30A, ST2 was also detected on cells in the stromal core of the floating villi. Furthermore, in the same image, it was clear that ST2 was localized only to the upper surface of CTB cells that were in contact with the STB layer. Localization on the side of CTB cells in contact with STB was clearly seen in sections from seven weeks (Figure 31A), nine weeks (Figure 32A) and 10 weeks (Figure 33B) of gestation.

ST2 was detected on trophoblast cells forming the anchoring columns of first trimester placentas. This localization pattern was seen at seven weeks (Figure 31B), nine weeks (Figure 32B), 10 weeks (Figure 33A) and 11 weeks (Figure 34A) of gestation. A stronger ST2 signal was detected in the area of the cell columns close to the villous core

(Figure 32B, Figure 33A and Figure 34). Furthermore, in the regions close to the cell columns, ST2 was commonly found on CTB on both the side in contact with the STB and that exposed to the villous core. The isotype negative controls for placentas between gestational ages of 6+1 to 12+1 showed no background staining in all sections examined (Figure 36).

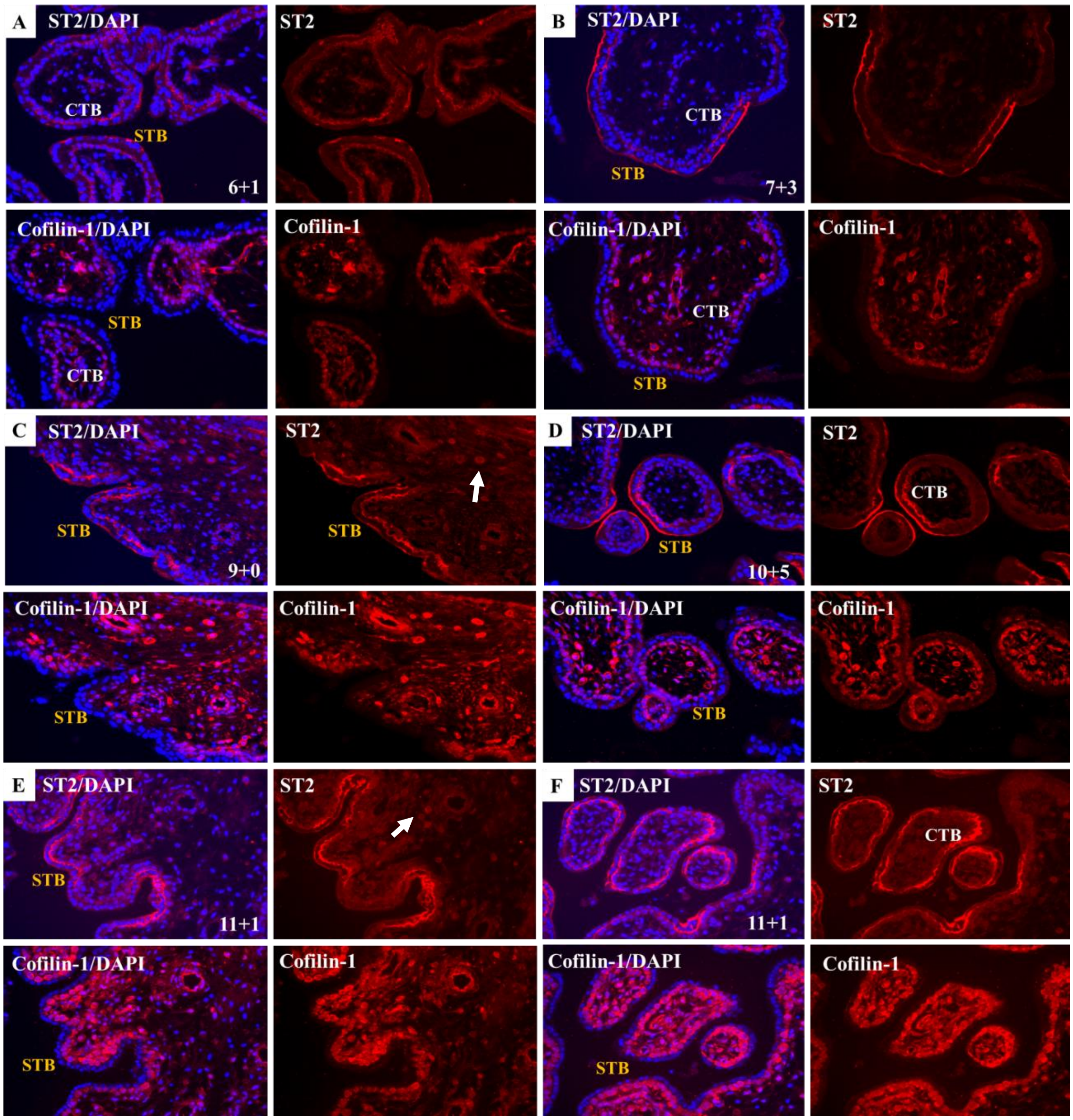


Figure 29. ST2 was detected at the apical and basolateral surfaces of the STB layer in first trimester placenta. Placental gestational ages are indicated at the lower right side of each ST2 (red)/DAPI (blue) overlay. Cofilin-1 (red) and DAPI (blue) overlays are placed to bottom right side of each panel. Cofilin-1 was used to distinguish STB layer from the underlying CTB layer. All images represent floating villi where this pattern of localization is commonly seen across the different placental sections. Scale bar = 100 µm.

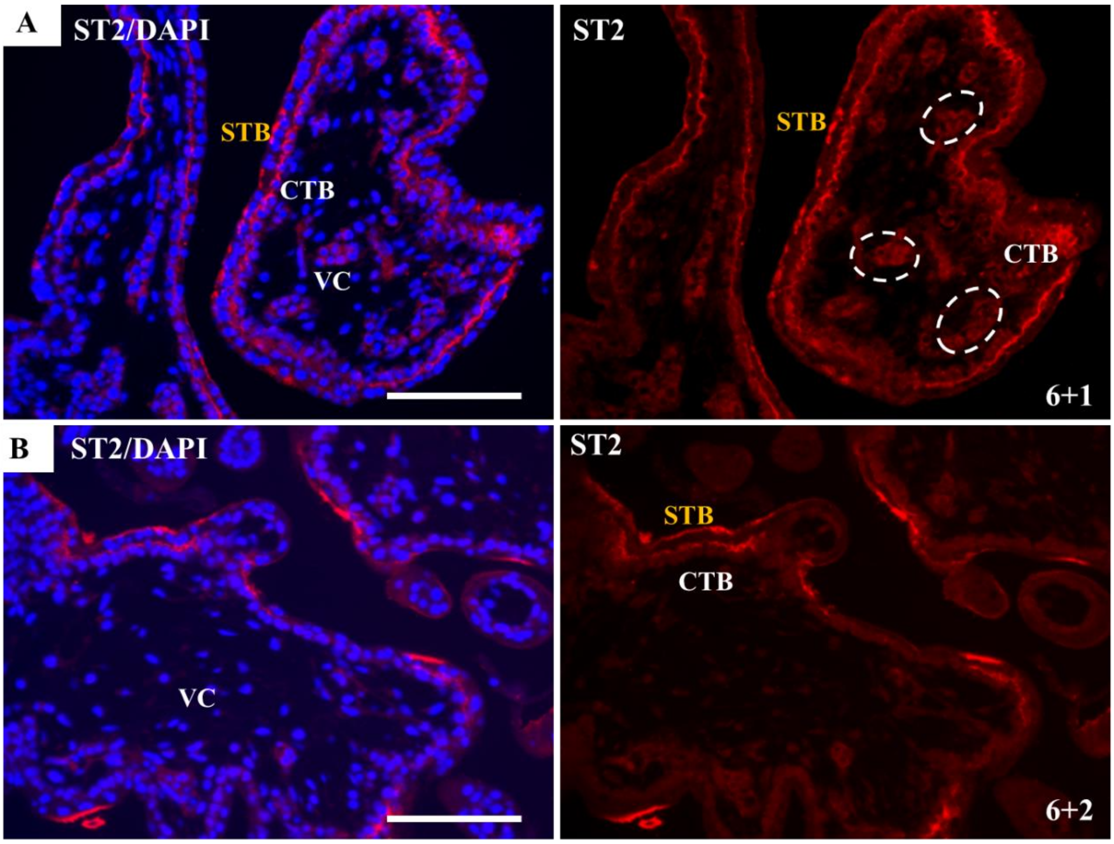


Figure 30. Localization of ST2 at 6+2 weeks of gestation. Overlay of ST2 (red) and DAPI (blue) at 6+1 (A) and 6+2 (B) weeks of gestation. To the right of both panels, is the red channel for ST2 only. ST2 was detected in both patient samples on STB (yellow) and the upper side of the CTB (white) in contact with the STB of the floating villi. Cells in the villous core (VC) of (A) also stained for ST2 (round white circles). Scale bars =100 µm.

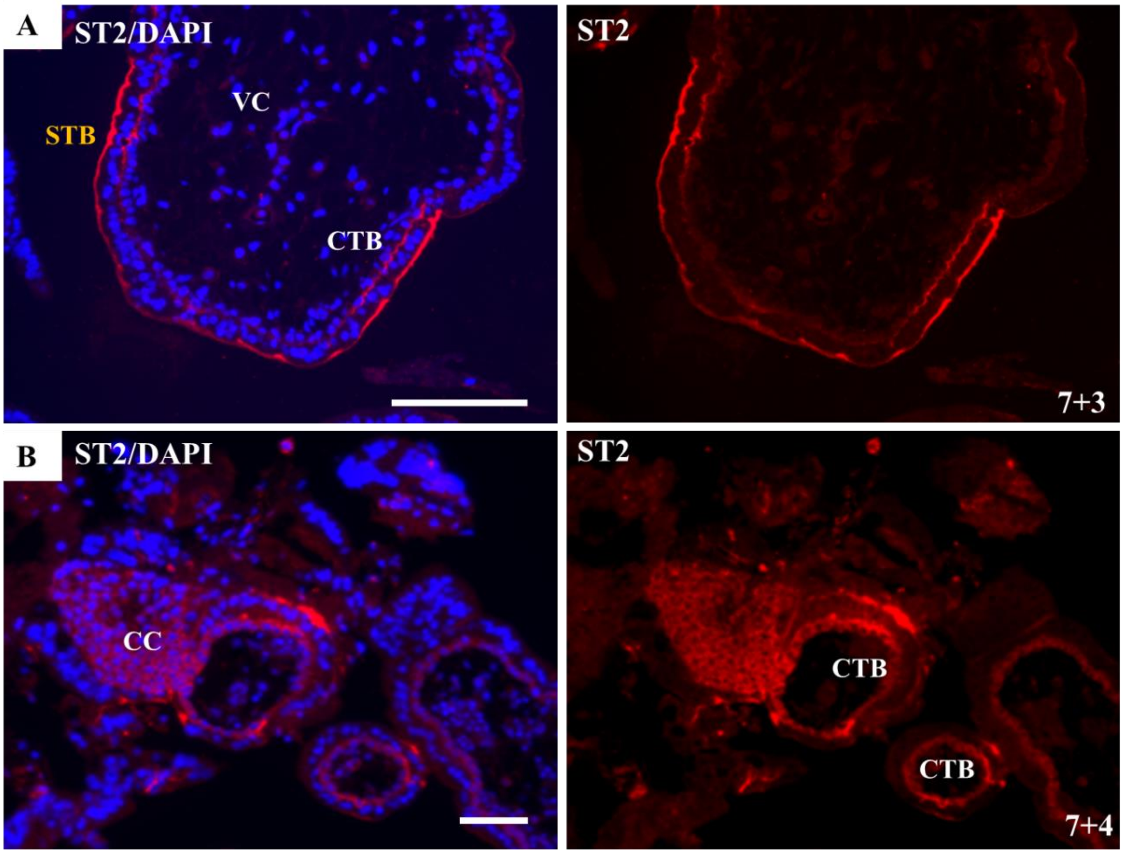


Figure 31. Localization of ST2 at seven weeks of gestation. Overlay of ST2 (red) and DAPI (blue) for (A) 7+3 and (B) 7+4 weeks placentas. (A) Localization of ST2 to the apical and basolateral surface of STB (yellow). (B) ST2 was detected on cell columns (CC), CTB and STB. VC: villous core. Scale bars = 100 µm.

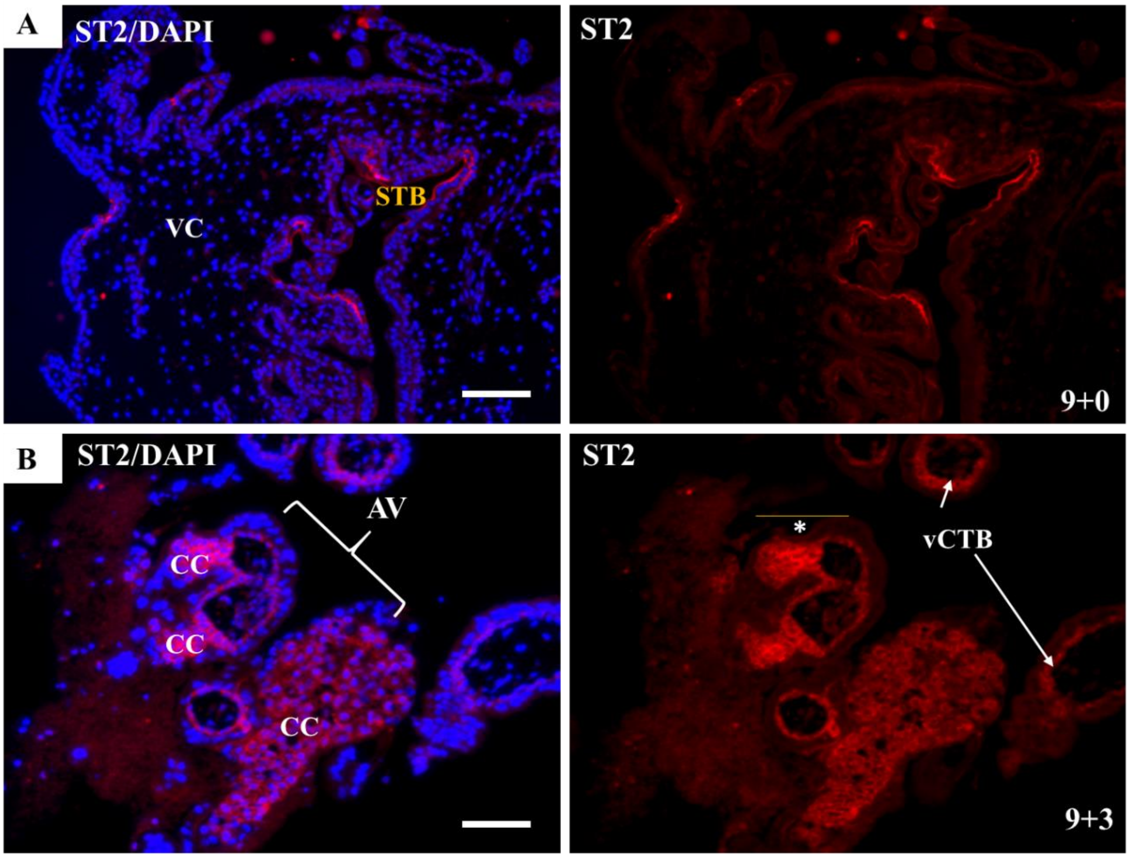


Figure 32. Localization of ST2 at nine weeks of gestation. The left panels (A) and (B) are overlays of ST2 (red) and DAPI nuclear staining (blue). The right panels are for ST2 only with gestational ages for each placental section displayed at the lower right side. (A) 9+0 placenta showed ST2 at the STB and on some of the underlying CTBs.
(B) ST2 was found on the trophoblast cell columns (CC) of the anchoring villi (AV) and on villous CTB as pointed to by the white arrows.
(*) highlights a region of strong ST2 signal intensity. VC: villous core.
Scale bars =100 µm.

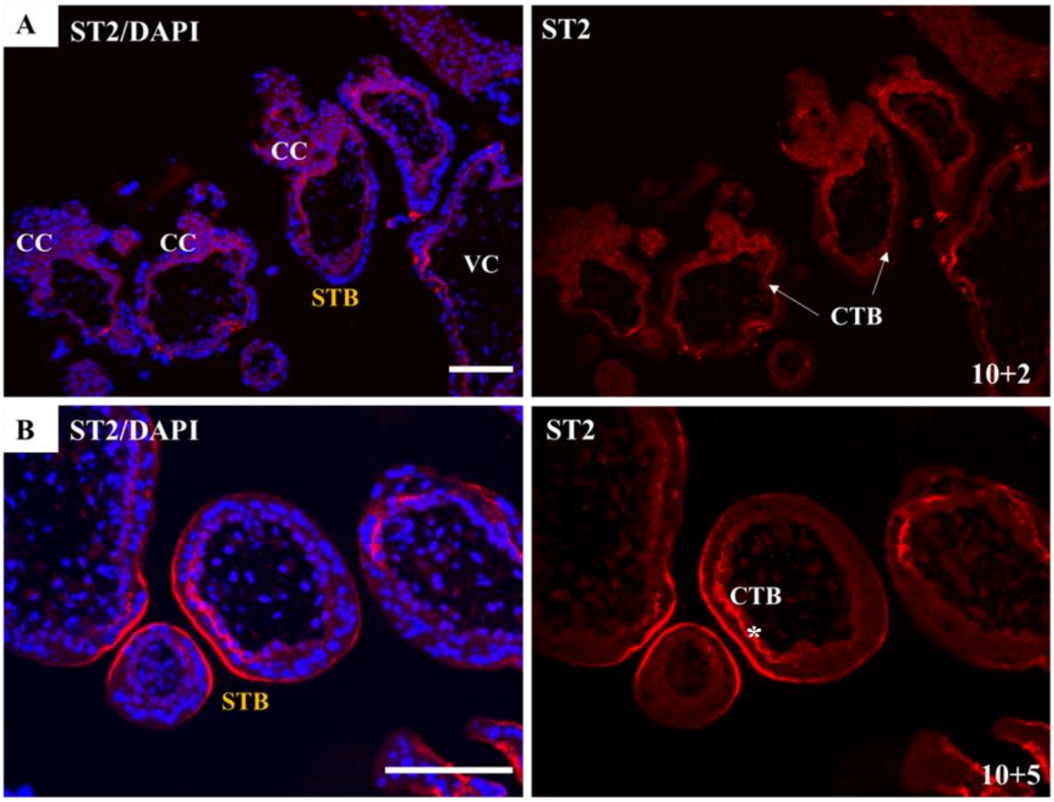


Figure 33. ST2 localization at 10 weeks of gestation. Left panels ST2 (red) and nuclear stain DAPI (blue) overlays.

(A) ST2 is detected on cell columns (CC) of 10+2 weeks of gestation placenta. The apical surface of the STB layer in all CC of the anchoring villi (AV) did not show ST2 expression, but ST2 was detected in the CTB cells of the (AV).

(B) ST2 localization to the apical and basolateral surface of the STB. White star (*) indicates regions of intense ST2 staining signal on the apical surface of the floating villi. Scale bars = 100 µm.

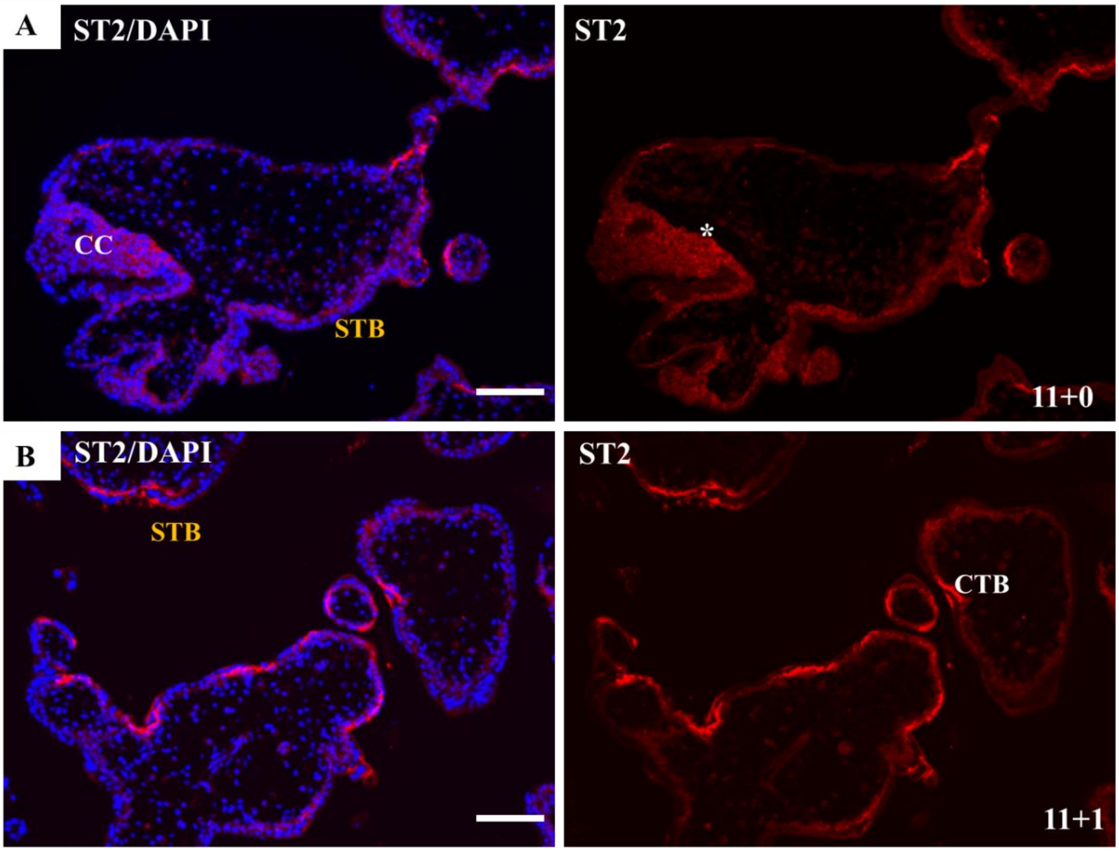


Figure 34. ST2 localization at 11 weeks of gestation. Overlay of ST2 (red) and DAPI (blue) in the left panel. (A) ST2 localized to trophoblast cells forming CC and CTB cells in 11+0 weeks. (B) ST2 localization to STB and CTB in 11+1 week placenta. Scale bars = 100 µm.

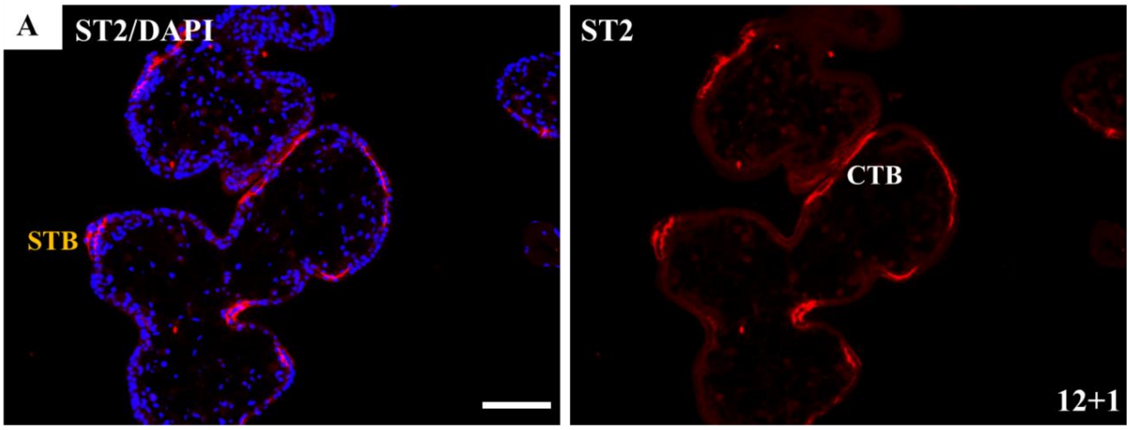


Figure 35. Localization of ST2 at 12 weeks of gestation. (A) Overlay of ST2 (red) and nuclear stain DAPI (blue). ST2 was localized to different regions of STB and CTB. Scale bars = 100 µm.

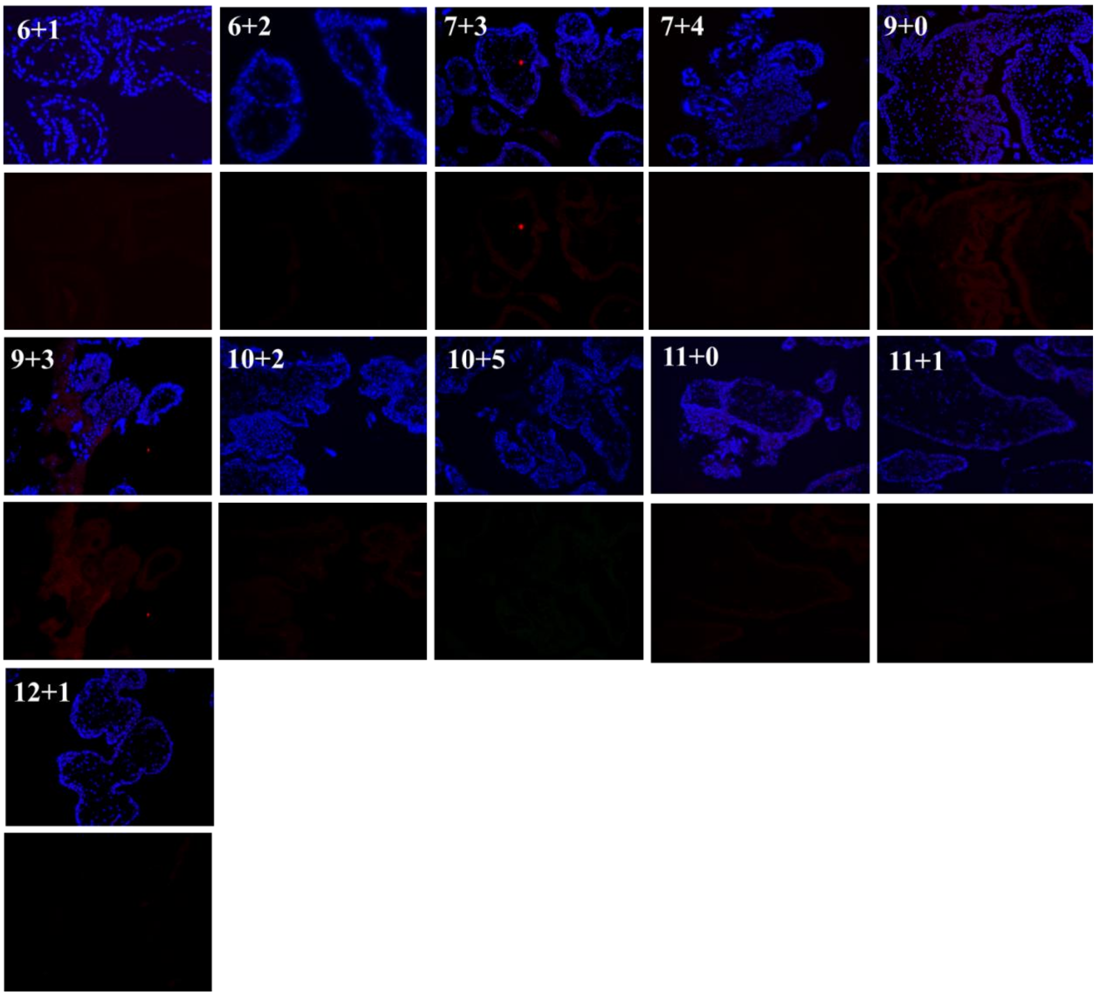


Figure 36. Isotype controls for placental sections from each gestational age. All controls displayed as overlays of the red channel and DAPI (blue). The lower panel under each overlay corresponds to the red channel only. Each isotype control image was taken with the same microscope and software parameters as that in the ST2 overlay images.

2.3.3.6 ST2 localization in term placenta

The previous section described the localization of ST2 expression in first trimester placenta, which has characteristic structural features such as larger cell columns and anchoring villi. These are reduced in size in term placenta where there are more floating villi. Three normal term placental sections from different patients were stained for ST2 as performed on first trimester sections. These experiments were carried out twice and the images presented in (Figure 37) are from of one of these experiments. Strong ST2 staining was detected on STB and CTB cells (Figure 37A and C). In Figure 37B, the ST2 signal was mainly localized to the basolateral side of the STB cells. Cells in the stromal core displayed a weak ST2 signal in large villi. The isotype controls corresponding to each placenta are shown in Figure 38.

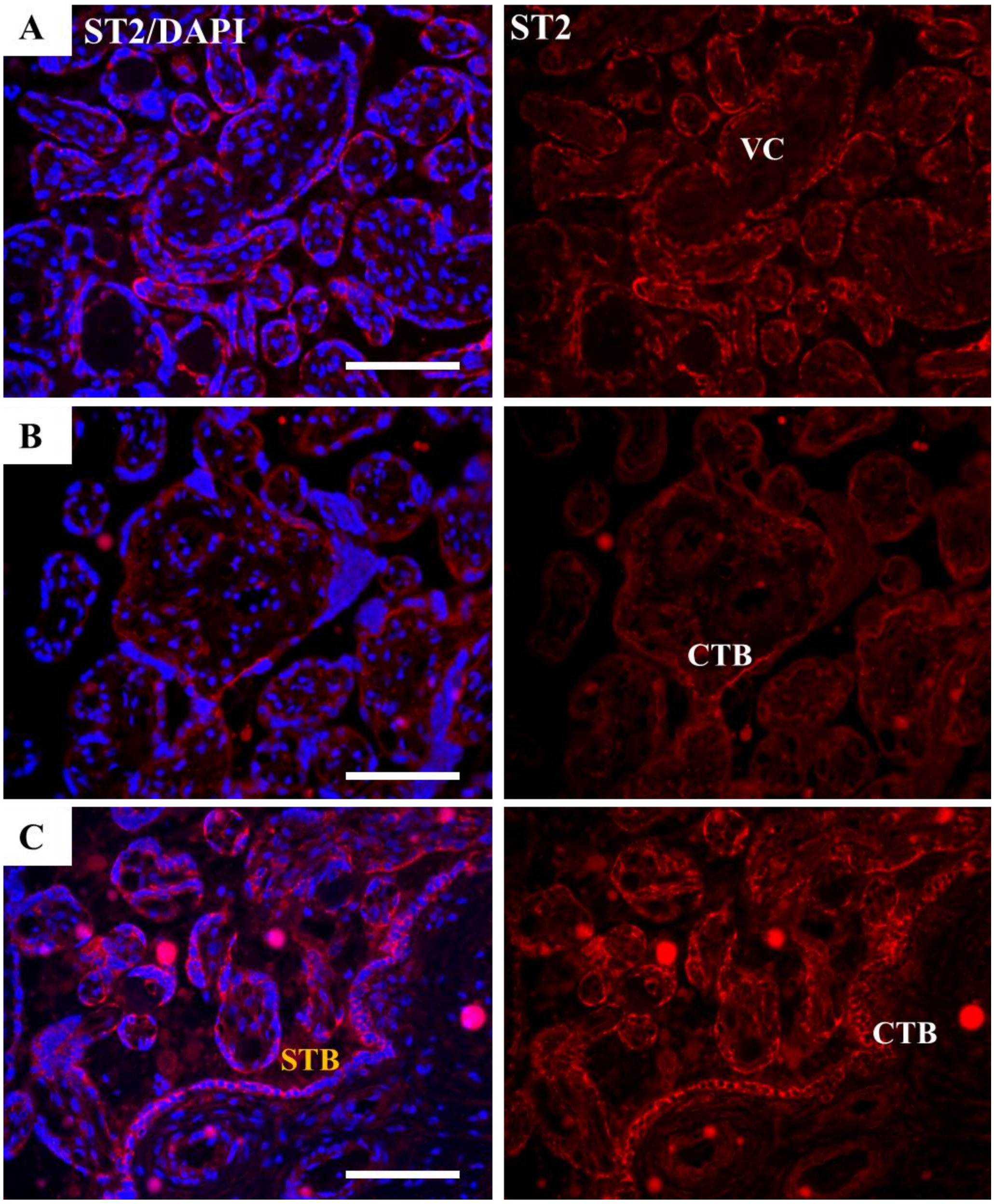


Figure 37. ST2 immunofluorescence staining in term placenta. The left panels are overlays of ST2 (red) and DAPI nuclear stain (blue). Left panels show images taken with TRITC channel (red) showing ST2 localization. VC: villous core of the floating villi. CTB: cytotrophoblast. Images taken with x20 magnification. Scale bars = 100 µm.

