Characterisation of Oestrogen-Responsive MicroRNAs Regulating Coagulation Factors

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Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

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Abstract

Oestradiol (E_2) regulation of microRNAs (miRNAs) is well characterised in breast cancer, but poorly understood in regulating coagulation. We previously reported that miR-494 directly downregulates Protein S gene (*PROS1*) expression in E_2-treated human liver carcinoma cells (HuH-7) (Tay et al. 2013). Subsequent miRNA array analyses (NanoString nCounter®) identified numerous E_2-responsive miRNAs in HuH-7 cells, which may be involved in regulating thrombotic factors. Therefore, this study sought to validate the E_2 responsiveness of the candidate miRNAs and investigate their direct effects on coagulation gene targets. Twelve E_2-responsive miRNAs were selected for validation and their potential coagulation gene targets predicted using four computational tools. HuH-7 cells were cultured -/+ 10nM E_2 for 12h followed by RT-qPCR quantitation of E_2-mediated expression of the twelve selected miRNAs and predicted gene targets. The direct interaction between validated miRNAs and their common coagulation gene targets were measured via dual-luciferase reporter assays. In concordance with NanoString nCounter® results, expression of let-7f-5p, miR-26b-5p, miR-128-3p, miR-365a-3p, miR-455-3p and miR-548aa were significantly downregulated following E_2 treatment ($p<0.05$). An associated increase in common predicted targets Tissue Factor (*F3*) and Factor VIII (*F8*) mRNA levels was also observed. Furthermore, miR-365a-3p was identified to have a direct binding site on *F3*-3'UTR. MiR-365a-3p was down-regulated by E_2 and it could directly bind to *F3*-3'UTR, suggesting down-regulation of miR-365a-3p may lead to up-regulation of Tissue Factor to promote thrombosis in high circulating E_2 levels during pregnancy or contraceptive pill use.

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Lists of Abbreviation

Ago	argonaute family protein
APC	Activated Protein C
ATIII	antithrombin III
CSS	charcoal stripped fetal bovine serum
DALYs	disability-adjusted life year
DMEM	dulbecco's modified eagle's medium
dNTP	deoxynucleotide triphosphate
E_2	(17β-) oestradiol
ER	oestrogen receptor
ERE	oestrogen-responsive element
FBS	fetal bovine serum
FVL	Factor V Leiden
HRT	hormone replacement therapy
IHD	ischaemic heart disease
LB	lysogeny broth
mRNA	message RNA
miRNA/miR	microRNA
miRNP	microribonucleoprotein
NEAA	MEM non-essential amino acids
OC	oral contraceptive
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PC	Protein C
pre-miRNA	precursor miRNA
PS	Protein S
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SP-1	Stimulating Protein-1
SVR	support vector repression
TF	Tissue Factor
TFPI	tissue factor pathway inhibitor
t-PA	tissue plasminogen activator
u-PA	urokinase-type plasminogen activator
UTR	untranslated region
VTE	venous thromboembolism
v/v	volume/volume
vWF	von Willebrand factor
WACTH	Western Australian Centre for Thrombosis and Haemostasis

Chapter 1

Introduction

Blood is an essential circulating fluid that transports oxygen and nutrients to tissues throughout the body and delivers wastes to kidneys. As such, any injuries to blood vessels must be contained via haemostasis to ensure a closed circulation. Platelet plug formation and blood coagulation are two primary mechanisms that constitute haemostasis, and in order to prevent an excessive or unnecessary clot formation (thrombosis), haemostasis is regulated by anticoagulants and fibrinolysis (Boron & Boulpaep 2012). In other words, a deregulation of haemostatic balance can lead to an abnormal blood clotting to trigger an onset of severe cardiovascular diseases, such as ischaemic heart disease and venous thromboembolism. Furthermore, various inherited and acquired risk factors can also increase the risk of an individual developing thrombosis.

1.1. The coagulation cascade and venous thrombosis

1.1.1 Haemostasis and coagulation cascade

Haemostasis is a well-established process that acts to cease bleeding when blood vessels are injured. In response to vascular injury, vascular smooth muscles immediately contract and the release of chemicals called endothelins by endothelial cells and pain receptors from the site of injury will trigger an activation of primary and secondary blood clotting pathways (*Hemostasis* 2013).

Primary haemostasis refers to the formation of platelet plug(s), which includes a process of platelet adhesion, activation and aggregation that is facilitated by different factors such as von Willebrand factor (vWF), collagen, platelet receptor (glycoprotein Ia/Ib), fibrinogen and thrombin (Figure 1.1-A) (Boron & Boulpaep 2012).

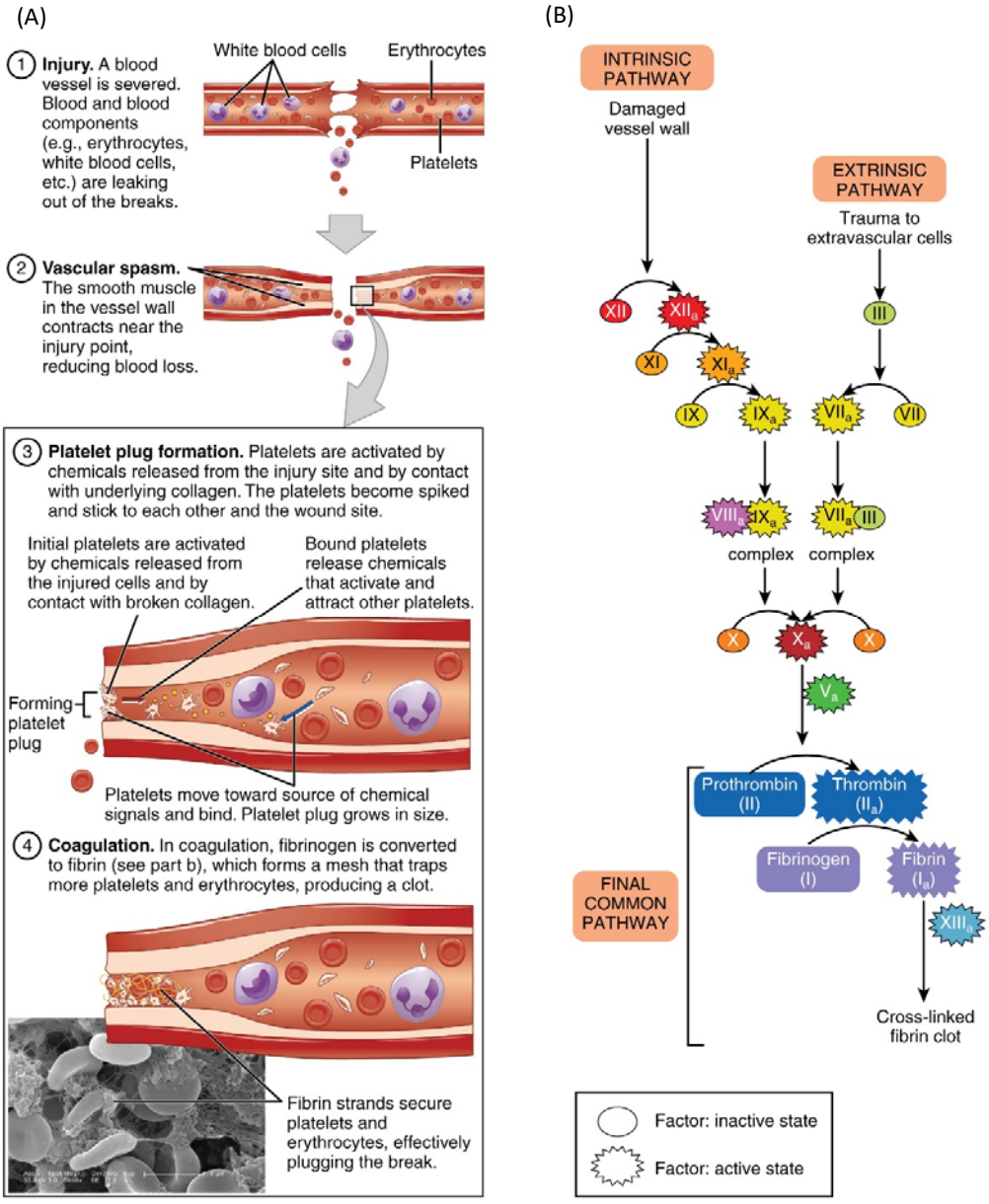


Figure 1.1 Blood clotting pathways. (A) Clotting overview including primary haemostasis. (B) Secondary haemostasis. (Adapted from *Hemostasis* 2013)

In addition, the secondary clotting pathway, or coagulation cascade, is initiated simultaneously. This is an important haemostatic mechanism that ultimately results in the generation of a stable intravascular blood clot (thrombus). Mechanism for the clotting pathway is derived from the waterfall sequence theory (Davie & Ratnoff 1964; Macfarlane

1964), which described how the clotting cascade would be mediated both inside (intrinsic) and outside (extrinsic) of the vascular system (Figure 1.1-B).

The intrinsic pathway, also known as the contact activation pathway, is initiated by the exposure of collagen from a damaged vessel wall to blood and an activation of Factor XII to Factor XIIa. Factor XIIa then proteolytically cleaves Factor XI to Factor XIa that further stimulates Factor IXa (*Hemostasis* 2013). The activated Factor IX converts Factor X into Factor Xa by the recruitment of Factor VIIIa and calcium ions. The extrinsic pathway, however, is induced by a non-proteolytic mechanism. When trauma arises in extravascular cells, Tissue Factor (TF) is released to bind to activated Factor VIII. The interaction between TF and Factor VIIIa together with calcium ion, forms a complex to stimulate an activation of Factor X to Factor Xa. Beyond this point, the intrinsic and extrinsic pathways merge into the common pathway. The conversion from prothrombin to thrombin is activated by the activities of Factor Xa and Factor Va. Thrombin then triggers the cleavage of fibrinogen to fibrin monomers, and the fibrin monomers ultimately generate a fibrin clot with the assistance of Factor XIIIa (Figure 1.1-B) (Boron & Boulpaep 2012).

This classical coagulation pathway was originally characterised in 1964 (Davie & Ratnoff 1964; Macfarlane 1964) and over the years scientists have found that the intrinsic and extrinsic pathways are not independent of one another. Hoffman (2003) established a cell-based model for coagulation, highlighting the importance of TF expressed on the surface of cells; like fibroblasts or monocytes in the cascade (Figure 1.2). Based on this model, blood

clot formation is initiated by TF that is bound to Factor VIIa, causing the subsequent activation of Factor X to Factor Xa that cooperates with Factor Va to generate thrombin.

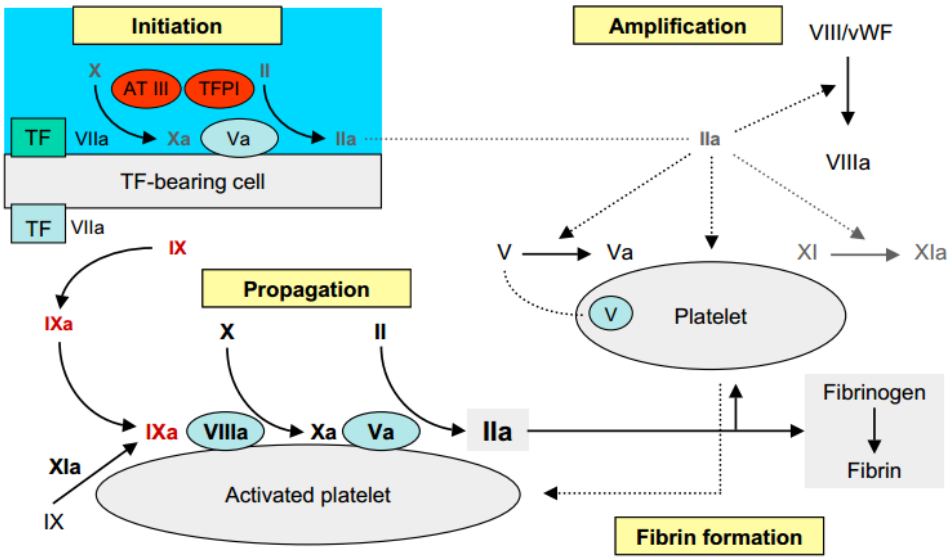


Figure 1.2 Hoffman's cellular model of coagulation cascade. (Adapted from Innerhofer & Kienast 2010)

The subsequent fresh thrombin produced contributes to the activation of adherent platelets that release Factor V; meanwhile, Factor VIII and Factor XI are to be stimulated by thrombin. Activated Factor XI can further convert Factor IX into IXa to increase the production of Factor Xa and Factor V stimulation occurs when thrombin is present in the system. The interaction between Factor Xa, Factor Va and activated platelet results in a "thrombin burst", which promotes fibrin clot generation and recycle to the cascade pathway to further induce platelet activation (Figure 1.2) (Hoffman 2003).

However, fibrin clot formation is not the terminal step of haemostasis, as excess clotting can lead to the obstruction of blood flow and cause thrombosis. Therefore, antithrombotic factors (or anticoagulants) along with fibrinolysis act to prevent unnecessary thrombus formation to maintain haemostatic balance (Figure 1.3).

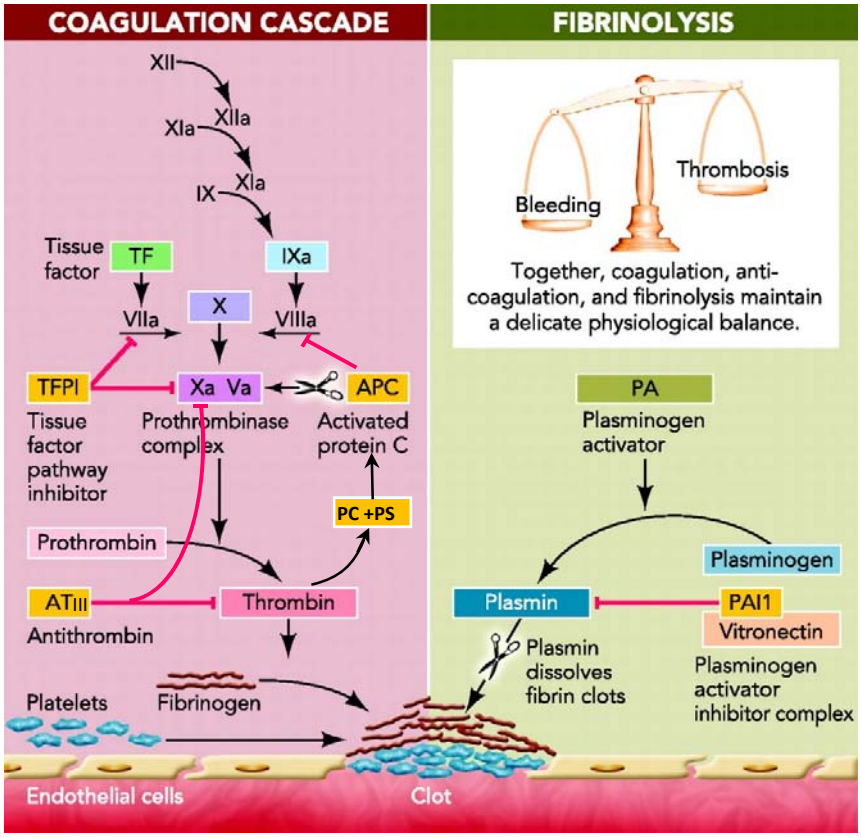


Figure 1.3. The anticoagulant and fibrinolysis pathways in haemostasis. (Adapted from Sun 2006).

Tissue factor pathway inhibitor (TFPI), antithrombin III (ATIII), Protein C (PC) and Protein S (PS) are four major anticoagulant proteins that inhibit the activity of distinct coagulation factors (Innerhofer & Kienast 2010; *Hemostasis* 2013). When thrombin generation exceeds the levels required for blood clot formation, the surplus thrombin interacts with

thrombomodulin, a receptor located on endothelial cells, to convert the inactivated PC into Activated Protein C (APC), which then complexes with the APC cofactor, PS, to repress Factor Va and Factor VIIIa activity. Fibrin clots are mainly lysed by plasmin (Figure 1.3), which is activated by two types of plasminogen activator (PA) –– tissue PA (t-PA) and urokinase-type PA (u-PA) from its precursor protein. The activity of PA is inhibited by plasminogen activator inhibitor (PAI), with stabilised PAI-1 targeting both t-PA and u-PA via binding to glycoprotein vitronectin, while PAI-2 is specific for the suppression of u-PA (Boron & Boulpaep 2012).

1.1.2 Venous Thrombosis

1.1.2.1 Venous and arterial thrombosis

Thrombosis is a pathological condition that causes ischaemic (or coronary) heart disease (IHD), cerebrovascular disease (stroke) and venous thromboembolism (VTE). Based on the latest report released by the Global Burden of Diseases, Injuries, and Risk Factors (Murray et al. 2012; Naghavi et al. 2015), in 2013, IHD and stroke were responsible for 8.1 and 6.4 million deaths respectively, and they were ranked as the diseases with the highest worldwide disability-adjusted life year (DALYs) in 2010, a new quantified term adopted by the World Health Organisation to more accurately evaluate the mortality and morbidity of diseases (*Metrics: Disability-Adjusted Life Year (DALY)* 2015).

As VTE-associated data was absent in the Global Burden of Diseases, Injuries, and Risk Factors study, the recent population-based studies of VTE was reported by the International Society on Thrombosis and Haemostasis Steering Committee, which indicated that

individuals who lived in developed western countries have a 10 to 16 times higher risk of developing thrombosis compared to individuals living in East Asia (Jha et al. 2013; ISTH Steering Committee for World Thrombosis Day 2014). Furthermore, VTE was considered the largest risk factor contributing to increased DALYs in seven adverse disorders investigated in this study, which constituted 2.3 million and 5.4 million of DALYs lost in high-income countries and low to middle-income countries (Jha et al. 2013), respectively. In Australia, 78,408 cases of DALYs lost was estimated in 2008 (*The burden of venous throboembolism in Australia* 2008).

Due to this strong disease burden contributed by thrombosis, it is imperative to have more cost-effective strategies in managing the disease. Thrombosis is primarily caused by distinct changes within the vascular system and is categorised into two subclasses - vein and arterial thrombosis (Schouwenburg et al. 2012). Venous thrombosis is mainly caused by abnormal blood flow and blood composition, whereas arterial thrombosis is affected by the changes in the vessel wall which triggers IHD and stroke (Virchow 1856; Rosendaal et al. 2003). Like other haemostatic disorders, thrombosis can be diagnosed using diverse coagulation assays, consisting of prolonged activated partial thromboplastin time, prolonged prothrombin time, prolonged thrombin time, abnormal clot solubility, rapid clot lysis and thrombin generation test (Longo & Harrison 2011).

As the result of the over production of blood clots, thrombosis can be treated by agents that either down-regulate the activity of platelets or coagulant factors. Aspirin (anti-platelet), heparin and warfarin (anti-coagulant) are well-known first generation medications; which

provide significant efficacy in treating thrombosis; whereas, the effect of heparin (enhances antithrombin activity) and warfarin are too strong to be self-controlled, therefore they require monitoring and may induce different side effects like bleeding (Gresele & Agnelli 2002; Roemisch et al. 2002). To date, second generation agents as well as novel approaches have been used as clinical or preliminary thrombotic treatments (Table 1.1).

Table 1.1. Antithrombotic agents. (Adapted from Gresele & Agnelli 2002).

	Antiplatelet agents[a]	Anticoagulant agents
First generation	Aspirin Thienopyridine (ticlopidine and clopidogrel)	Heparin Warfarin
Second generation	GPIIb/IIIa antagonists Aspirin–clopidogrel combination	Low-molecular-weight heparins Hirudin
Novel approaches	Inhibitors of vWf–GPIb interaction Inhibitors of collagen–platelet interaction Inhibitors of thrombin-induced activation Direct ADP receptor antagonists Nitric-oxide-releasing antiplatelet substances	Inhibitors of tissue-factor–factor-VIIa pathway Selective factor Xa inhibitors Selective thrombin inhibitors Human activated protein C Soluble recombinant thrombomodulin

[a]Abbreviations: GP, glycoprotein; vWf, von Willebrand factor.

1.1.2.2 Risk factors for venous thrombosis

1.1.2.2.1 Inherited risk factors

Venous thromboembolism (VTE), comprising deep-vein thrombosis and pulmonary embolism (Schouwenburg et al. 2012), is a group of complicated disease that is driven by various inherited and acquired risk factors. Inherited risks of VTE can be differentiated by two pathomechanisms: gene mutations leading to loss of antithrombotic function (deficiency in ATIII, PC and PS) or those leading to gain-of-prothrombotic-function (Factor V Leiden (FVL), Prothrombin G20210A, dysfibrinogenaemia, high Factor VIII level and non-O blood group) (Martinelli, De Stefano & Mannucci 2014). The prevalence of ATIII, PC and PS

deficiencies is relatively low in the average population, however, they are still significant contributors to VTE development, accounting for 15-20 times greater risk compared to non-deficient individuals (Lijfering et al. 2009).

Overall, over 200 mutations (Reitsma et al. 1995; Lane et al. 1996; García de Frutos et al. 2007) have been discovered in these anticoagulant genes, the majority of which result in decreased protein production or impaired function of normal protein. ATIII and PC deficiencies are divided into two sub-types. Patients with low antigen and activity levels are considered as type I, while type II sufferers have normal antigen levels but reduced activity. The anticoagulant, PS is present in the circulation in two forms with a large proportion of circulating PS (60-70%) being non-functional when bound to C4b-binding protein and only 30-40% of PS which circulates freely is functional (Dahlback & Stenflo 1981; Griffin, Gruber & Fernandez 1992). Hence, deficiencies in PS are divided into three subtypes on the basis of total and free PS concentrations, and their general activity. Approximately 95% of PS deficient individuals are type I and type III familial patients, or in the mixed type I/III due to the shared gene defect (Zoller, Garcia de Frutos & Dahlback 1995; Simmonds et al. 1997).

1.1.2.2.2 Acquired risk factors

In addition to congenital risk factors, a range of acquired risk factors also contributes to increased thrombotic risk as well. First, the risk of VTE is believed to be greatly elevated with age (Næss et al. 2007). Other risk factors of VTE can be grouped into weak, moderate and strong (Figure 1.4) that refer to the relative risk ratio (Bonita, Beaglehole & Kjellstrom 2006) for developing disease between affected and unaffected people.