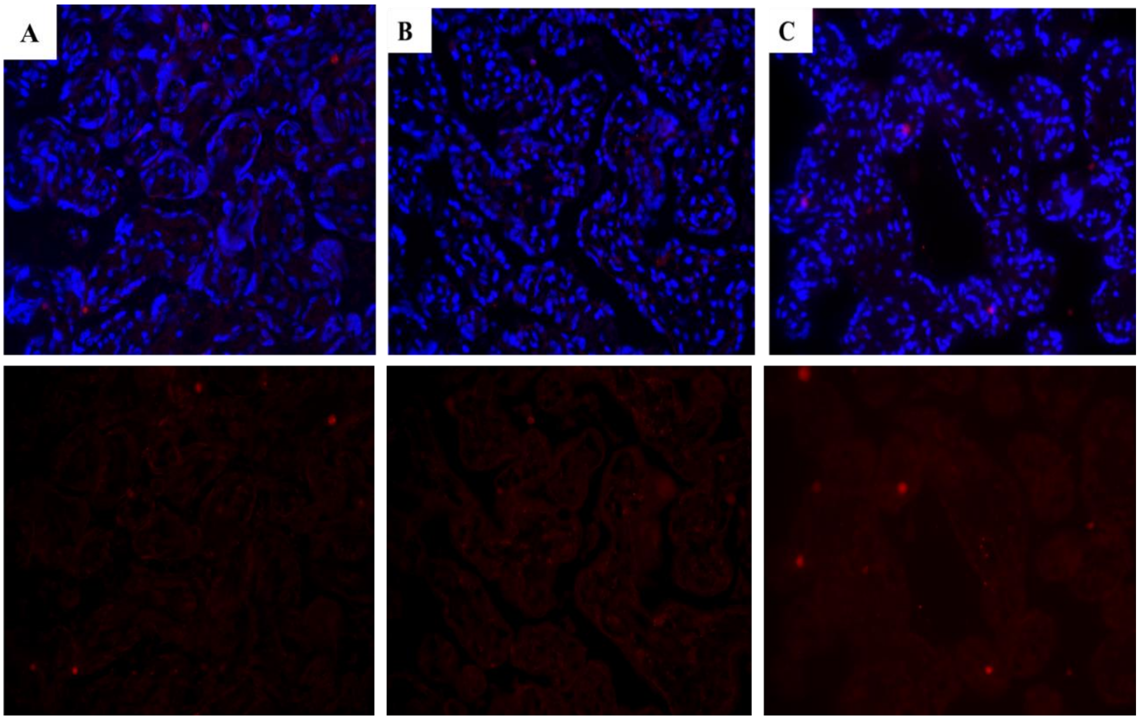


Figure 38. Immunofluorescence images of term placenta stained with rabbit IgG isotype control. Upper panel are overlays of Alexa 594 channel images (red) with DAPI nuclear stain (blue). Lower panels are for the red channel alone. Each isotype control image was taken with the same microscope and software parameters as that in the ST2 overlay images in Figure 38.

2.3.3.7 ST2 localization in extravillous cytotrophoblast of first trimester placenta

In first trimester placenta, anchoring columns express HLA-G (207). Anchoring columns are the site of CTB cells differentiation into EVT cells. As ST2 was expressed in the anchoring columns of placental villous structure, it was next determined whether ST2 was also expressed by EVT cells, which are invasive and uniquely express HLA-G. Therefore, HLA-G staining was used to discriminate EVT cells from other non-invasive cells in the cell column (see Figure 10). This was performed to investigate whether ST2 may have a functional role in trophoblast invasion.

Immunofluorescence analysis was performed on first trimester and term placental sections, which were stained with primary antibodies against ST2 and HLA-G. The staining was performed three times on sections of gestational ages ranging from 6+1 to 12+1 and on three normal term placental sections. Placental sections with positive HLA-G staining are shown in Figure 39-Figure 47.

EVT trophoblast as early as 6+1 weeks of gestation that labelled for HLA-G were also positive for ST2 in some regions (Figure 39). These regions were also positive for CK-7 - further proof of trophoblast origin. The ST2 signal was more intense on the CTB at the base of the cell columns and those located beneath the STB layer. Cofilin-1 was used to discriminate the STB layer, where ST2 localization was found on some regions of the apical surface.

The intense ST2 signal on the CTB at the base of the cell columns was not only found at 6 weeks of gestation (Figure 39 and Figure 40), but was also detected in nine week placental samples (Figure 42, Figure 43 and Figure 44). A more intense ST2 signal was detected near the base of the column and on CTB cells that underlie the STB layer

(Figure 40). This was evident in all cell columns that made up the anchoring villi. However, no ST2 was detected on the apical surface of the STB on any of these cell columns (Figure 40). ST2 was also detected on EVTs of seven week placentas and on CTBs that were not positive for HLA-G (Figure 41). This localization pattern of ST2 on EVT cells and on non-EVT ones such as the cell column trophoblast, STB and the CTB cells underlying them was consistently seen throughout the first trimester placentas (Figure 42-Figure 44). In these figures, EVT cells can be seen making up the anchoring villus structures which are important for placental attachment to the maternal endometrium.

By week nine of gestation, the anchoring columns are larger and occupy a larger area than the floating villi around them. It was clear from these images that ST2 was localized to different trophoblast cell types and the intense signal at the base of the CC was only detected in ST2 stained cells, but not those which labelled for cofilin-1, which was used to further assess this gradient of signal in these sites. Furthermore, by 10 weeks of gestation EVT cells were also positive for ST2 (Figure 45). This staining pattern for ST2 suggests that it may be involved in trophoblast functions such as proliferation and invasion.

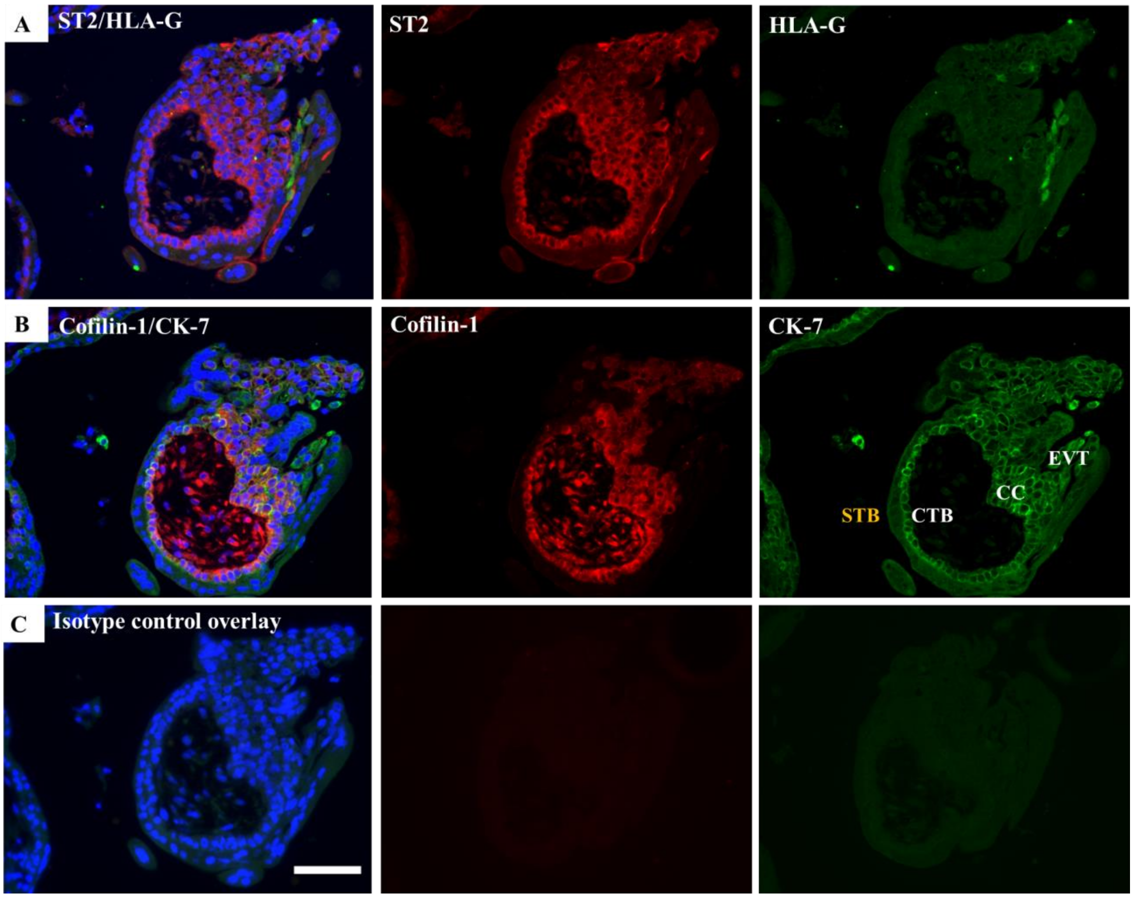


Figure 39. ST2 localization on EVT cells at 6+1 weeks of gestation. Immunofluorescence image of (A) Overlay of ST2 (red), HLA-G (green) and DAPI nuclear stain (blue). (B) Overlay of Cofilin-1 (red), CK-7 (green) and DAPI (blue). (C) Isotype control overlay of the red, green channels and DAPI (blue). Scale bar = 100 µm.

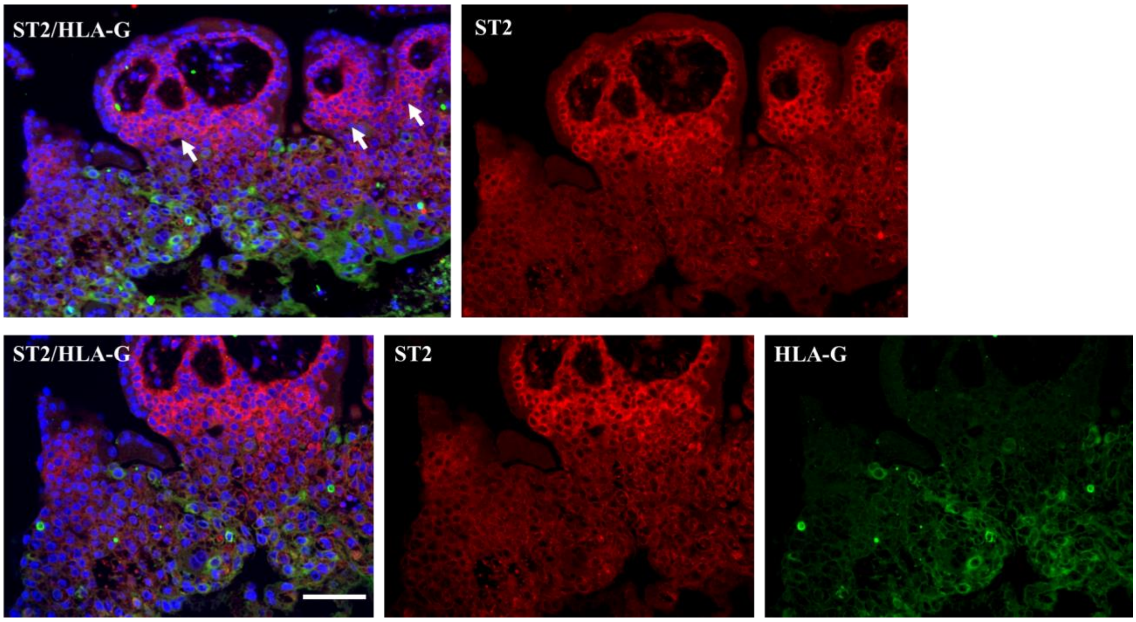


Figure 40. ST2 localization on EVT cells at 6+2 weeks gestation. Upper panel: 10X image of an overlay of ST2 (red), HLA-G (green) and DAPI (blue). To its right is the ST2 displayed with red channel only. Lower panel: x20 magnified image of ST2 (red) and HLA-G (green) and DAPI on an anchoring column. White arrows point to the base of the CTB cells present in the CC. Scale bar = 100 µm.

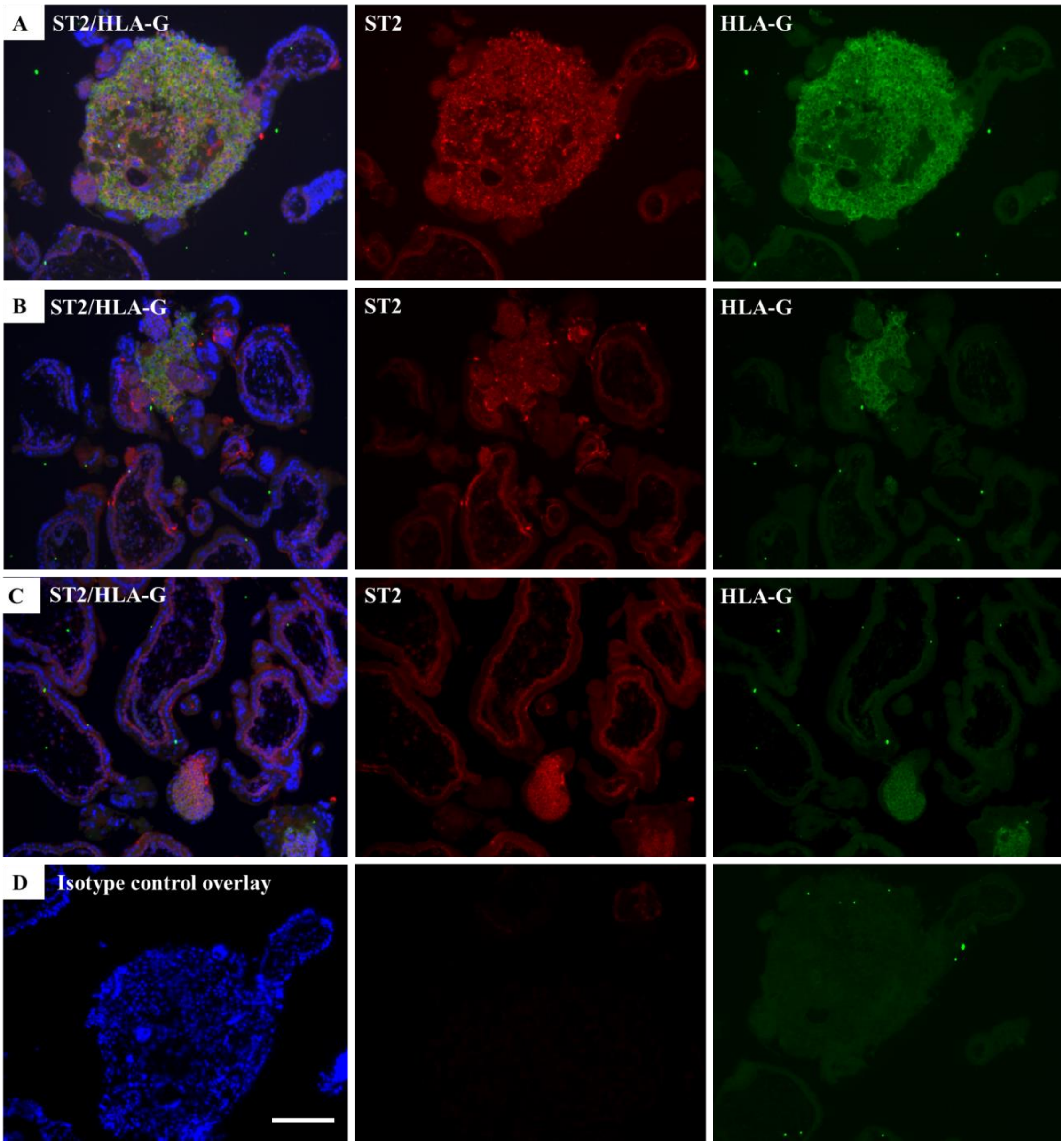


Figure 41. ST2 and HLA-G localization on EVT cells at 7+4 weeks gestation. (A), (B) and (C) are overlay images of ST2 (red), HLA-G (green) and DAPI (blue) from the same placenta taken at different locations in the same section. Scale bar = 100 µm.

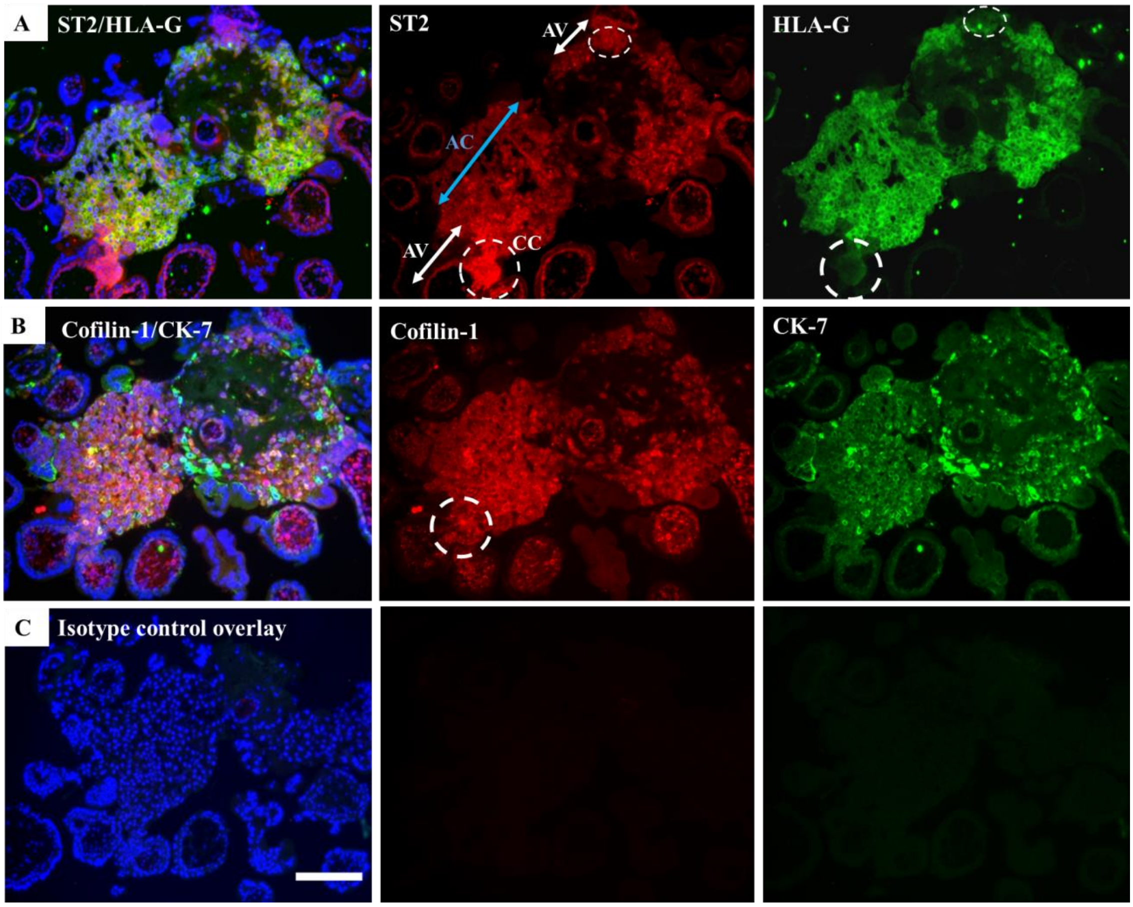


Figure 42. Co-localization of ST2 and HLA-G on 9+3 weeks gestation placenta. (A) Overlay of ST2 (red), HLA-G (green) and DAPI (blue). (B) Overlay of cofilin-1 (red), cytokeratin-7 (green) and DAPI (blue). (C) Isotype control overlay of the red, green and DAPI channels.

Anchoring villi (AV), trophoblast cell column (CC) displayed a gradient in ST2 signal expression in the base of the anchoring column (AC). This intense signal was not seen in cofilin-1 staining at the same site and very weak or almost undetectable levels of HLA-G were present at this site. Scale bar = 100 µm.

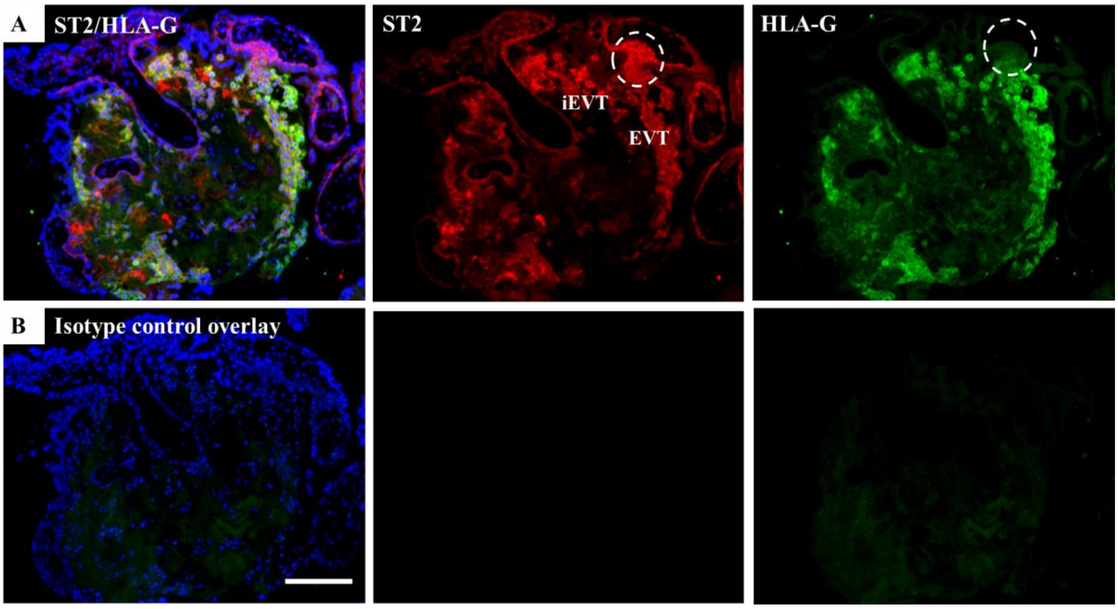


Figure 43. ST2 and HLA-G localization on 9+3 weeks placenta. (A) Overlay of ST2 (red), HLA-G (green) and DAPI (blue). Trophoblasts in the CC (white circle) display intense ST2 staining signal and no HLA-G was detected in this region. iEVT, interstitial trophoblasts, a subtype of EVT. (B) Isotype control overlay of the red, green and blue (DAPI) channels taken at the same image parameters. Scale bar = 100 µm.

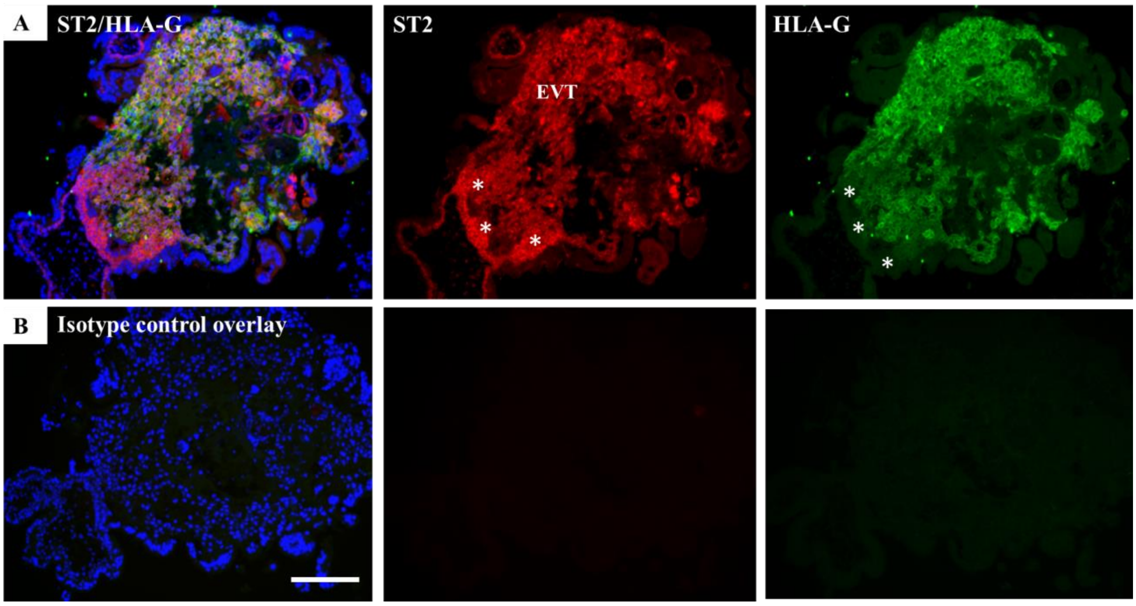


Figure 44. Co-localization of ST2 and HLA-G in placenta at 9+3 weeks of gestation. (A) ST2 (red) and HLA-G (green) with DAPI (blue). (B) Overlay of the isotype control. White star (*) show the CC trophoblasts with dense ST2 signal and no HLA-G staining. EVT cells (positive stain for ST2 and HLA-G) detached from the CC and migrate into the decidua. Scale bar = 100 µm.

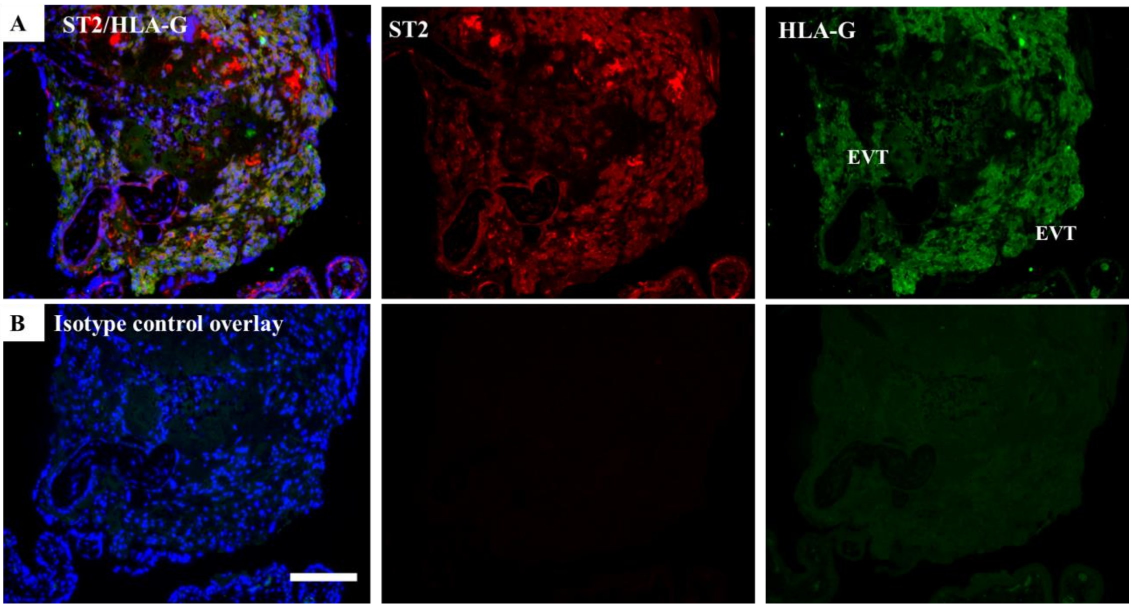


Figure 45. ST2 and HLA-G co-localization in 10+2 week placenta. (A) Overlay of ST2 (red), HLA-G (blue) and nuclear staining with DAPI (blue). (B) Isotype control overlay of the red, green and DAPI channels. Scale bar = 100 µm.

Third trimester placenta ST2 and HLA-G localization

Differences in the localization of IL-33 or ST2 between first trimester and term placenta were investigated to understand the possible role these proteins may play in placental maintenance. In first trimester placenta, ST2 was detected not only in the STB and the underlying CTB cells, but also in cell column trophoblast and EVT cells. The localization of ST2 on both EVT and non-EVT cells was interesting as it suggests multifunctional roles of this protein, which seems to be important during early stages of pregnancy. However, it was not known if the same localization pattern was present in term placenta.

Immunofluorescence analysis of ST2 on EVT cells was performed on term placental sections as for the first trimester placenta. Results for the co-localization of ST2 relative to HLA-G are shown in Figure 46 and Figure 47. Each of these results represents one of two experiments performed on three term placental sections. Identification of structures such as anchoring columns in term placenta is much harder than for first trimester ones as they are smaller in size. Cell columns forming the anchoring villi were therefore rarely found on the placental sections examined. In the only one instance when a cell column was detected (Figure 46), the ST2 signal in the CC was weaker than that seen on the STB cells. But consistent with first trimester findings, EVT cells staining positive for HLA-G were also positive for ST2 on different sites of term placental section (Figure 46) and on interstitial EVT cells that invaded the maternal decidua (Figure 47).