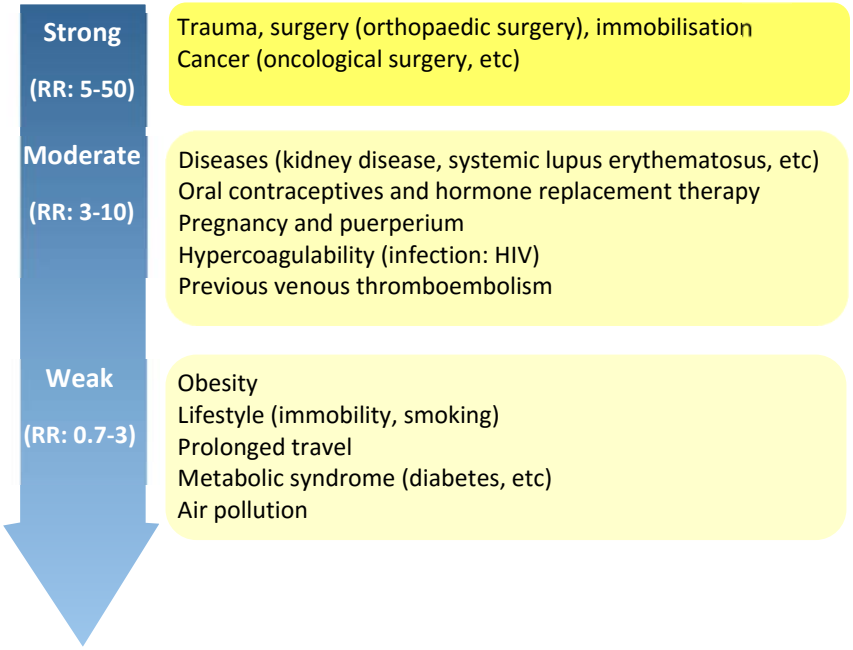


Figure 1.4. Acquired risk factors of venous thrombosis. (Adapted from Lijfering et al. 2011; Previtali et al. 2011).

Trauma, surgery, immobilisation and cancer are seen as strong risk factors for VTE (Danilenko-Dixon et al. 2001; Rosendaal et al. 2005). People with systemic lupus erythematosus or kidney disease, individuals with high circulating oestrogen levels arising from hormone therapy or who are pregnant, or those with an acute infection or who have previously experienced a VTE episode, are at moderate risk of developing VTE compared to individuals without these conditions (Lijfering et al. 2011; Previtali et al. 2011). Obesity, smoking and metabolic syndrome like diabetes are weak risk factors for VTE (Figure 1.4).

One of the acquired risk factors, high oestrogen levels affects women who are taking an oral contraceptive (OC), undergoing hormone replacement therapy (HRT) or who are pregnant. Currently, the most commonly available OC in the market is a combined formula with 20-40

μg of synthetic oestrogen like ethinylestradiol and two types of progestogen (second and third generation) (Bleker, Coppens & Middeldorp 2014). Studies investigating the risk of OC use and VTE have reported a 2 to 6 fold increased risk of VTE in users (Farmer et al. 1997; Lidegaard et al. 2009; van Hylckama Vlieg et al. 2009). The highest risk of VTE is observed in the first three months to a year of OC use, and there are conflicting evidence on whether the risk of VTE persists in women who have ceased OC use (Bloemenkamp et al. 2000; van Hylckama Vlieg et al. 2009; Bleker, Coppens & Middeldorp 2014).

The levels of various coagulation factors have been reported to be altered by OC use, including the up-regulation of Factor II, Factor VII, Factor VIII and Factor X (procoagulants) levels and down-regulation of ATIII and PS (anticoagulants) levels (Meade 1982; Burkman et al. 1991; Norris & Bonnar 1997; Koenen et al. 2005; Previtali et al. 2011). These studies showed that the use of OC is able to enhance resistance to APC, which shifts the overall haemostatic balance to become prothrombotic.

HRT can be prescribed as either oestrogen-only or combined oestrogen-progestin preparations and can be administrated differently (Bleker, Coppens & Middeldorp 2014). Generally, HRT is provided to postmenopausal women who fail to produce normal level of oestrogen for their age group, thus the absolute risk for this cohort is believed to be higher than those using OC, although several studies consistently revealed that the risk of VTE is 2-3 fold higher in women using HRT (Hemminki & McPherson 1997; Roberts et al. 2008). Furthermore, the risk of VTE is greater if OC or HRT users have an inherited VTE risk,

especially individuals with FVL that results in 2 to 5 times increase of VTE risk compared to those without FVL (Vandenbroucke et al. 1994; Herrington et al. 2002).

The frequency of VTE in pregnant women leading to maternal mortality occurs in 1-2/1000 pregnancies and the risk of women developing VTE during pregnancy will increase 4 to 5 times higher in comparison to non-pregnant women (Heit et al. 2005; Bleker, Coppens & Middeldorp 2014). FVL carriers have an 11-to-52 fold increased risk of VTE if they are pregnant, this increased risk is also present in carriers of congenital VTE risk factors (Martinelli et al. 2002; Doggen et al. 2008).

During pregnancy, circulating oestrogen levels progressively rise and this is associated with changes in the levels of haemostatic factors to a hypercoagulable state (Previtali et al. 2011; Bleker, Coppens & Middeldorp 2014). Multiple procoagulants (TF, Factors V,VII, VIII, IV, X, XI, fibrinogen, vWF and thrombin marker D-Dimer) are elevated and levels of circulating PS that lead to reduction of APC sensitivity are decreased (Figure 1.5).

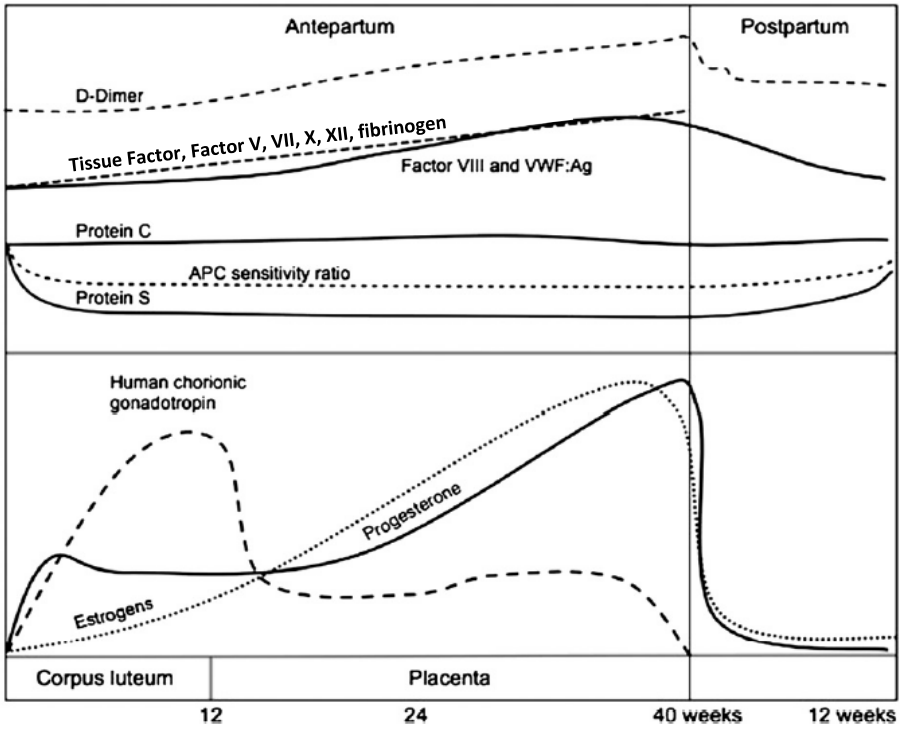


Figure 1.5. Haemostatic and hormone change during normal pregnancy. (Adapted from Bleker, Coppens & Middeldorp 2014).

These changes serve to ensure a smooth delivery of the baby where the hypercoaguable state can prevent excess bleeding during birth; nevertheless, there is also the potential to promote unnecessary thrombus formation due to the imbalance of procoagulant and anticoagulant factors (Bleker, Coppens & Middeldorp 2014). Although the association between elevated oestrogen levels and hypercoaguablility is frequently observed in the clinic, the mechanisms of oestrogen-mediated regulation of these haemostatic factors are poorly studied.

1.2. Oestrogen and oestrogen regulation

1.2.1 Oestrogen, oestrogen receptor and oestrogen synthesis

Oestrogen is an essential female steroid hormone responsible for female sexual development and the maintenance of bone strength and body homeostasis. There are three main classifications of oestrogen: oestrone, oestradiol (E_2 or 17β-oestradiol) and oestriol; with E_2 being the primary-form in women (Cui, Shen & Li 2013). The ovary is the dominant site of oestrogen synthesis and the production of oestradiol can be catalysed from androgens via a tissue specific enzyme, aromatase in ovarian granulosa cells (Figure 1.6).

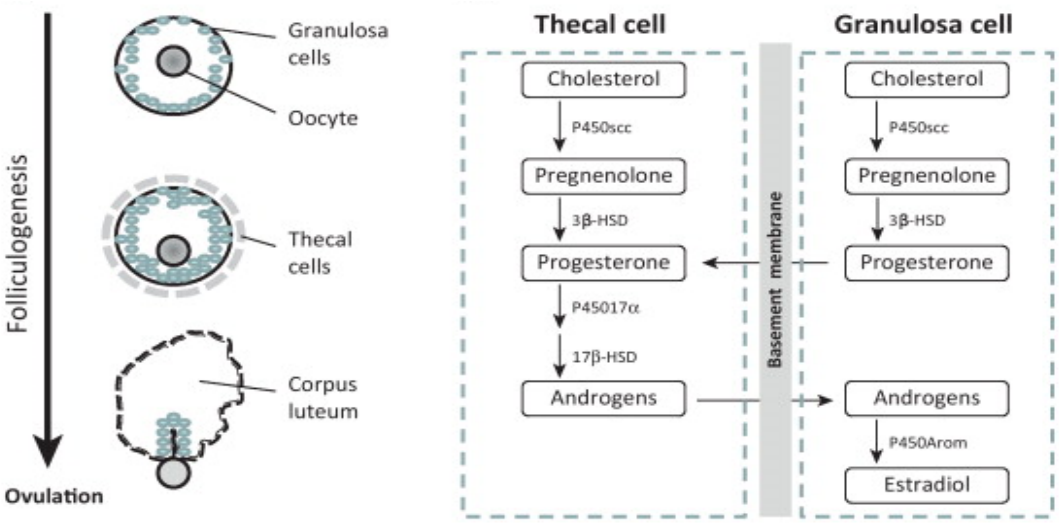


Figure 1.6. Oestrogen synthesis in ovary. (A) Folliculogenesis. (B) Oestrogen synthesis in ovary. The synthesized pathway initiates after cholesterol is catalysed into pregnenolone via cytochrome P450 side-chain cleavage enzyme; pregnenolone located in both thecal and granulosa cells is converted into progesterone by another enzyme, 3β-hydroxysteroid dehydrogenase; further conversion of progesterone can be achieved via cytochrome P450 17α-hydroxylase recruited with 17β-hydroxysteroid dehydrogenase to yield androgens in thecal cell. Lastly, the major form of E_2 can be catalysed by aromatase in granulosa cell. (Cui, Shen & Li 2013)

Besides the ovary, other non-gonadal tissues, such as brain, adipose tissue, breast and osteoblast cells (Miller 1991; Shozu & R. Simpson 1998; Garcia-Segura et al. 1999) can also synthesise oestrogen via the aromatase pathway to benefit post-menopausal women who have limited oestrogen production in the ovaries (Gruber et al. 2002). E_2 is secreted into the bloodstream via binding to carrier proteins, with 60% albumin-bound and 38% SHBG-bound (Figure 1.7-A); only 2% of oestrogen freely circulates (Boron & Boulpaep 2012).

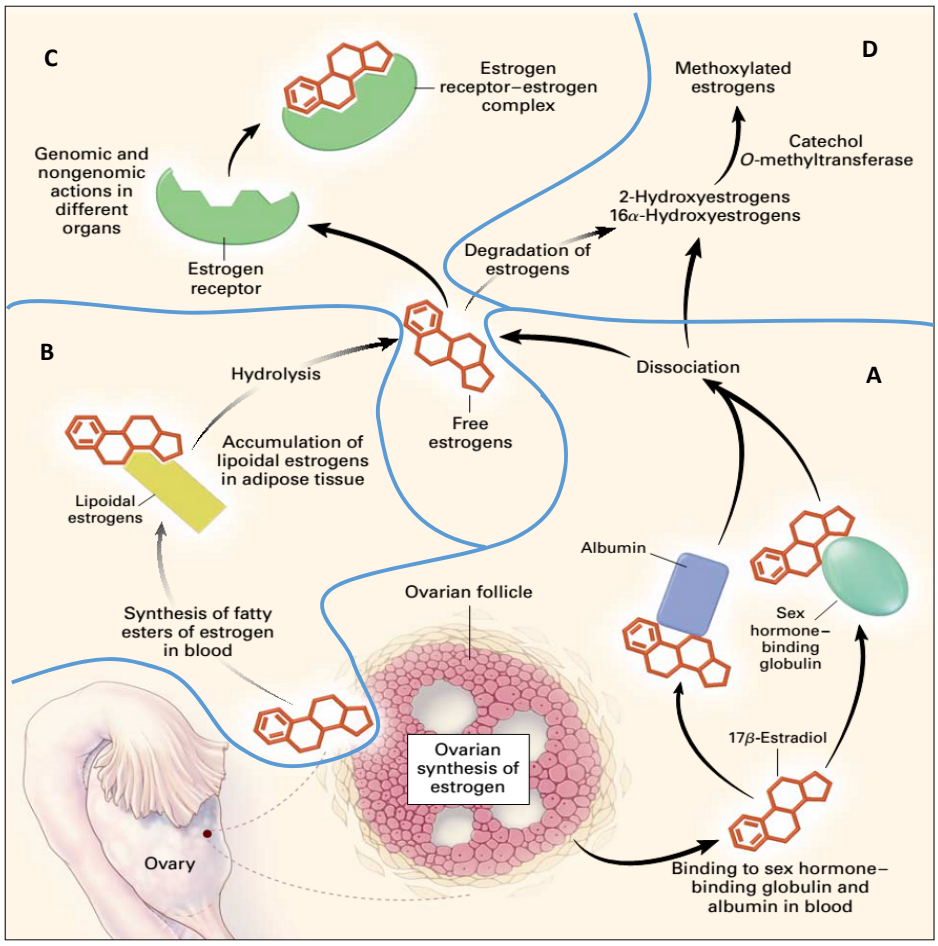


Figure 1.7. Oestrogen synthesis, transport and metabolism. A) oestrogen is syntheses in the ovary and is transported via carrier proteins; B) lipoidal oestrogen accumulates in adipose tissue; C) free oestrogen binds oestrogen receptors in the target tissue; D) metabolism of oestrogen. (Adapted from Gruber et al. 2002).

When oestrogen reaches the target tissue, it dissociates from the carrier proteins and directly diffuses across the cell membrane to interact with oestrogen receptor (ER) to activate its regulatory effect on the tissue (Figure 1.7-A, C). In addition to the ovary, oestrogen can also be synthesised in blood and other tissues. For example, lipoidal oestrogen is accumulated in adipose tissue to be further hydrolysed into free oestrogen (Figure 1.7-B). Non-functional oestrogen is converted into methoxylated oestrogen and excreted via bile or urine (Figure 1.7-D) (Gruber et al. 2002).

Oestrogen receptor (ER) transduces oestrogen-mediated signals. The receptor can be classified into nuclear-initiated (or genomic) ER (ERα and ERβ) and membrane-initiated (or non-genomic) ER (GPR30 and ER-X). Oestrogen receptor α and ERβ are encoded by *ESR1* and *ESR2* genes respectively, and are located on different chromosomes (Gosden, Middleton & Rout 1986; Enmark et al. 1997). Oestrogen receptor α is expressed in a variety of cerebral and peripheral tissues; while the tissue distribution of ERβ is more restricted to the ovary, which has the highest ERβ expression (Table 1.2) (Couse & Korach 1999; Hiroi et al. 1999).

Table 1.2. Primary tissues expressing oestrogen receptors variants. (Cui, Shen & Li 2013)

ER subtypes	Primary distribution in various tissues
ERα	Brain, breast, bone, epididymis, kidney, Leydig cells of testes, liver, stroma of prostate, theca and interstitial cells of ovary, uterus and white adipose tissue
ERβ	Bladder, brain, bone marrow, colon, epithelium of prostate, granulosa cells of ovary, lung, testis and vascular endothelium
GPR30	Brain, detected in adrenal medullar, ovary and renal pelvis
ER-X	Enriched in the fetal baboon brain, the neocortex, lung and uterus of the postnatal rodent; almost undetectable in the normal adult

The oestrogen-mediated activation through nuclear-initiated ER can occur within hours or even days, but the response of membrane-initiated ER can be much quicker, within seconds to minutes (Pedram, Razandi & Levin 2006).

1.2.2 Oestrogen signalling pathway and function

Oestrogen signalling is initiated after oestrogen diffuses through the cell membrane and binds to the ER. Ligand-bound ER molecules dimerise and are phosphorylated before translocation into the nucleus. The DNA-binding domain of the oestrogen-ER complex subsequently binds to specific DNA sequences (5'-GGTCACAGTGACC-3') namely oestrogen-response elements (EREs), within the promoter region of target genes (Figure 1.8) (Gruber et al. 2002).

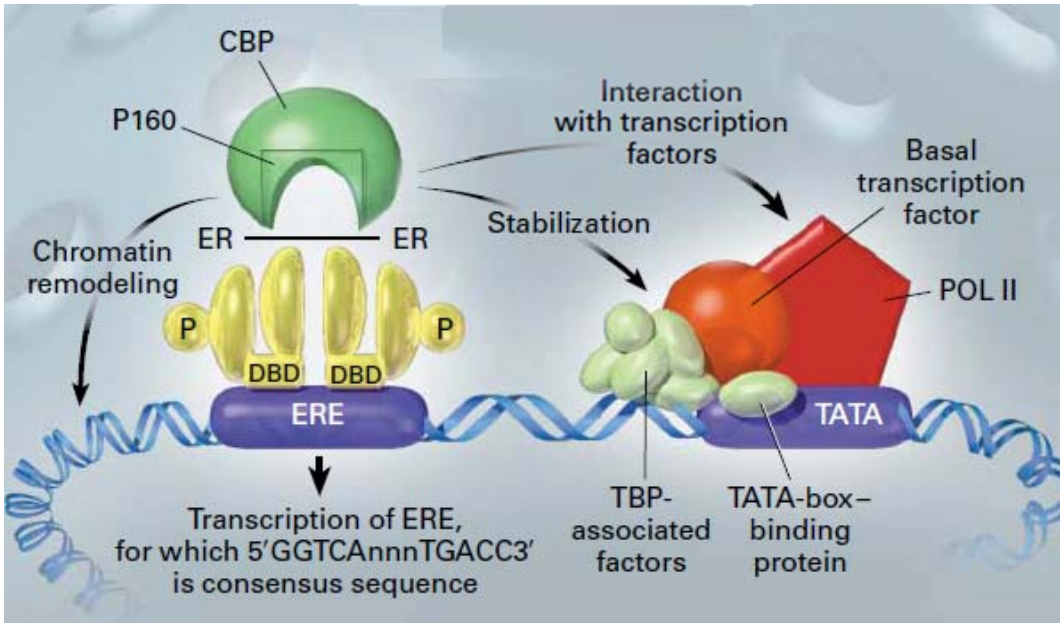


Figure 1.8. Key elements to be involved in oestrogen genomic signalling. (Adapted from Gruber et al. 2002).

This interaction stimulates target gene transcription through the action of the preinitiation complex. Transcription preinitiation factors are a large group of proteins essentially required for RNA polymerase II, a universal enzyme for RNA transcription, which consists of basal transcription factors, an assembly of several proteins like TATA-box-binding protein and ER's coactivators that enhance transcriptional activity (Horwitz et al. 1996) (Figure 1.8). Examples of coactivator include P160 and p300-cyclic AMP response-element-binding protein, which act to stabilise the preinitiation complex, remodel chromatin and interact with other transcription factors.

When all the conditions are in place, gene transcription will proceed (Figure 1.9 pathway 1-A) (Gruber et al. 2002). In addition to direct binding of ligand-bound ER on EREs on the target promoter, ER can also regulate target gene transcription indirectly via binding with transcriptional factors including Stimulating Protein-1 (SP-1), Activator Protein 1 and Nuclear Factor κB on the gene promoter (Figure 1.9 pathway 1-B) (Porter et al. 1996; Paech et al. 1997; Ray et al. 1997; Gottsch et al. 2009).

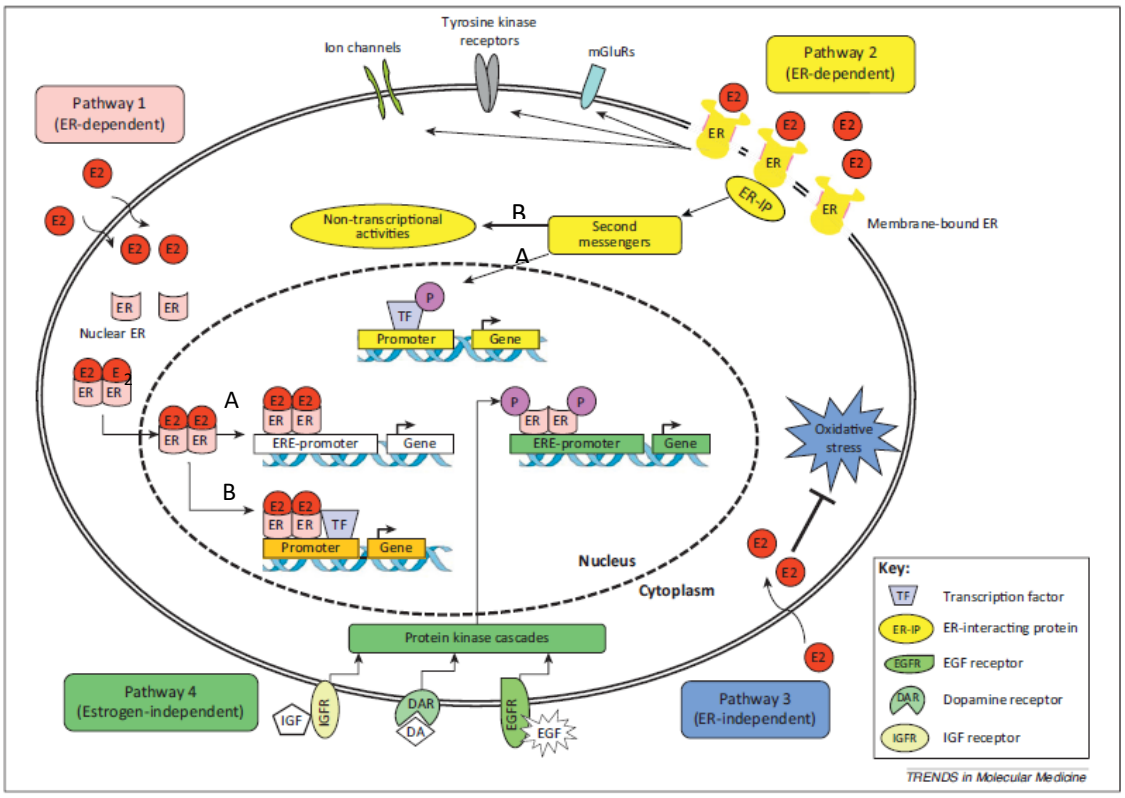


Figure 1.9. Oestrogen signalling pathways. Pathway 1: the nuclear-initiated ER bound to E2 diffuses into the nucleus, either interacts with EREs (A) or regulates TF (B) to stimulate gene transcription. Pathway 2: the membrane-initiated ER can trigger the activity of other membrane receptors and second messengers that can enhance (A) or inhibit (B) the transcription. Pathway 3: E2 can also independently suppress oxidative stress. Pathway 4: the phosphorylated (P) ER can be induced by protein kinase cascades instead of E2. This is mediated by insulin-like growth factor (IGF), dopamine (DA) and epidermal growth factor (EGF). (Adapted from Cui, Shen & Li 2013).