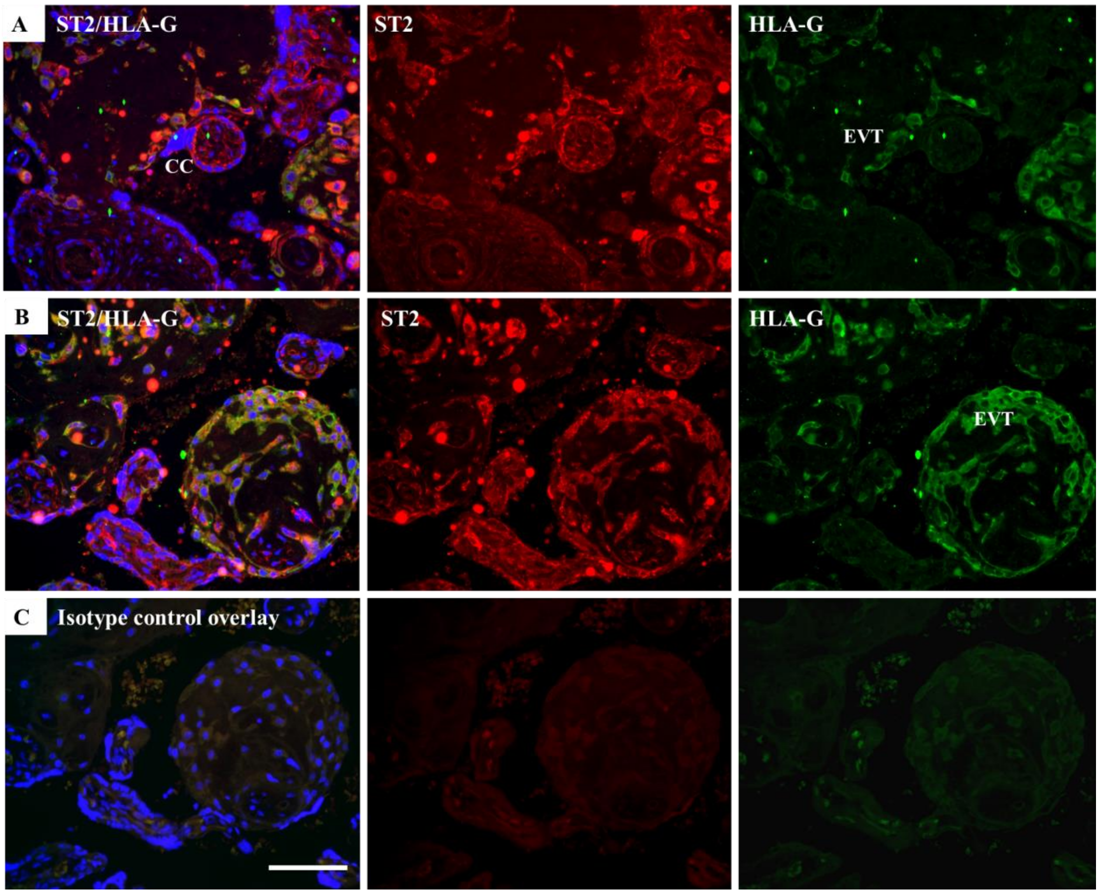


Figure 46. ST2 expression on EVTs in term placenta. (A) and (B) overlays of ST2 (red), HLA-G (green) and DAPI (blue) from different sites on the placental section. (C) Isotype control overlay of the red, green and DAPI channels with individual channels displayed to the right. Scale bar = 100 µm.

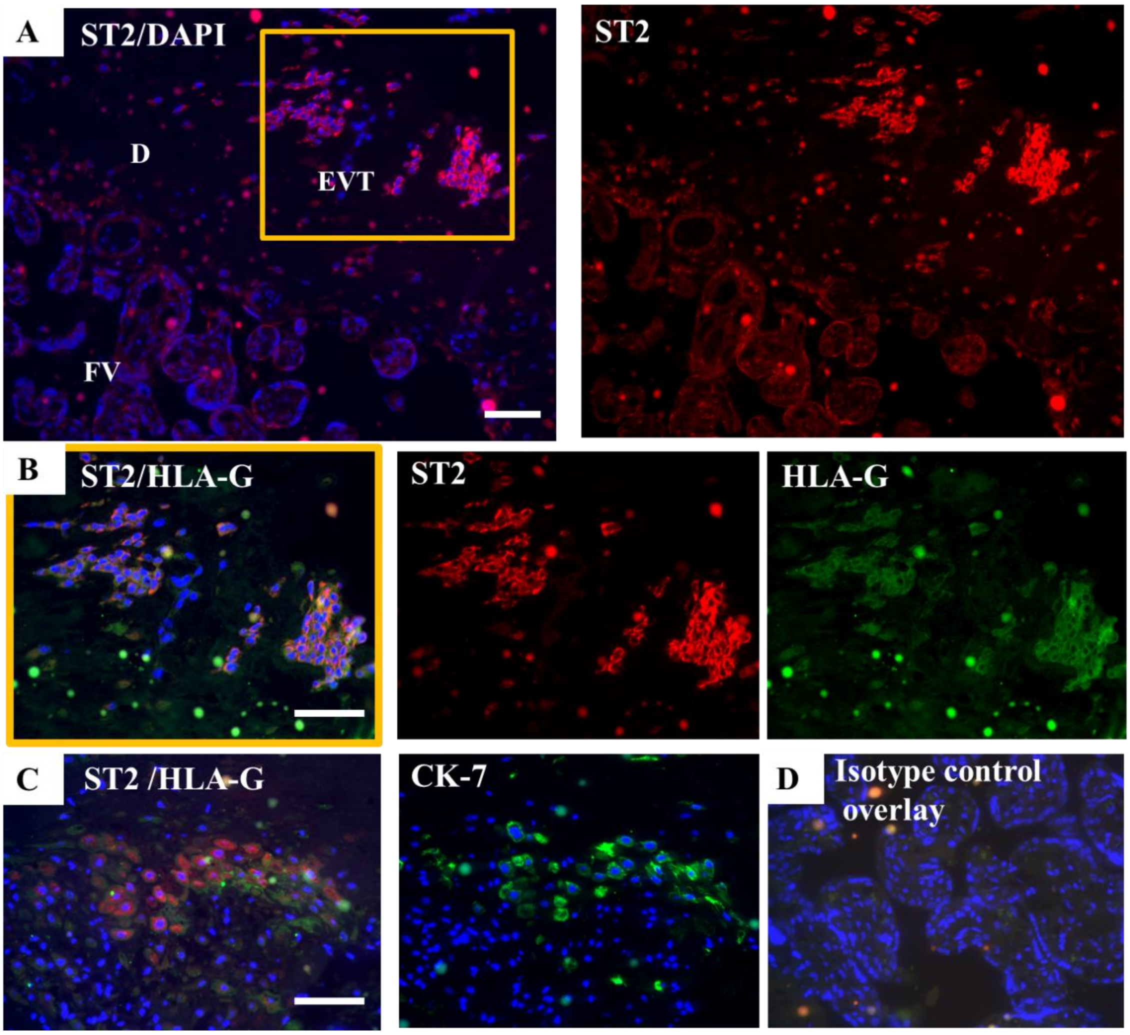


Figure 47. Co-localization of ST2 and HLA-G in term placenta. (A) Overlay of ST2 (red) and DAPI nuclear stain (blue). Interstitial EVT circled in yellow box. (B) Yellow box enlarged for demonstration showing an overlay of ST2 (red), HLA-G (green) and DAPI (blue). (C) Overlay of ST2 (red), HLA-G (green) and DAPI (blue). To the right is an overlay of cytokeratin-7 and DAPI. (D) Isotype control overlay with red, green and blue channels. Floating villi - FV, Decidua - D. Scale bar = 100 µm.

2.3.4 IL-33 and ST2 secretion from human placenta

2.3.4.1 IL-33 and ST2 secretion in first trimester placental chorionic villous explant culture

Explants of human placenta have been used to study many aspects of the maternal-fetal interface. The literature has shown their usefulness in studies related to different placental functions such as transport of molecules, endocrine function and metabolism. As the ST2 gene and protein detected in first trimester placenta corresponded to the soluble isoform of ST2 (sST2) and immunofluorescence staining identified the presence of ST2 at the apical surface of the syncytiotrophoblast layer, it is possible that sST2 is released from placental villi into the maternal blood in the intervillous space. Culture supernatants of chorionic villous explants, with and without EVT outgrowth, from three first trimester placentas were assessed for sST2 secretion by ELISA (Figure 48). Isolated chorionic villus explants were cultured for 48 h and culture supernatant was collected. As IL-33 was only expressed by cells within the villous stroma of the placenta, it is unlikely that it would be secreted by villous explants. Indeed no IL-33 release was detected (data not shown). In contrast, sST2 was detected in supernatants of explants both with and without EVT outgrowth (Figure 48).

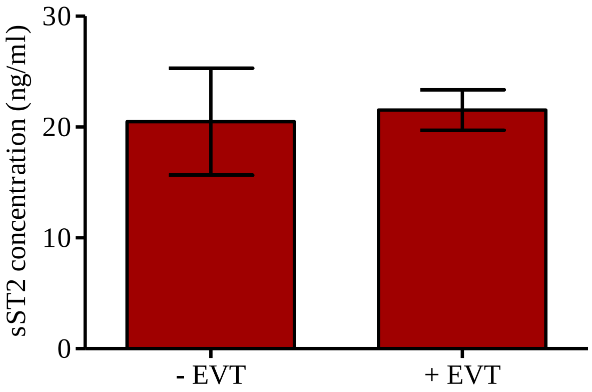


Figure 48. sST2 secretion from first trimester placental explants. ELISA analysis of ST2 secreted from first trimester placental explants without (-EVT) and with (+EVT) outgrowth from three chorionic villous explants at gestational ages of 7+6, 8+0 and 9+3 cultured for 48 h.

2.3.4.2 IL-33 and ST2 expression in primary trophoblast cells isolated from term placenta

To determine whether trophoblasts are the specific source of sST2 secretion by the placenta, primary trophoblast isolated from placentas were examined. Once isolated, villous cytotrophoblast from term placenta rapidly lose their proliferation capacity and undergo morphological differentiation forming syncytiotrophoblast. In the experiments described above, chorionic villous explants from first trimester placentas were found to secrete sST2. To determine the cellular source of sST2, ideally, villous trophoblast cells would have been isolated from first trimester placentas, but unfortunately tissues were not available. Term placentas were however readily available and villous trophoblast cells isolated from them were assessed for sST2 secretion by ELISA after 24, 48 and 72 h of culture (Figure 49). sST2 was secreted by all preparations and the highest levels were detected in 24 h culture supernatants. No IL-33 was detected in any of the trophoblast cell supernatants (data not shown).

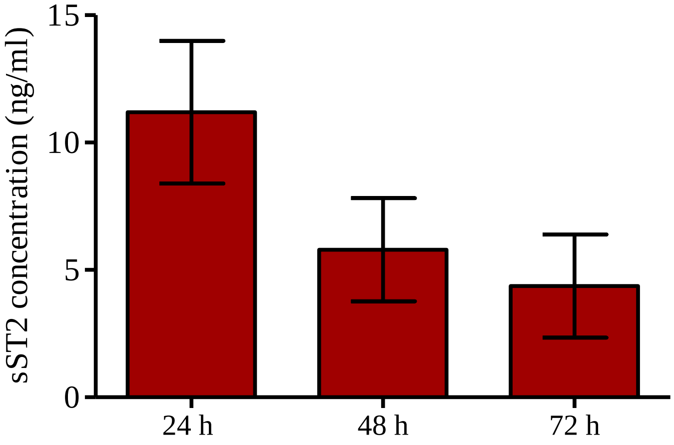


Figure 49. sST2 secretion by primary villous trophoblast cells. Primary villous trophoblast cells isolated from term placenta secreted sST2 following 24, 48 and 72 h in culture. ELISA was carried out using the Presage ST2 assay (Critical Diagnostics). Primary trophoblast cells from four different normal term placentas were studied. Culture media alone was used as a negative control and contained no sST2 (data not shown).

2.4 Discussion

Following reports on the detection of IL-33 and sST2 in pregnancy by our group (108), other groups have shown the presence of IL-33 and sST2 in the circulation of pregnant women in normal and pathological pregnancies, such as pregnancy loss (208), pre-eclampsia (209) and preterm birth (210). IL-33 levels were increased in the circulation of pregnancies that were lost before 20 weeks of gestation, the increase in IL-33 serum levels were evident as early as six weeks of gestation (208). In a transcriptomic study investigating gene expression profiles of spontaneous and preterm pregnancies, increased levels of ST2 transcript can be detected in the circulation of women who had preterm birth (210). Finally, consistent with our findings, sST2 levels in the circulation of women with pre-eclampsia were significantly higher compared to those in women with normal pregnancy (209).

2.4.1 IL-33 and ST2 mRNA transcript expression

The results of quantitative gene expression analyses performed in this chapter showed that IL-33 and ST2 mRNA were present at all gestational ages of first trimester placenta and in term placentas. The mRNA expression levels of IL-33 and ST2 were variable between samples. Total ST2 mRNA transcript levels were higher than IL-33 in all first trimester samples. Higher IL-33 transcript expression was detected in first trimester placenta compared to term placenta, whereas term placenta expressed higher ST2 mRNA levels than the first trimester. IL-33 mRNA expression has recently been reported in term placenta (211), but until now there have been no published reports comparing the mRNA and protein expression of both IL-33 and ST2 in first trimester placenta.

The hypoxic environment during the first 10 weeks of pregnancy plays an important role in trophoblast differentiation (212). By approximately 10 weeks of gestation, the placenta is exposed to maternal blood flow (213). An interesting observation was the low IL-33 mRNA levels in 10+2 weeks placental tissue samples were associated with the highest IL-33 protein expression. This may indicate that IL-33 is needed to induce local inflammation during the very early stages of placental exposure to maternal blood flow which occurs at this time.

2.4.2 IL-33 and ST2 protein expression

Western blot analysis detected the full length form of IL-33 in first trimester and term placentas and very low or almost undetectable levels of the cleaved form. The ST2 band detected in both first trimester and term placental lysate samples corresponds to the size of the sST2 isoform. The expected protein sizes of sST2, ST2L and ST2V are 50, 80, and 40 kDa respectively (150). The reported sizes correspond to the mature form of the proteins which are heavily glycosylated. Upon deglycosylation, the protein sizes shifts to approximately 35 (sST2), 60 (ST2L), and an approximately 25-30 (ST2V) kDa, as reported by the same authors.

However, in a recent report (214) which investigated ST2, but not IL-33, protein expression in primary first trimester trophoblasts, sST2 and ST2L were detected at approximately 35 and 65-70 kDa protein size respectively, which corresponds to the un-glycosylated form of the protein. ST2 has been detected in several other tissues and cells, corresponding to the glycosylated protein form (148, 215, 216). Enzymatic de-glycosylation performed by me on first trimester placental tissue lysates resulted in an apparent molecular weight band shift from 50 kDa to 37 kDa, corresponding to the molecular weight of the deglycosylated ST2 protein (data not shown). Glycosylation is the most common posttranslational modification of proteins, and expression of un-glycosylated proteins has been reported to be an indication of cell stress or nonviability (217).

2.4.3 Localization of IL-33 in human placenta

The localization of IL-33 in first trimester placenta was concentrated in stromal regions. Term placentas showed greater expression around fetal capillaries, consistent with the localization pattern reported previously (108, 211). The IL-33 was located in the cytoplasmic regions of cells, and in some sections was more intense on cells that were morphologically round, which could be placental macrophages as recently reported (214).

In patients with systemic sclerosis, it was found that nuclear IL-33 expression was lost in microvascular endothelial cells and the expression of ST2 was increased compared to that found in tissue sections from healthy individuals. This was detected not only in the skin, but also in other organs, one of which was the placenta (218). IL-33 was reported to be constitutively expressed in high levels by resting endothelial cells in healthy individuals, but dermal endothelial cells from patients with systemic sclerosis had decreased IL-33 mRNA expression. This decrease was suggested to be a result of the rapid secretion of IL-33 into the extracellular space following epithelial or endothelial cell damage (97, 110).

In a recent study the localization pattern of IL-33 protein in umbilical cord, and across the maternal (basal plate) and fetal (chorionic plate) sides of term placenta with preterm acute or chronic inflammation was investigated (211). Detection by IHC localized IL-33 protein to the nuclei of smooth muscle and endothelial cells found on the chorionic villi, basal and chorionic plate of normal term placenta. Double staining with IL-33 and the cytokerain-7 marker for trophoblast showed no staining for IL-33 in either villous or extravillous trophoblast cells.

IL-33 protein was detected in the nuclei of decidual endothelial cells found in normal chorioamniotic membranes. However trophoblast in the chorion leave, amnion epithelial cells and decidual cells were negative for IL-33 expression.

IL-33 protein was also localized to the nuclei of smooth muscle cells and endothelial cells in the arteries and veins of the umbilical cord. Nuclear localization of IL-33 protein was detected in stromal cells of the Wharton’s jelly (the gelatinous substance in the umbilical cord which holds the umbilical vein and arteries) of some normal placentas delivered at term. Cytoplasmic localization of IL-33 protein was also detected in myofibroblasts found in Wharton’s jelly, but no IL-33 nuclear and cytoplasmic localization was detected in CD14 positive macrophages contained therein.

IL-33 detected in first trimester and term placenta sections in this report may therefore be primarily of endothelial or smooth muscle cell origin (211).

IL-33 was previously detected in smooth muscle cells of bronchial (219), vascular (220) and visceral (111) human tissues. As Topping et al 2013 suggested, IL-33 expression may play a role in vascular biology of chorionic villous and umbilical vessels during placental development. This is supported by the localization of IL-33 to fetal vessels in the term placenta seen here and in our previous study (108). In contrast to previous reports of IL-33 expression in epithelial cells from a variety of different tissues (111), none was found in amnion epithelial cells.

Macrophages are common immune cells present at the maternal-fetal interface (221, 222). Low levels of IL-33 were expressed by activated macrophages (83) and it was found that LPS stimulation sequesters IL-33 in the nuclei of apoptotic monocytes (128). There are no reports of IL-33 expression in non-activated macrophages and double staining of IL-33 with the macrophage marker CD14 showed no staining for IL-33 in macrophages from normal placentas (211). Positive expression was found in inflamed chorioamniotic and umbilical tissues and it was therefore suggested to be a result of apoptotic or activated macrophages which are found in inflamed, but not normal tissues (suggesting inflammation caused by acute preterm labour activates local macrophage populations) (211).

However, a more recent report (214) contradicted these findings. IL-33 protein secretion was detected in placental and decidual macrophages isolated from first trimester pregnancies. The secretion of IL-33 from placental and decidual explants was significantly increased with IL-1β treatment (214). IL-33 was localized in the cytoplasm of macrophages in first trimester placental and decidual tissue sections. Immunoblotting analysis revealed the presence of both the full length (approximately 31 kDa) and a shorter cleaved form (approximately 18-20 kDa) in placental and decidual cell lysates.

2.4.4 ST2 localization in placental sections

In first trimester placentas, ST2 signal was detected on the apical surface of the syncytiotrophoblast, the underlying villous cytotrophoblasts and trophoblasts in the anchoring columns, EVTs of the cell columns and EVTs invading decidua. A similar pattern of localization towards the syncytium and the underlying villous cytotrophoblast was also observed in term placental tissue sections. However, these placentas showed no ST2 localization on trophoblast cell columns.

The significance of the localization to the syncytiotrophoblast and villous cytotrophoblast might suggest that ST2 is directly involved in the interaction with maternal tissues and that ST2 plays a role in the formation of the different subtypes of trophoblast cells. ST2 was localized to proliferating trophoblast in the anchoring column and also to the distal invasive trophoblast that are positive for HLA-G. Invasive trophoblast cells that migrate into the decidua lose their capacity to proliferate. Trophoblasts at the base of the anchoring villous column show dense ST2 staining with no HLA-G signal.

2.4.5 Localization of ST2 and HLA-G

The extravillous trophoblasts anchor the placenta to the uterus and invade into the maternal tissue and vascular system (28). The invading trophoblasts remodel the maternal spiral arteries by displacing endothelial cells and destroying the muscular tissue lining of these arteries (223, 224). Extravillous trophoblasts express HLA-G and this expression is recognized by the maternal NK cells which interact with trophoblast cells to promote their invasion of maternal decidua (23). Higher levels of HLA-G expression correlate with increased invasiveness (207). At different gestational ages of first trimester placenta, an ST2 staining gradient was seen in extravillous trophoblasts of the anchoring columns, with the most intense signal where the cells columns meet the anchoring villi. These cells were negative for HLA-G. ST2 was also detected on EVTs closer to the maternal decidua, which do express HLA-G. In term placenta, ST2 was detected on EVTs expressing HLA-G cells which invaded the decidua. Interestingly, ST2 levels on trophoblast columns in term placentas were low and these columns were not HLA-G positive.

Our results were consistent with the recently published report (214) who found ST2 in both proliferating and invasive trophoblasts, that they identified using HLA-G and Ki67 (a marker of proliferation). However, in contrast to the data presented here, no ST2 was detected in the STB and the cell columns did not display a gradient of ST2 expression. Trophoblast cells isolated from first trimester placentas cultured on fibronectin were reported to express both sST2 and ST2L proteins as detected by Western blot analysis. A large increase in sST2 protein was detected after 72 h of *in vitro* culture. However, the majority of the ST2 protein signal detected corresponded to the unglycosylated forms of sST2 and ST2L, while a very weak signal was detected at approximately 50 kDa (214).

Flow cytometric analysis also revealed the presence of ST2 on the surface of primary trophoblast cells isolated from first trimester placenta (214). Therefore, it was suggested by the authors that trophoblast cells are a major target for IL-33 at the maternal-fetal interface. In support of this, treatment with a recombinant full length IL-33 increased primary trophoblast proliferation in first trimester placental explants and this effect was also detected in first trimester primary trophoblast cells. Furthermore, recombinant IL-33 treatment enhanced placental explant ‘outgrowth’ by increasing EVT cell migration.

However the same effect was not detected when tested on primary EVT cells isolated from first trimester placenta (214). There is a need for further investigation of the role of IL-33 in trophoblast cell behaviour as it is known that placental explants are composed of different types of cells and the effects may not be directly on trophoblast cells, but mediated via other cell types.

The authors then examined a potential downstream signalling pathway that may be responsible for the increased proliferation by assessment of phosphorylation of the extracellular signal-regulated kinase (ERK), a signalling molecule activated when IL-33 binds ST2L. When primary trophoblast cells were treated with IL-33 alone, an increase in ERK phosphorylation was detected, but there was no activation of NF$_k$B. Moreover, a marked decrease in ERK phosphorylation was detected with primary trophoblast cells when treated with sST2 alone.

IL-33 induces downstream signalling once bound to ST2L by recruiting the myeloid differentiation primary response protein 88 (MyD88) complex, which activates two independent pathways, the mitogen activated protein kinase (MAPK) pathways leads to the activation of ERK, p38 and JUN N-terminal kinase (JNK) and the other pathway is through the phospholipase D (PLD)-sphingosine kinase (SPHK) that initiates Ca^{2+} mobilization and activation of the transcription factor nuclear factor $_k$B (NF-$_k$B) (87). Therefore, IL-33 activates the MAPK pathway which is responsible for the induction of cytokines and chemokines, including IL-5, IL-13, CCL5, CCL17 and CCL24 (87). Although recombinant human IL-33 treatment of primary trophoblast cells isolated from first trimester placenta increased ERK phosphorylation (214), the up-regulation of the downstream molecules described above has not been examined. This will be the ultimate test for a trophoblast response to IL-33.

The expression of ST2L and the functional effects of IL-33 on trophoblast cells remain to be confirmed as we were not able to detect ST2L protein in placenta tissue samples. Based on the localization of ST2 seen in this study, we propose that ST2 is important for cell proliferation at the fetal side and is important for cell invasion at the maternal side. ST2 may therefore play a role in trophoblast cell differentiation towards either a villous or an extravillous phenotype. This hypothesis will be investigated in Chapter 3.

2.4.6 IL-33 and ST2 protein secretion

The placenta secretes molecules from its surface which is composed of villous cytotrophoblasts and the syncytiotrophoblast layer that comes into direct contact with the maternal blood. Placental villous explants are often used for *in-vitro* studies. In this chapter we analysed first trimester placenta villous explant cultures (225) for the presence of secreted IL-33 and ST2. No secretion of IL-33 was detected in any of the explant culture supernatants. This is not surprising as IL-33 is known to be secreted as an alarmin signal as a sign of cell damage. IL-33 has recently been shown to be actively secreted by placental macrophages (214), but no studies showing IL-33 release from first trimester placental explants have been reported.

The levels of sST2 secretion in first trimester placenta have not previously been investigated. Our results show active secretion of sST2 from first trimester placental explants and primary trophoblasts isolated from term placenta. It was reported earlier that the ST2 protein is growth and proliferation dependent, meaning that actively growing cells secrete sST2 while arrested ones do not (226, 227). This may explain the decrease of sST2 secreted levels in trophoblasts of term placenta with the increase in culture time.

2.5 Summary of findings in this chapter

1. IL-33 and ST2 mRNA and protein were detected in first trimester and term placentas.
2. The ST2 mRNA detected in first trimester and term placentas corresponded predominantly to the sST2 isoform.
3. IL-33 protein was detected in cells present at the villous core of floating villi in first trimester pregnancy.
4. ST2 was detected in various trophoblast cell types; STB and the underlying CTB, cell column trophoblast and EVT cells.
5. Both first trimester placental explants and term villous trophoblast cells secreted sST2.

Chapter 3

The role of IL-33 and ST2 in trophoblast cell proliferation and invasion

3.1 Introduction

IL-33 has been shown to exert various biological effects depending on the target cells expressing its receptor, ST2L. In the previous chapter, no ST2L protein was detected in samples from first trimester placenta. The majority of gene and protein detected was of the soluble ST2 form, which was secreted from first trimester placental explants. Therefore, it is possible that the placenta and more specifically trophoblast cells are the source of sST2 at the maternal-fetal interface. This was supported by the localization of ST2 to proliferating, invasive and syncytialized trophoblast cell populations in first trimester placental sections. Trophoblast cell lines were used to study the function of ST2 in this chapter as we were not able to access primary trophoblast cells from first trimester placenta.

3.1.1 Trophoblast cell lines for studying placental functions

A variety of molecules and signalling pathways have been described in the literature which play key roles in regulating the various functions of trophoblast cells (proliferation, syncytialization, adhesion, migration and invasion). The identification of these molecules and pathways has been through the use of *in vitro* models such as placental explant cultures, primary cells, and trophoblast cell lines (13). As our laboratory did not have direct access to fresh first trimester placental tissue, this project used a number trophoblast cell lines which have previously been proven to be useful for studying the molecular and cellular behaviour of human trophoblast cells.

The BeWo choriocarcinoma cell line (established by co-culturing choriocarcinoma tissue with human decidual explants) was used here to model the syncytiotrophoblast and cell fusion (228). The choriocarcinoma cell lines, JEG-3, JAR and AC1M59 were used as models for EVT (229-231). The AC1M59 cell line were generated by fusing AC1-1 (a JEG-3 mutant) with term chorion cells (232).

The SGHPL-5 cell line differs from the other cell lines in that it was derived from first trimester chorionic villous explants from normal human placenta by transfection with the SV40 virus T-antigen and has been shown to have the characteristics of primary EVT (233, 234). When plated on Matrigel, these cells express cytokeratin-7 and HLA-G and invade into fibrin-embedded spiral arteries *in vitro* (235). The generation of SGHPL-4 (a clone of SGHPL-5 cells) spheroid cultures was shown to improve their invasive properties and has been used to study the effect of different molecules such as growth factors and cytokines on these cells (236). Trophoblast cell lines have therefore contributed significantly to the understanding of the role of different cytokines, chemokines and growth factors identified at the maternal-fetal interface. However, the expression of IL-33 and ST2 in trophoblast cell lines and their functions have not been described before.

3.1.2 IL-33 and ST2 target cells and cell lines

IL-33 was originally shown to predominantly promote Th2 responses (83). It became evident that signalling via its receptor ST2L, IL-33 stimulated effector functions of both T helper type 1 (Th1) and type 2 (Th2) cells (168). Human basophils, eosinophils and mast cells, but not resting Th2 cells, are the primary targets of IL-33 (169, 237, 238). Activated Th2 cells express low levels of ST2L in comparison to sST2 (239). IL-33 activates the NF_kB and MAP kinase signalling pathways to promote its cellular functions depending on the cell type. Downstream functional effects of IL-33 include production of chemokines, cytokines and growth factors, also altering cell morphology and inducing migration and adhesion (reviewed in (91)).

Studies on IL-33 and its downstream effects are mainly carried out in primary tissues and cells as there are few human cell lines expressing ST2L (240). The megakaryoblastic leukemia cell line UT-7 (241) and the mast cell leukemia cell line LAD2 (242) express ST2L, but the function and signalling of IL-33/ST2L in these cell lines is not well understood. Only recently the KU812 cell line, basophil precursor cells isolated from myelogenous leukaemia (243), was found to constitutively express ST2L and was utilized as an *in vitro* model for studying IL-33/ST2L interactions (244). High levels of ST2L surface expression on KU812 generated greater inflammatory response after IL-33 stimulation. Although activation of the ERK1/2, p38, JNK, and NF-κB pathways were detected in mast cells and basophils following IL-33 stimulation (245), no ERK1/2 activation was detected following IL-33 treatment in KU812 cells (244).

In chapter 2, IL-33 gene and protein expression were detected in human first trimester placenta, however localization was in non-trophoblast cells. As sST2 was the dominant protein form expressed in first trimester placenta, the IL-33 activation of inflammation via its ST2L receptor was not expected to play a key role at this stage as no ST2L protein and very low gene expression was found in all first trimester placental samples. The lack of ST2L protein expression in placental lysates of first trimester placenta indicates the possibility that IL-33, localized to non-trophoblast cells, does not target trophoblast cells, but can act on immune cells present at the maternal-fetal interface. The expression of IL-33 and ST2 in trophoblast cell lines has not been investigated elsewhere. Therefore one of the aims of this chapter was to test gene and protein expression in commonly used trophoblast cell lines.

3.1.3 Soluble ST2 activation of cell motility and invasion independent of its ligand IL-33

As the project progressed, new evidence was published demonstrating a role of ST2 that is independent of IL-33. ST2 was reported to promote cancer cell invasion in both mouse and human studies. ST2 expression had previously been reported on T cell lymphomas and leukemic cell lines (241, 246). In the new studies, ST2 deficiency in mouse breast cancer cells was found to reduce tumour growth and metastasis (247) and sST2 induced human breast cancer cell migration by targeting ErbB2 (240). This led us to speculate whether ST2 was strongly expressed both on proliferating CTB at the base of the anchoring villi and also on invasive EVTs, this might indicate a novel role for ST2 in trophoblast proliferation and invasion.

A striking similarity between trophoblast cells and cancer cells in properties such as proliferation, migration and invasion has been reported. Normal trophoblast cells and cancer cells share transcriptional and translational activities that control gene expression and protein production (248). A result of the similarities to cancer cells, trophoblast cells were referred to as ‘physiological metastatic’ tissue (249, 250). However, in contrast to cancer cells, trophoblast proliferation and invasion are tightly regulated (13). Trophoblast and malignant tumours express proto-oncogenes such as EGFR/ErbB1 and HER2/ErbB2 which, once activated, are able to induce cell migration (251, 252). Furthermore, both trophoblast and tumour cells express receptors for growth factors such as EGF (253), VEGF (254) and the granulocyte-macrophage colony-stimulating factor (GM-CSF) (255). Numerous other genes expressed by trophoblast cells are also found to be overexpressed in malignant cells (256).

3.1.3.1 IL-33 and ST2 expression in tumour tissues

IL-33 expression was found in endothelial cells of healthy organs, but not in endothelial cells isolated from tumour tissues (110). In contrast, as discussed above, ST2 is expressed by tumours and induces human breast cancer cell migration by targeting the epidermal growth factor receptor ErbB2 (240).

The epidermal growth factor receptor family consists of four ErbB receptor tyrosine kinase members; ErbB1, ErbB2, ErbB3 and ErbB4 (257, 258). These receptors have been implicated in a variety of cellular processes including proliferation, differentiation and apoptosis (259). No ligand has been identified for ErbB2 (unlike other family members) (260). Activation of ErbB2 occurs by its own overexpression (261). ErbB2 can also be activated by heregulins (HRGs) (262) which are a family of polypeptide growth factors derived from alternatively spliced genes. HRGs bind to ErbB3 or ErbB4, thereby inducing heterodimerization of ErbB3/ErbB4 complex with ErbB2 leading to receptor tyrosine phosphorylation and activation of downstream signal transduction (263).

The ErbB receptor tyrosine kinases are overexpressed in two-thirds of human breast cancers and are associated with malignant transformation (264). ErbB2 overexpression is detected in aggressive form of breast cancers with high metastasis and correlates with poor prognosis, but the mechanism involved in ErbB2 receptor activation in human breast cancer remains poorly understood (260).

SKBr3 breast carcinoma cells expressing ErbB2 secrete low amounts of sST2 and treatment with heregulin-1 increased sST2 gene expression and protein secretion (240). Treatment with ST2-blocking antibody reduced heregulin-1 induced SKBr3 cell migration. Therefore sST2 was identified as a target for ErbB2-induced cancer cell migration. ST2L isoform gene expression was not detected in SKBr3 cells which indicated that the secreted sST2 was not acting as a decoy receptor for IL-33. It was also shown that the recombinant human (rh)ST2-Fc, a chimera made out of the extracellular domain of ST2 that is fused to the Fc IgG fragment, was able to bind the surface of SKBr3 cells. Furthermore, high levels of sST2 secretion were detected in metastatic breast cancer cells. Therefore, the secreted sST2 appeared to promote migration by binding to a specific site on the cell surface and inducing intracellular signalling in this breast cancer cell line (240).

ErBb2 is expressed in first trimester EVTs and was also identified in trophoblast cell lines such as JEG-3, JAR and a clone of SGHPL-5 (265, 266). Also, treatment of first trimester placental villous explants with culture media from decidual explants resulted in the down-regulation of proliferative markers (Ki67, Connexin 40 and EGF receptor) and in the up-regulation of invasive markers (α1 integrin and ErBb2) (267). Collectively, results from the studies above supported the novel hypothesis examined in this chapter that as sST2 is secreted by first trimester placenta, it might bind to the surface of trophoblast cells and modulate their functions such as proliferation and invasion.

3.1.4 Aims of this chapter centred on answering the following questions:

1) Do trophoblast cell lines provide useful models for studying IL-33 and ST2 in syncytiotrophoblast and extravillous CTB?
2) Are IL-33 and ST2 expressed and secreted by trophoblast cell lines?
3) Do trophoblast cell lines bind sST2?

4) Does sST2 play a role in trophoblast proliferation and/or invasion?

3.2 Materials and methods

3.2.1 Cell lines and cell culture

JEG-3, BeWo and JAR choriocarcinoma cell lines were obtained from the European Collection of Cell Cultures. The AC1M59 cell line was a gift from Dr Peter Sedlmayr (University of Graz, Austria) and the SGHPL-5 trophoblast cell line was from Dr Judith Cartwright (St. George's Hospital and Medical School, University of London). Human umbilical vein endothelial cells (HUVECs) (known to expresses IL-33 and ST2 mRNA and actively secrete sST2 and therefore used as a positive control (226)) were isolated by collagenase treatment from three normal term placentas by Dr Dionne Tannetta, University of Oxford, following the protocol described previously (268).

JEG-3, AC1M59 and BeWo cells were maintained in Ham's F-12 medium. JAR and RAJI (kindly given by Dr Demin Li, University of Oxford) cells were maintained in RPMI 1640 medium. SGHPL-5 cells were cultured in Ham's F-10 media. HUVECs were cultured in M199 medium supplemented with endothelial cell growth supplement (30 µg/ml), heparin (90 µl/ml) and grown on 1% gelatin coated culture flasks. SKBr3 (kindly given by Dr Helen Sheldon, University of Oxford) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). All culture media were supplemented with 10% fetal bovine serum (PAA laboratories), penicillin (100 IU/ml)-streptomycin (100 µg/ml) and 2 mM L-glutamine (referred to as complete culture media).

Cells were maintained in 75 mm^2 flasks under standard culture conditions of 5% CO_2, 20% oxygen and 37°C with medium renewal every 3 days. At approximately 80-90% confluence, trophoblast cells were detached with Accutase (PAA Laboratories) after incubation for 3-5 minutes at 37°C.

3.2.2 Total RNA extraction, reverse transcription (RT) and quantitative Real-time PCR

Total RNA was isolated from $1x10^6$ cells using Qiagen RNeasy Mini Kit according to the manufacturer's protocol. The isolated RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and stored at -80°C.

cDNA was synthesized by reverse transcription from 1 µg of total RNA using the qScript cDNA SuperMix (Quanta Biosciences) following the protocol described earlier in Chapter 2 Section 2.2.4.

The quantitative real-time PCR was performed using a Corbett Rotor Gene Q system (Qiagen) with the same cycling conditions and using the same TaqMan Gene Expression Assays for total ST2, sST2, ST2L, IL-33 and β-actin as those described in Chapter 2 Section 2.2.4. No RT for each cell line and NTC controls were included to evaluate DNA contamination.

The relative gene expression was analysed by using the $2^{\wedge -(\Delta Ct)}$ formula described in Chapter 2 Section 2.4.4. The fold change in gene expression was analysed according to the same formula 2^(- (delta Ct treatment)-(delta Ct control (no treatment))) (200).

3.2.3 Analysis of IL-33 and ST2 protein expression on human trophoblast cell lines

3.2.3.1 Preparation of whole cell lysates

Cells were lysed with 125 µl of lysis buffer and processed for SDS-PAGE and Western blot as described in Chapter 2 Section 2.2.5.

3.2.4 Recombinant human ST2 proteins

Human recombinant chimera protein sST2-Fc (523-ST-100, R & D Systems) and the sST2 from Sino Biological Inc (10105-H08H) were used for functional studies. Both proteins were fused with a polyhistidine tag at the C-terminus.

3.2.5 ELISA

Human ST2/IL-1 R4 DuoSet ELISA (R & D Systems) detection range 31.2 - 2,000 pg/mL and Human IL-33 DuoSet ELISA (R & D Systems) with detection range of 23.4 - 1,500 pg/mL were used for protein detection in the culture supernatants of the trophoblast cell lines. Culture media alone was used as a negative control. Plasma samples from women previously shown to be positive for IL-33 and sST2 were used as positive controls (108).

3.2.6 BeWo cell fusion assay

To model syncytiotrophoblast formation by villous cytotrophoblast fusion, the BeWo cell line fusion assay was used (269). 7.5 $x10^4$ BeWo cells per well were seeded in a 4-well plate and suspended overnight in Ham F-12 culture media. Cell fusion was induced by replacing normal culture media with low glucose DMEM supplemented with 2.5% FCS supplemented with 1mM of dibutyryl cyclic AMP (dbcAMP) for 24 or 48 h as previously described (269). Control cells had no dbcAMP treatment. Following each treatment, control and treated cells were either pelleted for RNA isolation or lysed for immunoblot analysis. The culture medium from the control and treated cells was centrifuged for 30 seconds at 10,000 x g and was stored at -80°C for analysis by ELISA.

3.2.7 SGHPL-5 cell growth on Matrigel

To promote an extravillous cytotrophoblast phenotype, 5 x10^5 SGHPL-5 cells were cultured in a 4-well plate on 150 µl layer of diluted Matrigel (6mg/ml) (BD Biosciences) in complete culture media. Cells were either cultured under standard culture conditions (20% oxygen) or under hypoxic conditions (1% oxygen).

3.2.8 Cytokine, growth factor and serum treatment

A combination of 100 ng/ml of IL-3, IL-4, IL-1α and tumour necrosis factor-α (TNFα) (Peprotech), IL-1β (BD Biosciences) cytokines, GM-CSF chemokine (Peprotech) and LPS (Peprotech) was used for BeWo and SGHPL-5 treatment under standard oxygen culture conditions. SGHPL-5 cells were treated with 1 nM of the growth factor, heregulin-1(HRG) (R & D Systems). SGHPL-5 cells were also stimulated with 1% or 20% FCS under standard and hypoxic culture conditions.

3.2.9 Flow cytometry

3.2.9.1 Surface protein binding assay

To determine whether sST2 binds to trophoblast cell lines, 2 x10^5 cells were stained for 30 minutes at 37°C with an anti sST2 antibody (R & D Systems or Sino Biological Inc). An antibody to actin regulatory protein CAPG (capping protein gelsolin-like) (Abcam) was used as a negative control. Unstained cells and cells only incubated with secondary antibody were used as further controls to assess the binding. Cells were washed three times with 1 ml of 2% FCS in PBS and incubated with 0.5 µg/ml of secondary antibody (anti-His-APC (Abcam)) for 20 minutes at 4°C. Cells were washed three times with 1 ml of 2% FCS in PBS and suspended in 300µl PBS (2% FCS) for flow cytometry analyses. The breast adenocarcinoma cell line SKBr3 and the human Burkitt's lymphoma cell line RAJI were used respectively as a positive and negative control for sST2 binding as previously demonstrated (240).

3.2.10 Cell proliferation

BeWo and SGHPL-5 proliferation was assessed using the Vybrant MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (Molecular Probes, Invitrogen). 5×10^3 cells were seeded in 96-well plate and cultured overnight in phenol-red free complete DMEM/F-10 media. The medium was then removed and replaced with 100 µl of media containing different ST2 (R & D Systems) protein concentrations in duplicate. The cells were incubated for a further 24 h after which the medium was replaced with medium containing 12 mM MTT. Cells were cultured with MTT for 4 h at 37°C and then treated with DMSO which was used to dissolve formazan produced by live cells. The concentration was determined by its optical density at 540 nm using FLUOstar Optima (BMG LabTech) plate reader. The relative proliferation was measured by the ratio of the absorbance values of the treated cells to the control untreated cells.

3.2.11 SGHPL-5 spheroid invasion assay

3.2.11.1 Preparation of 'hanging drops'

Hanging drops were prepared following the protocol described by (270). Each drop contained 750 SGHPL-5 cells suspended in methylcellulose and 22.5 µl of complete growth medium. 30 µl drops were placed on the lid of a bacterial grade culture plate. A small amount of sterile PBS was placed into the deeper part of the plate. The lid with the cell drops was carefully inverted on top and was cultured for 24 h under normal culture conditions. Figure 50 displays how cells aggregate to form the spheroid.

3.2.11.2 Fibrin gel formation and spheroid invasion

After 24 h incubation of the ‘hanging drops’, 100 µl of fibrin gel was prepared by adding 2.5 mg/ml Fibrinogen, 1:50 of 100 KI units/ml Aprotinin, and 1:200 of 1.25 Units/ml Thrombin to serum free media. The spheroids from the hanging drops were gently added to the fibrin gel and allowed to settle for 20 minutes at 37°C. 100 µl of 1% FCS supplemented treatment media alone or containing 100 ng/ml of sST2 (Sino Biological Inc) 100 ng/ml CAPG, or 10 ng/ml EGF (Peprotech) was loaded on the gels and spheroids were incubated under normal culture conditions. Spheroids images were taken with an inverted microscope between 4-20 h. Analysis of spheroid invasion was carried out using Open Lab Software (PerkinElmer) and Figure 50 shows an example of how the analysis was performed.

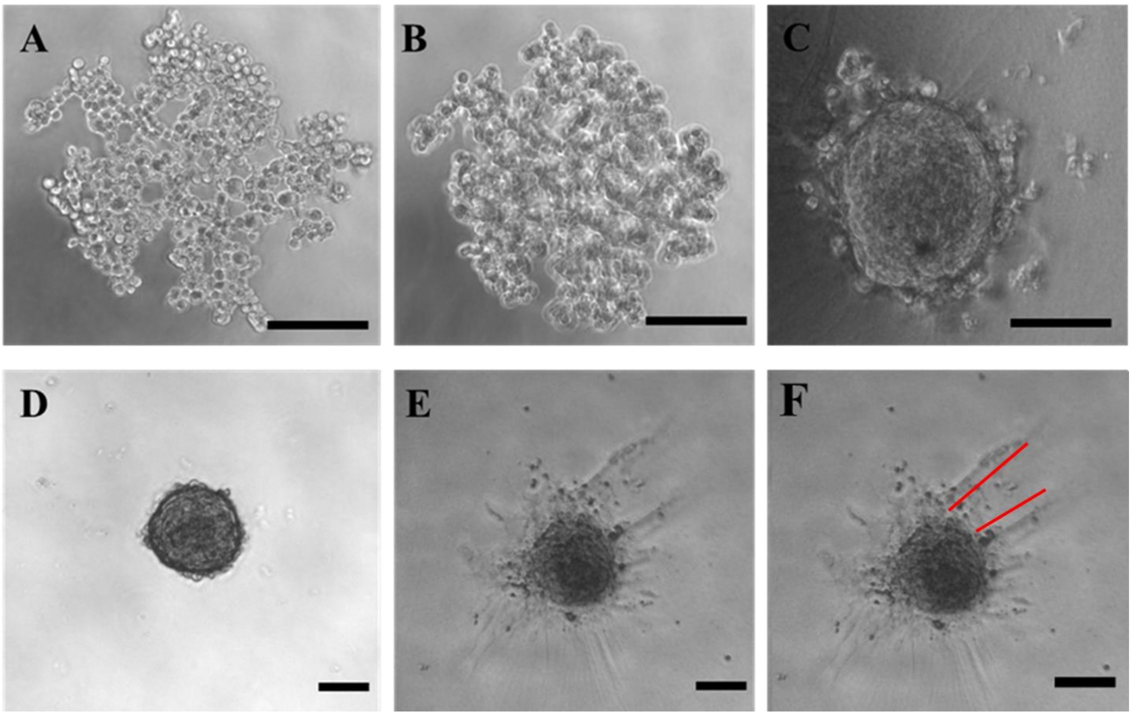


Figure 50. Formation of spheroids and analysis of cell invasion. (A-C) formation of SGHPL-5 spheroids in hanging drops after 10 minutes (A), 1 h (B) and 8 h (C). (D) 24 h SGHPL-5 spheroid on fibrin gel. (E-F) Analysis of spheroid outgrowth: red lines point to two outgrowths which length (µm) and number of were determined using Open Lab Software. (Scale bar =100 µm).

3.2.12 Statistical analysis

Data were plotted as the mean +/- the SEM using Graphpad software. Student's *t*-test with Welch's correction was used when comparing between two groups, while ANOVA with Bonferroni's correction was used for comparisons between three or more groups. p-values of <0.05 were considered significant *, very significant (p <0.01) ** or highly significant (p<0.001) ***.

3.3 Results

In this chapter, different trophoblast cell lines were investigated for their potential use to model the various types of trophoblast cells found *in vivo*. To model cell fusion and formation of the syncytium, the BeWo cell line was used. To model EVT, JEG-3, JAR, AC1M59 and SGHPL-5 cells were used. It was hypothesized that the trophoblast cell lines would express ST2 in the same way as first trimester placenta trophoblasts. To investigate this, the trophoblast cell lines, JEG-3, BeWo, SGHPL-5, AC1M59 and JAR were first assessed for their expression of IL-33 and ST2 genes by qRT-PCR, and protein by Western blot and ELISA.

3.3.1 IL-33 and ST2 mRNA transcript expression in trophoblast cell lines

Fluorescence quantitative PCR was used to determine IL-33 and ST2 mRNA expression in trophoblast cell lines. Cells were grown under normal culture conditions and 1x10^6 cells were pelleted and frozen at -80 °C until RNA isolation. The isolated RNA was processed for reverse transcription and quantitative RT-PCR as described in the materials and methods. RNA isolated from first trimester placenta and HUVEC cells was used as positive controls for IL-33 and ST2 gene expression.

RNA levels for IL-33 were undetectable in all trophoblast cell lines except SGHPL-5 which had low expression (Figure 51A). Similarly, the total ST2 mRNA transcripts were very low in all cell lines apart from SGHPL-5 (Figure 51B). Quantification of the expression levels for both IL-33 and ST2 mRNA are shown in Figure 51. ST2 mRNA was ten-fold higher in SGHPL-5 than IL-33. IL-33 transcript was 4 times higher in first trimester placenta and HUVEC than that in SGHPL-5. Also, the total ST2 level in HUVEC cells was over 2000 times higher than that detected in SGHPL-

5 and first trimester placenta. There was more sST2 detected in SGHPL-5 than the ST2L isoform Figure 52.

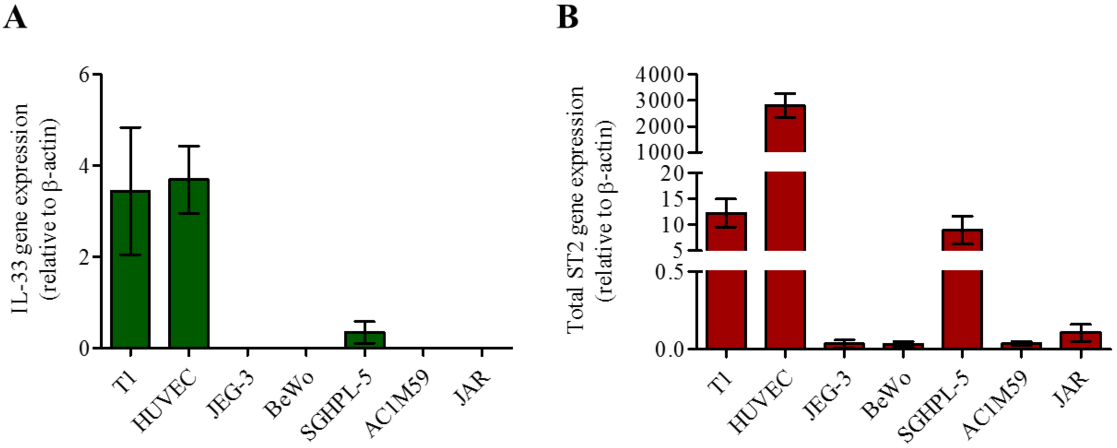


Figure 51. Levels of IL-33 and total ST2 gene expression in trophoblast cell lines measured by qRT-PCR. (A) IL-33 and (B) total ST2 gene expression levels in T1 (first trimester placenta), HUVEC, JEG-3, BeWo, SGHPL-5, AC1M59 and JAR cell lines. Columns represent the mean Ct values normalized to levels of house-keeping gene β-actin. The error bars represent the SEM of 5 independent experiments.

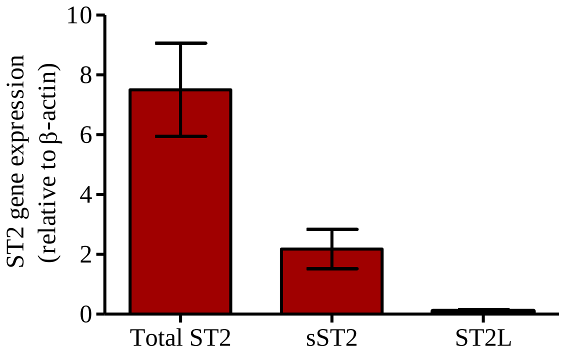


Figure 52. Total ST2 in SGHPL-5 corresponds more to the sST2 than ST2L isoform measured by qRT-PCR. Total ST2, sST2 and ST2L gene expression normalized to β-actin. Columns represent the mean of three independent experiments of three consecutive cell passages.

3.3.2 IL-33 and ST2 protein expression in trophoblast cell lines

IL-33 protein was not detected in any of the trophoblast cell lines (Figure 53) by immunoblotting. Similarly, no bands corresponding to the ST2L or sST2 isoforms were detected in any of the trophoblast cells (Figure 54). The previous chapter demonstrated that placental tissue lysates from first trimester placenta expressed IL-33 and sST2 proteins and therefore this was used as a positive control.

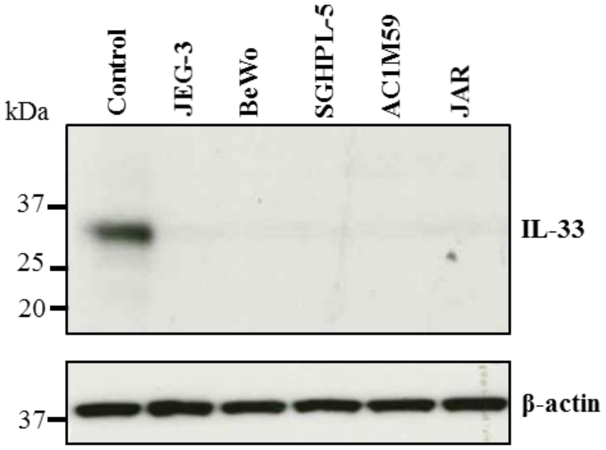


Figure 53. IL-33 protein detection by immunoblotting in trophoblast cell lines.
15 µg of protein from control (1^{st} trimester placental tissue lysates) and the indicated cell lines was tested for IL-33 expression. β-actin was used as a loading control. The blot is representative of three independent experiments.

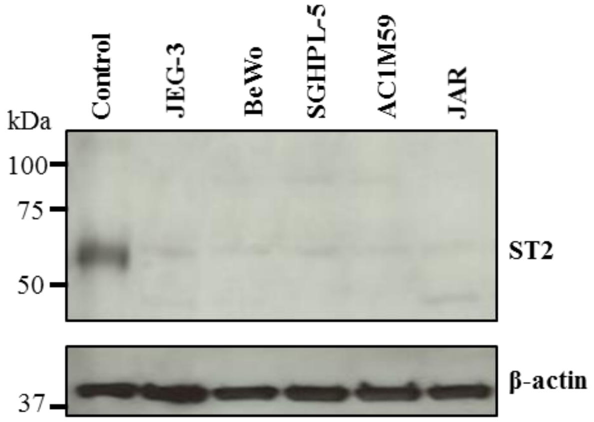


Figure 54. ST2 protein expression in trophoblast cell lines. First trimester placental tissue lysates (control) used as positive control for examining ST2 expression in trophoblast cell lines. β-actin was used as a loading control. The blot is a representative of three independent experiments.

3.3.3 IL-33 and ST2 protein secretion

The secretion of IL-33 and sST2 under normal culture conditions was analysed using a commercial ELISA (Figure 55). HUVEC and plasma samples from a woman with detectable levels IL-33 and sST2 were used as positive controls and culture media alone was used as a negative control. No secreted IL-33 or sST2 was detected in any of the trophoblast cell lines tested.

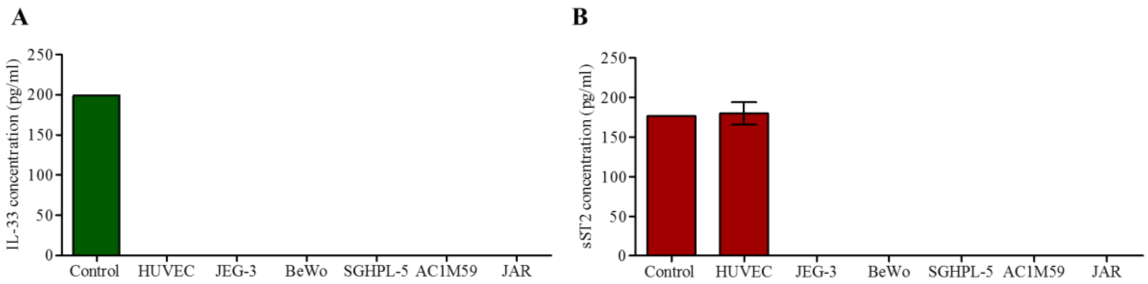


Figure 55. IL-33 and sST2 are not secreted by trophoblast cell lines. Culture supernatants from 1x10^5 trophoblast cells and HUVECs were tested for IL-33 (A) and sST2 (B) secretion by ELISA. The analysis was performed three times for all cells. Culture media alone was also tested and no IL-33 or sST2 was detected (data not shown). Controls are plasma samples from a woman in the third trimester of pregnancy previously shown to be positive for IL-33 and sST2 (108).

3.3.4 Effect of syncytialization and invasion on IL-33 and ST2 gene expression in BeWo and SGHPL-5 cell lines

In chapter 2, ST2 protein was detected on the apical surface of the syncytiotrophoblast. It was also localized to trophoblasts of the cell columns in the anchoring villi and to extravillous trophoblasts (EVTs). In this section, BeWo and SGHPL-5 cell lines were used to model STBs and EVTs respectively. Gene expression analysis showed no IL-33 and very low total ST2 gene expression in the BeWo cell line. On the other hand, SGHPL-5 cells displayed higher IL-33 (but still very low and undetectable in some preparations) and total ST2 gene expression than that detected in BeWo. Under normal culture conditions, both cell lines did not express or secrete IL-33 and ST2 proteins. It was therefore hypothesized that stimulation with factors which induce fusion and invasion or changes in culture conditions might up-regulate the expression of IL-33 and ST2. These pilot experiments were carried out either once or twice, and gene expression determined by qRT-PCR. Where up-regulation in gene expression was detected, samples were then tested by immunoblotting and ELISA for presence of IL-33 and ST2 protein.

3.3.4.1 BeWo cell fusion and its effect on IL-33 and ST2 gene expression

The BeWo choriocarcinoma cell line was used as a model of cytotrophoblast cell fusion to form syncytiotrophoblast. BeWo cells are able to fuse and form a multinucleated syncytial layer when treated with dbcAMP (271). No IL-33 RNA was detected in either the dbcAMP treated or non-treated groups (data not shown). There was no difference in total ST2 mRNA detected after 24 h of dbcAMP treatment compared to the control (Figure 56).

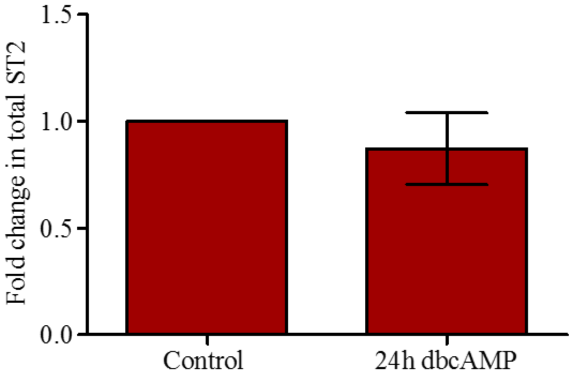


Figure 56. Effect of BeWo fusion on total ST2 gene expression. 24 h dbcAMP treated BeWo cells displayed no difference in total ST2 transcript expression to control untreated cells. Error bars represent the SEM of two independent experiments.

3.3.4.2 SGHPL-5 cell growth on Matrigel in standard and hypoxic conditions, and their effect on ST2 gene expression

SGHPL-5 cells were grown on a thick layer of Matrigel to promote an invasive phenotype, where cells detach and lose contact with each other (Figure 57). Matrigel is a reconstituted basal membrane that is similar to the decidual ECM - it is rich in laminin and some growth factors and cytokines and relatively poor in collagen IV (272). SGHPL-5 cells were grown on Matrigel under hypoxic and normal culture conditions, examples of cultures are shown in Figure 57. After 24 and 48 h in culture, these cells acquire an endothelial cell-like capillary tube structure in comparison to the control cells grown for the same time without Matrigel. The total ST2 gene expression was not increased after 24 h of culture on Matrigel under both normal and hypoxic conditions (Figure 58). No IL-33 transcript expression was induced by growth on Matrigel (data not shown).

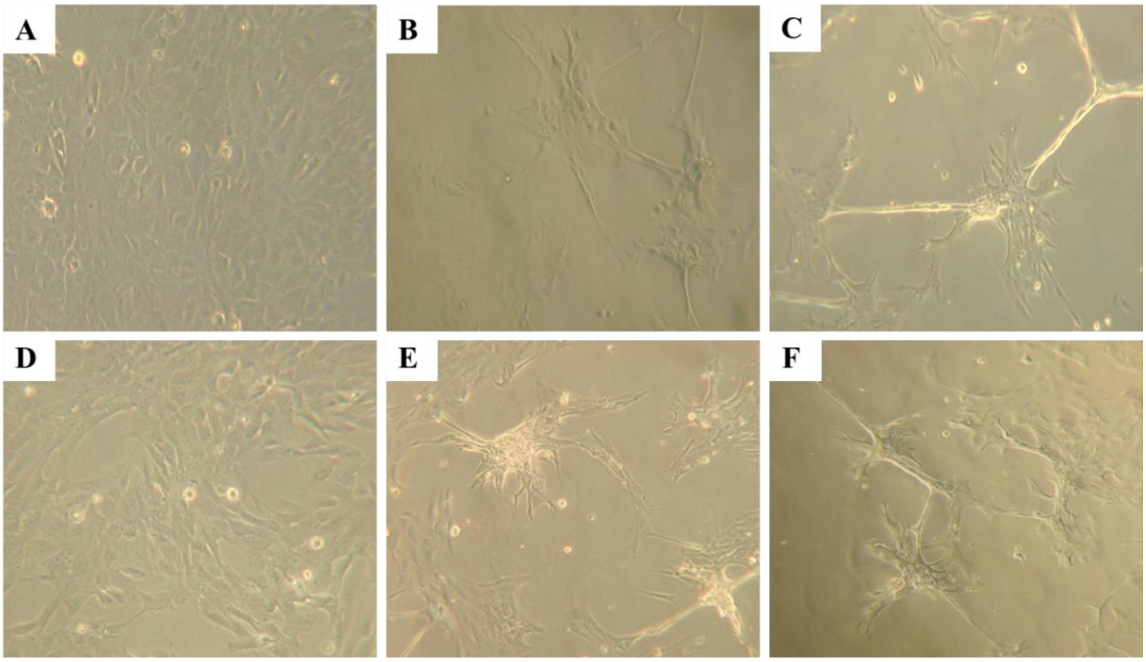


Figure 57. SGHPL-5 cells change phenotype with growth on Matrigel. Upper panel shows cells grown under normal culture conditions (20% O_2 level). (A) Control cells grown without Matrigel (48 h), (B) 24 h growth on Matrigel and (C) 48 h growth on Matrigel.

Lower panel cells were incubated under hypoxic conditions (1% O_2 level). (D) Control cells without Matrigel (48 h). (E) 24 h and (F) 48 h growth on Matrigel.

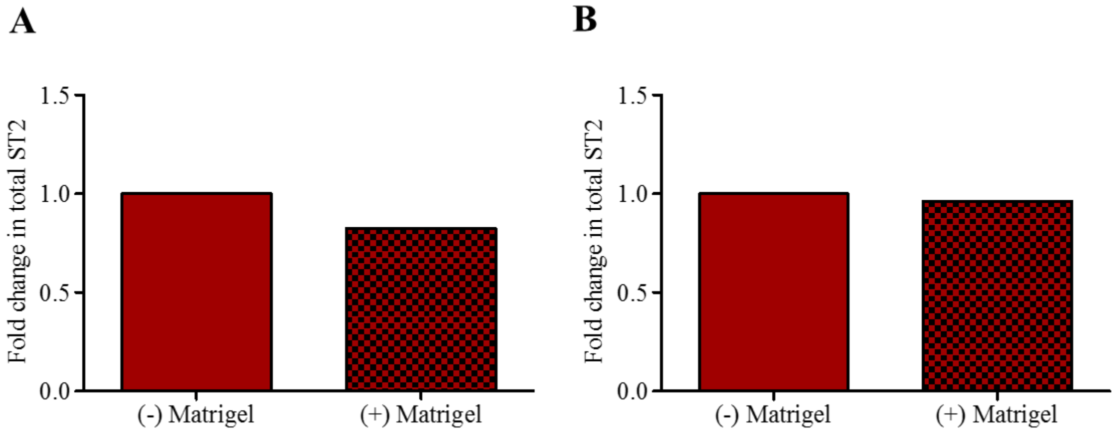


Figure 58. Fold change in total ST2 mRNA level in SGHPL-5 after 24 h growth on Matrigel. (A) Standard culture condition (20% O_2), (B) hypoxic culture conditions (1% O_2), (n=1).