Apart from transcriptional regulation, ligand-bound ER can participate in non-genomic activities that are driven by other mechanisms. The activated membrane-initiated ERs (Figure 1.9 pathway 2) is able to interact with other membrane receptors, such as ion channels, tyrosine kinase receptors or metabotropic glutamate receptors (Huang & Woolley 2012), in order to induce their activities. Furthermore, a number of the intracellular protein kinase cascades can be activated through non-genomic oestrogen pathways. Besides two crucial ER-

dependent pathways, oestrogen signalling can also be initiated without oestrogen or ER (Figure 1.9 pathway 3 and 4) (Cui, Shen & Li 2013).

Genomic and non-genomic activities of oestrogen signalling can regulate target gene expression in a variety of organs, with E_2 specific effects on brain, eye, cardiovascular system, bone, breast, colon, ovary and skin (Gruber et al. 2002). Oestrogen-expressing breast cells can benefit through E_2 signalling to accelerate proliferation (Porter 1974); but the same effect applied to cancer cells is detrimental (Yue et al. 1999). In the brain, oestrogen signalling is crucial in maintaining normal function (Green, Bishop & Simpkins 1997).

More importantly, oestrogen signalling follows a tissue-specific pathway. The specificity of nuclear ER signalling highly depends on the precise binding between EREs and ERs, which means a slight change on the binding partner can influence the interaction with co-regulators, leading to activity reduction (Marino, Galluzzo & Ascenzi 2006). For instance, ERα will have more favourable interaction with steroid receptor coactivator-2 if it binds to vitellogenin A2 EREs rather than the same element from vitellogenin B1 gene (Wood et al. 2001). Although the pathology of oestrogen-induced thrombosis, especially VTE can be explained based on Virchow's model, the actual mechanism of oestrogen signalling in causing thrombosis is still poorly understood at the molecular level.

1.3. MicroRNAs

1.3.1 MicroRNA biosynthesis

Prior to the end of the 1990s, non-coding RNAs were often referred to as “junk RNA” that had no ascribed biological function. This perspective has totally changed since the discovery of the first microRNA, *lin-4* in *Caenorhabditis elegans* (Lee, Feinbaum & Ambros 1993), indicating non-coding RNAs can also play an essential role in gene regulation. MicroRNAs (miRNAs), are short non-coding RNAs of ~22 nucleotides in length that bind with the 3’untranslated region (UTR) of the target genes to cause mRNA degradation or inhibit mRNA translation (Cech & Steitz 2014).

MiRNA synthesis is initiated in the nucleus from the miRNA genome (Figure 1.10). Within the genome, intergenic region (space between exon and intron) accounts for half of miRNA genes; 40% and 10% of miRNA coding genes are located within the intron and exon respectively (Rodriguez et al. 2004; O'Carroll & Schaefer 2013).

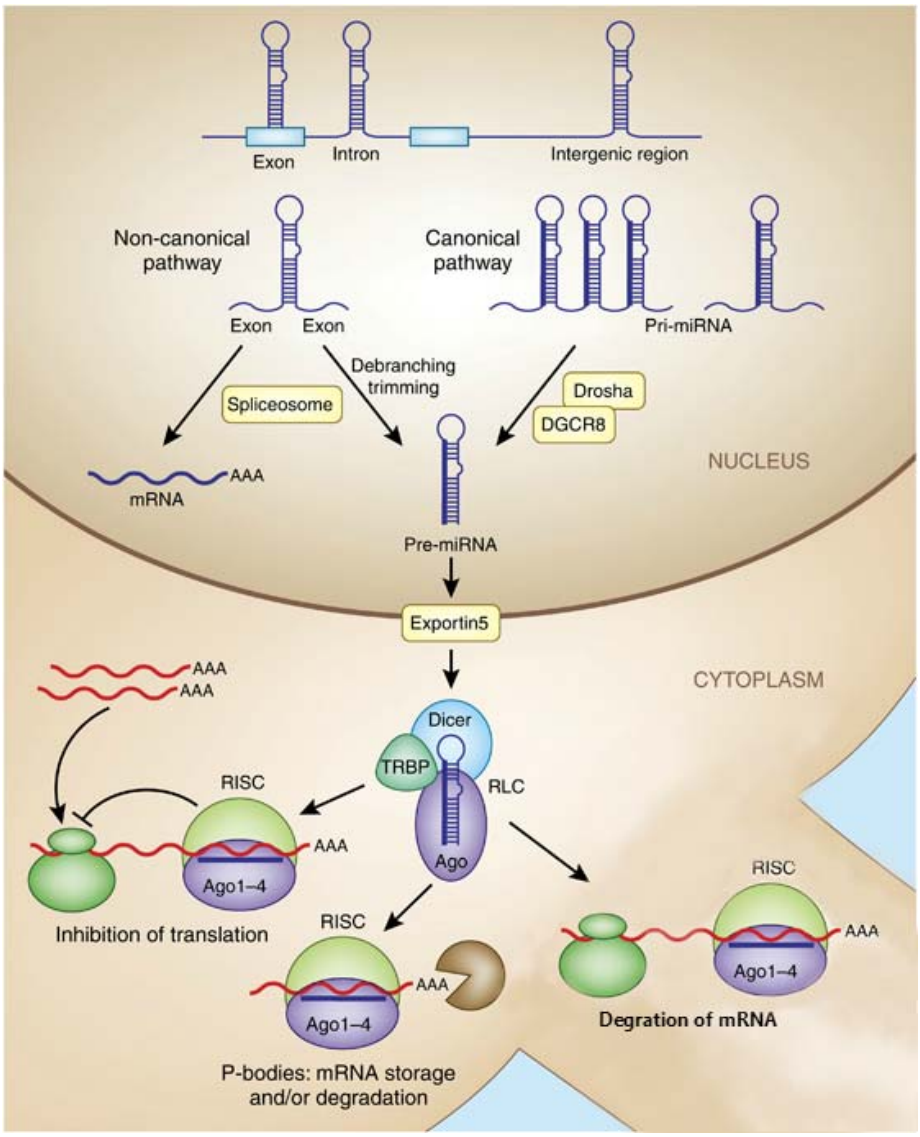


Figure 1.10. Canonical and non-canonical pathway of microRNA biosynthesis. (Adapted from O'Carroll & Schaefer 2013).

The gene is transcribed into a primary miRNA via RNA polymerase II, and the hairpin double stranded primary miRNA is recognized by two core proteins, Drosha (ribonuclease III enzyme) and DGCR8 (RNA binding protein), resulting in cleavage to give rise to a ~60-to-70 nucleotides long precursor miRNA (pre-miRNA) (He & Hannon 2004; Bozzoni et al. 2008). After which, the pre-miRNA is exported from nucleus to cytoplasm by Exportin-5, a

nucleus and cytosol shuttling protein. Once in the cytoplasm, the pre-miRNA is cleaved by another ribonuclease III enzyme, Dicer with the recruitment of TRBP2, a subunit of RNA-induced silencing complex Loading Complex, to form a duplex strand containing a template (or functional) miRNA and a transient (or non-functional) miRNA (Figure 1.10) (Filipowicz, Sonenberg & Bhattacharyya 2008). The latter is degraded by one of the Argonaute family protein (Ago) members, Ago2 (Filipowicz, Sonenberg & Bhattacharyya 2008), while the former is further cleaved by Dicer to generate a ~21-to-25 nucleotide-long single stranded miRNA (Kim et al. 2002). Ultimately, the mature miRNA is assembled into RISC or microribonucleoprotein (miRNP) where it comprises Ago, to create a miRNA-induced silencing complex that silences the target gene via mRNA degradation, translation repression, or processing bodies localization (Figure 1.10) (Meister 2013; Pedroza-Torres et al. 2014).

Besides the major canonical pathway as discussed above, miRNA can additionally be synthesised by a Drosha-independent approach, via debranching or trimming of a pri-miRNA molecule into a pre-miRNA or using spliceosome to act as an intron removal tool to produce a mature miRNA (Figure 1.10). However, only a small number of miRNAs are produced by these non-canonical pathways (Berezikov et al. 2007; Rasmussen et al. 2010). Furthermore, miRNA expression is highly tissue specific. Guo *et al.* (2014) identified tissue-specificity of 116 miRNAs in 12 distinct tissues, noting that almost 90% of analysed miRNAs were specifically expressed in a single tissue type while the expression of the remaining miRNAs investigated were detected in more than one tissue: miR-1, for example, displayed the expression in both heart and skeletal muscle. Similar findings that prove miRNA specificity have been announced 8 to 10 years before by Sood *et al.* (2006) and Babak *et al.* (2004)

respectively; but Guo and his colleague (2014) offer more details in miRNA and transcription factor controlled networks.

1.3.2 MicroRNA-message RNA interaction

The interaction between miRNA and its target mRNA can occur via perfect and imperfect complementary base pairing. Target recognition by miRNA requires 2-7 nucleotides as "seed" region to base-pair with the transcribed gene mRNA target (Lewis et al. 2003). The target site of miRNA binding is located in the mRNA 3'UTR, while increasing evidence also shows that binding of miRNA-mRNA can occur in the 5'UTR or the central coding region of the gene target (Easow, Teleman & Cohen 2007; Lytle, Yario & Steitz 2007; Bartel 2009). Furthermore, the degree of interaction is related to complementarity between the 3' end of mRNA and 5' end of miRNA, which forms the basis for the differentiation of the miRNA binding sites into three classes: canonical (Figure 1.11-A~E), 3'-supplementary (Figure 1.11-F) and 3'-compensatory sites (Figure 1.11-G) (Bartel 2009; Witkos, Koscianska & Krzyzosiak 2011).

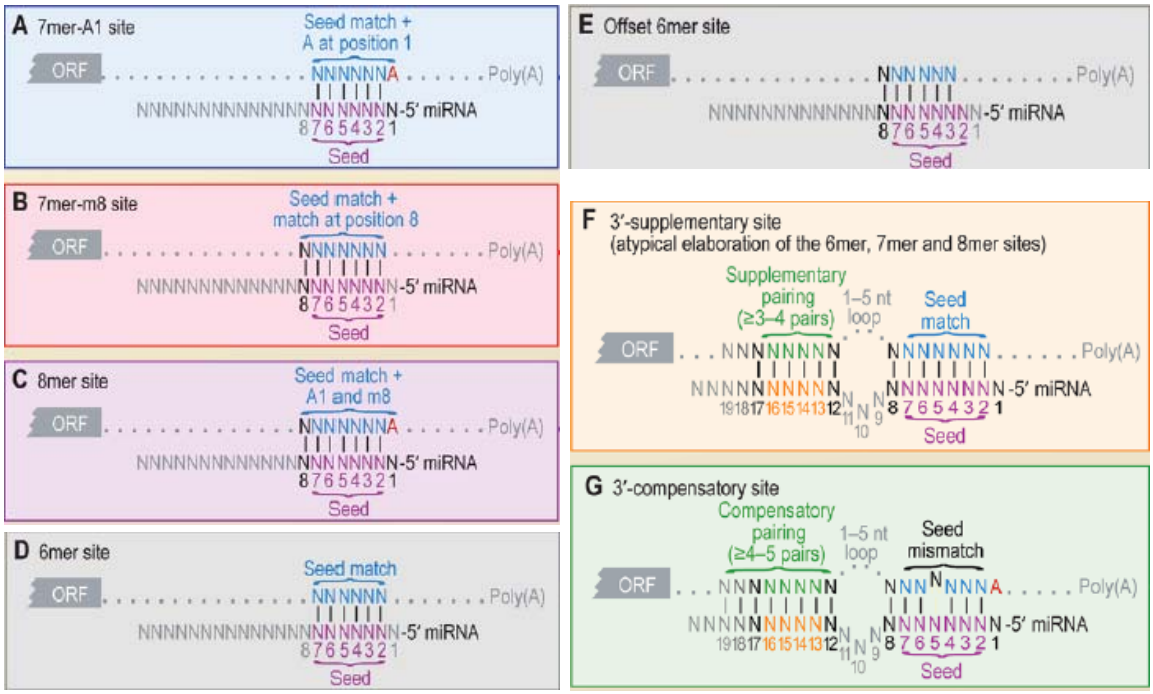


Figure 1.11. Classification of miRNA target sites. Vertical dashes represent single Watson-Crick pairing; seed region on 5'miRNA (purple); Adenine at position 1 (red); complimentary sequence of seed region on 3'mRNA (blue); extending nucleotide at position 12-16 of miRNA strand (orange); the base-pairing of extending nucleotide on mRNA strand (green); irrelevant sequences in miRNA: mRNA interaction or middle bridging mismatched loop in 3' supplementary and 3' compensatory site (grey). (Bartel 2009)

Canonical sites can be further divided into 3 sub-types: 7mer-A1 site that contains an adenine nucleotide at position 1 of the 3'section of mRNA (Figure 1.11-A); 7mer-m8 site that has an additional match at position 8 (Figure 1.11-B); and 8mer site that involves both features mentioned in previous two sites (Figure 11-C). A shorter site named 6mer site (position 2-7) can also be regard as target seed, despite its putatively modest function (Figure 1.11 D-E). If additional complementary base-parings are present at position 13-16 of the seed region, they are 3'-supplementary sites (Figure 1.11-F); however, the presence of mismatched seed can be replaced by similar extension numbers of base-pairing to form 3'-compensatory sites (Figure 1.11-G) to complete miRNA-mRNA interactions. In general, canonical sites form

the majority of miRNA target sites and 7mer-m8 sites tend to be targeted by highly conserved miRNAs (Friedman et al. 2009).

The miRNA-mRNA interaction can be summarized into three rules to improve specificity (Figure 1.12) (Filipowicz, Sonenberg & Bhattacharyya 2008).

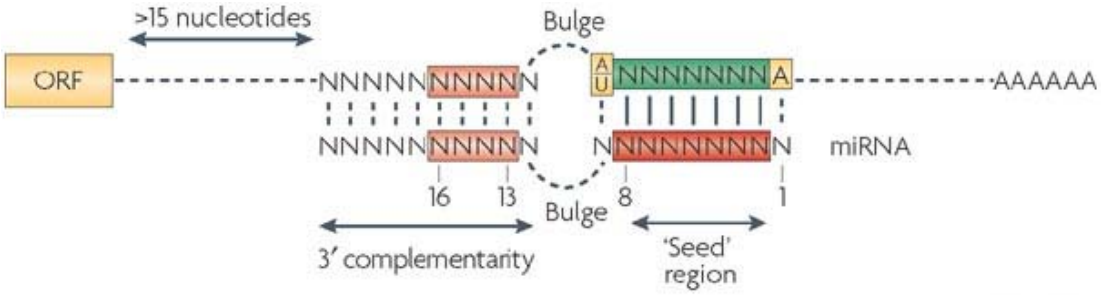


Figure 1.12. Major rules of miRNA target sites. Vertical dashes represent single Watson-Crick pairing; seed region on 5'miRNA (red); adenine at position 1 and adenine or uracil at position 9, ORF (open reading frame) (orange); complimentary sequence of seed region on 3'mRNA (green); extending nucleotide at position 12-16 of miRNA and mRNA strand (pink); irrelevant sequences in miRNA: mRNA interaction or middle bridging mismatched loop in 3' supplementary and 3' compensatory site (black). (Filipowicz, Sonenberg & Bhattacharyya 2008)

Rule 1, the seed region is a key element to achieve the occurrence of interaction. Additionally, an adenine located at position 1 of the seed region, and an adenine or uracil at position 9, are able to enhance binding site efficiency. Rule 2, Ago-induced cleavage of mRNA can be eliminated if the mistakes are present in the central region of miRNA-mRNA complex (position 10-12 of miRNA). Rule 3, complementarity between mRNA and miRNA at miRNA 3'half end can stabilise the interaction, since mismatches and bulges are more

tolerated here than the seed region, especially in the position of 13-16 that may shift into a more important binding site once the seed region is not the optimal choice.

As the pre-requisite region for binding between miRNA and mRNA binding is short, each miRNA is able to target multiple gene transcripts and vice versa. The same mRNA can be targeted by a single miRNA or miRNA clusters that almost share the identical sequence at their 5'UTR, which may be regulated at the transcriptional level via particular transcription factors and modified chromatin (Bartel 2009; Hausser & Zavolan 2014). If miRNA clusters are within the target gene in the range of 8-40 nucleotides, each miRNA can perform independently and simultaneously with each other, resulting in greater repressed effect than the total effect from two sites, suggesting miRNAs are able to cooperate with each other (Grimson et al. 2007; Sætrom et al. 2007). Interestingly, the positive effect of miRNA on mRNA was described in two studies, showing that miRNA-373 and miRNA-10a, in turn, induced the target gene expression and translation (Ørom, Nielsen & Lund 2008; Place et al. 2008). To date, miRNAs have been found to regulate proliferation, differentiation, apoptosis and development of normal and abnormal cells in eukaryotes (Friedman & Jones 2009). Taken together, these features establish a complicated miRNA-dependent gene regulation network that may be highly tissue specific.

1.3.3 Haemostasis-associated microRNAs

The role of miRNAs in diseases, cancers in particular (Friedman & Jones 2009), has been thoroughly investigated with accumulating evidence illustrating their importance in controlling the pathological process. Furthermore, miRNAs have been discovered as a stable element with diagnostic biomarker potentials (Schwarzenbach et al. 2014). In contrast to tumour-related miRNAs, the association between haemostasis and miRNAs has only been reported in recent years and the mechanism on how miRNAs regulate haemostatic elements remains poorly understood. Herein, miRNAs that have been reported to target different haemostatic genes will be addressed (Table 1.3) and the potential haemostatic gene target is shown in appendix 1.

Table 1.3. Known haemostasis-associated miRNAs and their corresponding targets.

Haemostatic factors	miRNAs	Human cell types/animal tissues	Regulation on target mRNAs	References
Platelet function				
$P2Y_{12}$ protein	miR-223	Embryonic kidney cells; megakaryocytes	↓ *$P2Y_{12}$*	(Plante et al. 2009)
Coagulation Factors				
Factor XI	miR-181a-5p	Liver cells	Direct ↓ *F11*	(Salloum-Asfar et al. 2014)
Fibrinogen	miR-29a, b, c; miR-409-3p	Liver cancer cells	Indirectly ↓*FGA, FGB, FGG* (via HNF4α); Directly ↓*FGB-β*	(Fort et al. 2010; Hatziapostolou et al. 2011; Lukowski et al. 2013)
Fibronectin 1	miR-1	HeLa derivative cells	Direct ↓*FN1*	(Wang et al. 2011)
Tissue factor	miR-19a; miR-19b, miR-20a; miR-223	Monocytic cells; breast cancer cells; colon cancer cells; vascular endothelial cells	Directly ↓ *F3*	(R. Teruel et al. 2011; Yu et al. 2013; Li et al. 2014a; Li et al. 2014b)
Anticoagulation Factors				
Antithrombin	miR-18a,b; miR-200a	Livers of mice	Regulate *SEPRINC1;* Indirectly ↓ *SERPINC1* (via St3gal3, St3gal4)	(Raúl Teruel et al. 2011; Teruel et al. 2013)
Fibrinolysis				
Plasminogen activator inhibitor-1	miR-30c, miR-301a; miR-421	Umbilical vein endothelial cells; pulmonary endothelial cells	Direct ↓*SEPRINE1*	(Patel et al. 2011; Marchand et al. 2012)
Others				
Thrombospondin 1	Lef-3g; Lef-7a, miR-18a, miR-194, miR-221	Umbilical vein endothelial cells & knock-out mice; HeLa derivative cells & malignant glioma cell	Direct ↓ *THBS1*	(Dogar et al. 2014; Liao et al. 2014)
VKCORC1	miR-133a	Liver cancer cells	Directly ↓	(Pérez-Andreu et al. 2012)

Legend: VKCORC1 (vitamin K 2,3-epoxide reductase complex subunit 1).

$P2Y_{12}$, is a G protein-coupled receptor involved in platelet aggregation (Cimmino & Golino 2013), its gene expression was reported to be regulated by microRNA 223 (miR-223) in human embryonic kidney 293 cells and megakaryocytes (Plante et al. 2009). This result further supports the close relationship between platelet and miR-223, as the former is the main source of circulating miR-223 (Plé et al. 2012; Willeit et al. 2013). Interestingly, a contrasting result was described by Leierseder *et al.* (2013), where mice displayed normal platelet activity despite a lack of miR-223; miRNAs levels were also found to be variable in men and women, suggesting the complexity of miR-223 function in platelets (Simon et al. 2014).

In terms of coagulation factors, Salloum-Asfar *et al.* (2014) showed that miR-181a-5p is able to directly inhibit the expression of Factor XI at both mRNA and protein level and this miRNA was suggested to specifically target *F11* gene, since no correlation was found between miR-181a-5p and *F9* gene, which encodes for Factor IX that is activated by Factor XI. Fibrinogen, the inactive precursor of the coagulation factor fibrin, can be both directly and indirectly regulated via distinct miRNAs and it is encoded by three gene, *FGA, FGB* and *FGG*. Fort *et al.* (2010) published the first paper describing miRNA regulation of fibrinogen-genes and demonstrated that miR-29 members (including subtypes miR-29a, b, c) can indirectly down-regulate all of the three fibrinogen genes; while *FGB-β* was validated to bind with the seed region of miR-409-3p to achieve the direct reduction of target gene expression.