3.3.5 Effect of cytokine treatment on BeWo and SGHPL-5 cells on IL-33 and ST2 gene expression

As the maternal-fetal interface is an environment rich in cytokines it was postulated that these molecules may enhance IL-33 and ST2 gene expression in BeWo and SGHPL-5 trophoblast cell lines. IL-4, IL-5 and GM-CSF have previously been shown to up-regulate IL-33 expression in eosinophils (170) and IL-4 induced ST2 expression in human endothelial and epithelial cells (273). Trophoblast cells express the toll like receptor-4 (TLR4) which acts through MyD88 intracellular signalling pathway, activates NF_kB and subsequently induces an immune response by increased cytokine secretion (274). TLR4 has the ability to recognize pathogen associated molecular patterns, such as bacterial lipopolysaccharide (LPS) (275). Activation of TLR4 by LPS in trophoblast cells results in the production of pro-inflammatory cytokines such as IL-6 and IL-8 (276).

Both BeWo and SGHPL-5 were treated with a combination of cytokines (IL-3, IL-4, IL-1α, IL-1β, TNFα (all at 100 ng/ml)), GM-CSF growth factor (100 ng/ml) and LPS for 24 h. While no change in total ST2 expression was detected in BeWo, these cytokines induced a two-fold increase in total ST2 mRNA in SGHPL-5 to that found in the control (untreated cells) (Figure 59).

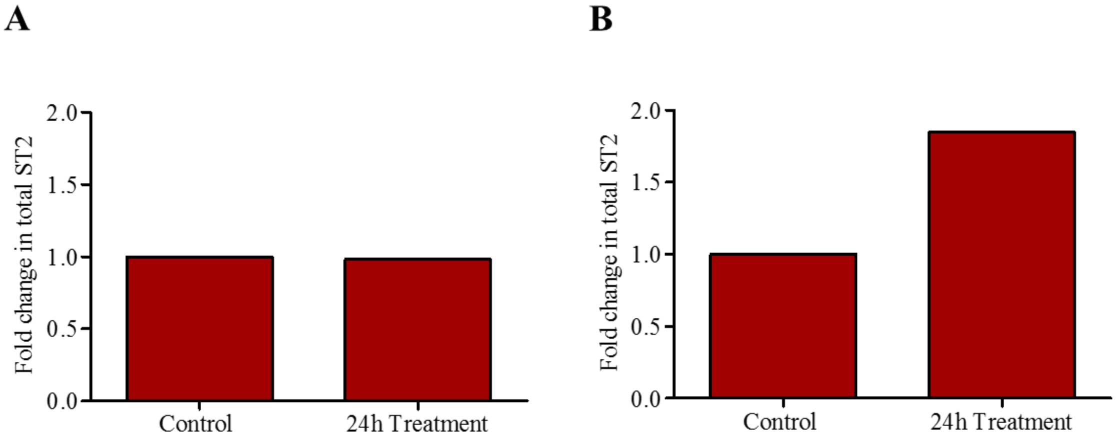


Figure 59. Change in total ST2 mRNA expression measured by qRT-PCR in BeWo and SGHPL-5 following cytokine treatment. BeWo (A) and SGHPL-5 (B) were subjected to 100 ng/ml of IL-3, GM-SF, IL-4, IL-1α, IL-1β, TNFα and LPS) for 24 h under normal culture conditions (n=1).

3.3.6 Serum concentration and growth factor effects on ST2 mRNA expression in the SGHPL-5 cell line

To test the possible effect of serum concentration on ST2 expression, SGHPL-5 were cultured with 1% or 20% FCS and gene expression was compared to control cells cultured with 10% FCS. As the very early stages of placental development take place under hypoxic conditions, treatments were also performed at 1% O_2. Under standard culture conditions, no change in ST2 gene expression was detected in any of the different FCS concentrations (Figure 60A). However, under hypoxic conditions, a three-fold increase in transcript expression was detected in cells cultured with 1% FCS (Figure 60B).

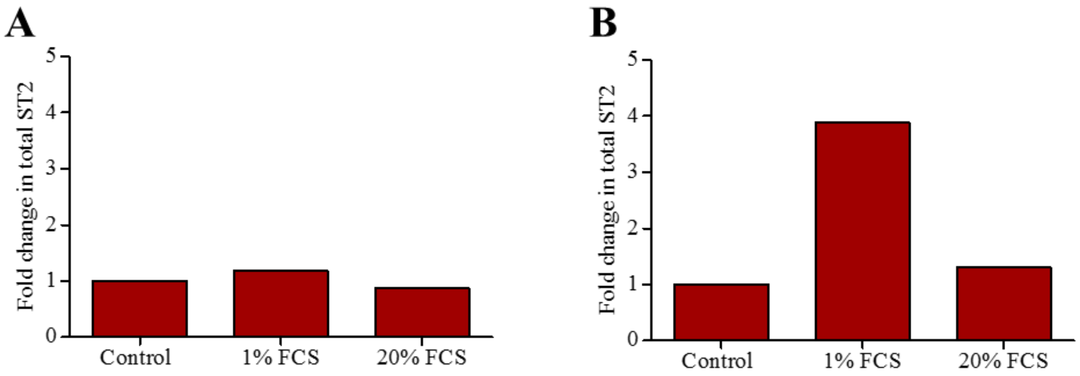


Figure 60. Serum concentration and its effect on total ST2 gene expression in SGHPL-5 cells. Cells were cultured for 24 h in 10% (control), 1% or 20% FCS in normoxic (20% O_2 standard) conditions (A) or 1% O_2 hypoxic conditions (B). ST2 mRNA levels detected by qRT-PCR, n=1.

The growth factor heregulin-1 (HRG) has been shown to induce an increase in ST2 mRNA and sST2 protein secretion in the metastatic breast cancer cell line SKBr3 (240). The receptor for HRG was found on EVT cells (266). In a similar approach to that performed on the breast adinocarcinoma cell line SKBr3 (240), SGHPL-5 cells were stimulated with 1nM of recombinant human HRG for 4, 8 and 24 h. Results displayed in Figure 61 shows a small increase in total ST2 mRNA level at all treatment time points but no major change in the level of expression was detected between the different treatment time points.

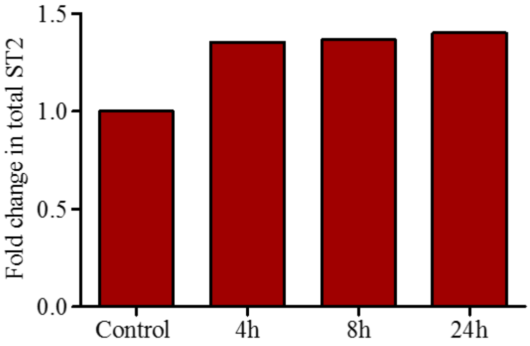


Figure 61. Effect of recombinant human heregulin-1 protein on total ST2 mRNA expression in SGHPL-5 cells. HRG (1nM) treatment of SGHPL-5 cells for 4, 8 and 24 h induce a small increase in total ST2 gene expression (n=1).

3.3.6.1 ST2 and IL-33 protein expression following stimulation experiments

The next step following gene expression analysis was to examine protein expression after treatments that induced an increase in the total ST2 transcripts. Here, BeWo induced to syncycialize with dbcAMP for 24 and 48 h, SGHPL-5 treated with the cytokine ‘cocktail’, SGHPL-5 cultured with 1% FCS under hypoxic conditions, SGHPL-5 cells grown on Matrigel and those treated with HRG were investigated by Western blot analysis for the expression of IL-33 or ST2 proteins. None of these stimulation experiments induced ST2 protein expression in the SGHPL-5 cell line (Figure 62 and Figure 63).

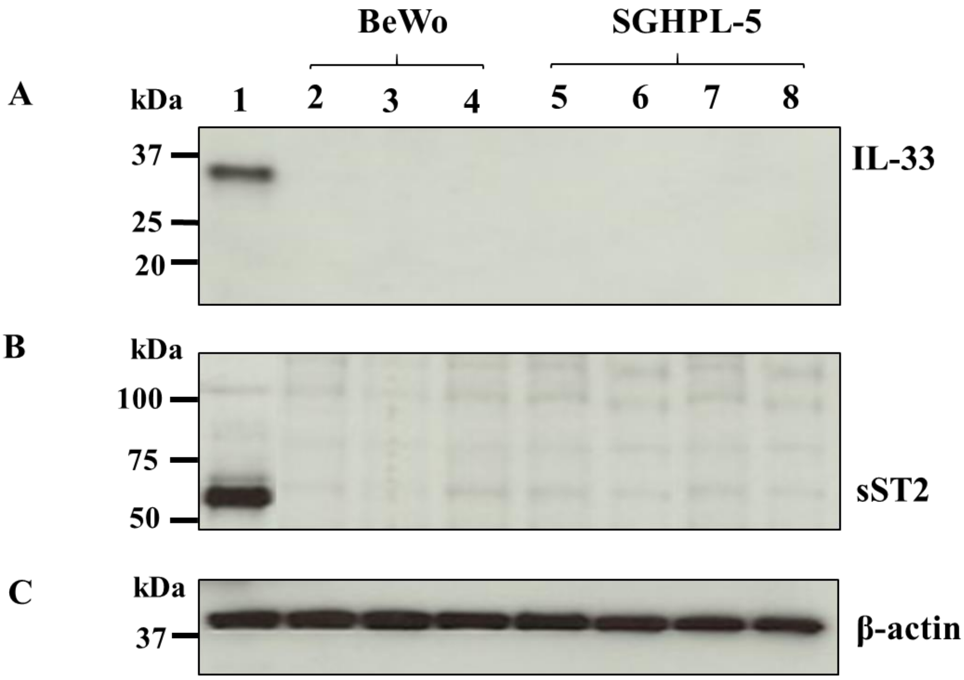


Figure 62. IL-33 and ST2 protein expression in BeWo and SGHPL-5 cell lines following the different stimulation experiments. BeWo or SGHPL-5 were analysed for IL-33 (A) or ST2 (B) expression by immunoblotting.

Control first trimester placental lysates (1). BeWo untreated cells (2), 24 h dbcAMP (3) and 48 h dbcAMP (4) treatments.

Untreated SGHPL-5 (5) and 24 h cytokine treatment (6).

SGHPL-5 cultured in hypoxic conditions in 10% FCS (7) and in 1% FCS (8). β-actin was used as a loading control (C), n=1.

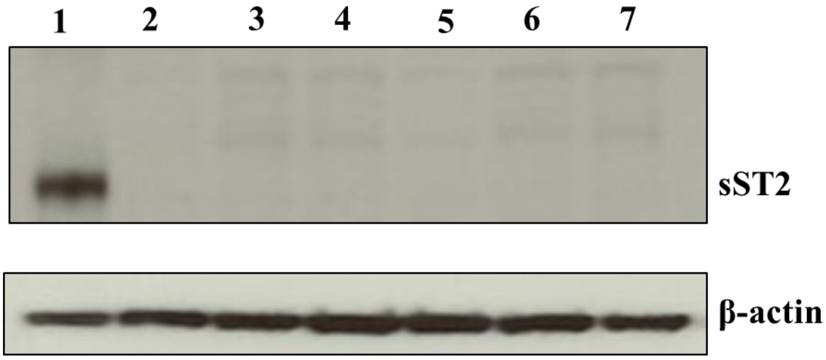


Figure 63. ST2 protein analysis by immunoblotting in SGHPL-5 cells following matrigel culture or heregulin-1 treatment. (1) Control first trimester tissue lysate. SGHPL-5 cultured without (2) and with Matrigel (3). HRG treated SGHPL-5 (5) 4, (6) 8, (7) 24 h and untreated cells (4).

3.3.6.2 IL-33 and sST2 secretion following stimulation experiments

Culture supernatant from all of the treated cells was assayed for IL-33 and sST2 secretion using the R & D systems ELISA. No secretion of IL-33 and sST2 was detected by any of the stimulated cells (data not shown).

3.3.7 sST2 protein binding to trophoblast cells

The experiments described above provided no evidence for the production of IL-33 and ST2 by the cells used in our experimental models. During these studies, a paper was published that suggested an alternative function of ST2 (240). Rather than acting as a membrane or a decoy receptor, sST2 can interact through an unknown receptor to stimulate cell motility independent of IL-33. sST2 binds the surface of breast cancer metastatic cells SKBr3 and induce cell motility (240). I hypothesized that sST2 may have a similar function on trophoblast cells. The remainder of this chapter followed the experimental strategy described by that group to determine the binding of sST2 to trophoblast cell lines by flow cytometry and its influence on cell invasion. Here, SKBr3 and RAJI cells were used as positive and negative control respectively. The actin regulatory molecule CAPG was used as a negative control and unlabelled and secondary antibody-only labelled cells were used to assess non-specific binding.

Human recombinant (hr) sST2 (Fc-sST2-His (R & D Systems)) protein associated with all trophoblast cell lines (Figure 64). The highest shift in mean fluorescence intensity was detected on SGHPL-5 cells indicating they are able to bind the highest level of sST2. sST2 did not bind to RAJI cells, but high levels associated to SKBr3 cells. The His-tag labelled only cells showed higher binding to SKBr3 cells, but this was not seen in any of the trophoblast cell lines.

SGHPL-5 cells were then used to test the binding of two different hrsST2 proteins (Figure 65) to further confirm the binding of recombinant sST2. sST2 binding was greater as recombinant protein levels increased (Figure 65). As expected, the CAPG control protein (contains His-tag) did not associate to SGHPL-5 indicating the specificity of sST2 binding.

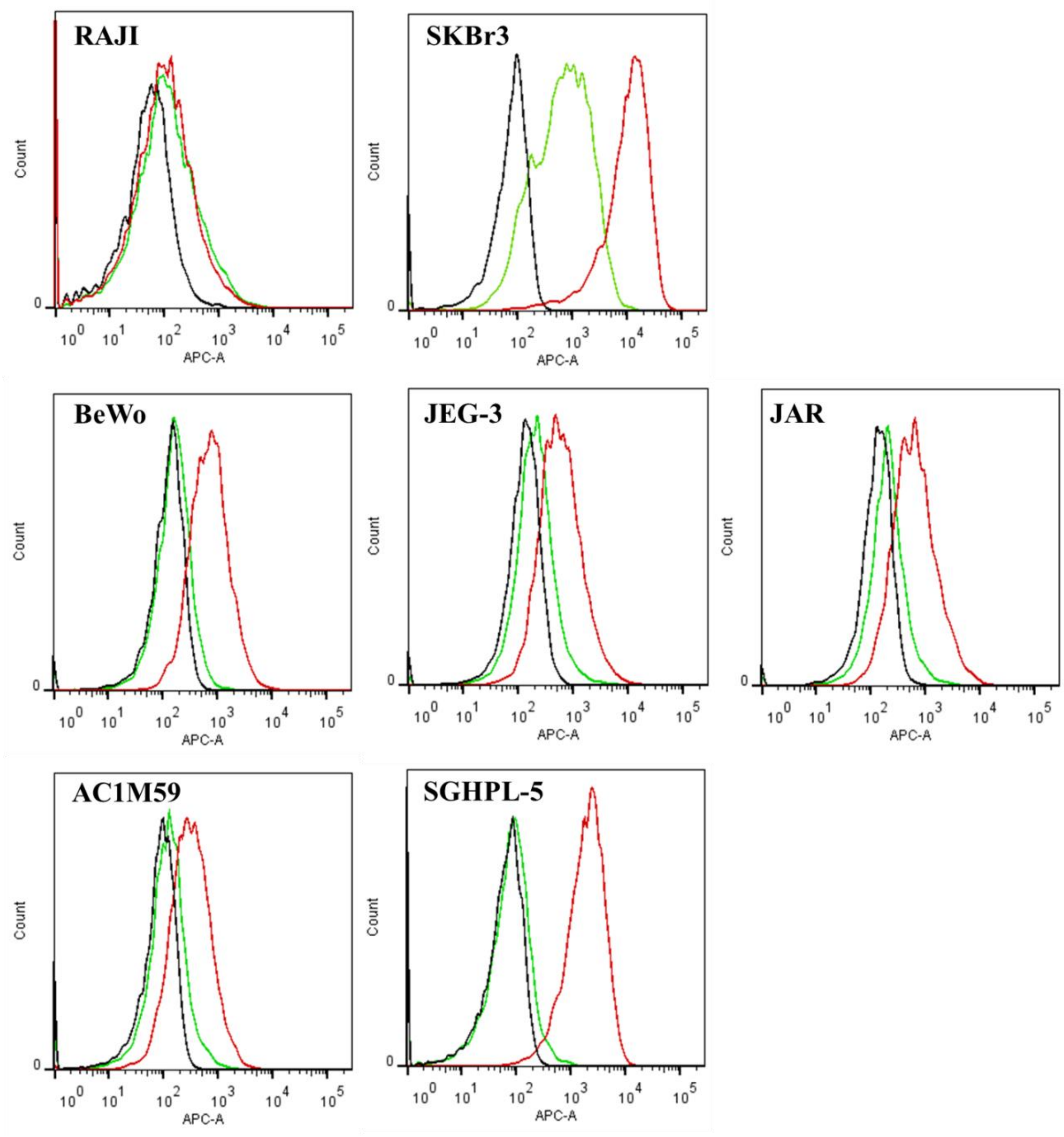


Figure 64. Recombinant human Fc-sST2-His binds the surface of all trophoblast cell lines. The graphs displayed are a representative of one out of 4 independent experiments. sST2 (red), anti-His APC antibody (cells stained with secondary antibody only (green) and unstained cells (black).

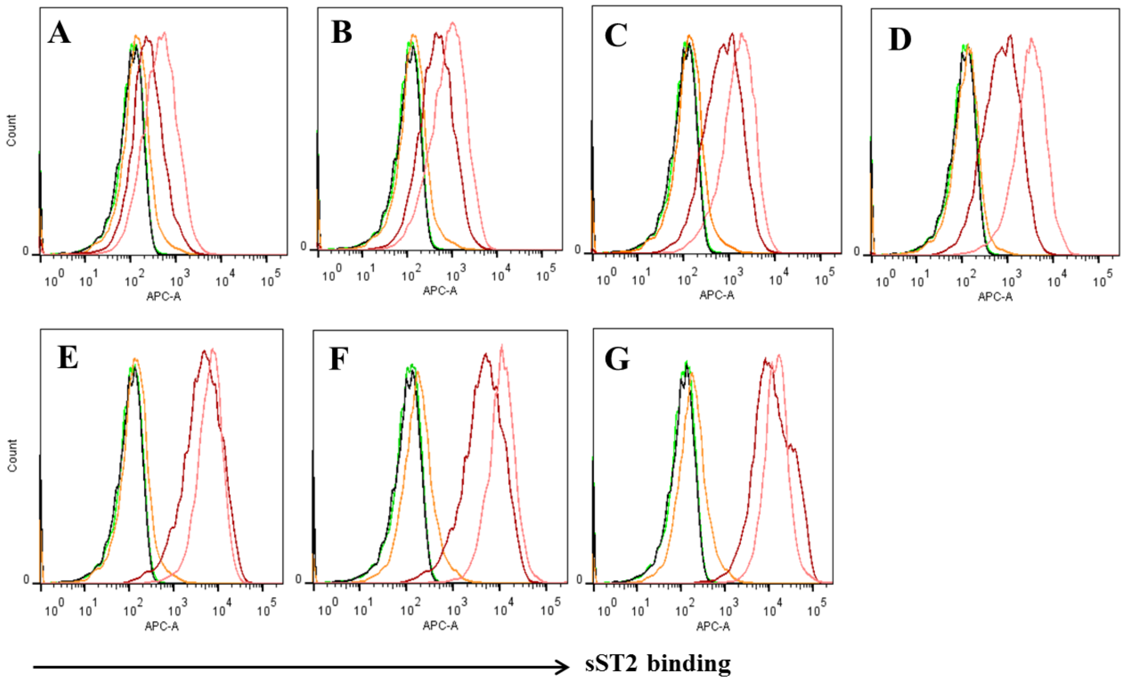


Figure 65. Titration of sST2 recombinant protein binding to SGHPL-5 cells. SGHPL-5 were incubated with either the Sino Biological Inc His-Tagged sST2 protein (dark red), R & D Fc-sST2-His-Tag protein (pink). CAPG negative control (orange), secondary antibody only (green) or left untreated (black). The concentrations of recombinant proteins from (A-G are: 78.125 ng/ml, 156.25 ng/ml, 312.5 ng/ml, 625 ng/ml,1.25 µg/ml, 2.5 µg/ml and 5 µg/ml), (n=1).

3.3.8 sST2 effect on trophoblast cell proliferation

The positive results obtained in the binding experiments and the unique localization of ST2 on primary first trimester placental tissue sections detected in Chapter 2 supported the idea that sST2 may be involved in regulating trophoblast functions such as proliferation and invasion. This section tested the effect of sST2 on trophoblast cell proliferation using BeWo and SGHPL-5 cell lines under normal and hypoxic O_2 conditions. Proliferation was measured using a colorimetric MTT assay. No significant changes in BeWo cell proliferation was observed under hypoxic and normal culture condition (Figure 66 A and B). However, at concentrations of 1 µg/ml and higher, sST2 significantly reduced SGHPL-5 proliferation. This reduction was observed under both hypoxic and normal culture conditions (Figure 66 B and C).

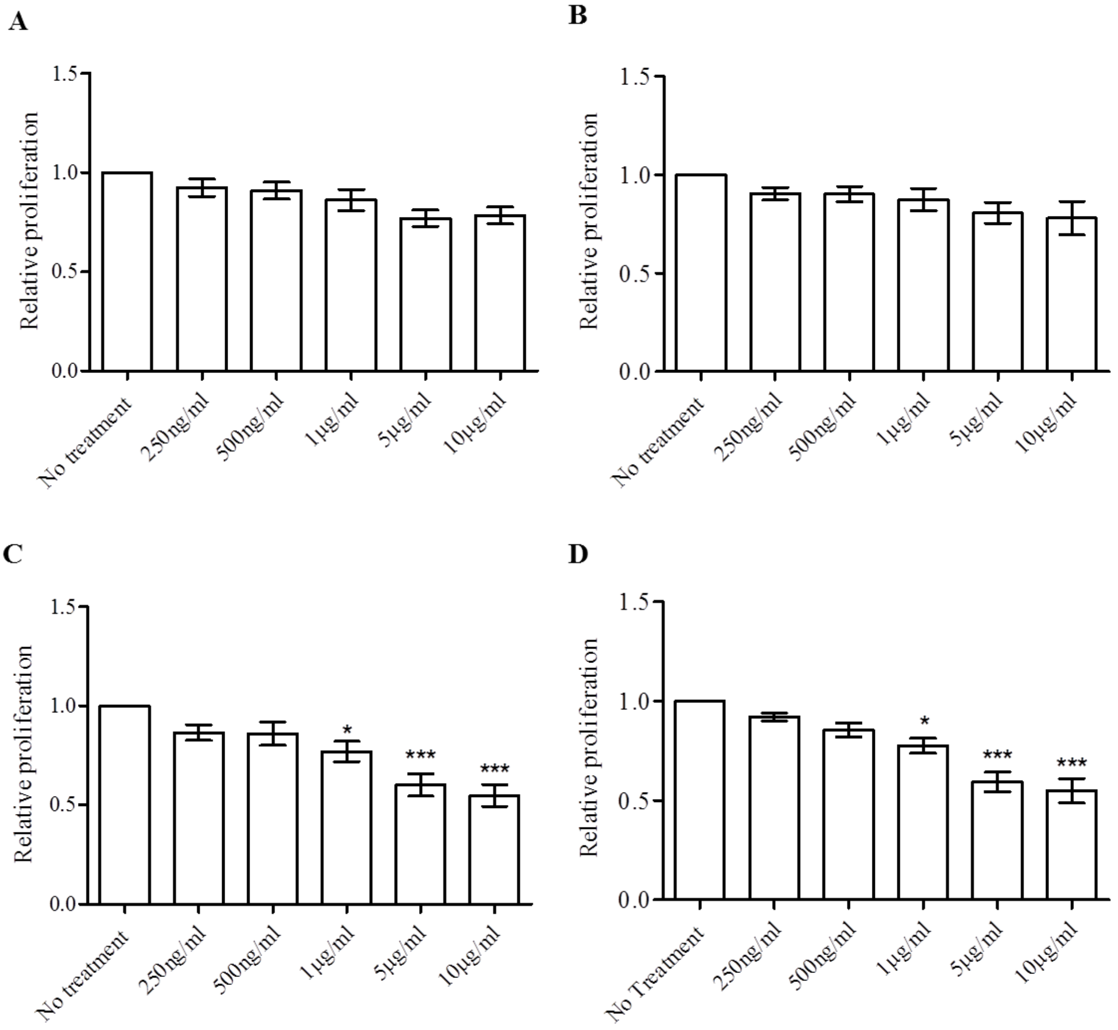


Figure 66. sST2 effect on SGHPL-5 and BeWo cell line proliferation tested by MTT assay (A) BeWo cells cultured under normal culture conditions. (B) BeWo cells in hypoxic conditions. (C) SGHPL-5 cells under normal culture conditions and (D) in hypoxic conditions. Cell proliferation was measured after 24 h of hr sST2 (R & D) treatment, n=4. Asterisks (* and ***) denote statistically significant differences between control and treated cells. p-value of <0.05 was considered significant * or highly significant (p<0.001) ***.

3.3.9 sST2 effect on SGHPL-5 spheroid invasion

Inhibition of proliferation is a step preceding differentiation into an invasive phenotype. As EVTs migrate away from the cell column towards the decidua they obtain an invasive phenotype and lose the ability to proliferate. As sST2 was found to inhibit SGHPL-5 trophoblast proliferation, the effect of human recombinant sST2 on SGHPL-5 spheroid invasion of fibrin gel was tested. Spheroid outgrowth in response to sST2, EGF and CAPG was tested over time from 4 to 20 h, representative images are shown in Figure 67. This analysis was performed twice and both experiments displayed clear outgrowth in cells treated with sST2 and the positive control protein EGF at 8 h of treatment. Longer treatment (Figure 67 C-E) up to 20 h resulted in an increased outgrowth size of the control (untreated cells) and the negative binding control (CAPG) (Figure 68). The effect of sST2 on spheroid outgrowth was evident in 4-16 h of treatment, at later time points sST2 induced outgrowth is close to that induced by the CAPG control. Therefore further experiments were analysed at the 8 h time point (Figure 69). Both the mean outgrowth length and the number of outgrowths were higher with EGF and sST2 treatment than in the controls, untreated and CAPG treated cells (Figure 70). The results obtained with the positive EGF control treatment were similar to that previously reported by our collaborators (personal communication, Dr Alison Wallace and Dr Judith Cartwright).

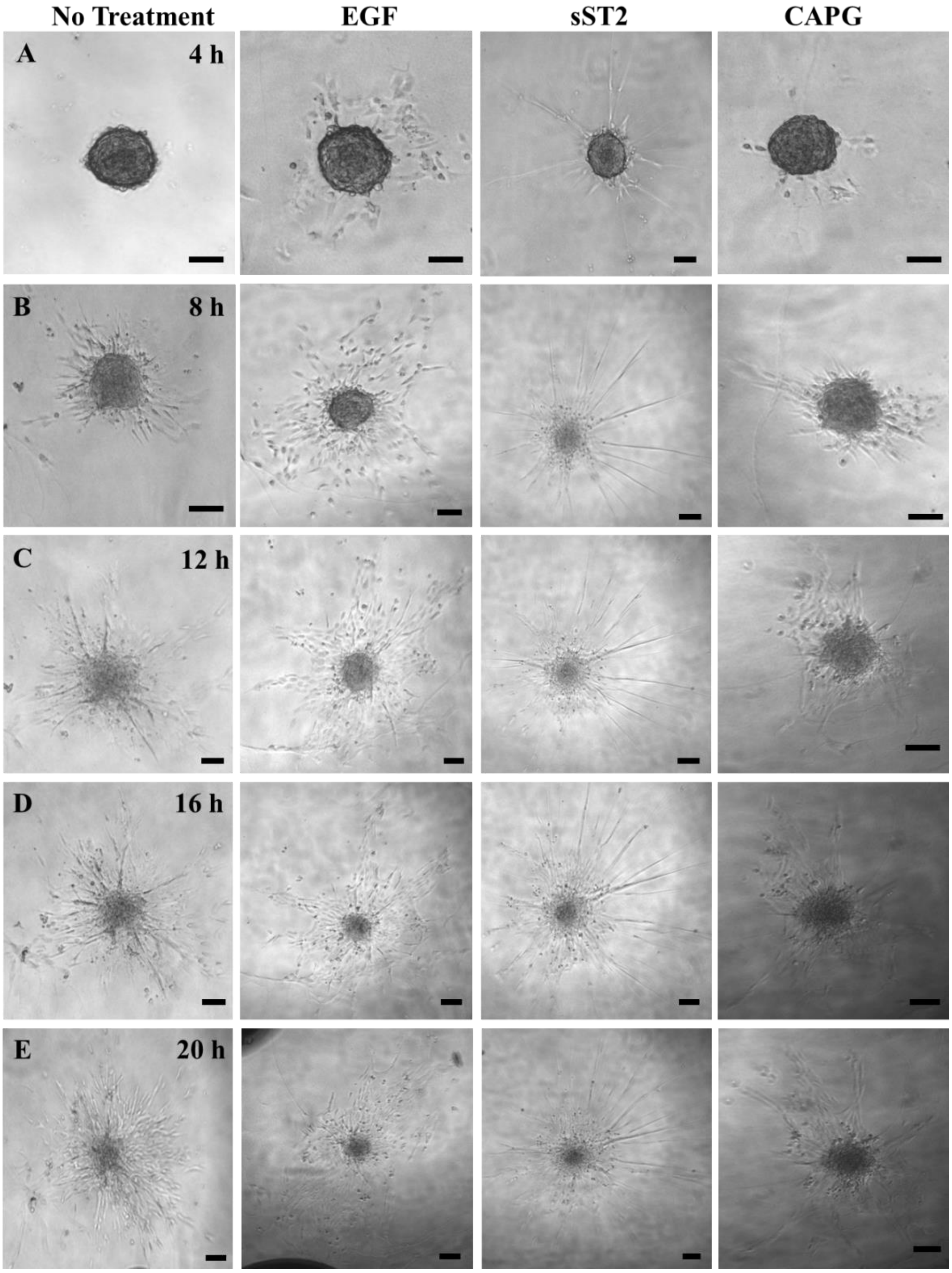


Figure 67. Time points for studying sST2 induced invasion of SGHPL-5 spheroids. SGHPL-5 spheroids were formed by the ‘hanging drop’ method. After 24 h of culture in complete growth medium, spheroids were plated on fibrin gel in 96-well plates and treatment with 10 ng/ml EGF (positive control, previously shown by Dr. Cartwright’s group to induce spheroids outgrowth), 100 ng/ml of sST2 (Sino Biological Inc) and 100 ng/ml of the negative control CAPG (Abcam) protein were used to test the effect of sST2 on SGHPL-5 spheroids at 4, 8, 12, 16 and 20 h. Images are a representative of two independent experiments, Scale bar = 100 µm.

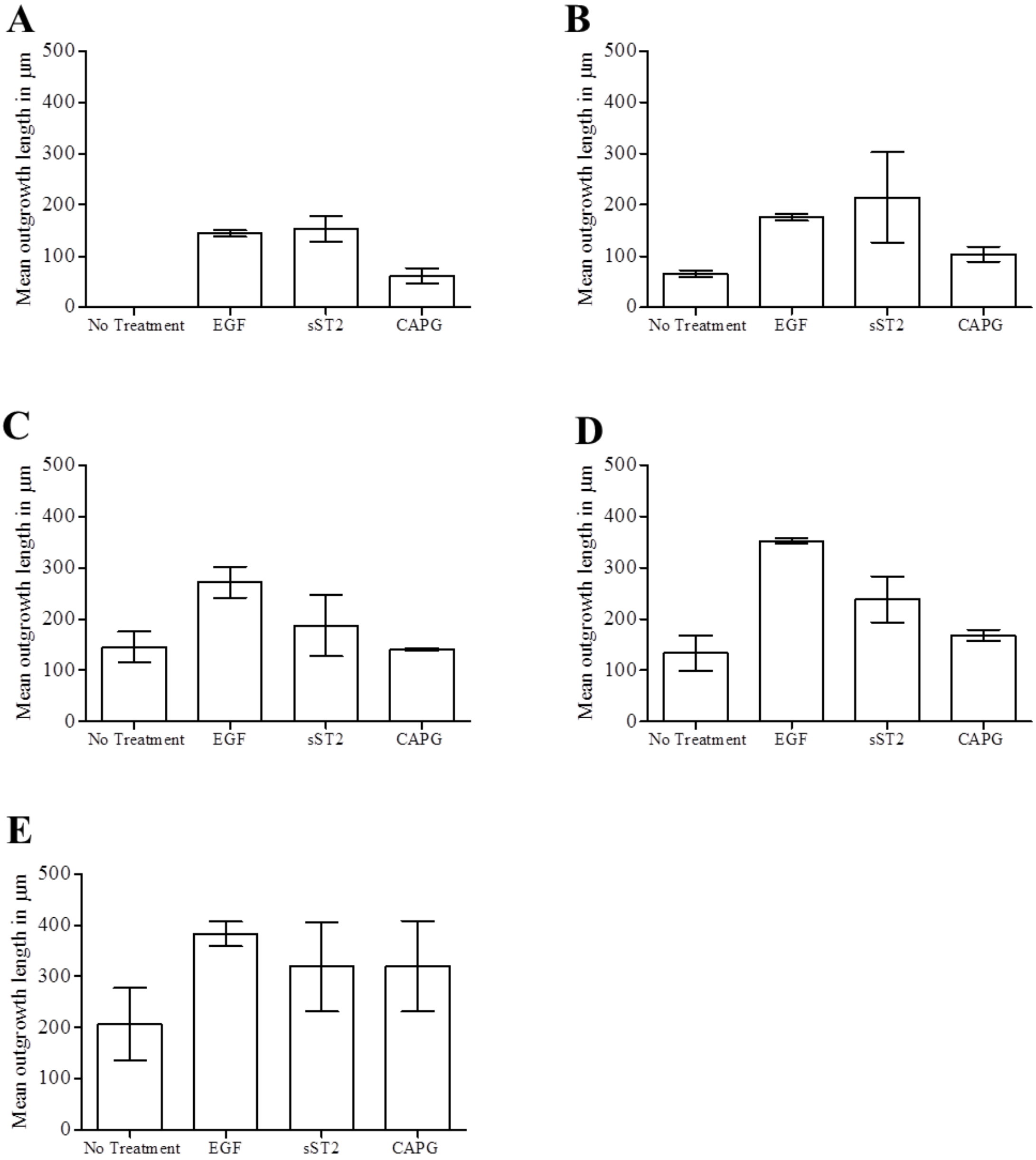


Figure 68. Time course analysis of SGHPL-5 spheroid outgrowth after EGF, sST2 and CAPG treatment. 4 (A), 8 (B), 12 (C), 16 (D) and 20 (E) h with sST2 human recombinant protein. Columns represent the mean of outgrowth length in two independent experiments and the error bars display the SEM.

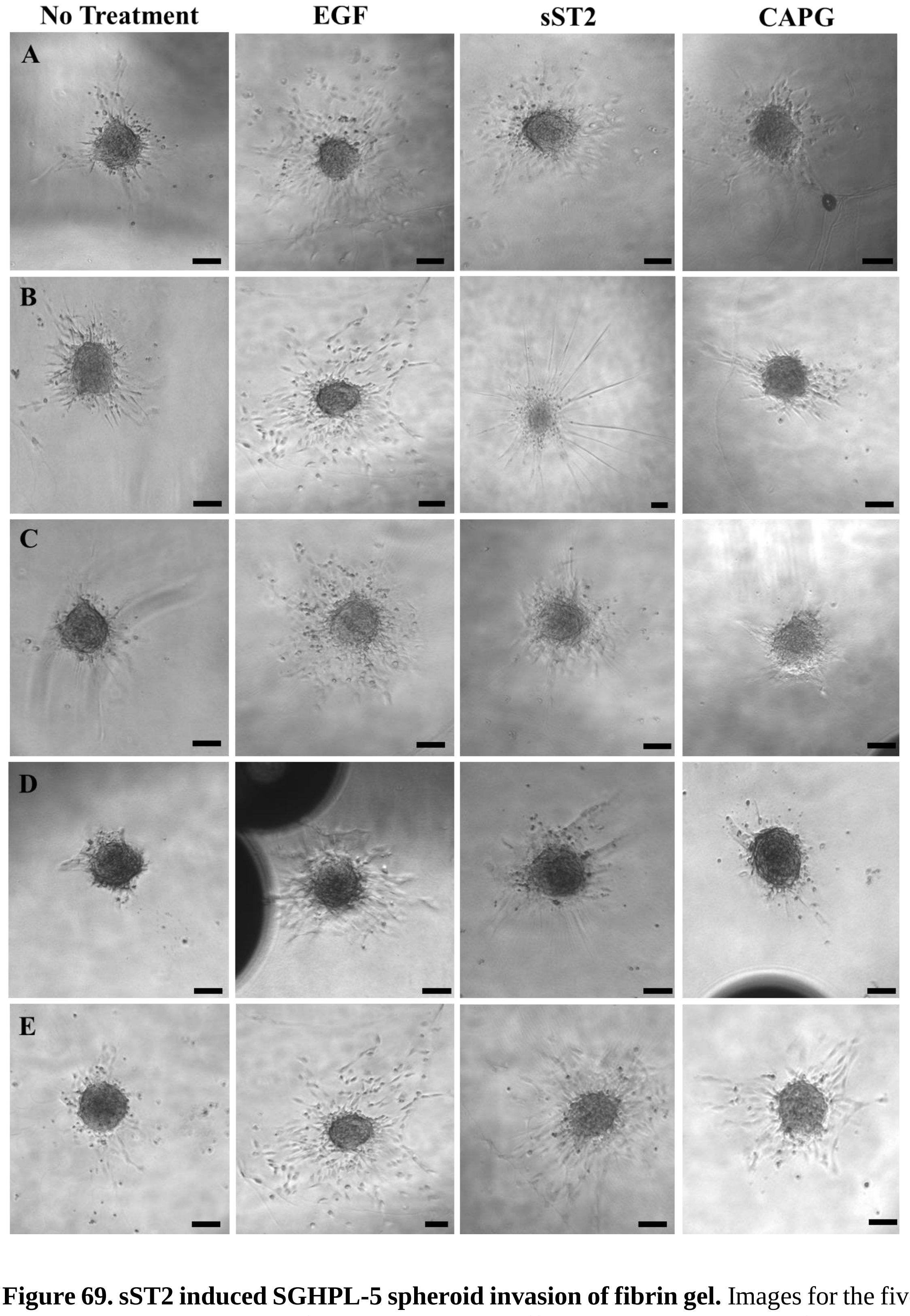


Figure 69. sST2 induced SGHPL-5 spheroid invasion of fibrin gel. Images for the five independent experiments performed to test sST2 effect on spheroid invasion following 8 h treatment. Variable degrees of outgrowth were induced by sST2 with spheroids in experiments (A), (B) and (E) displaying higher outgrowth than those seen in (C) and (D). Scale bar = 100 µm.

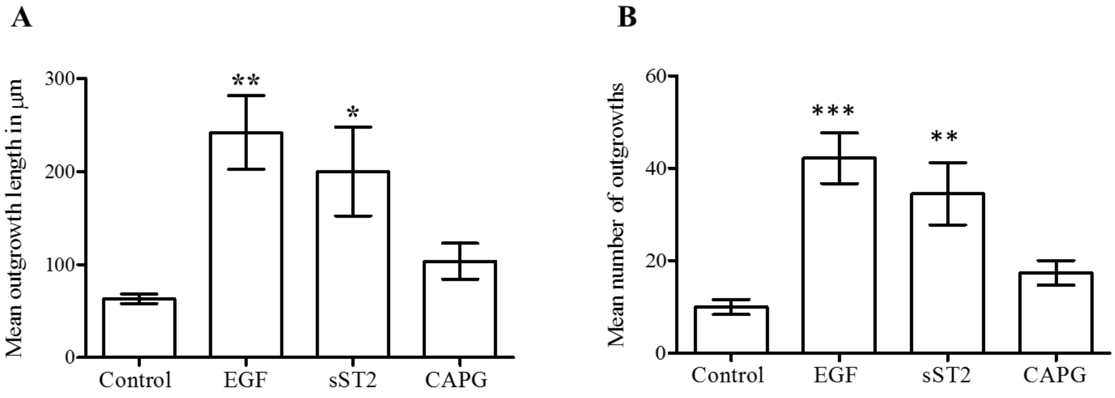


Figure 70. sST2 significantly increases SGHPL-5 spheroid outgrowth. Spheroids outgrowth length (A) and number (B) analysed after 8 h of control (untreated spheroids), EGF, sST2 and CAPG treatment. Columns represent the mean of outgrowth length in µm (A) or the number of outgrowths (B). Error bars represent the SEM of the 5 independent experiments. p-values of <0.05 were considered significant *, very significant (p <0.01) ** or highly significant (p<0.001) ***.

3.4 Discussion

The limited availability of first trimester placental tissues and their short lifespan demanded the use of cell lines to study the various trophoblast functions. In this chapter, trophoblast cell lines were explored as potential models for studying the role of IL-33 and ST2 in syncytiotrophoblast and extravillous CTB function. There are no available cell lines representative of trophectoderm. Although such cells from human embryonic stem cells have been induced by bone morphogenetic protein-4, they are not fully characterised in terms of functional activities (277). In this chapter, the trophoblast cell lines used, with the exception of SGHPL-5, reflect a carcinogenic cell type and differ from primary trophoblasts in many regards. Choriocarcinoma cells are malignant, immortal cells displaying non physiological carcinogenesis and they have developed mechanisms enabling them to escape from the control of the normal trophoblast invasion process (278).

3.4.1 IL-33 and ST2 mRNA transcript and protein expression by trophoblast cell lines

IL-33 mRNA and protein is expressed in first trimester placenta (Chapter 2). However, only low levels of IL-33 transcript expression were detected by qRT-PCR in all trophoblast cell lines and no protein expression or secretion was detected by immunoblotting or ELISA in cell lysates or culture supernatants of trophoblast cells grown under normal culture conditions.

ST2 is expressed on syncytiotrophoblast, underlying CTB, cell column trophoblast and EVT cells in the first trimester placenta (Chapter 2). However, most of the trophoblast cell lines examined here had low ST2 gene expression. Total ST2 mRNA was only evident in the SGHPL-5 cell line compared to that detected in all other trophoblast cell lines. The total ST2 transcript level detected in SGHPL-5 cells was similar to those found in first trimester placenta. This may be due to the fact that the SGHPL-5 cells are closer in their characteristics to primary trophoblast than the other trophoblast cell lines which are derived from choriocarcinomas. Different methods of cell transformation may impact gene and protein expression in the resulting cell line (279), which may explain this phenomenon and lack of ST2 mRNA in the majority of cell lines.

sST2 protein expression and secretion were not detected in any of the cell lines. It was possible that as these cell lines are not exposed to cytokines, chemokines and growth factors that are normally present at the maternal-fetal interface (280-283) they may have down regulated ST2 expression. Also, as trophoblast cells grow in a complex environment, we tried to recreate physiological conditions for trophoblast culture. This lead to the next group of experiments in which trophoblast cells were subjected to different factors and conditions present at the maternal-fetal interface to determine whether they could induce ST2 expression.

It is important to consider culture conditions and how different or similar they are to the normal physiology of *in vivo* cell growth, where a combination of many different factors, cells and oxygen tension (which changes at 10 weeks of normal placental development) may contribute to the difference in transcript and protein levels between primary cells and tissues and transformed cell lines.

Unlike the native trophoblast in the placenta, trophoblast cell lines express very low or undetectable levels of IL-33 and total ST2 mRNA, and neither are detectable at the protein level. Furthermore, treatments with different range of stimuli failed to increase the mRNA or protein expression of IL-33 and ST2. The close resemblance of SGHPL-5 cell line, in terms of total ST2 mRNA expression, to first trimester placenta distinguish these cells from the other carcinoma derived ones. We do not yet have an explanation of how ST2 protein production is inhibited in this cell line, but propose that the cell immortalization process (viral transformation) may cause some genetic changes responsible for halting the translational pathway associated with ST2.

3.4.2 Induction of ST2 expression in BeWo and SGHPL-5 cell lines

Trophoblast cells were treated to induce syncytialization, grown under different oxygen concentrations and treated with growth factors and cytokines, to determine whether such environmental factors may trigger ST2 up-regulation as follows.

3.4.2.1 BeWo fusion

No increase in ST2 transcript or protein expression or secretion was detected in BeWo 24 h after stimulating with dbcAMP cell fusion. A number of proteins such as the β subunit of human chorionic gonadotropin (hCG) and placental protein 13 (LGALS13 (member of the β-galactoside binding S-type galectin superfamily) have been shown to be up-regulated after trophoblast cell fusion (284). This however was not the case with ST2.

3.4.2.2 SGHPL-5 growth on Matrigel

To investigate the effect of extravillous CTB differentiation on IL-33 and ST2 expression, SGHPL-5 cells were grown on Matrigel. This has been previously shown to induce the expression of HLA-G and CD9 in primary trophoblast (285, 286). However, levels of ST2 transcript in SGHPL-5 cells grown on Matrigel under normal and hypoxic culture conditions did not change. Human primary trophoblast cultured on Matrigel for

48 h have been shown to increase surface c-ErbB2 and HLA-G expression, however the mRNA transcript level was not increased with 48 h culture on Matrigel (287). This finding indicates that induction of protein expression by Matrigel can take place without the need for an increase in the mRNA transcript. This however was not the case with SGHPL-5 grown on Matrigel as they did not express or secrete ST2 protein.

3.4.3 Cytokines, serum levels and growth factors effect on ST2 expression in BeWo and SGHPL-5 cells.

A number of cytokines, chemokines, and growth factors secreted by the various cells at the maternal-fetal interface promote trophoblast proliferation and differentiation. Previous studies have shown that IL-1β, TNF-α and LPS induced IL-33 expression in different human tissues and cells reviewed previously (92). IL-1α, IL-1β, and IFN-γ induced IL-33 expression by smooth muscle cells of human umbilical artery (220). The pro-inflammatory cytokines, TNF-α, IFN-γ and IL-1β, induced IL-33 mRNA and protein expression in cardiac myocytes, but no secretion was detected in unstimulated and undamaged *in vitro* cultured cells (288). Differential expression of IL-33 and ST2 was detected in primary amnion epithelial cells and HUVECs following treatment with IL-1β (211),with an increase in IL-33 and a decrease in ST2 mRNA. Therefore it might be possible to trigger a change in IL-33 and ST2 expression in trophoblast cells by treatment with pro-inflammatory molecules.

In this section, pilot studies were performed with a ‘cocktail’ of cytokines that have been shown to increase levels of IL-33 or ST2 in previous studies, as discussed above. BeWo and SGHPL-5 cells were treated with this combination of cytokines to test their effect on IL-33 and ST2 expression. No IL-33 gene or protein expression was detected in either BeWo or SGHPL-5 treated cells and the total ST2 transcript expression was not changed in BeWo treated cells to the control group. A small increase in total ST2 transcript was induced in SGHPL-5 cells after cytokine treatment, this however had no effect on the translation of protein as no intracellular or extracellular ST2 was detected.

3.4.3.1 Serum and growth factor induction of ST2 expression

sST2 was first identified as a serum and oncogene-induced gene in fibroblasts (88, 89). In a more recent report, an increase in sST2 mRNA and protein expression and secretion was found when metastatic SKBr3 cells were treated with heregulin-1 (HRG) (240), a growth factor that activates ErbB2 receptor tyrosine kinase, the over-expression of which is associated with aggressive (highly metastatic) form of breast tumours (289).

SGHPL-5 cells were treated with different concentrations of FCS under normal and hypoxic culture conditions. Cells were also treated with recombinant HRG for different time points. Normal culture conditions with variable FCS concentrations had no effect on total ST2 mRNA expression. However, a three-fold increase in the transcript was detected under hypoxic conditions with cells cultured in 1% FCS. Moreover, recombinant HRG induced a small increase in ST2 gene expression. However, the expression level after the different treatment times was not the same as that reported for SKBr3 cells (240). This group detected up-regulation of sST2 mRNA, protein expression and secretion during treatment times between 6-10 h, after which both mRNA and protein levels went down to the basal level, or close to that found in untreated cells. The results obtained here showed an increase in mRNA expression levels for HRG treated cells and maintenance of the same mRNA level at the 24 h time point. However, no ST2 protein expression or secretion was detected after the HRG treatment. This is a major difference between the two systems where the breast tumour cell line is, under normal conditions, able to produce low levels of sST2 which are enhanced with growth factor treatment, while, here, the trophoblast cells expressed ST2 mRNA, but this did not lead to the translation of this protein.

3.4.4 sST2 binding cell surface of trophoblasts

The binding of sST2 to the trophoblast cell surface was assessed by using two human recombinant sST2 proteins. Both recombinant proteins were shown to bind to the surface of SGHPL-5 in a concentration dependent manner. All other trophoblast cell lines displayed positive rhsST2 binding, but binding to SGHPL-5 was consistently higher than any other trophoblast cell line. As this cell line bears the closest resemblance of primary trophoblast, it is possible that *in vivo* sST2 is secreted by primary trophoblast cells and this secreted sST2 acts in an autocrine manner on different trophoblast subtypes, inducing either proliferation or invasion pathways at the different locations is it presented or secreted. Thus although the trophoblast cell lines were not a good model for protein expression and secretion of sST2, they retain a capacity to bind sST2 which is a specific feature of some but not all cell types.

Downstream effects of sST2 binding to trophoblast cells were investigated by looking at the proliferation and invasion processes, following treatment with the recombinant protein. rhsST2 had no significant effect on the proliferation of BeWo cells under normal and hypoxic culture conditions. On the other hand, SGHPL-5 cell proliferation was significantly reduced with increasing concentrations of rhsST2, under normal and hypoxic culture conditions. This observation was of significant relevance to the phenotypes induced under hypoxic and normal oxygen level. It is well known that early stages of placental and embryonic development take place in low oxygen environment (290). Hypoxia is critical for promoting trophoblast proliferation while normoxia or high O_2 levels inhibit proliferation and induce cell differentiation into an invasive phenotype (291). Therefore the results presented here suggest that sST2 inhibition of SGHPL-5 cell proliferation is possible mechanism for inducing invasion and cell migration.

Standard culture conditions do not reflect the normal *in vivo* growth of trophoblast cells, as CTBs interact with ECM and different cell types of the decidua (278). Spheroid invasion assays on fibrin gels are reported to be a more physiological way of determining SGHPL-5 invasion potential as they are cultured in a 3D-matrix (personal communication, Dr Judith Cartwright). In the 3D spheroid invasion assay, sST2 significantly increased both the length and number of outgrowths from the spheroids during the 8 h treatment period. We found similar positive effects of EGF on SGHPL-5 cell invasion as previously reported for SGHPL-4 cells (292, 293). The secretion of MMP9 is directly linked with trophoblast invasiveness, and MM9 has been shown to be up-regulated by EGF (294).

The mechanism employed by sST2 in inducing trophoblast cell migration in the SGHPL-5 spheroids model is not known. sST2 was reported to bind the surface of LPS activated monocytic cell line and inhibited the production of IL-6 cytokine (295). IL-6 was detected in primary CTBs, EVTs and various choriocarcinoma cells including BeWo, JEG-3 and JAR; reports on its effect however were conflicting (some indicate a positive promotion of proliferation and inhibition of invasion while others detect a role in promoting invasion) (74). It is therefore possible that sST2 may act on certain molecules which have a role in cell invasion and this will be further examined in future studies. sST2 protein has also been found to bind immature monocyte driven dendritic cells, and it is translocated from the cell surface to the cytoplasm where it inhibited signal transduction induced by LPS treatment (295). It remains uncertain what receptor sST2 binds to on the cell surface, but since trophoblast cell lines were able to bind sST2 they present a good model for studying a possible receptor.

3.4.5 Summary

Transformed trophoblast cell lines are not a good model for studying IL-33 or ST2 protein expression. However, they do retain a capacity to bind sST2 protein. This chapter demonstrated for the first time that sST2 was able to bind the surface of trophoblast cells. Functionally this has consequences for the trophoblasts, as sST2 causes SGHPL-5 proliferation inhibition and enhances SGHPL-5 spheroids invasion into fibrin gel.

Chapter 4

IL-33 and ST2 expression in human blastocysts and decidualized endometrial stromal cells

4.1 Introduction

It is clear that a maternal-embryo dialogue is essential for the initiation of embryo implantation and that the state of the blastocyst determines the implantation window there is however a long standing quest to identify specific embryo or endometrium derived signalling molecules that can functionally influence embryo implantation (65, 296).

In Chapter 2 it was shown that sST2 was present on invasive and non-invasive trophoblast of first trimester and term placentas as early as 6+1 weeks of gestation and sST2 secretion was detected from first trimester placental explants at 7+6 weeks of gestation. Further investigation in Chapter 3 identified a novel role for sST2 in trophoblast invasion.

Furthermore, ST2 gene and protein expression has been detected in mouse embryonic stem cells isolated from the inner cell mass (ICM) suggesting a role for ST2 in pluripotency and differentiation (297). The expression of ErbB2 has been shown in human blastocysts (298) and an increase in heregulin-1, activator of ErbB2, was detected in human endometrial stromal cells after decidualization (299). All of these observations suggest a possible involvement of ST2 in cell differentiation in the early embryo. Therefore the purpose of this chapter was to determine whether ST2 and its ligand IL-33 were present in pre-implantation human embryos and whether the endometrium and/or decidua could be a source of ST2 and IL-33 and play a novel role in embryo implantation and trophoblast invasion.

4.1.1 Aims of this chapter

1. To determine whether blastocysts express ST2 and IL-33 by qRT-PCR and immunofluorescence staining.
2. To determine whether ST2 and IL-33 are expressed in decidualized and non-decidualized endometrial stromal cells.

4.2 Materials and methods

4.2.1 Human blastocyst culture

Ethical approval for experimental use of human embryos was obtained from the Oxfordshire Research Ethics Committee (REF 04/Q1606/44) and the Human Fertilization and Embryology Authority (REF R0111). Embryos used in this project were donated for research with informed consent by patients treated in the Oxford Fertility Unit. Rapid thawing of embryos was carried out using the manufacturer's protocol for the sequential thawing media (Origio-Medicult Embryo Thawing Pack). According to the developmental day of the embryos (day 2 or 3 post fertilization), they were placed into either Sydney IVF Cleavage or Sydney IVF Blastocyst medium (Cook Medical) respectively. All embryos were cultured in 50 µl drops under oil (Origio-Medicult) at 37ºC in a humidified environment with 5% CO_2 in air. Human embryo culture and investigation were performed in the Nuffield Department of Obstetrics and Gynaecology and at the Institute of Reproductive Sciences, University of Oxford.

4.2.2 Blastocyst grading

Blastocysts were graded according to the criteria described previously (300). The grading was based on the degree of expansion (1-4 with the lowest grade (1) for early blastocyst and the highest grade (4) for fully expanded blastocyst with a blastocoel volume larger than half of the embryo). Blastocyst grading was based on the degree of development of the inner cell mass (ICM) and the trophectoderm (TE) layer, with each given a letter grade of A-C for the ICM, A= many tightly packed cells to C = a few, loosely packed cells; for TE, A = a cohesive layer comprised by many cells and C = loose epithelium layer comprised of a few cells. The quality of blastocysts used in this study were thus categorised into three groups; very good (4AA and above including hatching), good (3BB and above) and poor (2CC). This thesis examined the expression of IL-33 and ST2 in five very good, 23 good and nine poor quality blastocysts.

4.2.3 RNA isolation and whole transcriptome amplification

Once blastocysts were formed on day 5 or 6 post fertilization the zona pellucida was removed with acid Tyrode's solution and RNA was isolated using the RNeasy Plus Micro kit with genomic DNA eliminator columns (Qiagen), according to the manufacturer's instructions. RNA was eluted in a final volume of 12 µl and was quantified using the Nanodrop 1000 (Thermo Scientific). RNA was amplified as described in the protocol of the QuantiTect Whole Transcriptome Kit (Qiagen). The amplified cDNA was diluted 1/50 in H_2O and 2 µl of the amplified cDNA was used for the qRT-PCR reaction with cycling parameters as those described in the previous chapters using the same TaqMan gene expression assay (Applied Biosystems) sets for IL-33, ST2 and β-actin. The TaqMan gene expression assay for HLA-G (Hs03045108_m1) and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were used as positive controls. Also, a qPCR Human Reference cDNA (Clontech) was used to confirm positive detection of IL-33 and ST2 isoforms. Separate tubes with no-RT and a no cDNA template were used as negative controls.

4.2.4 IL-33 and sST2 secretion by human blastocysts

Conditioned culture media (50 µl drops) from 10 blastocysts were pooled for examination of IL-33 and sST2 secretion using a commercially available ELISA (R & D Systems). The conditioned culture medium from an additional 10 blastocysts was pooled and concentrated by a series of centrifugation steps using a Microcon 10 kDa centrifugal filter device (Millipore). The final volume was assayed for sST2 using the sST2 Presage assay following the manufacturer's instructions (Critical Diagnostics). Culture media alone was used as a negative control for all ELISAs performed.

4.2.5 Immunofluorescence staining of human blastocysts to detect IL-33 and ST2 by conventional and confocal microscopy

Blastocysts were fixed with 3% PFA for 10 minutes on day 5 or 6 post fertilization. They were washed and permeabilized for 10 minutes in permeabilization buffer (10 mM HEPES pH 7.4, 200 mM sucrose, 3 mM $MgCl_2$, 50 mM NaCl, 0.5% Triton X-100 and 0.2% Sodium Azide). Embryos were then washed with 1% BSA in PBS, blocked for 1 h at room temperature with 3% BSA in PBS and stained overnight at 4ºC with 2 µg/ml of antibody towards IL-33 (clone Nessy-1) (Enzo Life Sciences), ST2 (HPA007406) (Sigma-Aldrich) or the appropriate control antibodies; mouse IgG control or rabbit IgG control (Santa Cruz Biotechnology). Following this, embryos were washed three times for 5 minutes with 1% BSA in PBS and stained with 5 µg/ml antibodies towards mouse or rabbit IgG conjugated to Alexa Fluor 488 (green) or 594 (red) for 1 h at room temperature. Embryos were washed as before and transferred to a glass slide with 20 µl of Vectashield mounting medium with DAPI (Vector laboratories, USA). Embryos were examined with Leica DMIRE2 inverted fluorescence microscope and images were taken using a Hamamatsu Orca monochrome camera with Simple PCI software (C Imaging). Confocal microscope images were taken with an Olympus Fluoview 1000 with assistance from Dr Alan Wainman (University of Oxford). Confocal microscopy images were analysed using FIJI software (Distribution of ImageJ).

4.2.6 Human endometrial stromal tissues for isolation of endometrial stromal cells

Endometrial stromal tissue samples were obtained with informed consent in accordance with the ethical requirements of the Central Oxford Research Ethics Committee (REF C02.358). Endometrial tissue was isolated from three fertile patients with regular menstrual cycles after undergoing hysterectomy or sterilization for benign reasons. None of the patients had hormonal treatments for three months prior to tissue sampling, which was obtained during the mid-secretory stage (days 20-22) of the menstrual cycle. Endometrial stromal cells were isolated by collagenase digestion and purified through a percoll gradient according to the protocol described previously (301). Cell isolation was performed by Ms Janet Carver (University of Oxford).

4.2.7 Endometrial stromal cell culture and decidualization

Endometrial stromal cells were cultured in Dulbecco's modified essential medium supplemented with 10% FCS (PAA Laboratories), 100 IU/ml penicillin and 100 µg/ml streptomycin and maintained at 37ºC in a humidified environment with 5% CO_2 in air. Decidualization of endometrial stromal cells was induced by treating confluent cells with 0.5 mM 8-Br-cAMP (a cAMP analogue) with or without 1 µM Progesterone for 2 to 8 days with media changed every 48 h. Cells were used between passages 3 and 5.

4.2.8 Gene and protein expression in endometrial stromal cells

$1x10^5$ cells were plated in 4-well dishes and following 8-Br-cAMP or progesterone treatments, they were washed with PBS, lifted with Accutase (PAA Laboratories), pelleted and frozen at -80 °C for RNA extraction or protein analysis. RNA isolation, reverse transcription, quantitative gene expression and Western blot analyses were performed as described in Chapter 2.

4.2.9 ELISAs to measure IL-33 and ST2 in conditioned culture media from endometrial stromal cells

Following treatment times (indicated in each experiment), conditioned culture media was centrifuged at 10,000 x g for 10 seconds and the cell-free medium was frozen at -80°C until analysed by ELISA. Cell differentiation was confirmed by the secretion of the decidualization marker prolactin from endometrial stromal cells using a commercially available ELISA (R & D Systems). The presence of IL-33 or sST2 in the culture supernatant of decidualized and non-decidualized stromal cells was examined by using commercially available ELISAs (R & D Systems) according to the manufacturer's instructions.

4.2.10 Statistical analysis

Data are plotted as the mean only or the mean +/- the standard error of the mean using Graphpad software.

4.3 Results

4.3.1 IL-33 and ST2 expression in human blastocyst

The first aim of this chapter was to determine whether IL-33 and ST2 are present during the early embryonic stages of pregnancy. The presence of IL-33 and ST2 in human blastocysts was examined by using qRT-PCR and immunofluorescence analysis. Furthermore, the secretion of both molecules was analysed by ELISA.

4.3.1.1 Analysis of IL-33 and ST2 gene expression in human blastocysts

As the number of frozen IVF embryos donated for research was limited, examination of mRNA transcript expression included poor quality embryos along with good ones. Therefore day 5 blastocysts of good to poor quality were lysed in 50 µl RNA lysis buffer and RNA was isolated from pools of 3-5 blastocysts. The amounts of RNA isolated from the blastocysts were too low to perform conventional reverse transcription analysis used for cells and tissues described in earlier chapters. Therefore it was necessary to first amplify the mRNA to high enough levels to enable the detection of the fragments of interest. The QuantiTect Whole Transcriptome Kit (Qiagen) was used to achieve amplification of all mRNA transcripts. The kit should uniformly amplify as little as 1 ng RNA, so that the relative abundance of each transcript is preserved (preventing unbiased results in transcript quantification). Amplified cDNA was subjected to a qRT-PCR reaction protocol with cycling parameters as those used in Chapters 2 and 3.

Quantitative analysis of all the mRNA transcripts examined following amplification of 20 ng RNA (Figure 71) only detected signals for the control house-keeping gene β-actin. This representative plot is of a qRT-PCR of a pool of 3 good and two poor quality blastocysts. No signal for IL-33, total ST2, sST2 or ST2L was detected.