The transcriptional factor hepatocyte nuclear factor 4α was shown to be targeted by miR-29 members that may contribute to indirect fibrinogen regulation (Hatziapostolou et al. 2011;

Lukowski et al. 2013). Direct inhibition of *FN1* (appendix 1) gene through miR-1 was shown by Wang *et al.* (2011) and multiple studies indicated a direct down-regulation of TF via 4 miRNAs (Table 1.3), especially miR-19b that was reported to repress the *F3* gene in three different cell types (R. Teruel et al. 2011; Yu et al. 2013; Li et al. 2014a; Li et al. 2014b).

Teruel *et al.* (2011) discovered an inverse correlation between the antithrombin gene and miR-18a or miR-18b in newborn mice, suggesting miRNAs can modulate haemostatic genes during haemostasis development. Two years later, the same group published that another miRNA, miR-200a may target two enzymes, ST3 beta-galactoside alpha-2,3-sialyltransferase 3 and 4 (St3gal3, St3gal4) that act on antithrombin sialylation (Table 1.3), to indirectly control the post-translational process in antithrombin (Teruel et al. 2013). MiR-30c and miR-421 can both directly down-regulate PAI-1 activity (Marchand et al. 2012). Moreover, miR-30c and miR-301a were found to directly repress placental growth factor-mediated *PAI-1* gene (Patel et al. 2011).

Apart from the common haemostatic factors, other associated elements like thrombospondin 1 and VKCORC1, which is vital for vitamin-K dependent proteins (appendix 1), can be mediated by various miRNAs. Lef-7a, miR-18a, miR-194 and miR-221 are able to directly target the *THBS1* mRNA transcript and cause translational suppression (Dogar et al. 2014); lef-7g was shown to have a direct down-regulation effect in a dose-dependent manner (Liao et al. 2014). Mendell *et al.* (2006) reported that the Myc-mediated miR-19 showed direct repression of thrombospondin 1 protein, whereas, miR-19 has not been reported to directly

regulate *THBS1*. Interestingly, VKCORC1 was observed to be directly suppressed by miR-133a (Pérez-Andreu et al. 2012).

1.4. Oestrogen-induced microRNAs in haemostasis

1.4.1 Oestrogen signalling and microRNA

Nuclear ERs are capable of regulating target gene transcription via direct interaction within the target promoter region, as well as indirectly via other posttranscriptional regulators like miRNAs. Oestrogen signalling has been reported to regulate the expression of approximate 260 different miRNAs (Campbell et al. 2002). Activated ER can regulate the expression of miRNA genes by directly binding to miRNA promoter (P. Bhat-Nakshatri et al. 2009) or with other transcription factors that regulate the promoter (Castellano et al. 2009). Despite breast cancer cells being the model cell line used for most studies, it is not without controversy.

For instance, E_2-mediated miRNA let-7f effect was demonstrated by 6 individual laboratory groups with 50% showing E_2 up-regulation and the other 50% indicating E_2 down-regulation (Blenkiron et al. 2007; Kovalchuk et al. 2007; P. Bhat-Nakshatri et al. 2009; Klinge 2009; Maillot et al. 2009; Di Leva et al. 2010). On the other hand, miRNAs can also control ERα gene expression, miR-206 (Adams, Furneaux & White 2007), miR-221/222 (Cochrane et al. 2010) have been shown to cause suppression of ERα activity. Furthermore, several studies indicate that oestrogen signalling (E_2 and ERα) is associated with modulating miRNA biosynthesis.

1.4.2 Mir-494 and Protein S expression

The increase in oestrogen levels is a significant VTE risk factor for women who use OC or who are pregnant. Oestrogen signalling can target specific genes presented in various tissues, and increasing evidence suggests that genomic ERα signalling can trigger the up- and down-regulation of miRNA levels. MiRNA control of haemostatic diseases is still not fully understood. In breast cancer cells, numerous oestrogen-regulated miRNAs have been identified, yet few studies mention the effect on haemostatic diseases, except for two papers on miRNA and PS deficiency that were recently published (Suzuki et al. 2010; Tay et al. 2013).

Suzuki *et al.* (2010) asserted that genomic ERα signalling can inhibit PS gene *PROS1* expression and PS normal activity. Suzuki and his colleagues also discovered E_2 regulation was closely related to two adjacent GC-rich motifs located on the *PROS1* promoter segment, which was further confirmed to interact with transcriptional factors SP1 and SP3 with ERα. Moreover, the ERα-SP1 protein-promoter interaction was also found to involve the recruitment of receptor-interacting protein 140 and the nuclear receptor corepressor-silencing mediator for retinoid and thyroid hormone receptors-Histone deacetylase 3 complex. This suggests the ERα-Sp1 complex as described above contributes to the levels of PS gene reduction (Suzuki et al. 2010).

Three years later, Tay *et al.* (2013) demonstrated a down-regulation of *PROS1* gene with increased expression of E_2-induced miRNA, miR-494, which is able to directly target the 3'UTR of the *PROS1* gene and induce translational inhibition. Moreover, Tay *et al.* (2013)

demonstrated that oestrogen signalling can also be expressed in non-gonadal tissues, such as liver carcinoma cells (HuH-7), where E_2-responsive miRNAs target the anticoagulant PS gene. Taken together, these studies provided experimental evidence that suggests the potential function of oestrogen signalling or oestrogen-modulated miRNAs to regulate the genes attributed to haemostasis or thrombosis.

1.4.3 NanoString nCounter® micro array

NanoString nCounter® micro array is a recent technology capable of quantifying the total amount of nucleic acids via stoichiometric hybridization. NanoString workflow comprises three major procedures: hybridisation, purification and counting (Figure 1.13) (*nCounter Workflow* 2015). The target nucleic acid sequence is hybridised by two specific probes: capture probes and reporter probes. Both of them are complementary to the target sequence: capture probes carry an affinity tag bound to the 5'end while reporter probes have a distinct color-coded tag at the 3'end sequence. Different types of nucleic acids will use their own specific probes in NanoString; for instance, if studying the miRNAs in a human sample, a specific probe is provided to target this category of RNA. After applying the hybridisation step to all the target DNA or RNA species, excess probes are removed and the purified target/probe complex is immobilised upon a slide surface. Lastly, the immobilised complex is measured by counting the specific colour barcodes in the microarray.

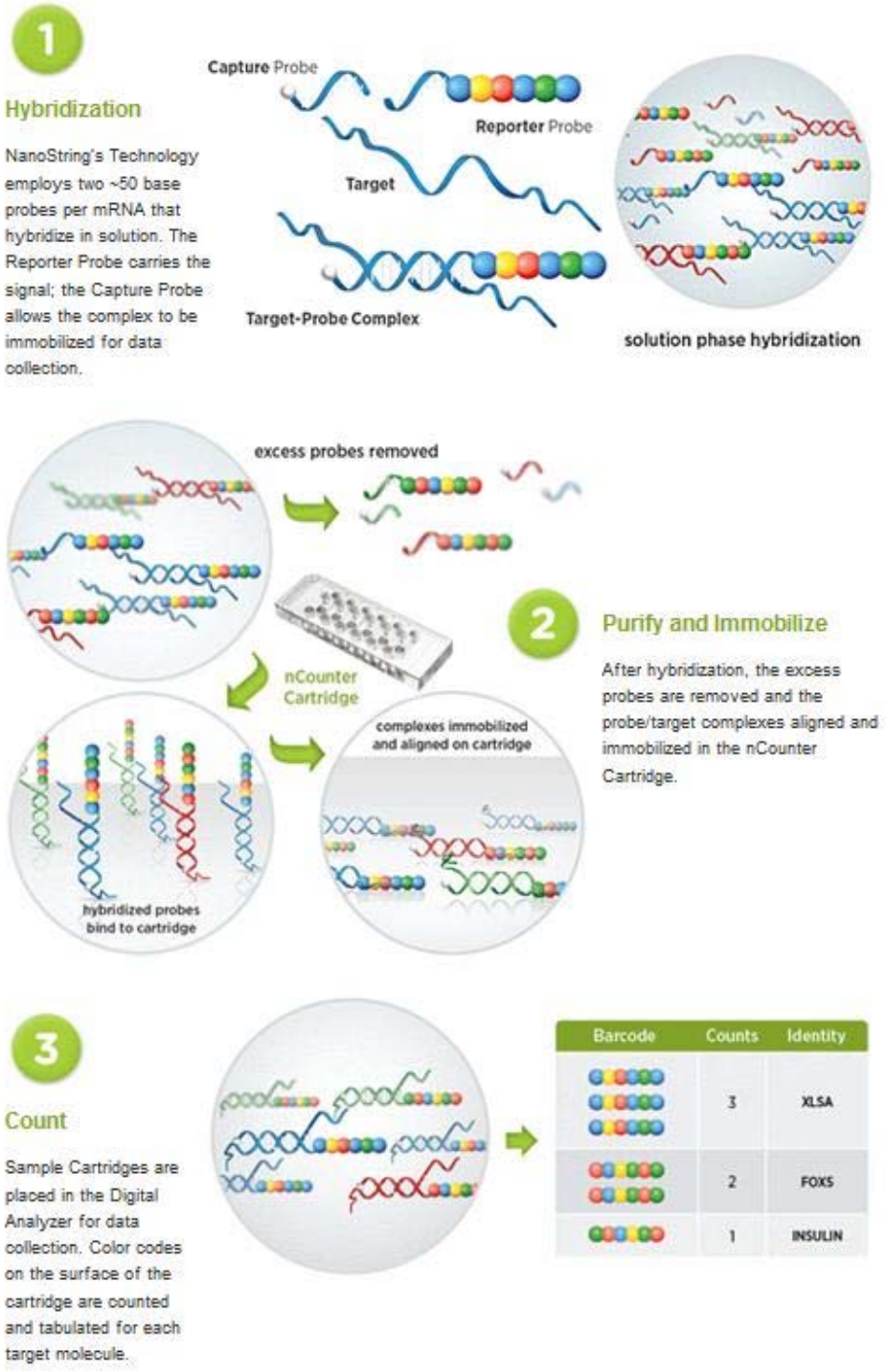


Figure 1.13. Workflow of NanoString nCounter® micro array. (*nCounter Workflow* 2015)

By measuring the sample directly, NanoString prevents genetic-specific 3' bias. Likewise, NanoString contains greater sensitivity than normal microarray as examined samples are in the aqueous state instead of being bound to a surface. The digital readout in NanoString can also be advantageous in exhibiting more explicit signals, and a relatively large-scale screening in contrast to traditional microarray as well as reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is another advantage of NanoString (Oliveri et al. 2008).

NanoString technique had been employed to analyse the target sequence in various diseases, especially potential gene targets contributing to carcinoma development (Singh et al. 2011; Prat et al. 2013; Quek et al. 2015). Regulatory small RNAs like miRNAs also become a popular application field for NanoString (Wang et al. 2012; Liu et al. 2015). As the Western Australian Centre for Thrombosis and Haemostasis (WACTH) is interested in oestrogen-regulated haemostasis and thrombosis, Dr. Jasmine Tay previously performed two NanoString arrays on different cell lines and plasma samples to search for novel E_2-responsive miRNAs.

1.4.4 Hypothesis and aim

Based on unpublished results, approximately 31 miRNAs were identified as significantly E_2-responsive in HuH-7 cells. These results, however, require further validation by alternative detection methods. We hypothesise that oestrogen-mediated thrombotic diseases, like PS deficiency, are likely caused by ER regulation of a network of miRNAs, which may target a number of coagulation factors to trigger a hypercoaguable state under conditions of high E_2 concentration.

To test this hypothesis, the primary aim is to validate the oestrogen-responsiveness of candidate miRNAs identified in the microRNA array analysis using RT-qPCR. The secondary aim is to identify the coagulation factors that may be regulated by these E_2-responsive miRNAs via several computational analyses and to characterise whether these miRNAs directly target their putative coagulation genes using dual-luciferase reporter assay.

Chapter 2

Materials

2.1 Reagents and suppliers

2.1.1 Cell culture

Reagents	Catalogue No.,Suppliers
0.25 % Trypsin-EDTA	25200-056; Gibco
0.4% Trypan blue stain	T10282; Invitrogen
Charcoal stripped fetal bovine serum (CSS)	12676-011; Gibco
Dulbecco's modified eagle's medium (DMEM)	21063-029; Gibco
Fetal bovine serum (FBS)	10099-141; Gibco
Konakion® MM phytomenadione (10mg/mL)	84-80-0; Roche
MEM non-essential amino acids (NEAA) (100X)	11140-050; Gibco
Penicillin streptomycin (10,000 U/mL)	15140-122; Gibco
Sodium pyruvate (100mM)	11360-070; Gibco

2.1.2 Beta-oestradiol treatment

Reagents	Catalogue No. and Suppliers
Absolute ethanol	5077-20G PL; Ajax Finechem
Beta-oestradiol	E8875-1G; SIGMA-ALDRICH

2.1.3 Cell harvesting and RNA extraction

Reagents	Catalogue No. and Suppliers
Absolute ethanol	5077-20G PL; Ajax Finechem

mirVana™ PARIS™ Kit	AM1556; Ambion
RNaseZap® RNase decontamination solution	AM9780; Ambion
RNA*later*® stabilisation solution	AM7021; Ambion

2.1.4 DNase treatment and RNA concentration measurement

Reagents	**Catalogue No. and Suppliers**
Amicon ultra-0.5 centrifugal filter devices	UFC500324; Merck Millipore
TURBO DNA-*free*™ kit	AM1907; Ambion

2.1.5 Reverse transcription of total RNA

Reagents	**Catalogue No. and Suppliers**
50μM Random hexamers	N8080127; Invitrogen
5,000 units RNaseOUT™ recombinant ribonuclease inhibitor	10777-019; Invitrogen
10,000 units SuperScript® III reverse transcriptase: - 5X First-Strand Buffer, - 0.1M DTT, - 200units/μL SuperScript™ III Reverse Transcriptase.	18080-044; Invitrogen
Sigma water	W4502; SIGMA-ALDRICH
TaqMan® microRNA reverse transcription kit - 100mM dNTPs,	4366596; Applied Biosystems

- MultiScribe Reverse Transcriptase,
- 10X RT buffer,
- RNase Inhibitor.

2.1.6 Quantitative polymerase chain reaction

Reagents	**Catalogue No. and Suppliers**
Sigma water	W4502; SIGMA-ALDRICH
TaqMan® gene expression assays: - *PROS1* (ID: Hs00165590_m1), - *ESR1* (ID: Hs00174860_m1), - *F2* (ID: Hs01011988_m1), - *F3* (ID: Hs01076029_m1), - *F5* (ID: Hs00914120_m1), - *F8* (ID: Hs00252034_m1), - *18S* (ID: Hs99999901_m1), - *GAPDH* (ID: Hs02758991_m1), - *ACTB* (ID: Hs99999903_m1).	4331182; Applied Biosystems
TaqMan® miRNA assays: - hsa-let-7f-5p (ID: 000382), - hsa-miR-128-3p (ID: 002216), - hsa-miR-18b-5p (ID: 002217), - hsa-miR-26b-5p (ID: 000407), - hsa-miR-326 (ID: 000542),	4427975; Applied Biosystems

- hsa-miR-338-3p (ID: 002252),
- hsa-miR-365a-3p (ID: 001020),
- hsa-miR-423-5p (ID: 002340),
- hsa-miR-4455 (ID: 463355_mat),
- hsa-miR-455-3p (ID: 002244),
- hsa-miR-494-3p (ID: 002365),
- hsa-miR-548aa (ID: 463041_mat),
- hsa-miR-98-5p (ID: 000577),
- RNU6B (ID: 001093),
- RNU44 (ID: 001094),
- RNU48 (ID: 001006).

TaqMan® universal master mix II, no UNG	4440040; Applied Biosystems

2.1.7 Dual-luciferase reporter assay

Reagents	**Catalogue No. and Suppliers**
Ampicillin sodium salt	A9518; SIGMA-ALDRICH
Isopropanol	1407174409; Ajax Finechem
Metafectene® PRO	T040-1.0; Biontex
Nuclease-free Water (1.75mL)	AM9914G; Ambion
NucleoBond® xtra midi kits	740410.100; MACHEREY-NAGEL
pMIR-REPORT™ miRNA expression reporter vector system	AM5795; Invitrogen

pRL-SV40 Vector	E2231; Promega
RapidReporter® Firefly glow assay kit	GeneStream

2.2 Equipment

Equipment	**Catalogue No. and Suppliers**
0.2mL Maximum Recovery PCR tube	PCR-02-L-C; Axygen
1.5mL Mikro-Schraubröhre tube	72.692.005; Sarstedt
1.7mL MaxyClear microcentrifuge tube	MCT-175-L-C; Axygen
2mL MaxyClear microcentrifuge tube	MCT-200-C-S; Axygen
5mL Serological pipette	606 107; Greiner Bio-One
10mL Serological pipette	607 107; Greiner Bio-One
15mL Falcon tube	188271; Greiner Bio-One
24 well Cell culture plate	353047; Falcon
30mL Tube with yellow screw cap	60.9922.212; Sarstedt
50mL Falcon tube	227261; Greiner Bio-One
75cm^2 Tissue culture flask	353136; Falcon
96 well Cell culture microplate	655098; Greiner Bio-One
96-well PCR-Cooler	eppendorf
100*20mm Cell culture dish	664160; Greiner Bio-One
Allegra X-15R Centrifuge	Beckman Coulter
Allegra 25R Centrifuge	Beckman Coulter

ART™ barrier pipette tips (10, 20, 200, 1000µL)	2140-05-HR, 2149-P-05-HR, 2770, 2179-05-HR; Thermo Scientific
Biopette® A, 8 channel (20-200µL)	P4612-200A; Labnet
C1000™ thermal cycler	BIO-RAD
CellGard ES class II biological safety cabinets	NU-480-400E; NuAire
Centrifuge 5430	eppendorf
CFX96™ and CFX384™ real-time PCR detection system	BIO-RAD
CFX Manager, version 3.1	BIO-RAD
CO_2 incubator	MCO-18AIC; SANYO
Countess™ automated cell counter	C10281; Invitrogen
Eclipse microscope	TS100; Nikon
EVE™ cell counting slide	EVS-050; NaNoEnTek
Incubator Shaker 4500	Bioline
IsoTherm-System (2mL)	eppendorf
Lab dancer tube shaker	IKA
Microfuge® 16 Centrifuge	Beckman Coulter
Microseal® 'C' Optical Film Film	#MSC1001; BIO-RAD
MoToPET pipette controller	Axygen
NanoDrop® Spectrophotometer	ND1000; Thermo Fisher Scientific
Nichipet 7000 multi-channel pipette (5-50µL)	NICHIRYO
Single pipettes (0.1-2µL, 0.5-10µL, 2-20µL, 20-200µL, 100-1000µL)	AP-2, AP-10, AP-20, AP-200, AP-1000; Axypet

Sprout® mini centrifuge	HS1000BC; Heathrow Scientific
Stirred thermostatic water bath	NET-4; Clifton
Two block digital dry block heater	DBH20D; Ratek
Victor™ light luminescence counter	1420-060; PerkinElmer
Wallac 1420 Manager (version 3.0)	PerkinElmer

2.3 Location of suppliers

Beckman Coulter Australia Pty Ltd, Lane Cove, New South Wales, Australia

Bioline (Aust) Pty Ltd, Alexandria, New South Wales, Australia

Biontex-USA, San Diego, California, USA

Corning Inc., Corning, New York, USA

Eppendorf South Pacific Pty. Ltd, North Ryde, New South Wales, Australia

Fisher Biotec, Wembley Western Australia, Australia

GeneStream, City Beach, Western Australia, Australia

GenScript, Piscataway, New Jersey, USA

Greiner Bio-One GmbH, Bad Haller, Kremsmünster, Austria

Heathrow Scientific LLC, Vernon Hills, Illinois, USA

IKA Works, Inc., Wilmington, North Carolina, USA

Labnet office, Edison, New Jersey, USA

MACHEREY-NAGEL Inc., Bethlehem, Pennsylvania, USA

Millipore Ireland B.V., Carrigtwohill, County Cork, Ireland

NaNoEnTek office, Guro-gu, Seoul, Korea

NICHIRYO office, Koshigaya-City, Saitama Prefecture, Japan

NuAire, Inc., Plymouth, Minnesota, USA

Nikon Instruments Inc., New York, USA

PerkinElmer office, Glen Waverley, Victoria, Australia

Promega office, Alexandria, New South Wales, Australia

Roche Australia Pty Ltd, Dee Why, New South Wales, Australia

Ratek Instruments Pty Ltd, Boronia, Victoria, Australi

Sarstedt, Inc., Newton, North Carolina, USA

SANYO Electric Co., Ltd, Moriguchi City, Osaka, Japan

SIGMA-ALDRICH Pty Ltd, Castle Hill, New South Wales, Australia

Thomas Scientific, Inc., Swedesboro, New Jersey, USA

Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Victoria, Australia

Chapter 3

Methods

3.1 Cell Culture

The human liver cancer cell line, HuH-7 was gifted by Associate Professor Mark Watson (Institute for Immunology and Infectious Diseases, Murdoch University). HuH-7 cells were maintained in DMEM media without phenol red and were supplemented with 10%(v/v) of FBS, 1nM sodium pyruvate, 100U/mL penicillin streptomycin and 1X NEAA. Cells were cultured in 75cm^2 flask and incubated at 37°C, 5% CO_2 incubator. Media was replaced every 2-3 days and cells were passaged once a week or when they reached 90% confluency.

To passage cells, the culture media was removed and cells were washed with sterile PBS (appendix 2); then the entire culture surface was covered with ~2mLs 0.25 % Trypsin-EDTA and incubated at 37°C for ≤ 2min for trypsin to dislodge the cells from the plastic surface. The trypsin solution was neutralised with equal or greater volume of culture media, and diluted cell suspension was transferred into a clean 75cm^2 flask that contained appropriate 10mL culture media. HuH-7 cells were passaged at a dilution of 1:15.

3.2 Viable cell counts

Homologous cell suspension (10µL) were mixed with 0.4% Trypan blue in 1:1 ratio and loaded into EVE^{TM} cell counting slide; total number per mL and viability of the cells were detected after inserting the slide into automated cell counter. The viable cells were unstained and calculated by total cell number/mL × viability (%) × volume of cell suspension (mL).