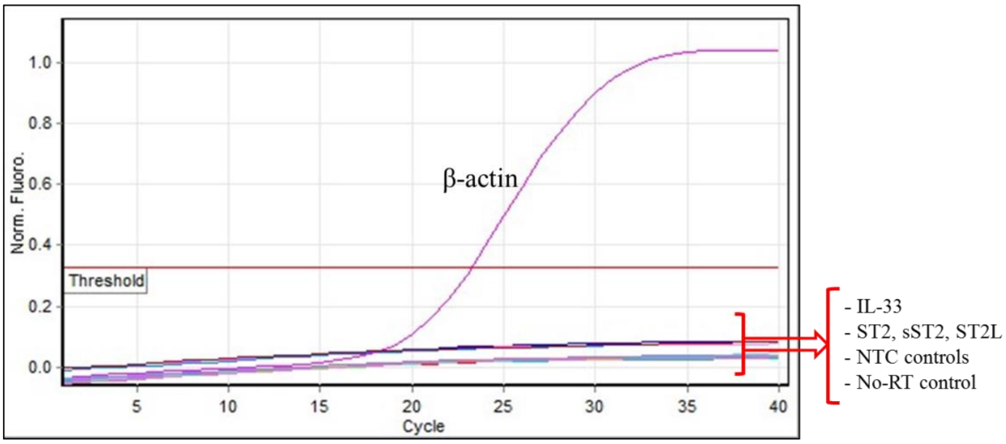


Figure 71. qRT-PCR amplification plot of pooled blastocysts for the detection of IL-33 and ST2. RNA was isolated from a pool of five blastocysts. 20 ng of RNA was subjected to reverse transcription, cDNA amplification and qRT-PCR. A rise in the fluorescence signal above the threshold level was detected for β-actin. The signals from IL-33, total ST2, sST2, ST2L and the negative controls remained below the threshold level.

Following that, two pools of five and three good quality blastocysts respectively were examined for their expression of IL-33 and ST2 by qRT-PCR (Figure 72). In the same qRT-PCR reaction, a reference cDNA was used as a positive control for the detection of IL-33 and ST2 isoforms. β-actin was detected in both the five and three pooled blastocyst samples, with no signal detected for IL-33 and ST2 in either. The control reference cDNA, as expected, gave a positive signal for IL-33, total ST2, sST2, ST2L and β-actin. Additional positive controls were included in the next experiment to ensure that the qRT-PCR could detect other genes such as GAPDH and HLA-G (Figure 73). 30ng of RNA isolated from five good quality blastocysts was subjected to reverse transcription, cDNA amplification and qRT-PCR. This preparation was positive for GAPDH, β-actin and HLA-G, but not IL-33 total ST2, sST2 or ST2L. Negative controls in all preparations remained at levels lower than the threshold.

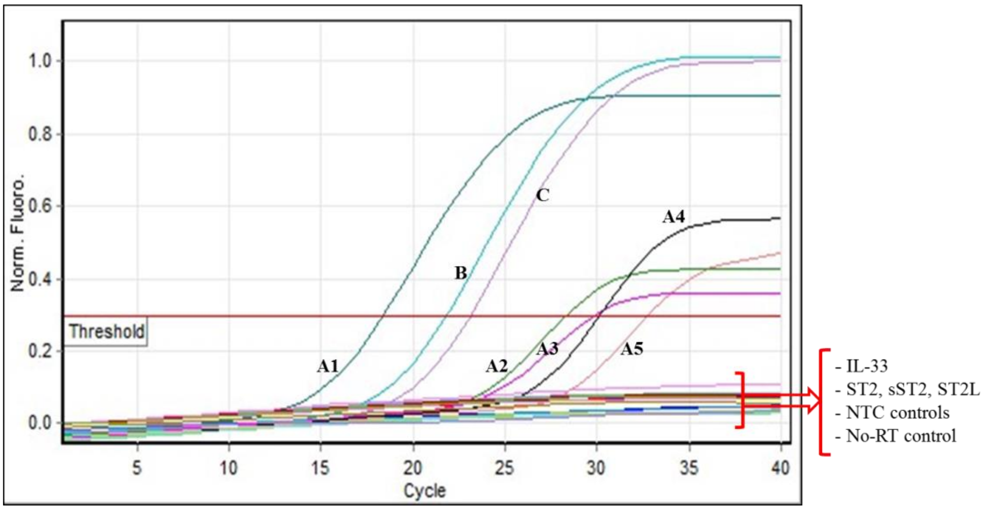


Figure 72. Detection of IL-33 and ST2 by qRT-PCR in human blastocysts. β-actin signal (A1), total ST2 (A2), sST2 (A3), ST2L (A4) and IL-33 (A5) for the reference human cDNA. 20 ng of RNA from pooled blastocysts of good quality was reverse transcribed and following cDNA amplification, a fluorescence signal from the qRT-PCR detected β-actin in five (B) and three (C) blastocysts. Signals for IL-33, total ST2, sST2 and ST2L remained under the threshold in the region where the negative controls were detected.

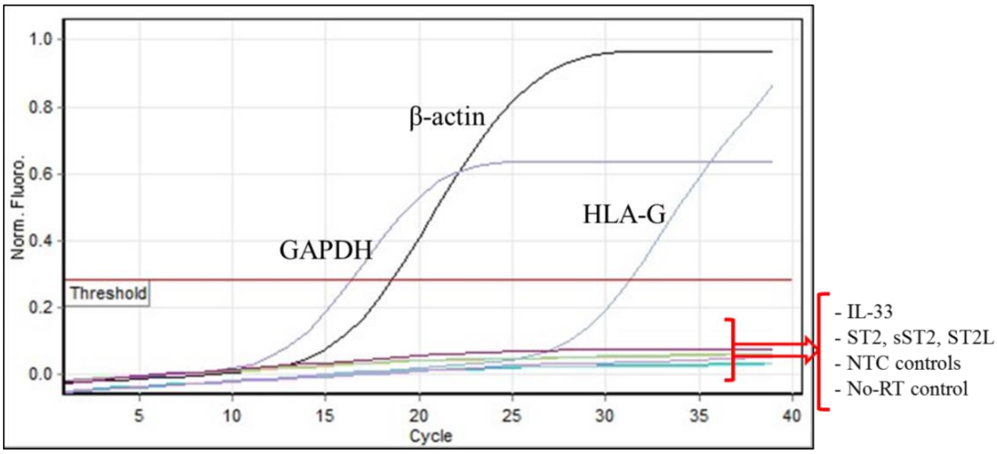


Figure 73. IL-33 and ST2 transcript detection in human blastocyst by qRT-PCR. The fluorescence signal for GAPDH, β-actin and HLA-G was higher than the threshold level in 30 ng of RNA isolated from a pool of five good quality blastocysts in two separate experiments. The fluorescence signal for IL-33, total ST2, sST2, ST2L and the negative controls remained below the threshold.

4.3.2 Immunofluorescence analysis of IL-33 and ST2 expression by human blastocysts

The challenges associated with studying mRNA expression in embryos in this study were not only limited to the number of frozen IVF embryos donated for research, but also to the quality of the blastocysts. Examination of transcript expression was therefore limited to blastocysts ranging from good to poor quality; this eventually affects the amount of the starting material available for carrying out the qRT-PCR reaction. Taking into consideration the technical challenges associated with transcript detection in embryos, detection of molecule expression should not only be based on gene analysis, but should include protein expression by IHC and ELISA. IHC has the advantage of localizing cellular sources of protein expression. In this section, IHC was performed on day 5 and 6 blastocysts either of very good, good or poor quality. Blastocysts were stained with antibodies towards IL-33 and ST2 or the appropriate isotype negative controls. The initial experimental approach using conventional fluorescence microscopy suggested possible ST2 protein localization around the cellular boundaries on blastocysts (Figure 74B). This staining pattern was not seen in the isotype control (Figure 74A). However, this ST2 localization pattern was only observed occasionally in very good and good quality blastocysts as opposed to poor quality blastocysts, as shown by further analysis with confocal microscopy.

It was decided that a positive signal in images taken by confocal microscopy was based on the detection of a specific localization pattern with fluorescence signal intensity higher than that detected in the cytoplasmic region. IL-33 displayed a diffuse signal that was equally distributed in ICM and TE regions with no special localization pattern (Figure

75). Further repeats of IL-33 staining showed a similar diffuse signal over all the blastocyst (data not shown), suggesting that the signal was non-specific.

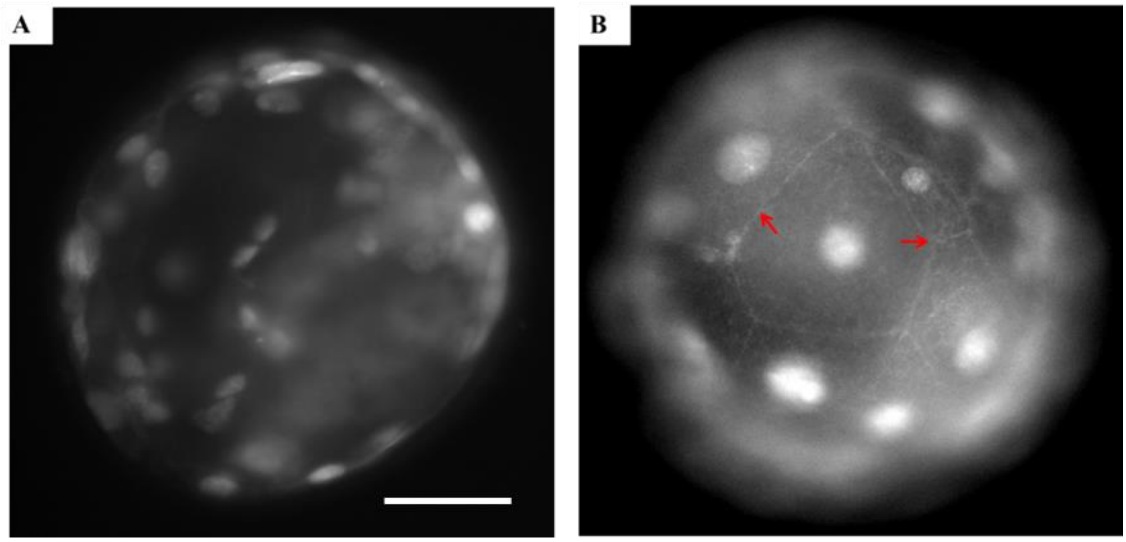


Figure 74. ST2 Immunofluorescence signal detected in human blastocysts using conventional fluorescence microscopy. (A) Isotype control and (B) ST2. Images were taken at x20 magnification. Red arrows point to ST2 positive cellular boundaries. White circles are DAPI stained nuclei. Scale bar = 50 µm.

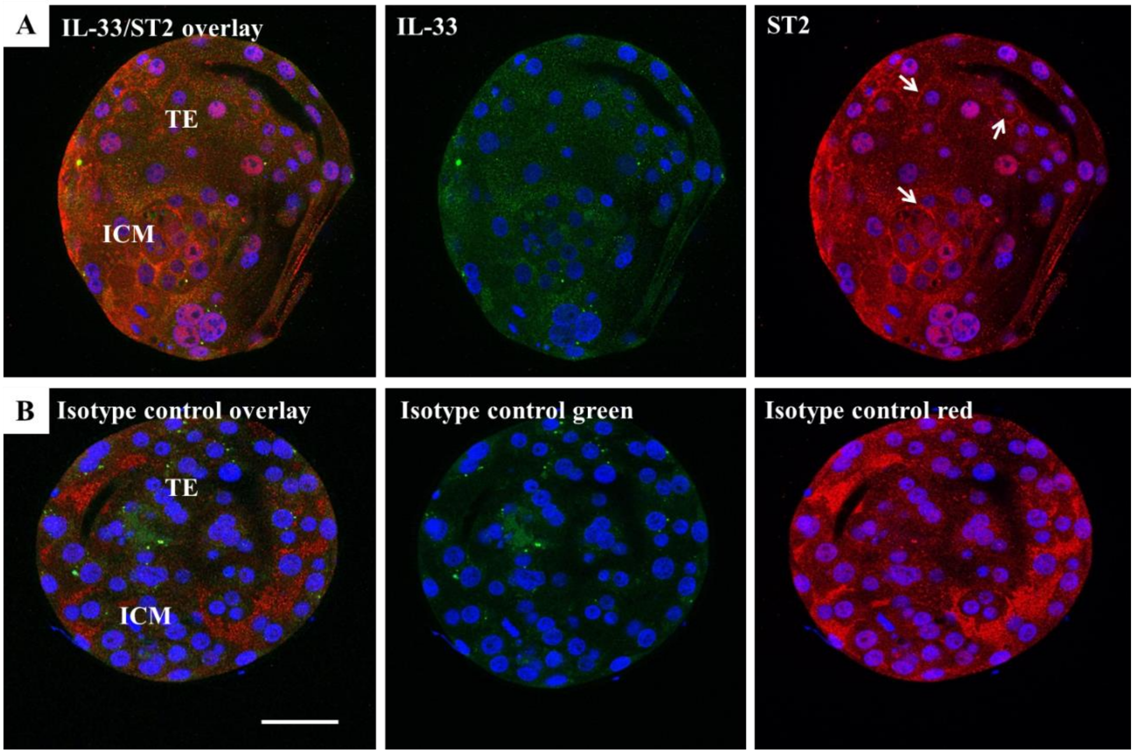


Figure 75. Confocal microscopy of images showing IL-33 and ST2 localization in human blastocysts. (A) Overlays of ST2 (red), IL-33 (green) and DAPI (blue) on expanded blastocysts. (B) Isotype control overlay with green, red and DAPI (blue) channels. White arrows point to cellular boundaries. ICM: Inner cell mass. TE: trophectoderm. Scale bar = 50 µm.

ST2 protein was detected on a number of blastocysts as shown in images taken by confocal microscopy (Figure 75, Figure 76 and Figure 77). ST2 was seen on the cell membranes of the blastocyst primarily on cells of the ICM, but also on cells of the trophectoderm. ST2 localization was detected in 70% (7/10) of blastocysts examined, of very good to good quality. This localization pattern was particularly intense in ICM regions of very good quality blastocysts (Figure 76A). Good quality blastocysts also displayed an intense ST2 signal on TE cellular boundaries where the cells were dense and tightly packed, but not in regions with fewer numbers of cells (Figure 76B).

The ST2 signal was less evident (Figure 77A) or undetectable (Figure 77B) in poor quality blastocysts. No staining with the same localization pattern was seen in the negative isotype controls (Figure 78), confirming the specificity of ST2 staining on the cellular boundaries.

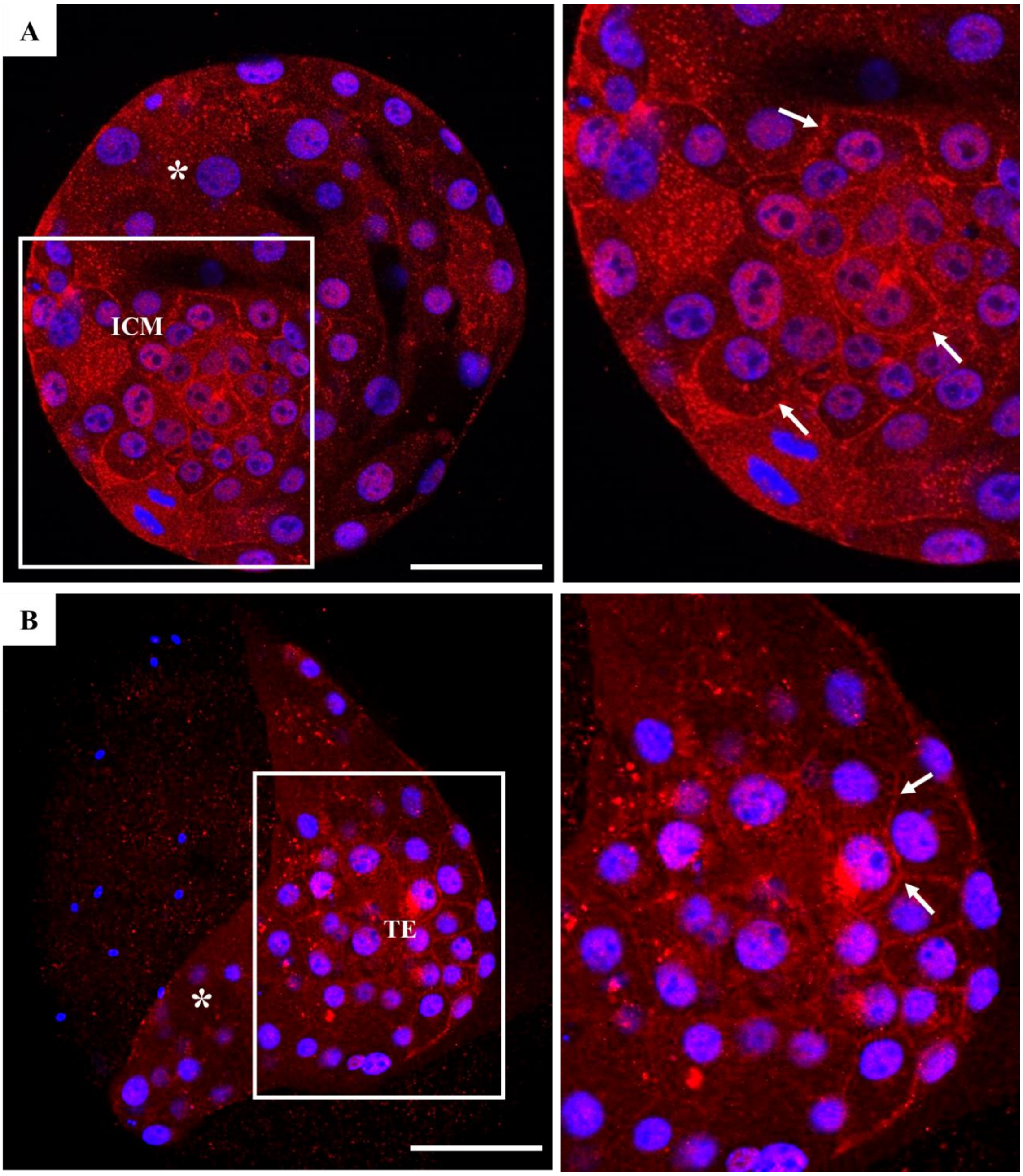


Figure 76. ST2 localization to ICM and TE in human blastocysts detected by confocal microscopy. To the left, overlays of ST2 (red) and nuclear staining with DAPI in very good quality (A) and good quality (B) blastocysts. White square inserts in (A) and (B) are enlarged on the right. * indicates regions with lower cell number and ST2 signal intensity. White arrows point to cell boundaries expressing ST2. Scale bar = 50 µm.

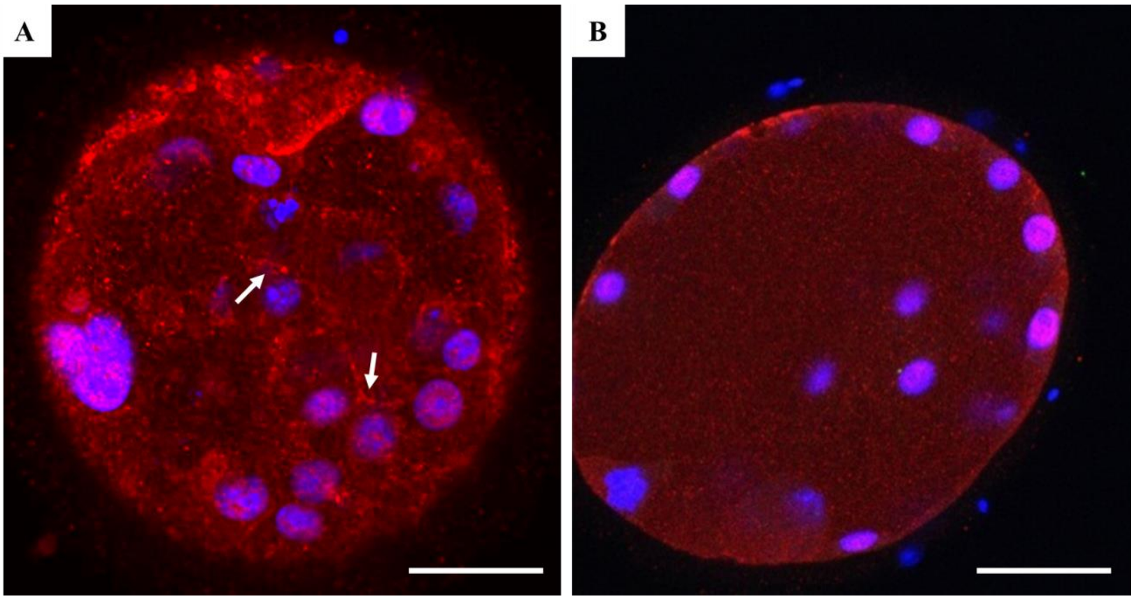


Figure 77. ST2 localization in poor quality embryos detected by confocal microscopy. Overlays of ST2 (red) and nuclear stain DAPI (blue) on two different embryos (A and B). ST2 signal on cellular boundaries indicated by white arrows. Scale bar = 50 µm.

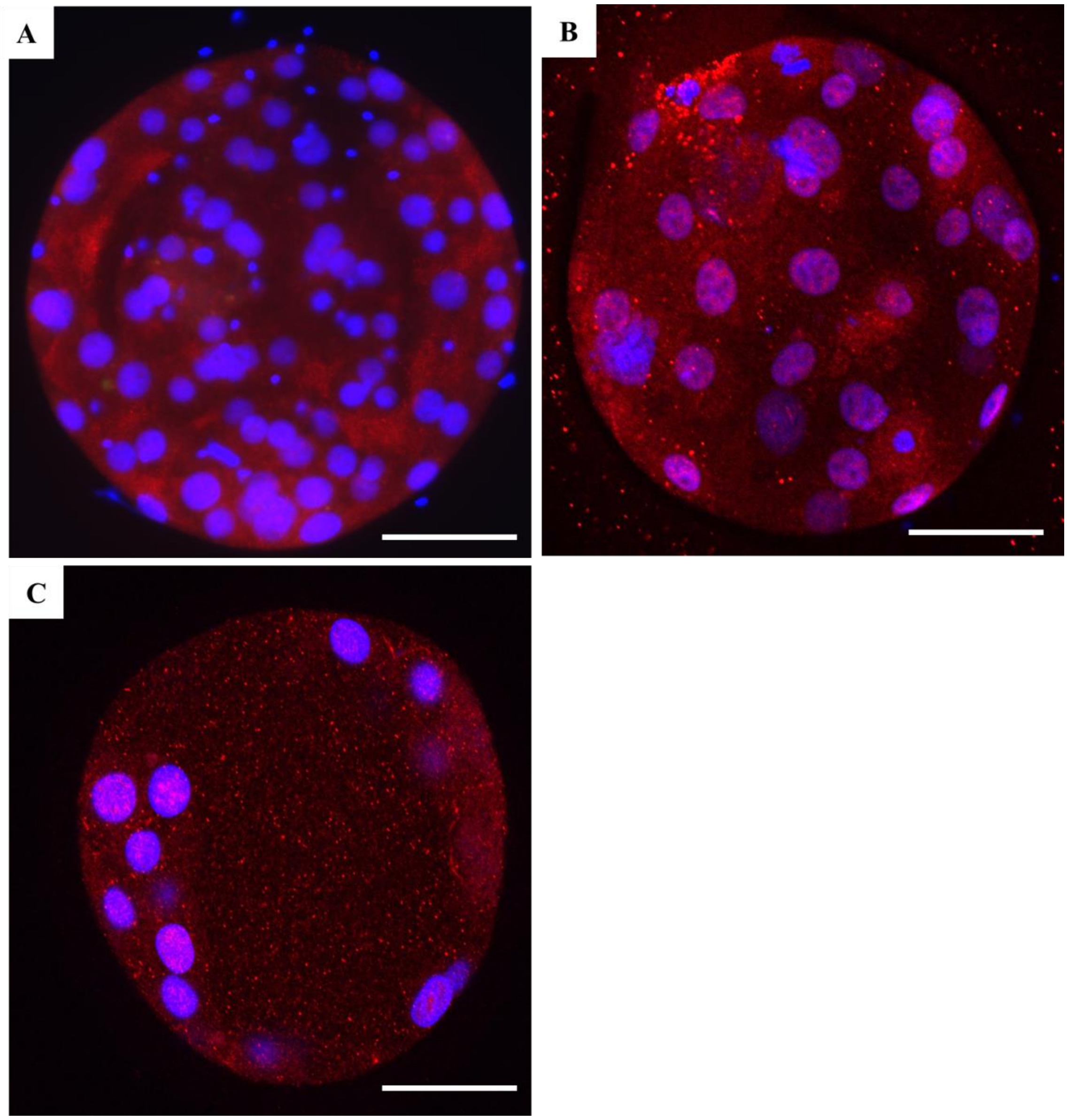


Figure 78. Confocal microscopy images of isotype control overlays. Overlays of red channel and DAPI (blue) in very good (A), good (B) and poor (C) quality blastocysts. Images were taken with the same microscope and software parameters as those in the ST2 overlay images. Scale bar= 50µm.

The results obtained by confocal microscopy for ST2 staining were variable between blastocysts. Within the blastocyst itself ST2 expression was patchy as the protein was detected in part, but not all, of the TE. It is not known if the staining of the cellular boundaries corresponds to the ST2L or the sST2 isoforms. If ST2L is present it may be important for IL-33 signalling, or if sST2 is present it may be secreted into the culture media. This was examined by ELISA (Figure 79). Initial analysis of both un-concentrated and concentrated blastocyst culture conditioned medium was performed using the R & D ELISA system, but no sST2 was detected (data not shown). Additional culture supernatants from 10 blastocysts were collected and concentrated five-fold for analysis with a more sensitive ELISA (Presage, Critical Diagnostics), but the sST2 levels were no different than those seen in the concentrated control culture medium (Figure 79).

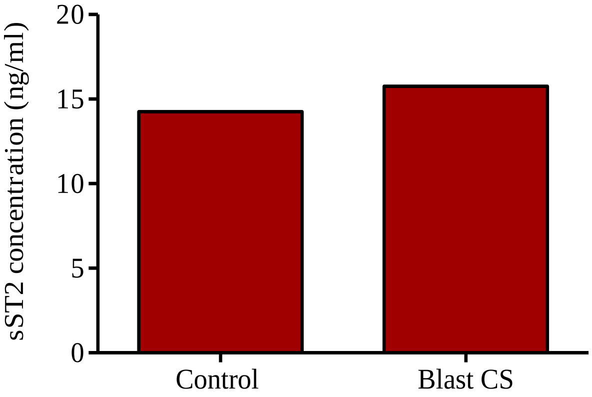


Figure 79. Human blastocysts do not secrete sST2. The conditioned culture supernatant of 10 blastocysts was pooled (Blast CS) and concentrated using 10 kDa Microcon centrifugal devices and was analysed for sST2 secretion using the sST2 Presage ELISA Assay protocol (Critical Diagnostics). Concentrated culture medium alone was used as a negative control.

4.3.3 IL-33 and ST2 expression in decidualized endometrial stromal cells

The second aim of this chapter was to examine the expression of IL-33 and ST2 in *in vitro* decidualized endometrial stromal cells isolated from endometrial tissues of normal patients during the secretory phase of the menstrual cycle. Treatment with 8-Br-cAMP induced a distinct morphological change between treated and non-treated endometrial stromal cells (Figure 80). The treated cells displayed a polygonal shape (Figure 80B) while untreated cells (Figure 80A) maintained a fibroblast-like appearance.

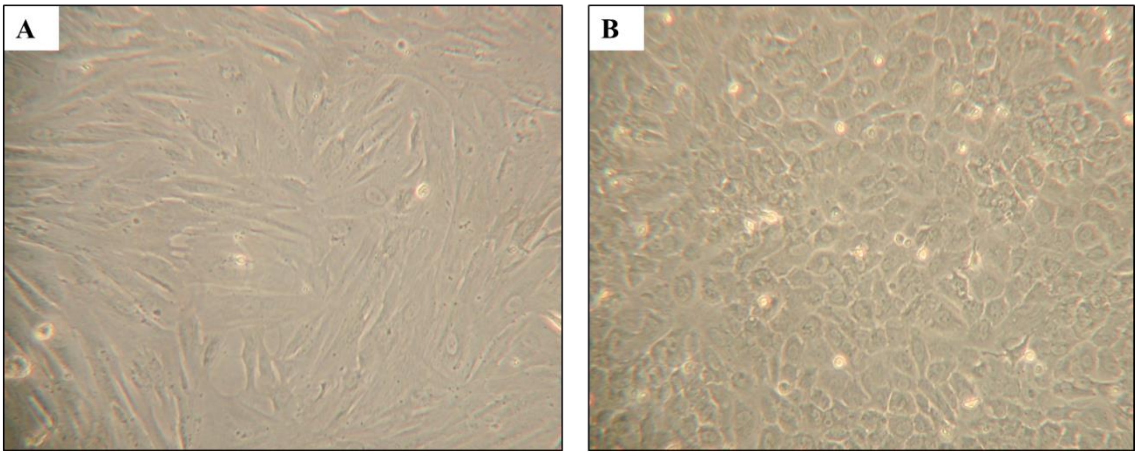


Figure 80. Decidualization of endometrial stromal cells by 8-Br-cAMP. Morphology of non-decidualized (A) and decidualized (B) human endometrial stromal cells. Endometrial stromal cells from normal patients were treated with 8-Br-cAMP (B) for 3 days or left untreated (A).

In addition to the use of 8-Br-cAMP as an inducer of decidualization (302), hormones such as progesterone can have similar effects on stromal cells (11). Activation of both 8-Br-cAMP and progesterone pathways are important for endometrial stromal decidualization, and the combination of both factors have been reported to induce higher prolactin RNA transcript expression from human endometrial stromal cells than treatment with 8-Br-cAMP alone (12). Endometrial stromal cells were therefore treated with 8-Br-cAMP with or without progesterone to test the effect on prolactin secretion.

Decidualization was confirmed by measuring the levels of secreted prolactin in 8-Br-cAMP treatments alone or in combination with progesterone (Figure 81). The concentration of prolactin was increased by day 2 in both treatments, peaked on day 4 and remained high until day 8 in cell treated with 8-Br-cAMP or progesterone alone. No additional prolactin release was seen when 8-Br-cAMP and progesterone were used in combination.

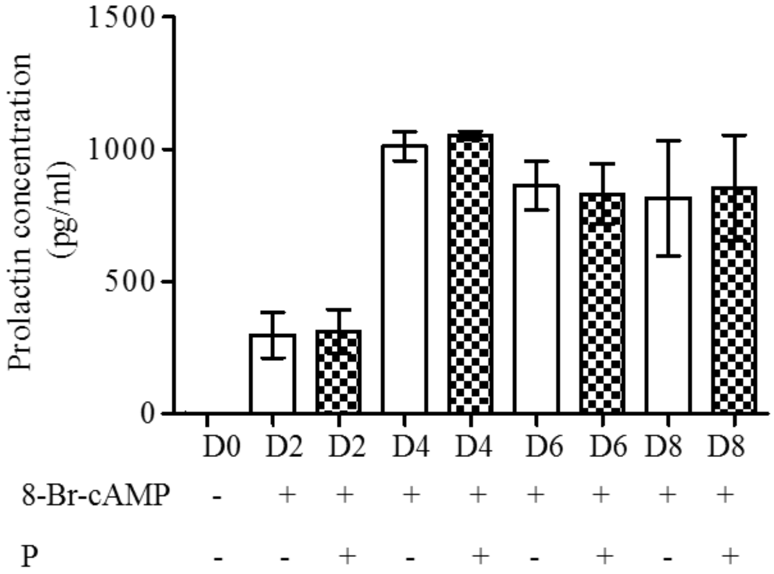


Figure 81. Prolactin release into culture supernatant of endometrial stromal cells treated with 8-Br-cAMP and progesterone. An ELISA specific for prolactin was used to measure the concentration of prolactin released from endometrial stromal cells isolated from endometrial tissues of three normal patients. Data represents the mean +/- the SEM of three independent experiments. Untreated cells (D0) and each treatment day (D2-8) is indicated. + or – denotes the presence (hatched bars) or absence (non-hatched bars) of progesterone.