3.3 Beta-oestradiol treatment

When the cell density of HuH-7 cells exceeded 9.0×10^5 cells/mL, they were evenly seeded (1.5×10^6 cells per well) into 100mm cell culture petri dishes, and maintained in DMEM media supplemented with 5%(v/v) charcoal stripped fetal bovine serum (CSS). The adjustment of media contents was to minimise oestrogen mimic (FBS) side-effect during subsequent beta-oestradiol (E_2) treatment and maximise *PROS1* expression by the cells. The HuH-7 cells were incubated at 37°C for 1.5~2 days.

When cell confluency was above 50% after 24h of incubation, the media in the wells was replaced with fresh media that contained 0.1%(v/v) absolute ethanol (vehicle group) or freshly prepared 10^{-8}M E_2 (treatment group) (Table 3.1). The fresh E_2 solution was serially diluted from a 10^{-2}M E_2 stock prepared by Dr. Quintin Hughes (appendix 2) on the day of the experiment. The E_2-treated HuH-7 cells were incubated at 37°C for 12h and 24h, and the treatment was initiated from the time of media replacement.

Table 3.1. Beta-oestradiol treatment for 12h and 24h.

	12h treatment	**24h treatment**
Components	0.1% absolute ethanol; 10^{-8}M β-oestradiol	0.1% absolute ethanol; 10^{-8}M β-oestradiol

3.4 Cell harvesting and RNA extraction

The vehicle-treated or E_2-treated cells were dislodged from the culture dish by trypsinisation (section 3.1) and the cell suspensions were transferred to clean 15mL tubes. Cell counting was performed and the cell suspensions were centrifuged at 200xg for 5min at room temperature to pellet cells. The culture supernatant was discarded and the cell pellets were resuspended in 1mL RNA*later*® stabilisation solution and stored in 4°C for a minimum of 24h and up to 7 days, prior to RNA extraction.

Cells in RNA*later*® were recovered by diluting the cell suspension with an equal volume of PBS (appendix 2) and centrifuging at 2000xg at room temperature for 5min to pellet the cells. The diluted RNA*later*® solution was carefully removed before proceeding with RNA extraction. Total RNA from each sample was extracted using *mirVana*™ PARIS™ Kit according to the kit protocol provided. Briefly, cell pellets were lysed with 500µL ice-cold Cell Disruption Buffer; then denatured with an equal volume of 2X Denaturing Solution and incubated on ice for 10min. Subsequently, an equal volume (1mL) of Acid-Phenol: Chloroform was added and the mixture was vortexed for 60s and centrifuged for 10,000xg for 5min to separate the organic and aqueous phases. The top organic-extracted aqueous layer (~800µL) was carefully transferred to a clean tube and mixed with 1.25 times volume (1mL) of absolute ethanol, mixed well by inversion and pipetted into a Filter Cartridge and centrifuged for 1min at 10,000xg to bind the total RNA. The flowthrough was discarded and the filter was washed once with 700µL Wash Solution 1 followed by two washes of 500µL Wash Solution 2/3. Before elution, the filter was centrifuged for 2min at 10,000xg to remove

any remaining wash solution. Finally, the filter was transferred to a clean 1.5mL tube and the total RNA eluted with 100µL Elution Solution heated to 95°C by centrifugation.

3.5 DNase treatment and RNA concentration measurement

To remove contaminating genomic DNA from the extracted total RNA, DNase treatment was performed using the TURBO DNA-*free*™ Kit. Firstly, 100µL of total RNA was combined with 10µL 10X TURBO DNase Buffer and 1µL TURBO DNase and incubated at 37°C for 30min. The TURBO DNase reaction was terminated by adding 10µL DNase Inactivation Reagent and incubated at room temperature for 5min with mixing every few times. The inactivated total RNA/DNase mixture was then centrifuged for 10,000xg for 90s and the supernatant containing DNase-treated total RNA was carefully transferred into clean 1.5mL tubes, aliquoted in 20µL volume and stored at -80°C freezer until required. The total RNA concentration and purity were analysed using NanoDrop® 1000 Spectrophotometer to determine 260nm/280nm and 260nm/230nm ratios.

If the 260nm/230nm ratio from an individual sample was below 1.5, total RNA samples from the same experimental set were mixed with deionised water up to 500µL and transferred to Amicon® Ultra-0.5 Centrifugal Filter Devices and centrifuged at 14,000xg for 30min at room temperature, in order to remove phenol contaminants. Afterwards, filter was placed upside down to a clean filtrate collection tube and concentrated RNA elutes were collected by centrifuging for 8,000xg for 5min. The quantity and quality of the washed RNA samples were measured again using NanoDrop® 1000 Spectrophotometer.

3.6 Reverse Transcription of Total RNA

3.6.1 Gene expression

Total RNA was converted to cDNA for gene expression qPCR analyses using the two-step reverse transcription approach with SuperScript™ III Reverse Transcriptase. For first strand synthesis, 1µg of total RNA was combined with 1µL of 50µM random hexamers and 1µL of 10mM dNTP mix (appendix 2), and the reaction volume was made up to 13µL with nuclease-free water and heated to 65°C in a thermal cycler for 5min. The sample were cooled on ice for 1min and subsequently combined with the SuperScript™ III mastermix which consisted of 4µL of 5X First-Strand Buffer, 1µL of 0.1M DTT, 1µL of SuperScript™ III Reverse Transcriptase and 1µL of RNaseOUT™ Recombinant RNase Inhibitor. The samples were mixed by pipetting and the reaction mix was returned to the thermal cycler and heated to 25°C for 5min, 50°C for 60min and the reverse transcription reaction inactivated at 70°C for 15min. The cDNAs were used for TaqMan® gene expression analyses the next day (section 3.7) or stored in -20°C freezer for up to two weeks until required.

3.6.2 MicroRNA expression

For the analysis of microRNA expression, total RNAs were required to be converted into cDNA. MicroRNA cDNA conversion was performed by one-step reverse transcription using TaqMan® MicroRNA Reverse Transcription Kit. In an individual reaction, 3µL of 100ng/µL total RNA was combined with TaqMan® MicroRNA Reverse Transcription mastermix, comprising of 6µL customised RT primer pool (appendix 2), 0.3µL 100mM dNTPs, 3µL MultiScribe Reverse Transcriptase, 1.5µL 10X RT buffer and 0.19µL RNase Inhibitor; the RT mixture was made up of nuclease-free water to yield 15µL final volume. The reaction

mix was placed in the thermal cycler and heated to 16°C for 30min, 42°C for 30min and the miRNA cDNA synthesis terminated at 85°C for 5min. The cDNAs were used for TaqMan® microRNA expression analyses the next day (section 3.7) or stored in -20°C freezer for up to two weeks until required.

3.7 Quantitative Polymerase Chain Reaction

Once cDNAs were produced, Taqman® gene expression assays and Taqman® Small RNA Assays were applied to quantify target gene expression (*PROS1, ESR1, F2, F3, F5* and *F8)* and microRNA expression (let-7f-5p, miR-128-3p, miR-18b-5p, miR-26b-5p, miR-326, miR-338-3p, miR-365a-3p, miR-423-5p, miR-4455, miR-455-3p, miR-494, miR-548aa and miR-98-5p) respectively via q-PCR. The total volume of q-PCR reaction mix was adjusted to 10µL, thus the volume of 20X Taqman® expression assay and 2X Taqman® expression master mix were halved (5 and 0.5µL per well respectively) in order to maintain the ratio of 10:1 described in the manuals. Complementary DNA samples were diluted to 1:7 (140µL and 105µL total volume for gene expression and microRNA expression respectively) using deionised water and 4.5µL of the diluted cDNA was loaded into each well of the 96- or 384-well reaction plate (Figure 3.1).

The plate was sealed with a film and the combined cDNA and mastermix was centrifuged at 200xg for 30sec. The qPCR plates were processed in a qPCR machine at the following conditions: denaturation at 95°C for 10min, annealing at 95°C for 15s and extension at 60°C for 1min. The normal numbers of cycles for annealing and extension step was 40, except for oestrogen receptor α analyses that required up to 44 cycles (Figure 3.1).

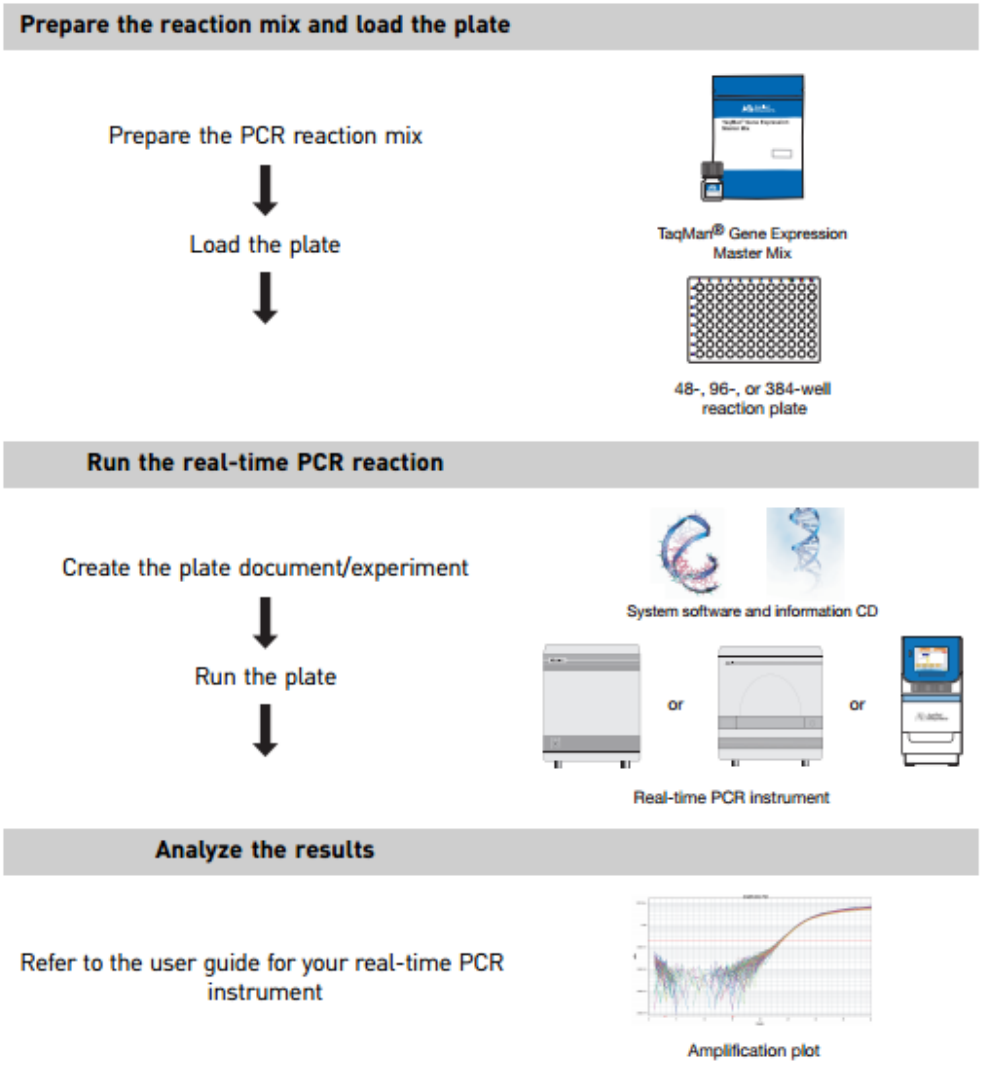


Figure 3.1. Quantitative polymerase chain reaction workflow.

For qPCR gene expression, each loaded sample was normalised against the expression of 18S ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*); while samples for the detection of miRNA expression were normalised against the expression of small-nucleolar RNAs reference genes *RNU6B, RNU44, RNU48*. The expression of control and E_2-treated cells were both calculated by 2^-ΔΔCt and their differences were analysed by using Student's t-test and confidence interval: a p-value of less

than 0.05 and no overlapping of confidence interval were considered as statistical significant. All analyses were performed using Microsoft Excel software and CFX manager 3.1. The fold change of target gene and microRNA expression was expressed as mean ± standard error of mean.

3.8 Computational Analysis

Twelve miRNA candidates that had been analysed in NanoString ncounter® micro array were selected for validation experiments. The candidate miRNAs were selected based on two criteria: miRNA raw counts had to be above 40 and E_2-response in HuH-7 cell lines was significantly different ($p<0.05$) through comparing the counts between vehicle and E_2-treated groups. A miRNA, miR-338-3p that exhibited a significant E_2-responsiveness in human plasma sample, was also included for validation. TargetScan 5.2, microRNA.org and miRWalk 2.0 were used as computational tools to predict the potential coagulation-associated gene targets (appendix 1) of these miRNAs. The predicted binding sites of the candidate miRNAs in the coagulation genes were determined in their 3'UTR, and predicted miRNA binding sites in the 5'UTR and central coding region of these genes were also determined when applying the search in miRWalk 2.0.

3.9 Dual-Luciferase Reporter Assay

3.9.1 Preparation of luciferase plasmid vectors

The pMIR-REPORT vector (appendix 3a) containing a full length 3'UTR sequence (*PROS1, F3* and *F8*-3'UTRs) (appendix 4), and a pRL-SV40 vector (appendix 3b) that encodes for

Renilla luciferase. Both pMIR-REPORT vector and pRL-SV40 vector were transformed into *Escherichia coli* (strain DH5-α; *E.coli*) and stored in glycerol (appendix 2) at -80°C freezer before use. In order to propagate the plasmid of interests, *E.coli* bacteria that have pMIR-REPORT vector and pRL-SV40 vector were initially grown in 5mL lysogeny broth (LB; appendix 2) containing 100mg/mL ampicillin in 1:1000 dilution, with shaking at 37°C overnight; after which overnight culture was transferred into 100mL LB supplemented with 100μg/mL ampicillin and returned to the shaker for 37°C to grow overnight.

Plasmid DNAs (pMIR-*PROS1*, *F3, F8*-3'UTR and pRL-SV40) were extracted and purified via NucleoBond® Xtra Midi EF kit. Bacterial cells were recovered by centrifuging at 4064xg, for 20min at 4°C and the cell pellets were resuspended with 8mL Resuspension Buffer (RES-EF) that was prepared according to the manual instruction. Cell suspension was subsequently mixed with equal volume of Lysis Buffer (LYS-EF) by gently inverting the tube 5 times, and incubated at room temperature for exact 5min. During the lysis, Column Filters were inserted into NucleoBond® Xtra Columns and the filters were equilibrated by adding 15mL of Equilibration Buffer (EQU-EF). The lysing procedure was terminated by the immediate addition of 8mL Neutralisation Buffer (NEU-EF) with gentle inversion until a white colour was presented in the tube, and incubated on ice for 5min. Once the lysates were confirmed to have become homogeneous, they were carefully loaded onto the Column Filters. The first wash step was to apply 5mL Filter Wash Buffer (FIL-EF) to the filtration system, then the filters were removed. The columns were further washed with 35mL Wash Buffer (ENDO-EF) and 15mL Wash Buffer (WASH-EF) in turn, and the plasmid DNAs were collected in 15mL tubes after pipetting 5mL Elution Buffer (ELU-EF). Crude eluates were precipitated

through centrifuging at 4064xg for 60min at 4°C after the addition of 3.5mL isopropanol, then 2mL of endotoxin-free 70% ethanol was used to wash the precipitates with centrifugation at 4064xg for 20min at room temperature. When majority of ethanol was removed, the pellets were dried at room temperature for 5-10min and later dissolved in 20µL endotoxin-free water (H_2O-EF). The quality and quantity of plasmid DNA were detected using NanoDrop® 1000 Spectrophotometer.

3.9.2 Transfection

HuH-7 cancer cells were seeded in a 24 well tissue culture plate at a density of 25,000 cells per well and incubated overnight at 37°C, 5% CO_2 until an 80-90% cell confluency was reached. Fresh transfection solutions were prepared 2h before the incubation was completed and these solutions were initially separated into Solution A that contained various plasmid DNAs and miRNA of interests, and Solution B that included a transfection reagent (Table 3.2).

Table 3.2. Components of transfection solutions for a single well.

	Solution A	**Solution B**
Components	50µL DMEM media only; 400ng 3'UTR of interests (*PROS1*[@], *F3, F8*-3'UTR); 100ng pRL-SV40[#]; 0.5µL no-miRNA or 50µM pre-miRNA precursors (miR-NC or miRNA of interests)	48µL DMEM media only; 2µL metafectene PRO[*]

Legend: [@] (*PROS1*-3'UTR experimental set was the positive control of efficient transfection), [#](encodes for Renilla luciferase and were used for normalising the transfection of each 3'UTR of interest), [*] (Solution A should be added into Solution B and pipetted up and down once for mixing). miR-NC (miRNA negative control).

50µM stocks of pre-miRNA precursor (appendix 2) in Solution A were prepared beforehand, distributed into 5µL aliquots and stored in -20°C freezer for long-term use. When the preparation of Solution A and B was finished, Solution A was added into Solution B, mixed once by gently pipetting and incubated at room temperature for 15-20min. Meanwhile, each well with adhered cells was replaced with 400µL fresh DMEM media. After incubation, cells were immediately transfected with 100µL DNA: miRNA-lipid mixture that contained 50nM miRNA of interests (Table 3.3), for ≥6h.

Table 3.3. Summary of pre-miRNA precursors that predicted to target their corresponding 3'UTR sequences.

	***PROS1*-3'UTR[#]**	***F3*-3'UTR**	***F8*-3'UTR**
Pre-miRNA precursors	miR-NC; miR-494	miR-NC; let-7f-5p; miR-128-3p; miR-26b-5p; miR-365a-3p	miR-NC; let-7f-5p ; miR-26b-5p; miR-365a-3p; miR-455-3p; miR-548aa

Legend: [#](used as positive control of efficient transfection), miR-NC (miRNA negative control).

Later, the transfected cells were dislodged from the 24 well cultural plate by the addition of 100µL EDTA-Trypsin per well and neutralised with 2.9mL DMEM media that supplemented with 10%(v/v) FBS. One mL pipette was used to resuspend the cells completely and each sample that had different 3'UTR plasmid and miRNA of interests were evenly re-seeded into 6 wells of a 96 well micro-assay plate for further transfection up to 24h.

3.9.3 Luciferase reporter assay and analysis

At 24h post transfection, HuH-7 cells were lysed by the addition of 20µL of 5X lysis buffer per well and incubation at room temperature for at least 30min. After lysis, each sample (6 wells) in the 96-well culture plate was divided into two groups: triplicate wells for examining Firefly luciferase activities and the other triplicate wells were used for Renilla luciferase assay measurement. For luciferase activity, 60µL of Firefly/Renilla luciferase reporter substrate (appendix 2) was added to the appropriate wells and incubated in the dark for 10mins to allow the reaction to reach a steady glow phase for measurement. After 10min assay stimulation, the fluorescence can reach the maximum signal without exposure to light, and the dual-luciferase reporter activity was immediately measured and analysed using Luminescence Counter and Wallac 1420 Manager 3.0 respectively.

The normalised Firefly luciferase activity of each sample was calculated by dividing the averaged Firefly signals in the triplicate wells by the mean of corresponding Renilla signals from the triplicate wells of the same sample. Then the inhibition of putative gene targets by different miRNAs, was calculated by Firefly activity of each candidate miRNA against the activity of miRNA negative control. Statistical analyses were done by comparing the signal between the negative control and specific miRNA-transfected samples. The luciferase activity was expressed as mean ± standard error of mean.

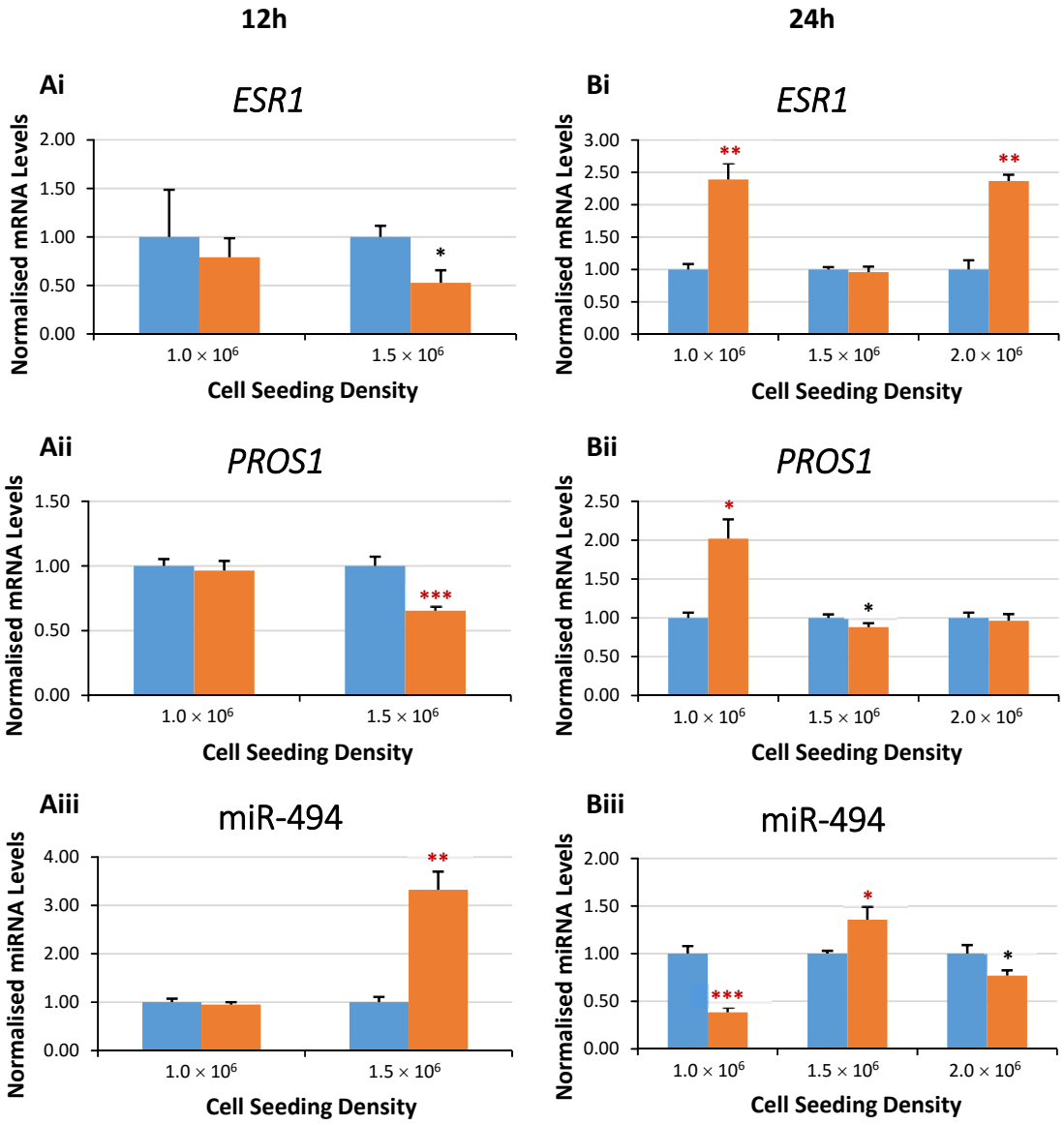
Chapter 4

Results

4.1 Optimisation of E_2 treatment in HuH-7 cells

In previous experiments performed in the WACTH laboratory, HuH-7 cells were treated with 10nM E_2 for 12h and additional miRNAs showing E_2-responsiveness were identified through NanoString® nCounter micro array. However, due to laboratory relocation, newer stocks of HuH-7 cells at lower passage numbers were obtained from Associate Professor Mark Watson, and treatment conditions had to be re-optimised. Re-optimised conditions include cell seeding density and E_2-induction time point (section 4.1). The optimal E_2-treatment condition was then applied to HuH-7 cells and E_2-responsive miRNA expressions were validated using RT-qPCR analysis (section 4.2). Following the identification of putative gene targets of these validated miRNAs, miRNA direct effect on their relative gene targets were determined (section 4.3).

To optimise E_2-treatment conditions, HuH-7 cells were seeded in increasing cell numbers in 10cm petri dishes and cultured in the absence or presence of 10nM E_2 for 12h or 24h (Figure 4.1). Samples exhibiting an up-regulation of miR-494 and associated down-regulation of PROS1 expression were used as positive controls for E_2-responsiveness experiments (Tay et al. 2013). Oestrogen signalling activation can also be directly measured by the level of oestrogen receptor α (*ESR1*) mRNA, because active-formed oestrogen receptor results in increasing turnover of the ER-α through ubiquitin proteasome pathway (Lonard et al. 2000), therefore a decrease in *ESR1* levels is indicative of bioactive E_2.



Legend: Vehicle, Treatment (10nM E_2). * p<0.05 (obtained in t-test or confidence interval only); * p<0.05, ** p<0.01, *** p<0.001 (obtained in both t-test and confidence interval).

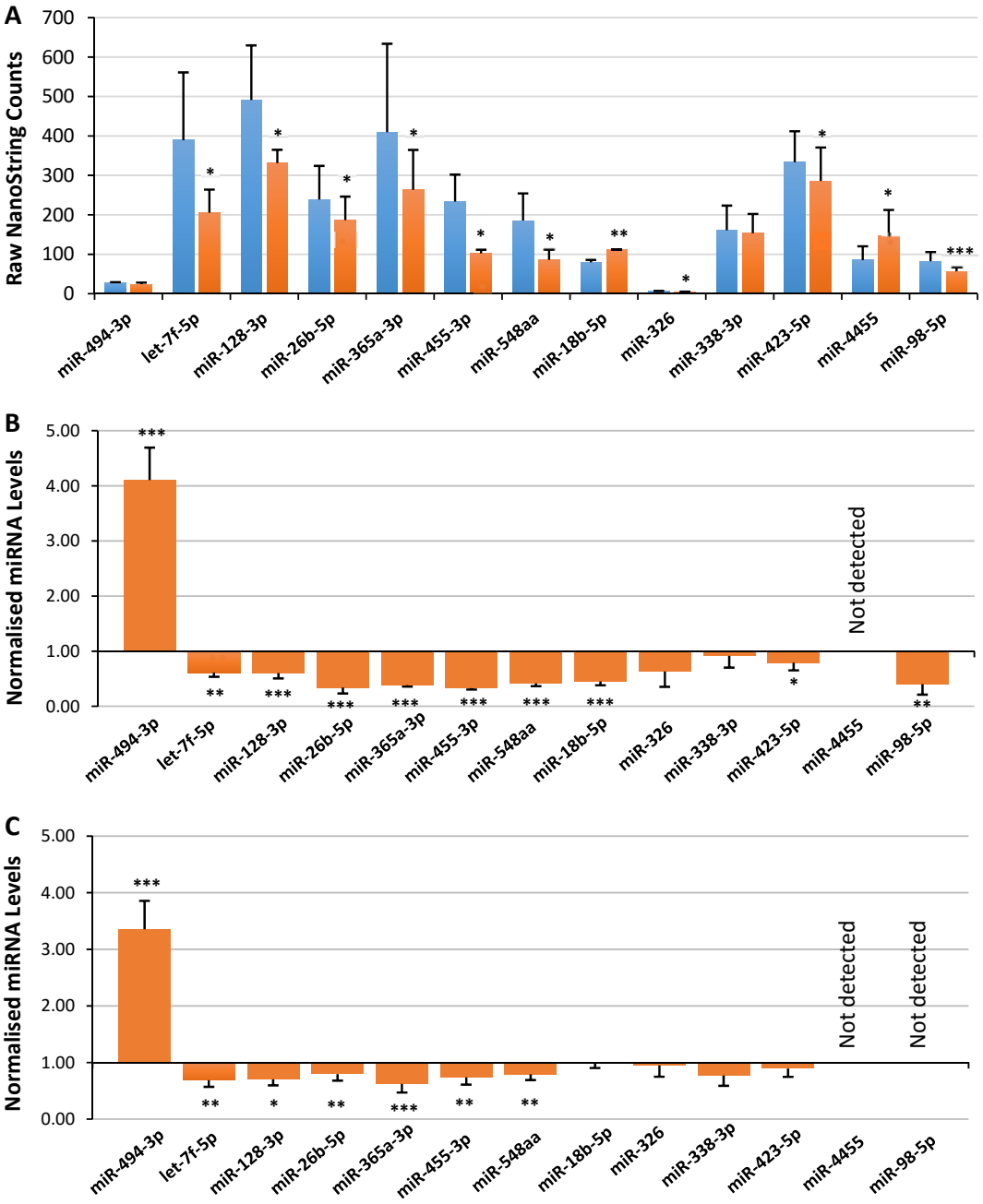
Figure 4.1. Effect of cell density and E_2 treatment timepoint on the mRNA levels of *ESR1, PROS1* and miR-494. HuH-7 cells were seeded in increasing cell numbers and cultured in the absence or presence of 10nM E_2 for (A) 12h and (B) 24h. Total RNAs for each sample were extracted and levels of *ESR1* and *PROS1* mRNA levels were analysed using Taqman® gene expression assay. MiR-494 expression was detected using Taqman® microRNA assay. (A-B) Single replicate (n=1) for optimisation, except 12h E_2 treatment with 1.5 × 10^6 cell seeding density, which contained two technical replicates (n=2) to ensure optimal conditions. The error bars indicate the standard deviation of each test condition.

For 12h E_2 treatment, no significant fold change was observed in the E_2-treated group compared to the vehicle, when cells were seeded in a density of 1.0×10^6. On the contrary, at the higher cell density of 1.5×10^6 cells, a greater than 3-fold increase of miR-494 levels and an associated 40% decrease in *PROS1* mRNA levels in E_2-treated cells were observed when normalised to vehicle control (Figure 4.1 Ai-iii). This indicates an expected E_2 response that was comparable to what was previously shown (Tay et al. 2013). E_2 treatment in 24h demonstrated an opposite trend in 1 and 2×10^6 cell seeding density. Specifically, a significant inhibition in miR-494 expression by 60% with an up-regulation of *PROS1* gene by 200%, was observed at the 1×10^6 cell seeding density, despite the fold of E_2 treated group being significantly increased by almost 2.5 times. The cell density at 1.5×10^6 could down-regulate (~10%) of *PROS1* expression by slight up-regulation of miR-494, however, there was no significant change in oestrogen receptor levels when compared to vehicle treatments (Figure 4.1 Bi-iii).

Moreover, at the time of harvest, low cell seeding (1×10^6) density sample displayed less viable cells and cells seeded at the high density (2×10^6) were over-confluent (data not shown). HuH-7 cells were also found to be responsive to E_2 treatment if the number of passages was less than 13 (data not shown). Following comparisons with positive controls, cell seeding density and oestrogen treatment period were optimised to 1.5×10^6 cells per cultural dish and 12h respectively.

4.2 Validation of E_2-responsive miRNAs

Thirty-one miRNAs were previously identified in the Nanostring ncounter® micro assay to be significantly up- or down-regulated by E_2 in HuH-7 cell ($p<0.05$), of which 10 miRNAs had raw Nanostring counts of more than 40. Although miR-338-3p lacked significant E_2-regulation in HuH-7 cells, it was found to be significantly E_2-reponsive in a separate NanoString analysis of human plasma samples and therefore, miR-338-3p was included in this study. From the NanoString analysis, 11 out of 12 miRNAs illustrated significant E_2-responsiveness. MiR-18b-5p and miR-4455 expression were up-regulated by E_2; the rest of the miRNAs (let-7f-5p, miR-128-3p, miR-26b-5p, miR-326, miR-365a-3p, miR-423-5p, miR-455-3p, miR-548aa and miR-98-5p) revealed E_2 down-regulation, although raw NanoString counts of miR-326 was less than 40 (Figure 4.2 A).



Legend: Vehicle, Treatment (10nM E_2). * p<0.05; ** p<0.01; *** p<0.001.

Figure 4.2. The expression of selected miRNAs in HuH-7 cells that were analysed via NanoString or RT-qPCR. HuH-7 cells were treated with vehicle or 10nM E_2 for 12h. Total RNA of individual samples were extracted and miRNA expression analysed using (A) NanoString or (B-C) Taqman® assay RT-qPCR. (A-B) Two technical replicates (n=2) for samples previously analysed in NanoString; (C) three technical replicates (n=3) for new extracted samples analysed in RT-qPCR. The error bars indicate the standard deviation of each sample. Vehicle expression in RT-qPCR was normalised as 1.

4.2.1 Predicted haemostatic gene targets of candidate E_2-responsive miRNAs

Potential coagulation gene targets (appendix 1) of the twelve miRNAs were predicted via three online tools (TargetScan 5.2, microRNA.org and miRWalk 2.0). The twelve candidate E_2-responsive miRNAs, except miR-4455, were predicted to bind to at least one haemostatic factor (Table 4.1).

Table 4.1. MiRNA target gene prediction.

MiRNA candidates	Predicted target coagulation genes
lef-7f-5p	*F3*[#], *F8*[#], *SERPIND1*
miR-128-3p	*F2*, *F3*, *F11*, *FN1*, *PROS1*, *SERPIND1*, *SERPINE1*, *TFPI*, *vWF*
miR-18b-5p	*ADAMTS13*, *F2*, *FN1*, *SERPINE1*, *THBD*, *THBS1*
miR-26b-5p	*ADAMTS13*, *F3*, *F5*, *F8*
miR-326	*ADAMTS13*, *F5*, *F9*, *F10*, *FN1*, *PROS1*, *SERPINF2*, *vWF*
miR-338-3p	*F2*, *F5*, *F7*, *F8*, *F10*, *F11*, *F12*, *FGB*, *FGG*, *PLAU*, *PLG*, *SERPINE1*, *TFPI*, *THBS1*
miR-365a-3p	*ADAMTS13*, *F2*, *F3*, *F5*, *F8*, *PLG*, *TFPI*
miR-423-5p	*ADAMTS13*, *SERPIND1*, *SERPINE1*, *SERPINE2*, *TFPI*
miR-4455	None
miR-455-3p	*F5*, *F8*, *F10*, *PROS1*
miR-548aa	*F8*
miR-98-5p	*SERPIND1*

Legend: [#] (only predicted in RegRNA 1.0), orange (the common coagulation gene targets for the validated miRNA candidates).

In the list, the majority of coagulation genes were targeted by miR-338-3p, miR-128-3p and miR-326: miR-338-3p contained 14 differentially putative gene targets, and the latter two miRNAs were predicted to bind to 9 and 8 haemostatic-associated genes respectively. Moreover, based on these computational analyses, it is possible for a miRNA to have an interaction with both pro-thrombotic and anti-thrombotic gene targets. For instance, there were 5 pro-thrombotic and 2 anti-thrombotic genes predicted to bind to miR-365a-3p.

Conversely, miR-548aa and miR-98-5p were merely predicted to interact with a single coagulation gene, *F8* and *SERPIND1* respectively.

4.2.2 Validation of E_2-regulated expression of miRNAs

RT-qPCR was used to check for concordance between the 12 selected miRNAs displayed and data obtained from NanoString. Herein, HuH-7 cells were treated with E_2 at the optimal condition (section 4.1) and miR-494 again was used as the positive control (Figure 4.2 B-C). First, an opposite trend was observed after amplifying the same samples that were analysed using NanoString. For instance, miR-494 expression was determined to have a four-fold enhancement with E_2 induction when analysed using RT-qPCR, compared to NanoString analysis result (Figure 4.2 A, B). In addition, miR-18b-5p was down-regulated to 55% by E_2 and miR-326 failed to show significant difference when shifted to RT-qPCR measurement. The concordant E_2-responsiveness between NanoString and RT-qPCR indicated in 9 miRNAs analysed, 8 miRNAs were suppressed by oestrogen signalling and miR-338-3p with no significant difference (Figure 4.2 B).

To confirm the E_2-responsiveness of miRNAs, technical replicates were performed and six miRNAs consistently presented oestrogen down-regulation (Figure 4.2 C). The miRNAs included let-7f-5p, miR-128-3p, miR-26b-5p, miR-365a-3p, miR-455-3p and miR-548aa. Among these six miRNAs, miR-365a-3p demonstrated the highest inhibition at 38% and this result was highly significant ($p<0.01$). Furthermore, more than 20% decrease in expression was found in let-7f-5p, miR-128-3p, miR-26b-5p, miR-455-3p and miR-548aa with E_2 treatment. Let-7f-5p, miR-128-3p, miR-26b-5p, miR-365a-3p, miR-445-3p and miR-548aa

demonstrated the concordance in both NanoString and RT-qPCR, therefore they were chosen for further investigation on the direct effects they have on their common coagulation gene targets (section 4.3).

4.2.3 E_2-regulated expression of coagulation genes

For the 6 miRNAs showing similar E_2-regulation trend in NanoString and RT-qPCR, their common *in silico* prediction coagulation targets genes are *F2, F3, F5* and *F8* (highlighted in orange and bold in Table 4.1). Hence, besides the detection of E_2-responsiveness as mentioned before, endogenous E_2-regulated expression of *F2, F3, F5* and *F8* were measured in both NanoString samples and technical replicates via RT-qPCR. Results indicated that the level of *PROS1* gene was significantly down-regulated by more than 30% in all samples, reflecting activation of oestrogen signalling in E_2-treated HuH-7 cells. Notably, *ESR1* gene was only detected in technical replicates with 45% reduction but not in NanoString samples (Figure 4.3). The absence of *ESR1* expression suggested ER-α gene was degraded in those samples.

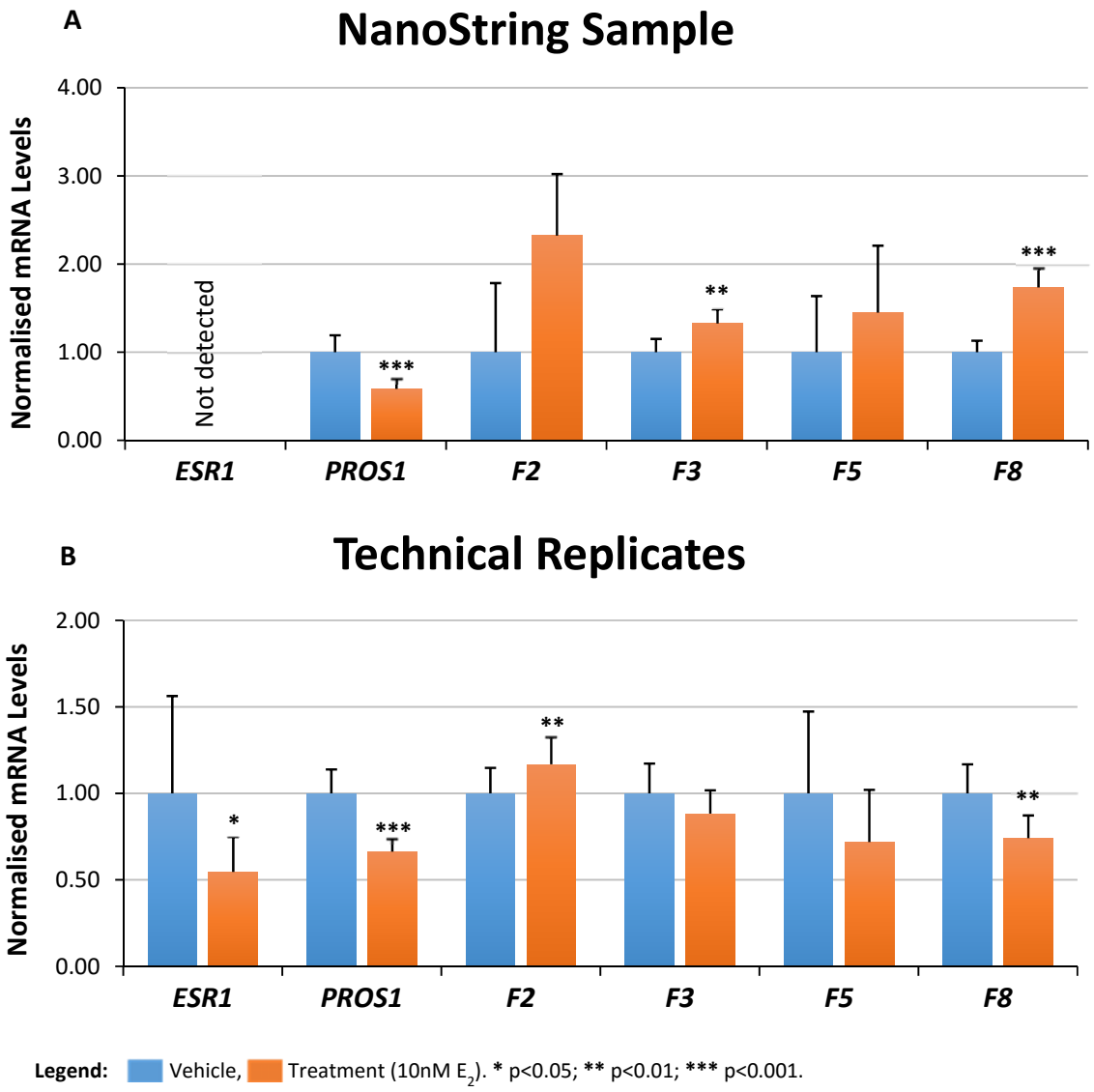


Figure 4.3. *ESR1*, *PROS1*, *F2*, *F3*, *F5*, *F8* gene expression of vehicle and E_2-treated HuH-7 cells. HuH-7 cells were treated with vehicle or 10nM E_2 for 12h. Total RNAs of individual sample were extracted and gene expression of *ESR1, PROS1, F2, F3, F5* and *F8* analysed using RT-qPCR. (A) Two technical replicates (n=2) for samples previously analysed in NanoString; (B) three technical replicates (n=3) for new extracted samples analysed in RT-qPCR, except *F2* and *F5* gene expression were detected twice only (n=2). The error bars indicate the standard deviation of each sample.

In terms of coagulation gene targets, Prothrombin (*F2*), TF (*F3*), Factor V (*F5*) and Factor VIII (*F8*) genes all revealed up-regulation in NanoString analysis of E_2-treated cells, particularly *F3* and *F8* which increased their corresponding expressions to 33% and 73% with a significant difference of $p<0.01$ and 0.001 respectively. Nevertheless, technical replicates displayed reverse E_2-regulation on *F3, F5* and *F8* gene by 12-28% and *F8* was the only one to have statistical significance ($p<0.01$) among these three genes. Even though a significantly increased level of *F2* gene shown in E_2 treatment group of replicates, the fold difference was much less than the change in *F3* and *F8* gene. To this end, *F3* and *F8* were selected for further investigation, in particular whether or not a direct interaction with miRNAs can occur (section 4.3).

4.3. Evaluation of miRNA effects on coagulation gene of interests

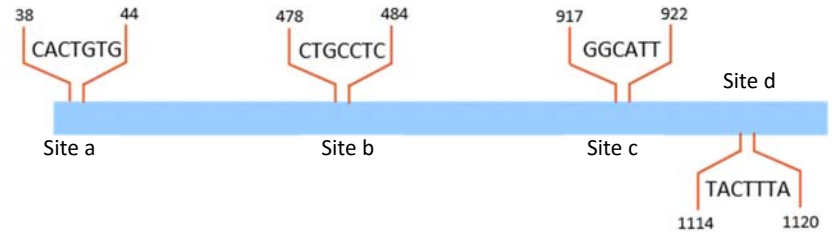
4.3.1 Putative miRNA effects on *F3* and *F8* 3'UTR

Six selected miRNAs, which revealed concordant E_2-responsiveness in both NanoString and RT-qPCR analysis, were predicted to interact with *F3* and/or *F8* gene via TargetScan 5.2, microRNA.org and RegRNA 1.0. According to the description of miRNA-mRNA interaction (section 1.3.2), miRNAs usually bind to the target gene mRNA 3'UTR through a "seed region".

Within the *F3*-3'UTR sequence, four sites were predicted to bind to miR-128-3p, let-7f-5p, miR-365a-3p and miR-26b-5p respectively (Figure 4.4-A). These miRNA-mRNA binding

sites are distributed at a certain distance and by comparing the putative score of three online prediction tools with other miRNAs, the strongest conserved interaction was found between miR-128-3p and *F3*-3'UTR.