4.3.4 Expression of IL-33 and ST2 transcripts in decidualized human endometrial stromal cells

As mentioned above, progesterone plays a vital role in the maintenance of the decidual phenotype which requires high levels of cAMP and sustained activation of the protein kinase pathway (11, 303). The downstream signalling effects induced by progesterone and cAMP result in the up-regulation or down-regulation of different genes, this had been reported by a number of groups using microarray gene expression analysis to identify factors important for decidualization (304-306). The expression levels of IL-33 and ST2 were therefore analysed in decidualized and non-decidualized stromal cells to determine whether they were a source of sST2 expression and secretion during the early stages of pregnancy.

RNA was isolated from decidualized and non-decidualized endometrial stromal from two patients and analysed for IL-33 (Figure 82) and ST2 (Figure 83) mRNA expression by qRT-PCR. Treatments with 8-Br-cAMP alone or in combination with progesterone resulted in an increase in IL-33 transcription starting day 2 of treatment. The up-regulation of IL-33 transcript was comparable between 8-Br-cAMP treatment alone or in combination with progesterone and ranged from a two- to five-fold increase compared to untreated cells. The highest fold increase was detected in cells treated following 8 days of culture.

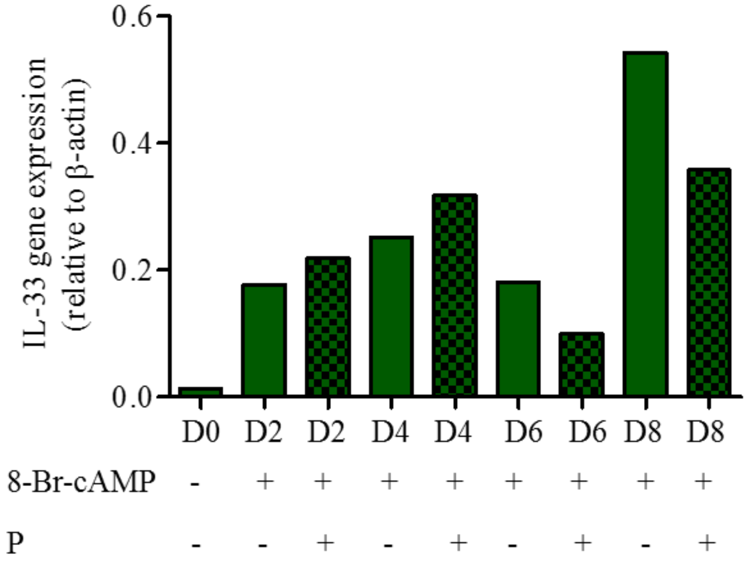


Figure 82. IL-33 gene expression levels in decidualized stromal cells detected by qRT-PCR. The levels of IL-33 transcripts were normalized to the endogenous house-keeping gene β-actin. Columns represent the mean of IL-33 gene expression from two patients. Untreated cells (D0) and each treatment day (D2-8) are indicated. + or – denotes the presence (hatched bars) or absence (non-hatched bars) of progesterone, n=2.

The levels of total ST2, sST2 and ST2L transcript expression were analysed in undifferentiated and decidualized stromal cells from two patients (Figure 83). 8-Br-cAMP alone or in combination with progesterone induced an increase in total ST2 (Figure 83A), sST2 (Figure 83B) and ST2L (Figure 83C) transcripts. The increase in total ST2 transcript was coupled increased with sST2 and ST2L mRNA expression. The increase in the total ST2 transcript was largely due to the sST2 isoform which was induced to much higher levels (between 10-30%) than that of the membrane isoform. Total ST2, sST2 and ST2L transcripts were higher on day 2 and 8 of treatment with 8-Br-cAMP and progesterone. Total ST2, sST2 and ST2L transcripts levels were almost undetectable in non-decidualized stromal cells.

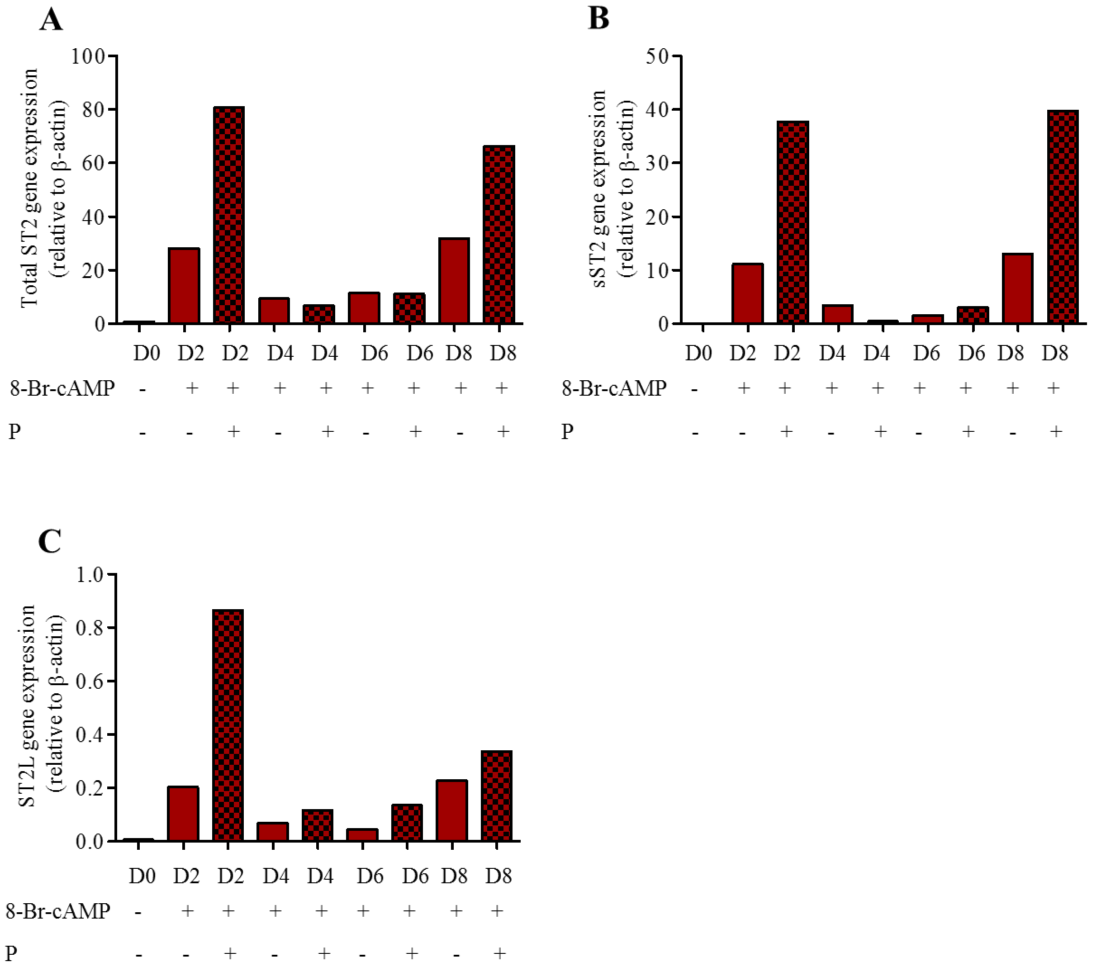


Figure 83. ST2 transcript detection in decidualized endometrial stromal cells. The expression of total ST2 (A), sST2 (B) and ST2L (C) isoforms was analysed by qRT-PCR and the relative expression level for each isoform was normalized to β-actin. Untreated cells (D0) and each treatment day (D2-8) are indicated. + or – denote the presence (hatched bars) or absence (non-hatched bars) of progesterone. Columns represent the mean of IL-33 gene expression from two patients, n=2.

It was next determined whether the up-regulation of the transcript was sufficient to induce protein expression. This was examined in the samples displaying higher ST2 transcript expression. However, Western blot analysis showed no ST2 protein in non-decidualized, 8-Br-cAMP only, and 8-Br-cAMP with progesterone decidualized stromal cells (Figure 84). Furthermore, the conditioned culture supernatants of decidualized and non-decidualized stromal cells for all treatments shown in (Figure 81) were analysed for IL-33 and sST2 secretion with a commercial ELISA, but no protein was detected in all samples tested (data not shown).

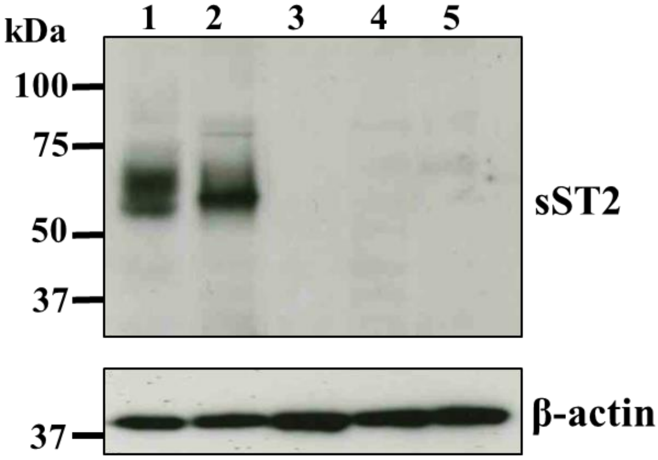


Figure 84. Analysis of ST2 protein expression in non-decidualized and decidualized endometrial stromal cells. Protein lysates (10µg) from first trimester placenta (1), HUVEC (2) used as positive controls for ST2 expression. 10µg of protein lysate from non-decidualized stromal cells (3), day 2 8-Br-cAMP (4) and day 2 8-Br-cAMP and progesterone (5). β-actin was detected and used as a loading control.

4.4 Discussion

The aim of this chapter was to investigate the expression of IL-33 and ST2 in blastocyst stage embryos and *in vitro* decidualized stromal cells, to identify any possible role these two proteins may play in the human endometrium, particularly during the implantation and decidualization stages.

The findings from the previous chapters suggest that IL-33 and ST2 are expressed by cells of placental origin and the majority of the ST2 detected corresponded to that of the soluble isoform, which was also secreted by explant cultures and term placental trophoblasts. Further investigation performed on trophoblast cell lines suggested a possible involvement of the soluble ST2 isoform in eliciting an effect on trophoblast proliferation and invasive potential. To follow up on the expression of IL-33 and ST2 in earlier stages of pregnancy, it was proposed that if sST2 is an important factor in very early stages of embryo implantation, it would be expressed either by the embryo or endometrium during the time of implantation. This hypothesis was investigated in this chapter by examining the expression of IL-33 and ST2 in human embryos at the blastocyst stage and endometrial stromal cells that were induced to decidualize *in vitro*.

4.4.1 IL-33 and ST2 expression in human blastocysts

IL-33 and ST2 gene expression was investigated with qRT-PCR and protein expression was assessed by immunofluorescence staining and ELISA. The gene expression analysis performed following amplification of blastocyst RNA showed no expression of either IL-33 or ST2 transcripts. Assessment of RNA transcripts in human blastocysts was challenging as a result of the small number of pooled blastocysts used. Also, blastocyst quality ranged from good to poor, reducing the amount of RNA isolated and therefore lowering the chance of detection of transcripts present at low copy numbers.

Previous protocols used for the detection of mRNA expression in human blastocysts after pooling large number of blastocysts and amplifying the cDNA or performed RNA amplification followed by cDNA amplification (307) or double amplification of the cDNA (308).

Gene expression in human embryos has been reported with conventional and quantitative RT-PCR methods (76, 309), generation of amplified cDNA libraries (310) and by using differential display and DNA sequence database interrogation (311, 312). No signal for IL-33 and ST2 was detected in previous reports examining the embryo transcriptome (313, 314).

Some methods allow the embryo lysis, reverse transcription and subsequent amplification of mRNA to be carried out on a single embryo, retaining mRNA abundance and avoiding sample loss (307, 315). Detection of β-actin mRNA has been previously shown to be suitable for use as a reference endogenous gene in human embryos (316). The protocol used in this section allowed the detection of β-actin mRNA in all preparations and also detected GAPDH and HLA-G. It is possible that the IL-33 and ST2 transcripts were present at very low levels but that amplification was not achieved. Future work would therefore focus on optimizing protocols for RNA amplification to confirm the presence or absence of both transcripts. It must also be remembered that human embryo gene expression under IVF conditions may not always reflect gene expression during *in vivo* embryo development (317).

Protein analysis by immunofluorescence revealed ST2 staining in very good quality blastocysts. An intense signal for ST2 was detected on cellular boundaries; however this was not uniform across blastocysts of different qualities. ST2 was localized to cell membranes in the ICM and the TE in very good and good quality blastocysts. It was more evident in regions where cells were densely packed. This pattern of expression adds to the challenges of mRNA transcript detection as the blastocyst quality needs to be considered and future work should focus on the selection of very good quality embryos for gene expression studies, however these can be difficult to obtain for research purposes.

A common feature of the transcriptome is the variation in transcript abundance which is caused by a burst-like stochastic activation of transcription (318). Episodes of mRNA synthesis last a few minutes and are followed by periods of transcriptional silence (318). As a result, cells selected randomly display great variation in their mRNA content ranging from those that have undergone a burst in transcription to those exhibiting complete mRNA degradation, as that detected for RNA polymerase II (319). Therefore it is possible that the blastocyst expresses ST2 mRNA transcript at a specific stage of blastocyst development after which the transcription is switched off and all ST2 mRNA is degraded, which may explain the negative PCR data but positive protein detection by immunofluorescence analysis.

Furthermore, no additional ST2 protein was found in concentrated blastocyst culture supernatants compared to the culture medium alone. However, the secretome of blastocysts co-cultured on human endometrial epithelial cells is reported to contain higher levels of sST2 than those detected in blastocysts cultured in sequential culture media (320). However, in this report sST2 was detected in both concentrated culture media alone or with blastocyst. This is most likely due to the fact that human IVF culture media contain proteins, macromolecules and undefined growth factors, collectively referred to as human serum albumin (321) (322). Therefore it is possible that sST2 is one of the components of the undefined proteins under the HSA label.

4.4.2 *In vitro* decidualization of endometrial stromal cells

Differentiation of endometrial stroma into decidua is controlled at the molecular level by the activation of progesterone-dependent genes and is characterised by a continued elevation of cellular cAMP levels (11, 323). Abnormal regulation of decidualization can lead to different pregnancy related disorders such as infertility and endometriosis (12). Decidual stromal cells are known to express different hormones, cytokines and their receptors, compared to undecidualized endometrial cells (324). *In vitro* cultured human endometrial stromal cells are induced to differentiate into decidualized cells by estrogen, progesterone and/or analogues of cyclic AMP ((325, 326). The differentiation reported is characterized by morphological and biochemical changes similar to those found in *in vivo* decidualized cells. The results presented in this chapter were consistent, in terms of the change in phenotype and biochemical secretion of prolactin in decidualized stromal cells, with those previously reported (12). The round polygonal phenotype was observed in cells treated with 8-Br-cAMP alone or in combination with progesterone. The morphological changes started from day 2 and were maintained until the last day of treatment by the continuous replacement of media and supplementation of fresh 8-Br-cAMP and progesterone.

It is important to note that endometrial stromal cell decidualization can also be induced *in vitro* by other factors such as relaxin, corticotrophin-releasing hormone, FSH and LH (327-331). These factors increase the levels of intracellular cAMP and activate the regulatory subunits of protein kinase A signalling pathway which lead to the differentiation of stromal cells. Also, endometrial stromal cells have been induced to decidualze in response to progesterone treatment alone when maintained in serum free media (332).

In addition to analysis of cellular morphological changes, biochemical assessment of decidualization was carried out by testing for prolactin release from all patients' cells following treatment with the differentiation inducing factors. As expected prolactin levels were increased in all patients' samples from day 2 following the treatment, in comparison to undifferentiated cells which showed very little or undetectable levels of prolactin. Therefore treatment with other factors known to induce decidualization was not performed as the treatments given here were deemed to be sufficient.

The biochemical response to decidualization characterized by the release of prolactin was different between patients. It was expected that the cells would maintain a similar characteristics with an increase in prolactin release with increasing days in culture. This however was not the case in one of the patients tested, whose cells showed an initial increase followed by a further increase, but had lower secretion at the end of the culture period. Despite the decrease, levels of prolactin secretion were still higher than at the start of the treatment, which was an indication of successful decidualization. It is important to note that this was a pilot experiment and only three patients were tested. Only the ones with a normal response were analysed for their IL-33 and ST2 expression.

4.4.3 Activation of molecular pathways in response to decidualization- are IL-33 and ST2 involved?

When this part of the study was designed, it was known that 8-Br-cAMP and progesterone increased the gene expression of ST2 from studies using microarray gene analysis. However, the first report of this finding did not show any data nor specified which isoform was detected (304). In a subsequent study, ST2 gene transcription was shown to be increased in decidual tissues isolated from first trimester pregnancy (333). Again, this study did not indicate which ST2 isoform was up-regulated and neither of these studies examined IL-33 expression in endometrial stromal cells. More recently, when decidualized endometrial stromal cells were treated with hCG and IL-1β, IL-33 mRNA was found to be up-regulated in cells treated with IL-1β alone, but the levels were decreased when cells were treated with a combination of hCG and IL-β (334).

Thus, the results presented here are consistent with previous findings in relation to the up regulation of ST2 gene expression following stimulation of decidualization. This up-regulation affected both isoforms; sST2 and ST2L. sST2 levels were higher than those of the membrane isoform and the increase in the level of expression peaked on days 2 and 8 of decidualization. IL-33 gene expression was also up-regulated and expression levels peaked on day 8. Despite the induction of high levels of ST2 transcript in treated endometrial stromal cells, no protein expression was detected following decidualization.

The ST2 gene up-regulation during decidualization found in this thesis supports previous reports in the literature (335-337), but it is difficult to compare these studies as each group used a different experimental approach and found the up-regulation of ST2 on different days to that which is reported here. A standardised approach to stromal cell decidualization (in terms of factors used or days of treatment)

is not described in the published reports. Some tested the effect on day 3 and others for up to 10 or even 15 days (338-340). Therefore, the variation between the reported results can be ascribed to the different *in vitro* culture conditions and experimental strategies employed by different investigators.

In conclusion, this chapter demonstrated an up-regulation of both IL-33 and ST2 gene expression by decidualization of endometrial stromal cells. However, this gene up-regulation was not followed by protein expression or secretion. Blastocyst stage embryos were examined for gene and protein expression and a distinct localization pattern of ST2 was detected in very good quality embryos. The significance of these findings is addressed in the final chapter.

Chapter 5

Final Discussion

5.1 General Discussion

Embryo implantation and the establishment of pregnancy are complex dynamic processes that involve the interaction between a receptive maternal endometrium and a competent blastocyst. An elaborate and coordinated network of molecules is involved in this maternal-embryo cross talk during the limited period of the window of implantation (341). Many studies have demonstrated major changes in these molecules within this period, which affect processes such as proliferation, invasion and the maternal response to the implanting embryo (324, 334, 342).

The starting point for this thesis was the identification of the cytokine IL-33 and its receptor ST2 in human term placenta (108). As the receptor for IL-33 was found on Th2, but not Th1 cells (186), we previously proposed that it plays a role in modulating the immune response during pregnancy (108). However, it is not yet fully understood how the IL-33/ST2 system contributes to pregnancy. The aim of the work presented in this thesis was to shed further light on this question by investigating the expression of these two proteins in early pregnancy, by studying first trimester placental samples, human blastocysts and endometrial stromal cells, together with trophoblast cell lines.

5.1.1 IL-33 in the first trimester placenta

In Chapter 2 it was shown that IL-33 is expressed at both the transcript and protein levels in first trimester placental tissue samples. Almost all first trimester placental tissues examined from 6 to 12 weeks of gestation expressed the full length IL-33 protein (approximately 30 kDa protein). It was previously thought that this form of IL-33 was inactive and that signalling via ST2L required the proteolytic cleaving of the full length

IL-33 (83), but this is now known not to be the case (95, 97, 99, 343). More recent reports have shown that full length IL-33 is processed by caspases 3 and 7 into a less bioactive form of 20-22 kDa (97, 343). Further studies have shown the situation to be even more complex as IL-33 was also found to be cleaved by elastase and cathepsin G into an 18-22 kDa protein, with a bioactivity that is tenfold higher than the full length active protein (99). Additionally an alternatively spliced variant of IL-33 was reported to result in a 5 kDa protein lacking the exons cleavable by caspases, but with a bioactivity similar to that of the full length IL-33 (95). However, none of these cleaved forms were found in the first trimester placenta.

Immunofluorescence labelling showed no IL-33 staining in the syncytiotrophoblast, villous cytotrophoblast or extravillous cytotrophoblast. It was however localized to the villous core of the floating villi, probably to endothelial or immune cells. Despite being found in many different tissues, IL-33 expression is usually restricted to endothelial and epithelial cells (85, 86, 110, 111). This is the case in a range of human organs and tissues including the brain (344), adipose tissue (345), atherosclerotic plaques (346) skin, kidney, liver lung and colon (110, 111). IL-33 expression has been reported on placental and decidual macrophages in first trimester placenta (214), but not on placental and decidual macrophages in term placenta (211).

The active secretion of IL-33 by placental and decidual macrophages has been reported (214). Activated macrophages and dendritic cells are said to be the only haematopoietic cells expressing IL-33 mRNA, albeit at low levels (83). However, active secretion of IL-33 has not been detected in cells cultured under normal conditions; its detection was always associated with cell death or stress (97, 98, 127, 128, 347), Collectively, these reports show that secretion of the full length IL-33 protein normally only occurs when cells are stressed and cell necrosis is initiated, as in pathological conditions such as infection (348) or rheumatoid arthritis (121). This was not the case in the placentas examined. We have found no evidence of IL-33 secretion from placental explants. This is consistent with our previous observation that IL-33 levels in the maternal circulation do not change significantly during pregnancy. Taken together the findings presented here do not support the hypothesis that placental IL-33 is involved in the maternal-fetal dialogue.

5.1.2 ST2 in the first trimester placenta

ST2 protein expression in the first trimester placentas correlated with the mRNA analysis, which showed higher expression of the sST2 soluble isoform compared to the ST2L membrane receptor. Indeed, no ST2L protein was detected in any of the first trimester placental tissue lysates examined, but sST2 was found in abundance. Immunofluorescence labelling showed that ST2 displayed a wide pattern of distribution, being present on both villous and extravillous trophoblasts. Further investigation showed that sST2, but not IL-33, was secreted by first trimester placental explants and primary term trophoblast cells.

These results were important as they revealed that ST2L, the receptor to which IL-33 binds and stimulates the release of Th2 cytokines, is missing at the maternal-fetal interface. This, together with the finding that IL-33 is localized to the villous stroma rather than to the trophoblast, leads to the conclusion that the original hypothesis that IL-33/ ST2L interactions in the placenta are important for successful pregnancy was unlikely. sST2 may however have other functions, and this became a new focus for the thesis.

In the Nuffield Department of Obstetrics and Gynaecology fresh first trimester placental tissue samples are not available; those used in Chapter 2 were processed in St George's Hospital, London, and were a kind gift from our collaborator, Dr Judith Cartwright. Trophoblast cell lines are an alternative option for research, and could be used as a model to investigate sST2 expression and secretion from placental cells. Surprisingly, none of the cell lines tested expressed sST2 (nor as expected IL-33) mRNA in significant quantities, except SGHPL-5 cells, which had levels of sST2 mRNA similar to those seen in first trimester placenta. However this did not appear to be translated into protein and no secretion of sST2 was seen by any of the cell lines. Stimulation of trophoblast differentiation, either along the syncytialization or invasive pathways, had no effect on sST2 expression in the BeWo and SGHPL-5 cell lines respectively. Furthermore treatment with pro-inflammatory cytokines, serum starvation and heregulin-1 had no effect on sST2 mRNA or protein production. Why these cell lines do not express sST2 protein or secrete it, whereas primary trophoblast do, is not known, but it could be that cell transformation and prolonged *in vitro* culture cause loss of gene expression, as has been documented for other proteins (279).

5.1.3 sST2 may have a role independent from IL-33/ ST2L axis inhibition

The localization of IL-33 and the lack of ST2L but predominance of sST2 in trophoblast cells indicated that sST2 is not produced to limit the IL-33/ST2L axis in the placenta. sST2 secreted from the STB directly into the maternal blood in later pregnancy is likely to act on the IL-33/ST2L inflammatory system in the mother, as previously reported (108). However sST2 secretion from EVT and STB in early pregnancy would act more locally, and may have an unknown role in placental development, either through immune cell inhibition (IL-33/ST2L blocking) or direct interactions with trophoblast. This latter idea came from previous studies that were performed to identify proteins that bind ST2 (215, 349). For many years since its discovery, ST2 was known as an orphan receptor with no identified ligand, despite one study that found that ST2 could bind to an unknown cell surface protein (349). IL-33 was identified as a ligand for ST2 (specifically ST2L) in 2005, with sST2 as a decoy factor, and the majority of the work that has followed focuses solely on this pathway and the initiation of Th2 immune responses. IL-1RAcP was found to be the co-receptor for the initiation of signalling post IL-33 binding (154, 155).

Another ST2L binding protein has since been identified - a protein known as tp24, p24γ_1 or TMED1 (350, 351). TMED1 is a membrane spanning protein located in the endoplasmic reticulum and Golgi lumen. Its interaction with ST2L was demonstrated to be via the TLR domain of ST2L found in the cytosol and it is thought to aid the trafficking of ST2L to the cell surface (350) or, as this group suggested, it could be involved in the internalization of ST2L after IL-33 binding which eventually results in the degradation of ST2L by FBXL19 (162). As both IL-1RAcP and TMED-1 bind ST2L through cytoplasmic domains, they cannot associate with sST2. However, extracellular sST2 was recently found to associate with monocyte-derived dendritic cells, resulting in sST2 internalization by endocytosis, but the protein to which sST2 binds, and the molecular pathways it is controlling, remain a mystery (295). Such investigations are starting to reveal the complexity of IL-33/ST2L/sST2 protein system - with each protein playing unique roles independent of the other.

sST2 binding to the cell surface has also been shown in different cancer cell lines (352). sST2 inhibited cancer cell colony formation in soft agar plates, but more recently, sST2 has been shown to enhance cancer cell motility and invasion after cell binding (240). As invasive trophoblasts bear many similarities to cancer cells, we sought to determine whether sST2 might have the same effect on trophoblast cell lines. We first showed by flow cytometry that recombinant sST2 bound to the surface of all the trophoblast cell lines tested, with particularly strong binding to the SGHPL-5 line. This suggested that there must also be a novel receptor for ST2 on trophoblast cells. We then studied the functional effects of sST2 and showed it to inhibit cell proliferation and promote SGHPL-5 migration in a 3D invasion assay. Thus this thesis demonstrates, for the first time, a novel function of sST2 in the placenta, which is independent of IL-33.

If sST2 is important for promoting trophoblast differentiation, and more specifically for inhibiting cell proliferation and enhancing cell motility, this would explain the localization of sST2 to both the invasive and non-invasive trophoblasts in the cell columns of the first trimester placenta. It may also be possible that sST2 controls cell fate in the pathway to syncytialization and this will be investigated in future studies.

5.1.4 IL-33 and ST2 in early embryos

If sST2 plays a role in trophoblast invasion then it might be expected to be present in pre-implantation embryos. IL-33 and ST2 gene expression in human blastocysts was investigated with qRT-PCR and protein expression was assessed by immunofluorescence staining and ELISA. The gene expression analysis performed following amplification of blastocyst RNA showed no expression of either IL-33 or ST2 transcripts and possible technical challenges were highlighted in the discussion of that chapter. In contrast, protein analysis by immunofluorescence revealed ST2 staining in very good quality blastocysts.

An intense signal for ST2 was detected on cellular boundaries where ST2 was localized to cell membranes in the ICM and the TE in very good and good quality blastocysts. However, sST2 secretion into the blastocyst supernatant could not be detected, either sST2 is not secreted or levels are too low to be detected by the assay system. It is not possible to study human embryos beyond the blastocyst stage in culture, and placental tissues are not available before six weeks gestation, therefore we are unable to determine when detectable levels of sST2 are first released by trophoblasts in humans.

5.1.5 The endometrium as a source of IL-33 and sST2

If trophoblast invasion is dependent on exogenous sST2 then it might be expected that endometrium is a source. However, although endometrial stromal cells decidualized *in vitro* had increased levels of both sST2 and IL-33 mRNA, no protein was detected either by Western blotting or ELISA. Salker et al 2012 have also reported an increase in IL-33 mRNA in decidualized endometrial stromal cells, but they also saw increased protein expression and secretion. They suggested that this may be a result of the morphological differentiation induced by decidualization, which stresses the cells in a similar way to that induced by mechanical strain (137). Furthermore, they were able to detect both ST2L and sST2 gene and protein expression in decidualized endometrial stromal cells, and they suggested that the IL-33/ST2 system functions in an autocrine manner in these cells. They showed that in un-differentiated endometrial stromal cells IL-33 is localized to the nucleus but upon decidualization, it is translocated to the cytoplasmic region and is then actively secreted as a result of cell differentiation (353). However, there are a number of concerns about this report which need addressing.

First, they reported the detection of IL-33 in the nuclei of undifferentiated stromal cells, which upon decidualization was released into the culture supernatant. ST2L expression was upregulated upon decidualization of endometrial stromal cells. However, upon close examination of their endometrial stromal cell immunohistochemistry, it is clear that these cells do not display the morphological phenotype characteristic of decidualized stromal cells. The examination of endometrial tissue sections detected high levels of IL-33 localization to nuclei of epithelial cells lining the uterine glands, therefore it cannot be ruled out that their ‘endometrial’ stromal cells preparations were contaminated with epithelial cells which as described earlier are major cellular source of IL-33 expression.

Secondly, their immunoblotting analysis did not distinguish the sizes of the ST2 and IL-33 proteins detected. This is important to determine which isoforms are present.

Thirdly, this study reported secretion of IL-33 and sST2 in undifferentiated and decidualized endometrial stromal cells isolated from endometrium of both normal women and women with recurrent pregnancy loss. This finding contradicts their theory that decidualization stresses the cell structure and leads to the secretion of IL-33 as they report active secretion from undifferentiated cells.

Finally, in this paper the levels of secreted IL-33 and sST2 reported were below the limits of detection of the ELISAs they used. Thus it is still not clear whether the decidualized endometrium is a source of functional IL-33 and sST2.

5.1.6 Future work

The key finding of this thesis is the role of sST2 in promoting the invasion of the SGHPL-5 trophoblast cell line. A next critical step would be to test the effect of sST2 on early human embryo development in 3D models of implantation. If sST2 was found to promote blastocyst invasion it may have therapeutic potential for IVF implantation failure. From a scientific perspective, we need to identify the receptor for sST2 on trophoblast, the signalling pathways that it activates and the downstream targets. In the longer term, we would want to study sST2 and its receptor on trophoblast and in particular the cell columns from pathological pregnancies such as pre-eclampsia and recurrent pregnancy loss, where defects in trophoblast invasion are found.

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