A



B

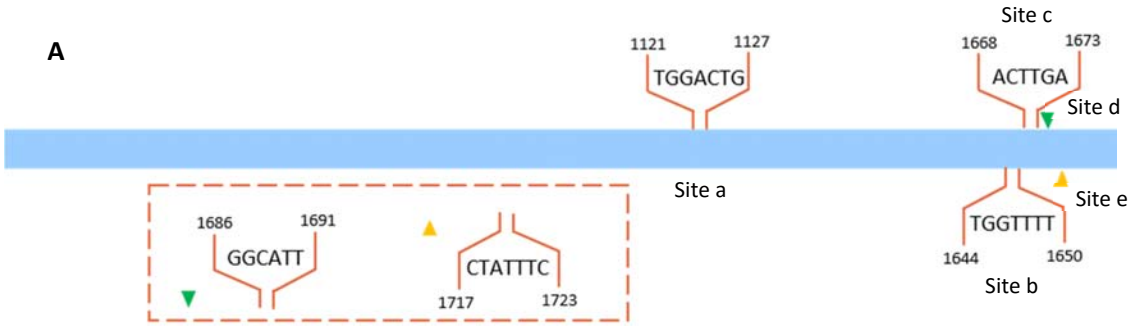
Putative miRNA binding sites	TargetScan (content + score)	microRNA. org (mirSVR score)	RegRNA (minimum free energy)
a 38 *F3*-3'UTR 5' AAATGCTATATTGCACTGTGA 3' miR-128-3p 3' UUUCUCUGGCCAAGUGACACU 5'	-0.37	-0.62	-12.00
b 478 *F3*-3'UTR 5' GTTCAAGCAATTGTCTGCCTCA 3' let-7f-5p 3' UUGAUAUGUUAGAUGAUGGAGU 5'	–	–	-15.20
c 917 *F3*-3'UTR 5' TTAACTGACTTAAGTGGCATTA 3' miR-365a-3p 3' UAUUCCUAAAAAUCCCCGUAAU 5'	-0.17	-0.13	–
d 1114 *F3*-3'UTR 5' TAATTTATTTAATATACTTTAA 3' miR-26b-5p 3' UGGAUAGGACUUA-AUGAACUU 5'	–	-0.16	–

Figure 4.4. Computational analyses of *F3*-3'UTR sequence predicted to bind to miR-128-3p, let-7f-5p, miR-365a-3p and miR-26b-5p. (A) Schematic of binding sites between *F3*-3'UTR and miR-128-3p, let-7f-5p, miR-365a-3p and miR-26b-5p seed region, which were located at position 38-44, 478-484, 917-922 and 1114-1120 downstream of *F3* coding region (5'→3') respectively. (B) Complementary pairing and prediction scores of putative miRNA-mRNA binding sites in *F3*-3'UTR. The seed region for each miRNA recognizing *F3*-3'UTR is shaded. The starting nucleotide pairing between miRNA and *F3*-3'UTR is also indicated. For all the prediction tools, stronger miRNA-mRNA binding is indicated by a lower negative score.

The seed region of miR-128-3p was located at position 38-44 of *F3*-3'UTR (5'→3'direction), which can be classified as an 8mer binding site (Figure 4.4-B). Compared to miR-128-3p-*F3* interaction, the predicted affinity between miR-365a-3p and 3'end of TF gene was relatively lower (-0.17 in TargetScan and -0.13 in microRNA.org). Although lef-7f-5p binding with *F3*-3'UTR was predicted in RegRNA 1.0, the putative binding strength was larger than miR-128-3p-*F3* if contrasting the score at the identical prediction website (Figure 4.4-B).

Let-7f-5p, miR-26b-5p and miR-365a-3p were also predicted to interact with *F8*-3'UTR, along with miR-455-3p and miR-548aa (Figure 4.5-A). Unlike the target site on the TF gene above, most of the putative miRNA-mRNA interactions on *F8* were concentrated towards the 3'end of the 3'UTR, except miR-455-3p binding site. MiR-455-3p was predicted to bind to the *F8*-3'UTR at the position of 1121-1127 (5'→3'direction) in all the three computational tools, and this seed region was 7mer-m8 site. Furthermore, the putative binding affinity between miR-455-3p and *F8*-3'UTR was highest among 5 miRNAs in microRNA.org, whereas, this trend could not be observed in another prediction software, RegRNA 1.0. Other miRNAs binding to Factor VIII gene were identified in one online tool only (Figure 4.5-B).

A



B

Putative miRNA binding sites	TargetScan (content + score)	microRNA. org (mirSVR score)	RegRNA (minimum free energy)
a 1121 *F8*-3'UTR 5' GTCAGAAGAAAATTGGACTGG 3' miR-455-3p 3' CACAUAUACGGGUACCUGACG 5'	-0.19	-0.53	-9.30
b 1644 *F8*-3'UTR 5' AACCCCAAAGGTGA-TATGGTTTTA 3' miR-548aa 3' ACCACGUUUUCAUUAACACCAAAAA 5'	–	–	-13.80
c 1668 *F8*-3'UTR 5' TCCTGTTATGTTTAACTTGAT 3' miR-26b-5p 3' UGGAUAGGACUUAAUGAACUU 5'	–	-0.11	–
d 1686 *F8*-3'UTR 5' TGATAATCTTATTTTGGCATTC 3' miR-365a-3p 3' UAUUCCUAAAAAUCCCCGUAAU 5'	–	-0.15	–
e 1717 *F8*-3'UTR 5' GACTATATACATCT-CTATTTCT 3' let-7f-5p 3' UUGAUAUGU-UAGAUGAUGGAGU 5'	–	–	-15.60

Figure 4.5. Computational analyses of *F8*-3'UTR sequence predicted to bind to miR-455-3p, miR-548aa, miR-26b-5p, miR-365a-3p and let-7f-5p. (A) Schematic of binding sites between *F8*-3'UTR and miR-455-3p, miR-548aa, miR-26b-5p, miR-365a-3p and let-7f-5p seed region, which were located at position 1121-1127,1644-1650, 1668-1673, 1686-1691, 1717-1723 downstream of *F8* coding region (5'→3') respectively. (B) Complementary pairing and prediction scores of putative miRNA-mRNA binding sites in *F8*-3'UTR. The seed region for each miRNA recognizing *F8*-3'UTR is shaded. The starting nucleotide pairing between miRNA and *F8*-3'UTR is also indicated. For all the prediction tools, stronger miRNA-mRNA binding is indicated by a lower negative score.

Analyses using microRNA.org illustrated that *F8* was targeted by miR-26b-5p and miR-365a-3p and their corresponding binding sites were separated by 12 nucleotides (Figure 4.5-B). Additionally, by compared with the prediction binding score, miR-365a-3p has a slightly better affinity with *F8*-3'UTR (score -0.15) than miR-26b-5p (-0.11). The remaining miRNAs (miR-548aa and let-7f-5p) were predicted to have target site on *F8*-3'UTR using RegRNA 1.0, however, the maximum predicted binding was presented in let-7f-5p that reached the score to -15.60, which was even higher than the prediction score of miR-455-3p (-9.30) in the RegRNA 1.0 analysis (Figure 4.5-B).

Interestingly, the affinity score of miRNA-mRNA interaction changes when prediction sites updated their analytical methods, such as in TargetScan (Table 4.2).

Table 4.2. Selected miRNAs prediction score of *F3* and *F8* gene in different versions of Target Scan.

miRNA	*F3*			*F8*		
	Version 5.2 Jun. 2011	Version 6.0 Nov. 2011	Version 7.0 Aug. 2015	Version 5.2 Jun. 2011	Version 6.0 Nov. 2011	Version 7.0 Aug. 2015
let-7f-5p	×	×	×	×	×	×
miR-128-3p	✓ (-0.37)	✓ (-0.30)	✓ (-0.39)	×	×	×
miR-26b-5p	×	×	×	×	×	×
miR-365a-3p	✓ (-0.17)	✓ (-0.14)	✓ (-0.11)*	×	×	×
miR-455-3p	×	×	×	✓ (-0.19)*	✓ (-0.16)*	×
miR-548aa	×	✓ (-0.07)*	✓ (-0.02)*	×	✓ (-0.25)*	✓ (-0.15)

Legend: Jun. (July); Aug. (August); Nov. (November); * (poorly conserved miRNA-mRNA binding site). () indicates the prediction score, a lower negative score represents a stronger miRNA-mRNA binding.

There was a mild fluctuation in the predicted interaction between miR-128-3p and *F3*-3'UTR (-0.37 ~ -0.39) among three TargetScan versions. Likewise, miR-548aa, which failed to have

putative target site on *F3*-3'UTR using TargetScan 5.2, displayed a bare interactive score in the latest two versions. In *F8*-3'UTR sequence, a putative binding site for miR-455-3p disappeared in TargetScan 7.0, yet the site could be previously predicted in TargetScan 5.2 and 6.0.

4.3.2 Confirmation of predicted miRNA-mRNA direct interaction

To confirm direct interaction of selected miRNAs and *F3* and *F8*-3'UTR, HuH-7 cells were transfected with pMIR-REPORT vector containing *F3* or *F8*-3'UTR sequence, and 50nM miRNA precursor for 24h. The effects of a selected miRNA on their putative gene targets *F3* and/or *F8* were conducted in dual-luciferase reporter assays. MiR-494 direct targeting on *PROS1*-3'UTR acted as a positive control.

In three independent replicates, *PROS1*-3'UTR dependent luciferase activity was inhibited by miR-494 by 21%, nevertheless, it did not reveal a statistical significance (Figure 4.6-A). For the predicted *F3*-3'UTR target miRNAs, only miR-365a-3p significantly down-regulated *F3*-3'UTR dependent luciferase activity by 21%; the other three miRNAs resulted in increased luciferase signals and miR-26b-5p in particular, showed more than 50% raise ($p<0.05$) of *F3*-3'UTR dependent luciferase activity (Figure 4.6-B). Moreover, comparable luciferase signals were also observed. Instead of exhibiting a strong inhibition as expected, miR-128-3p or let-7f-5p elevated *F3*-3'UTR-dependent activity up to 9% and 21% in turn. Noticeably, miR-128-3p had a small impact on *F3*-3'UTR mRNA sequence even though it was predicted to have the strongest affinity.

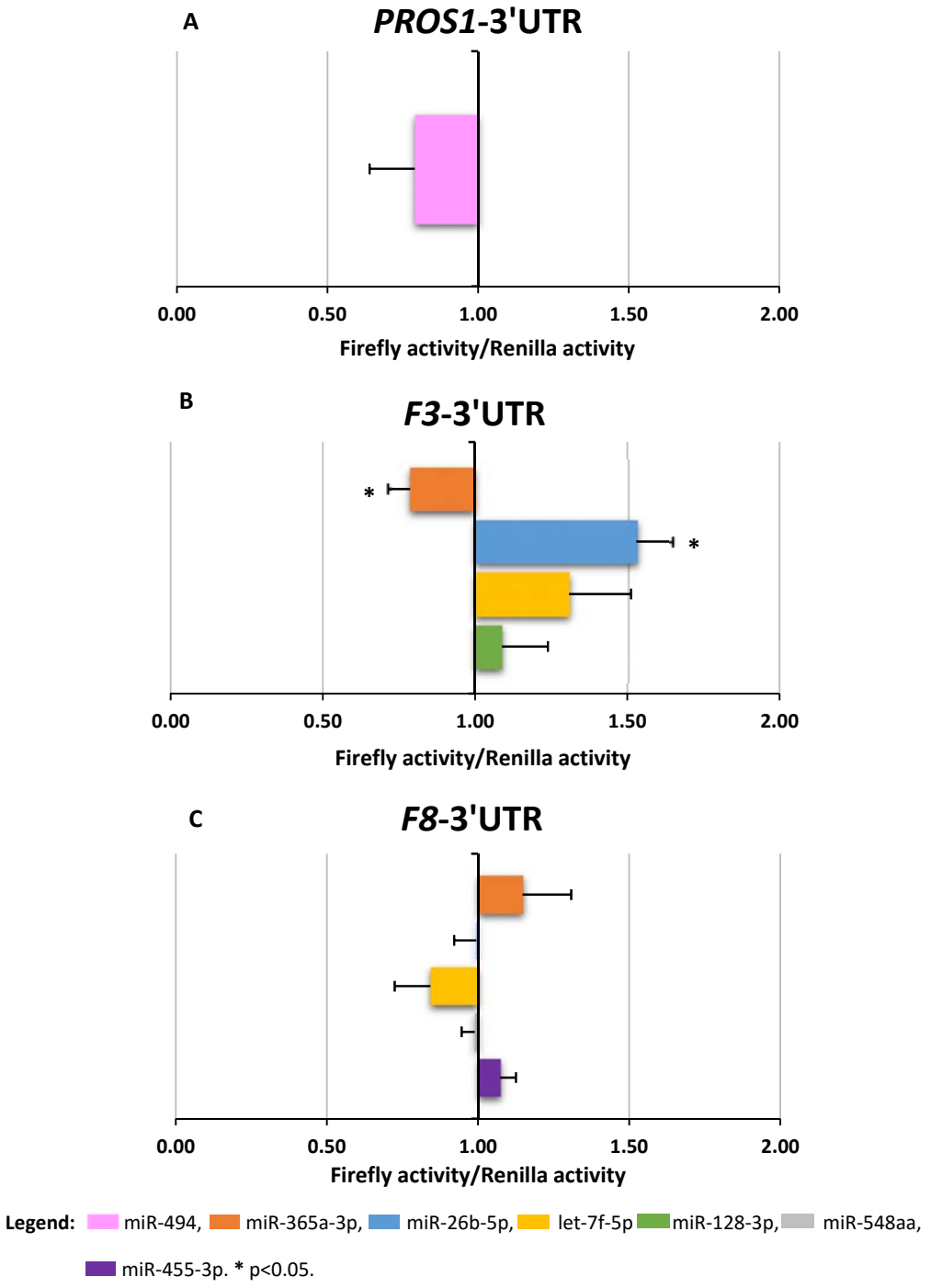


Figure 4.6. Direct interaction between miRNAs and their putative target genes. HuH-7 cells were transfected with both pRL-SV40 Renilla luciferase control vector and pMIR-REPORT Firefly luciferase vector with the 3'UTR of interests (A) *PROS1*-3'UTR (B) *F3*-3'UTR (C) *F8*-3'UTR. Transfected cells were treated with 50nM miRNA precursor for 24h. The level of transfection efficiency was measured via dual-luciferase reporter assay. (A-C) Three technical replicates (n=3) for dual-luciferase reporter assay. The error bars indicate the standard deviation of each sample. Negative control signal was set as 1.

There was no significant change of *F8*-3'UTR dependent luciferase activity, though an inhibition (16%) of luciferase activity presented when transfected with let-7f-5p construct (Figure 4.6-C). Counter to the prediction result (Figure 4.6-B), miR-455-3p was shown to slightly enhance *F8*-3'UTR dependent luciferase signal by 7%; a 15% increased luciferase activity was also exhibited in miR-365a-3p transfection. Although changes in luciferase activity was observed, these changes were not statistically significant. For miR-26b-5p and miR-548aa transfection, only ~1% of luciferase activity changes were detected.

Even though miR-548aa construct could not down-regulate the *F8*-3'UTR dependent luciferase activity, it was found to affect cell proliferation. First, less cell numbers were visually observed in miR-548aa transfection samples, by comparing to other *F8*-3'UTR targeted miRNAs, after re-seeding cells into a 96-well plate (data not shown). To make a comparison of viable cell numbers between miR-548aa and miR-NC construct, total cell numbers and viability were measured in three independent experiments. Based on these raw cell count data, a viable cell count ratio on miR-548aa against miR-NC was calculated, indicating 29-36% decline of cell viability when transfected with miR-548aa (Table 4.3).

Table 4.3. Viable cell numbers of negative control and miR-548aa transfected sample

	First replicates	Second replicates	Third replicates
***F8*-miR-NC**	$1.11*10^5$	$1.04*10^5$	$1.89*10^5$
***F8*-miR-548aa**	$7.37*10^4$	$7.38*10^4$	$1.20*10^5$
% decline	34%	29%	36%

Legend: miR-NC (miRNA negative control).

Chapter 5

Discussion

People with high circulating E_2 levels will have higher risks of VTE, as increased levels of various coagulation factors and a reduction of anticoagulant Protein S level are observed in pregnant women and oral contraceptive users. Nevertheless, the mechanism by which E_2 induces thrombosis at a molecular level is still not clear. Our laboratory previously identified a number of novel E_2-regulated miRNAs in HuH-7 cell lines and/or plasma samples using NanoString micro array. In this study, twelve miRNAs were selected from NanoString analysis and their E_2-responsiveness was validated via RT-qPCR. This showed 6 out of the 12 miRNAs had a robust and consistent down-regulation by E_2 treatment using NanoString and RT-qPCR detection methods. These validated miRNAs were predicted to target coagulation genes *F3* and/or *F8*, which were up-regulated in the E_2-treated samples. However, following a verification of miRNA predicted gene targets via dual-luciferase reporter assay, miR-365a-3p was shown to directly inhibit the *F3*-3'UTR luciferase activity and miRNAs that predicted to target *F8* all failed to have a direct interaction with *F8*-3'UTR.

5.1 Oestradiol treatment and factor of influence

To investigate E_2 regulation on haemostasis, we chose to use the HuH-7 human liver carcinoma cells line as the primary cell line, as the majority of pro- and anti-coagulants are synthesised in the liver (Boron & Boulpaep 2012). In addition, using HuH-7 liver cancer cells, an E_2-responsive miRNA, miR-494 that directly targets *PROS1* transcript was discovered (Tay et al. 2013). Thereby, in the current study, E_2 treatment was also performed in HuH-7 cells. Moreover, cultural media used for HuH-7 cells here distinguished to other cell cultures; the usage of 5%(v/v) CSS was due to two reasons: (i) optimal percentage of serum can provide sufficient nutrients for cells and assists cells to express consistently high

E_2 levels; (ii) non-polar lipids such as hormones have been removed but media remain salts, glucose and amino acids to maintain normal cell growth (Soto & Sonnenschein 1979; *Charcoal Stripped Fetal Bovine Serum* 2015).

Laboratory change can alter cell growth conditions, so E_2 treatment conditions need to be optimised. Cell seeding density and passage time have been reported to affect the oestrogen signalling response in the E_2-treated cells (Campbell et al. 2002). Campbell et al. (2002) used a murid pituitary tumour cell line to study the effect of cell growth conditions on regulating oestrogen signalling and observed that intracellular ERα level could reach at the peak when cell plating density was approximately 3000 cells per well. Apart from the cell plating number, Campbell's team (2002) also found that the expression of intracellular ERα in the early (passage <11) passage cells was higher than the late passage one (passage ≥15), indicating some cells may loss the abilities to be responsible to the oestrogen signalling when their ages increase. These similar results could obtain from current study as well; when HuH-7 cells were insufficient or over confluent and too many passages, their E_2-reponsive efficiency reduced compared to the cells with appropriate confluence and passage time.

Treatment time courses are other essential factors to be involved in the E_2-treatment experiments, particularly those associated with oestrogen-regulation (Frasor et al. 2003; Lin et al. 2004; Carroll et al. 2006; Lin et al. 2007). In these published studies, Frasor et al. (2003) discovered an interesting time pattern presented in 438 E_2-regulated genes expressed by MCF-7 breast cancer cells. At a shorter E_2 exposure times (4h and 8h), most of the E_2-induced genes expressed maximal fold change in the analysis; while the genes suppressed by

oestrogen seemed to have higher expressions when performing E_2-treatment for longer period (24h and 48h) (Frasor et al. 2003). Herein, HuH-7 cells exhibited greater sensitivity to oestrogen signalling when exposure period to 10^{-8}M E_2 was 12h, which referred to the expected results of *PROS1* and miR-494 expression (Tay et al. 2013); whereas, other treatment time points that excluded 12h or 24h can also be considered, perhaps at other E_2 treatment concentrations. To date, studies looking into the time-dependent effect of E_2-regulated genes in liver carcinoma cells have not been reported.

Besides the cell density and treatment time, E_2 concentration is also suggested to become the variant for optimisation, as the result of a close association with cell survivals in MCF-7 cells (Chow, Chan & Fung 2004). Furthermore, oestrogen overdose (i.e. 1000nM E_2) can decline the cell viability (Chow, Chan & Fung 2004) that may further influence the cell responsiveness of ERα (Soto & Sonnenschein 1979).

5.2 Oestradiol regulation on selected miRNAs and their predicted gene targets

MiRNAs can silence target gene translation and their functions in inhibiting tumour cell proliferation are being widely investigated. In this study, the majority of the candidate miRNAs investigated have been reported to target various tumour-associated genes or display abnormal expressions in carcinoma cell lines, in particular let-7f-5p. It is one of the pioneer miRNAs to modulate cell development in *Caenorhabditis elegans* (Pasquinelli et al. 2000). Nevertheless, miR-4455 and miR-548aa are two novel miRNAs whose functions have not been addressed in the literature. In addition, only 3 out of 12 selected miRNAs have an effect, either individually or combined effects, on haemostasis. According to the discovery

from Yu et. al. (2015), miR-326 can directly bind to anti-apoptotic protein Bcl-2 transcripts and may interact with another Bcl-2 family member Bcl-xL through an indirect mechanism to enhance platelet apoptosis. Furthermore, up- and down-regulation of miR-365a-3p and miR-98-5p level were observed in activated platelet samples respectively, suggesting there may be a potential role for them to participate in platelet activities (Osman & Fälker 2011).

Oestrogen signalling can directly or indirectly regulate miRNA expressions; or even affect miRNA synthesised proteins, including Ago2, Dicer, DGCR8, Exportin-5 and TRBP, to suppress or promote miRNA production (Table 5.1).

Table 5.1. Oestrogen signalling effect on microRNA biogenesis components.

microRNA biogenesis protein	Associated ER	Oestrogen effect on gene expression	References
Ago2	ERα	↓	(Adams, Claffey & White 2009; Cheng et al. 2009)
Dicer	ERα	↑	(P. Bhat-Nakshatri et al. 2009; Cheng et al. 2009; Redfern et al. 2013)
DGCR8	ERα	↑	(Nothnick, Healy & Hong 2010)
Exportin-5	ERα	↑	(Nothnick, Healy & Hong 2010)
TRBP	ERα	↑	(Cheng et al. 2009; Redfern et al. 2013)

Legend: DGCR8 (DiGeorge syndrome chromosomal region 8); TRBP (transactivation response RNA binding protein); Ago2 (Argonaute 2 protein).

Among these proteins, Dicer has been reported to display an induction when E_2 is present; the Dicer complex (Dicer, TRBP and protein kinase RNA activator) can couple with a steroid receptor RNA activator that target the promoter region of the steroid receptor gene to regulate the activity of nuclear receptors like ERα, suggesting ERα may be relevant in miRNA

biosynthesis through interacting with the Dicer complex (P. Bhat-Nakshatri et al. 2009; Redfern et al. 2013). Moreover, a preliminary result revealed that Ago2 expression may be inhibited by the oestrogen signalling; however further investigation is necessary to verify this finding (Adams, Claffey & White 2009). Within the twelve miRNAs selected in this study, let-7f-5p, miR-18b-5p, miR-26b-5p, miR-338-3p, miR-365a-3p and miR-98-5p have been shown to have down-regulation in E_2-treated or ER positive breast cancer cells (P Bhat-Nakshatri et al. 2009; Leivonen et al. 2009; Kodahl et al. 2014a; Kodahl et al. 2014b; Tan et al. 2014; Newcomb et al. 2015). Interestingly, there is one study where elevated level of miR-128-3p in a transfected MCF-7 cell line that only containing E_2 has been reported (Masri et al. 2010); while in this experiment miR-128-3p was down-regulated in HuH-7 cells following E_2 treatment, suggesting E_2 regulation may be tissue-specific. However, to our knowledge, there is no paper that has reported the E_2-regulation of these selected miRNAs in hepatoma cell lines, thus no reference can be used to judge the authenticity of these results.

Coagulation factors, prothrombin (Factor II), TF, Factor V and Factor VIII are involved in forming blood clots to arrest bleeding, and levels of all these haemostatic factors have been observed to increase during pregnancy (Bleker, Coppens & Middeldorp 2014). But in this study, there was an unexpected down-regulation of *F3, F5* and *F8* gene expression in technical replicates. In fact, this observation is common in cell-based experiments, as a similar cell line obtained from different laboratories may reflect the time-dependent genetic instability (Bensadoun et al. 2011; Frattini et al. 2015). This can explain the different E_2-regulated miRNAs and gene expression shown in RT-qPCR analyses here, because HuH-7 cells used for NanoString and repeated RT-qPCR analyses were derived from different sources.

5.3 NanoString micro array and RT-qPCR concordance

NanoString is a highly specific and reproducible technique, but it lacks sensitivity to differentiate miRNAs with only a single nucleotide difference as well as to measure low miRNA counts (Table 5.2) (Knutsen et al. 2013; Mestdagh et al. 2014; Polytarchou et al. 2015). Therefore, NanoString often requires a high sensitive technique such as RT-qPCR to validate miRNA expressions, in order to ensure the accuracy and reliability of analytical outcomes. RT-qPCR is a highly sensitive amplification technique for analysing miRNA reverse transcript copies, so there is no restriction for it to detect small amounts of cDNA from total RNA samples (Knutsen et al. 2013; Mestdagh et al. 2014).

Table 5.2. Level of distinct parameters in three miRNA expression analysed platforms

	NanoString	RT-qPCR	NGS
Accuracy	Moderate	High	Moderate-High
Flexibility	Moderate	Moderate	High
Reproducibility	High	Low-High	High
Sensitivity	Low	High	Moderate-High
Specificity	High	Moderate	High

Legend: NanoString (NanoString nCounter® micro array); RT-qPCR (reverse transcription – quantitative polymerase chain reaction); NGS (next generation sequencing).

In this experiment, there was a 75% concordance in the E_2-regulated miRNA expression between the NanoString and RT-qPCR analyses, if analysing the same RNA samples. Similar findings were also obtained by Mestdagh et. al. (2014), who showed 70-75% concordant miRNA levels could be detected between NanoString and Taqman card RT-qPCR platforms. However, combined with three technical replicates analysed in RT-qPCR, the overall

miRNA concordance reduced to 58%, which was likely due to passage effects of the carcinoma cell lines obtained from different laboratories.

Although RT-qPCR is a gold-standard validated cost-effective approach, it is limited to the specific probes or assays used in the platform (Mestdagh et al. 2014). This disadvantage has led to the development of applying next generation sequencing in miRNA profiling quantification, because there is no such vital weakness to impact on the performance accuracy (Table 5.2) and it contains a relatively extensive coverage of the targeted miRNAs referred to miRBase database and provides some additional information including individual heteroplasmy site of miRNAs or iso-miRNAs (Knutsen et al. 2013; Mestdagh et al. 2014). However, an average concordance between any two combination of hybridization, RT-qPCR and next generation sequencing to quantify a set of miRNA replicates was only 54.6% (Mestdagh et al. 2014).

Another research group from Norway compared miRNA expression profiles of breast cancer cell lines generated from different digital miRNA high-throughput technologies, and discovered that the results obtained using NanoString were less correlated to those produced by RT-qPCR based and next-generation sequencing methods, as the paired sensitivity declined to around 45%; whereas, a confirmation pairing between RT-qPCR and sequencing or within two different sequencing techniques reached to 65-73% (Knutsen et al. 2013). Besides the technological variation, other factors including low miRNA abundance, presence of iso-miRNAs and normalisation approach can cause a dissimilarity of miRNA expression as well (Chugh & Dittmer 2012). Taken together, the criteria to select the optimal miRNA

profiling platforms are associated with the cost, accuracy, precision and sample quality (Baker 2010), even though the concordance between analyses is moderate and only small proportions of miRNAs have been verified to date.

5.4 Computational analysis and verification concordance

Four computational analyses were used in this experiment, TargetScan 5.2, microRNA.org, miRWalk 2.0 and RegRNA 1.0. These predicted approaches applied in each program vary from each other, thereby it is no surprise that the miRNA-mRNA interaction sites can be predicted by some algorithms but not others, as well as the difference of predicted binding scores, which was also reflected in the present study. TargetScan is an algorithm to predict a conserved seeding region of the miRNA-mRNA binding and the binding affinity is expressed as a context score (Lewis, Burge & Bartel 2005; Grimson et al. 2007). On the contrary, microRNA.org is a new tool to score the efficiency of predicted target sites by analysing a number of miRNA transfection results via support vector regression (SVR). Hence, conservation does not need to be used as a prediction filter and mirSVR score is independent to the seeding categories, which provides more accurate and comprehensive predictions (Betel et al. 2010). MiRWalk is an online database to scan the putative miRNA target genes by combining the search from several established prediction software and published literatures (Dweep et al. 2011); while miRNA target sites prediction in RegRNA is mostly based on the data from miRBase and integrates another prediction tool miRanda (Huang et al. 2006).

Apart from the variance between prediction software, the update within a single tool may also affect the prediction outcomes, such as TargetScan (Table 4.2), because more potential miRNA-mRNA binding contributors are contained to improve the prediction performance. For example, a decreased proficiency was attributed to weak predicted seed-pairing stability and high target-site abundance in lsy-6 miRNA, hence, these two contributors are involved in TargetScan 6.0 (Garcia et al., 2011).

The putative miRNA-mRNA interactions between candidate miRNAs and *F3* and/or *F8* 3'UTR were verified using dual-luciferase reporter assays here. The results demonstrated that out of the 6 miRNAs predicted to target either *F3* and/or *F8*, only miR-365a-3p significantly down-regulated the *F3*-3'UTR-dependent luciferase assay compared to miRNA negative control treated cells, indicating that miR-365a-3p directly binds to the seed region in *F3*-3'UTR sequence. The discrepancy between prediction and verification results indicated that accurate miRNA target sites prediction is still a challenge at the present time, due to following reasons: (i) miRNA-mRNA interactions in animals are complicated and these binding mechanism are not fully defined yet; (ii) current predicted mRNA targets are mainly restricted in duplex sequencing form so it is possible that potential mRNA binding sites are hidden in their secondary and tertiary fold structures; (iii) non-conserved 3'UTR binding sites are overlooked in many conservation-filter algorithms; (iv) the number of verified miRNA prediction targets are insufficient in prediction database, which increases the difficulty to have adequate literatures for references (Betel et al. 2010; Witkos, Koscianska & Krzyzosiak 2011; Peterson et al. 2014).

An overlap of miRNA predicted target sites is not significant in distinct computational analyses as described above, so the prediction is usually performed in more than one computational analyses to explore all the possible miRNA-mRNA interaction sites. Herein, TargetScan and microRNA. org are two complementary approaches to predict miRNA target sites on the 3'UTR region; nevertheless, miRWalk failed to predict the majority of miRNA binding sites, as the rules employed by this algorithm may lead to the reduction of specificity albeit sensitivity is increased, which was not recommended by Witkos et al. (2011). Due to the inefficient prediction of miRWalk, it was replaced by RegRNA to predict the miRNA binding sites on *F3* and *F8.* Although three computational analyses were applied in this study, including microRNA.org that concerns the non-conserved binding sites, none of these analyses specifically perform the prediction according to the secondary structure of mRNA targets, suggesting that it may lose some putative miRNA-mRNA binding sites (Witkos, Koscianska & Krzyzosiak 2011).

5.5 Characterisation of miRNA-mRNA direct interactions

To date, miR-365a-3p has been discovered to target several genes where play a role in tumourigenesis, cell proliferation and apoptosis (Qi et al. 2012; Zhang et al. 2014); whereas, even though no paper particularly addresses miR-365a-3p regulation on coagulation genes, it was found to result in up-regulation in activated platelets (Osman & Fälker 2011) and patients with intracerebral haemorrhage, indicating the potential roles of miR-365a-3p in thrombotic-associated diseases (Guo et al. 2013). As TF mRNA targeted by miR-365a-3p is a preliminary result, further verification is required through site directed mutagenesis of deleting binding site of miR-365a-3p on *F3*-3'UTR.

Besides the significant inhibited effect, the transfections of some miRNAs were shown to enhance luciferase activities (Zhou et al. 2013). This suggests that those miRNA-mRNA interactions may stabilize the mRNA transcripts, which is contrary to the common knowledge of miRNA's function in triggering mRNA degradation and inhibiting protein translation. This enhanced luciferase activity was highest in the interaction between miR-26b-5p and *F3*-3'UTR that reflected a sharp signal raise of ~50% compared to the miR-NC control. Increased luciferase signals were also reported by Zhou et al. (2013), of which slight-moderate elevation of luciferase activities were shown in miR-141, miR-514 and miR-27a bound to their corresponding target genes. They proposed it may be owing to the interaction between miRNAs and their associated protein complexes miRNPs, leading to repressive miRNAs or abrogated miRNPs to stimulate gene expression. Five years prior to Zhou et al.'s study, miRNPs and miRNAs relationship was addressed in a paper with regard to AU-rich elements (Vasudevan, Tong & Steitz 2007). The authors suggested miRNPs can serve as translation activators on cell cycle arrest, as their interested miRNA miR-369-3 was able to directly recruit with Ago2-fragile X mental retardation-related protein 1 complex, which are two related factors of miRNPs, resulting in AU-rich elements translation up-regulation under the serum-starved condition.

However, it is also possible that the enhanced luciferase activities could be an artefact. This might be due to the vector used for this project, it has no destabilised elements so that the luciferase transcript will degrade much slower than the normal state to cause an accumulation of existing and newly-formed proteins (Dr Jim Tiao personal communication). According to this presumption, miR-365a-3p inhibited effect on *F3*-3'UTR might be a typical example to overcome the strong accumulation process, suggesting more significant suppression would

arise in the normal accumulation stage. In order to validate whether this accumulation is not produced artificially, *F3*-3'UTR is suggested to be re-cloned into vectors containing destabilised elements and to confirm the actual interaction with miR-26b-5p. For instance, the RapidReporter® pRR-High Vector, which contains both mRNA and protein destabilising elements can accelerate the degradation of luciferase proteins in order to eliminate the background noise from the accumulation of luciferase over time and to increase the accuracy of measurement (*RapidReporter Gaussia Luciferase Assay* 2007).

With the *F8*-3'UTR luciferase activity, the absence of significant regulation suggested that no direct binding site of these 6 miRNAs was found in *F8*-3'UTR transcripts. Interestingly, cell number change was observed in miR-548aa, a novel miRNA to be discovered recently, suggesting it may contribute to cell cycle mechanism such as apoptosis. Recently, a new member of miR-548 family, C2-miRNA, was identified in a tumour suppressor gene *FHIT*, and tumour cell growth could be inhibited by the interaction between C2-miRNA and several tumour cell growth genes (*CCND1, ERBB2, DNMT3A* and *DNMT3B*). Moreover, tumour cell development suppression was also discovered with the presence of miR-548 cluster, suggesting the important role of miR-548 family in tumour cell growth inhibition (Hu et al. 2014).

5.6 Conclusion and future directions

In conclusion, this project demonstrated that oestrogen can regulate both miRNAs and coagulation genes to trigger a negative correlation between miRNAs and their gene targets in HuH-7 cell models. Let-7f-5p, miR-128-3p, miR-26b-5p, miR-365a-3p, miR-455-3p and

miR-548aa were validated to be E_2-responsive miRNAs and elevated E_2 levels could down-regulate their miRNA expressions. The induction of E_2 treatment could also regulate several coagulation gene expression, in this case, TF (*F3*) and Factor VIII (*F8*) gene expression were significantly up-regulated in high E_2 concentration. Although these six E_2-responsive miRNAs were predicted to interact with *F3* and/or *F8* gene(s), only miR-365a-3p was shown to directly bind to the predicted target site in the *F3*-3'UTR sequence leading to a down-regulation of TF mRNA levels, indicating prediction software does not always reflect the actual binding sites between miRNAs and mRNAs.

The initial future direction of this study is to confirm the miR-365a-3p direct interaction with *F3*-3'UTR using site directed mutagenesis. Once *F3* mRNAs are verified to be the direct targets of miR-365a-3p, the association between *F3* and miR-365a-3p will be further investigated at the protein level. Likewise, miR-365a-3p may also regulate other coagulation genes; hence, the second direction is to characterise miR-365a-3p mechanism on other coagulation gene targets at both transcriptional and translational level. Lastly, other non-selected miRNAs that showed E_2-responsiveness from NanoString need to be validated and evaluation of the presence of direct miRNA-mRNA binding is required. The ongoing work may provide more evidences to prove whether a network of miRNAs that can be altered by E_2 to promote thrombosis and some robust E_2-responsive miRNAs may serve as bio-markers to diagnose thrombotic gene changes in the future.

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Appendices

1. Haemostatic-associated proteins and corresponding encoded genes

Pro-thrombotic proteins		
Name	Encoded Genes	Gene ID (Ensembl)
ADAMTS13	*ADAMTS13*	ENSG00000160323
Alpha 2-antiplasmin	*SERPINF2*	ENSG00000167711
Factor I (Fibronogen) Factor Ia (Fibrin)	*FGA, FGB, FGG*	ENSG00000171560; ENSG00000171564; ENSG00000171557
Factor II (Prothrombin#) Factor IIa (Thrombin)	*F2*	ENSG00000180210
Factor III (Tissue Factor)	*F3*	ENSG00000117525
Factor IV (Ca^{2+})	/	/
Factor V & Va	*F5*	ENSG00000198734
Factor VII# & VIIa	*F7*	ENSG00000057593
Factor VIII & VIIIa	*F8*	ENSG00000185010
Factor IX# & IXa	*F9*	ENSG00000101981
Factor X# & Xa	*F10*	ENSG00000126218
Factor XI & Xia	*F11*	ENSG00000088926
Factor XII & XIIa	*F12*	ENSG00000131187
Factor XIII & XIIIa	*F13*	ENSG00000213028
Fibrinetin 1	*FN1*	ENSG00000115414
Glia derived nexin (Protease nexin I)	*SERPINE2*	ENSG00000135919
Kininogen-1	*KNG1*	ENSG00000113889
Neuroserpin	*SERPINI1*	ENSG00000163536
Platelet factor 4	*PF4*	ENSG00000163737
Plasma kallikrein	*KLKB1*	ENSG00000164344
Protein Z	*PROZ*	ENSG00000126231
Thrombospondin 1	*THBS1*	ENSG00000137801
Thrombin-activatable fibrinolysis inhibitor	*CPB2*	ENSG00000080618
Von Willebrand factor (vWF)	*vWF*	ENSG00000110799

Anti-thrombotic proteins		
Name	Encoded Genes	Gene ID (Ensembl)
C4b-binding protein (C4BP)	*C4BPA, C4BPB*	ENSG00000123838; ENSG00000123843
Tissue plasminogen activator (t-PA)	*PLAT*	ENSG00000104368
Urokinase-type plasminogen activator (u-PA)	*PLAU*	ENSG00000122861
Plasminogen	*PLG*	ENSG00000122194
Protein C#	*PROC*	ENSG00000115718
Protein S#	*PROS1*	ENSG00000184500
Protein Z-related protease inhibitor	*SERPINA10*	ENSG00000140093
Plasminogen activator inhibitor-2 (PAI-2)	*SERPINB2*	ENSG00000197632

Antithrombin III (ATIII)	*SERPINC1*	ENSG00000117601
Plasminogen activator inhibitor-1 (PAI-1)	*SEPRINE1*	ENSG00000106366
Tissue factor pathway inhibitor (TFPI)	*TFPI*	ENSG00000003436
Thrombomodulin	*THBD*	ENSG00000178726

Legend: # (vitamin-K dependent proteins).

2. Buffers and Solutions

2.1. Beta-oestradiol stock (10^{-2}M, 1mL)

Beta-oestradiol	0.002724g
Absolute ethanol	1mL

2.2. Firefly luciferase reporter substrate (1X, 60µL)

10X Firefly substrate	6µL
50X Firefly enhancer	1.2µL
Firefly glow buffer	52.8µL

2.3. Glycerol bacterial stock (15%, 1mL)

Bacterial culture (contained plasmid)	800µL
30% v/v Glycerol (17904, Thermo Fisher Scientific): low-salt LB solution	200µL

2.4. Low salt lysogeny broth (LB; 1L)

Sodium Chloride (NaCl; X190-1kg, amresco®)	5g
Tryptone (J859-500G, amresco®)	10g
Yeast (J850-500G, amresco®)	5g
Tap distilled water (dH_2O)	1L

2.5. Lysogeny broth (LB; 1L)

Sodium Chloride (NaCl; X190-1kg, amresco®)	10g
Tryptone (J859-500G, amresco®)	10g
Yeast (J850-500G, amresco®)	5g
Tap distilled water (dH_2O)	1L

2.6. Nucleoside triphosphates (dNTPs, 10mM, 1mL)

2'Deoxy-adenosine-5'-triphosphate (dATPs); 2'Deoxy-cytidine-5'-triphosphate (dCTPs); 2'Deoxy-guanosine-5'-triphosphate (dGTPs); 2'Deoxy-thymidine-5'-triphosphate (dTTPs) (DN-25, 100mM, fisher biotec australia)	100µL per each
Sigma water (Cat. No. W4502; SIGMA-ALDRICH®, Life Technologies™)	600µL

2.7. Phosphate Buffer Saline (PBS, 0.01M, pH7.4, 200mL)

PBS (Cat. No. P4417-100TAB; SIGMA-ALDRICH®, Life Technologies™)	1 tablet or 1g
Tap distilled water (dH_2O)	200mL

2.8. Pre-miRNA precursor stock (50µM, 100µL; aliquot to 5uL)

Pre-miRNA precursors (Ambion®, Life Technologies™): - Pre-miR™ miRNA Precursor Negarive Control #1, Cat. No, AM17110; or - Pre-miR™ miRNA Precursor has-miR-494-3p, Cat. No. PM10902; or - Pre-miR™ miRNA Precursor has-let-7f-5p, Cat. No. PM12409; or - Pre-miR™ miRNA Precursor has-miR-26b-5p, Cat. No. PM12899; or - Pre-miR™ miRNA Precursor has-miR-128-3p, Cat. No. PM11746; or - Pre-miR™ miRNA Precursor has-miR-365a-3p, Cat. No. PM11133; or - Pre-miR™ miRNA Precursor has-miR-455-3p, Cat. No. PM11142; or - Pre-miR™ miRNA Precursor has-miR-548aa, Cat. No. PM19524	5nmole
1.75mL Nuclease-free Water (Cat. No. AM9914G; Ambion®, Life Technologies™)	100uL

2.9. Renilla luciferase reporter substrate (1X, 60µL)

200X Renilla substrate	0.3µL
Flash & Glow buffer	59.7µL

2.10. Taqman® Custom miRNA RT primer pool (2mL; aliquot in 100µL)

Taqman® miRNA RT primers: - cel-miR-39-3p (ID: 000200), - ath-miR-159a (ID: 000338), - RNU6B (ID: 001093), - RNU44 (ID: 001904), - RNU48 (ID: 001006), - hsa-let-7a-5p (ID: 000377), - hsa-let-7f-5p (ID: 000382), - hsa-miR-7-5p (ID: 000268), - hsa-miR-16-5p (ID: 000391), - hsa-miR-17-5p (ID: 002308), - hsa-miR-18a-5p (ID: 002422), - hsa-miR-18a-3p (ID: 002423), - hsa-miR-18b-5p (ID: 002217), - hsa-miR-19a-5p (ID: 002424), - hsa-miR-19b-3p (ID: 000396), - hsa-miR-21-5p (ID: 000397), - hsa-miR-25-3p (ID: 000403), - hsa-miR-26b-5p (ID: 000407), - hsa-miR-29a-3p (ID: 002112), - hsa-miR-98-5p (ID: 000577), - hsa-miR-122-5p (ID: 002245), - hsa-miR-128-3p (ID: 002216), - hsa-miR-149-5p (ID: 002255), - hsa-miR-188-5p (ID: 002320), - hsa-miR-190b (ID: 002263), - hsa-miR-192-5p (ID: 000491), - hsa-miR-208a-3p (ID: 000511), - hsa-miR-222-3p (ID: 002276), - hsa-miR-223-3p (ID: 002295), - hsa-miR-326 (ID: 000542), - hsa-miR-338-3p (ID: 002252), - hsa-miR-365a-3p (ID: 001020), - hsa-miR-367-3p (ID: 000555), - hsa-miR-378a-5p (ID: 000567), - hsa-miR-378e (ID: 463378_mat), - hsa-miR-423-5p (ID: 002340), - hsa-miR-455-3p (ID: 002244), - hsa-miR-488-3p (ID: 002357), - hsa-miR-489-3p (ID: 002358), - hsa-miR-494-3p (ID: 002365), - hsa-miR-515-5p (ID: 001112), - hsa-miR-518f-5p (ID: 002387),	20µL per each

- hsa-miR-520f-3p (ID: 001120), - hsa-miR-548aa (ID: 463041_mat), - hsa-miR-548n (ID: 002888), - hsa-miR-572 (ID: 001614), - hsa-miR-584-5p (ID: 001624), - hsa-miR-598-3p (ID: 001988), - hsa-miR-612 (ID: 001579), - hsa-miR-618 (ID: 001593), - hsa-miR-622 (ID: 001553), - hsa-miR-625-5p (ID: 002431), - hsa-miR-633 (ID: 001574), - hsa-miR-636 (ID: 002088), - hsa-miR-647 (ID: 001600), - hsa-miR-760 (ID: 002328), - hsa-miR-1183 (ID: 002841), - hsa-miR-1238-3p (ID: 002927), - hsa-miR-1272 (ID: 002845), - hsa-miR-1285-3p (ID: 002822), - hsa-miR-1289 (ID: 002871), - hsa-miR-1912 (ID: 121110_mat), - hsa-miR-2682-5p (ID: 463610_mat), - hsa-miR-4284 (ID: 241828_mat), - hsa-miR-4425 (ID: 462954_mat), - hsa-miR-4455 (ID: 463355_mat), - hsa-miR-4741 (ID: 464892_mat).	
TE buffer (pH 8.0, Cat. No. AM9858; Ambion®, Life Technologies™)	660µL

3. Plasmid map

a. pMIR-REPORT vector

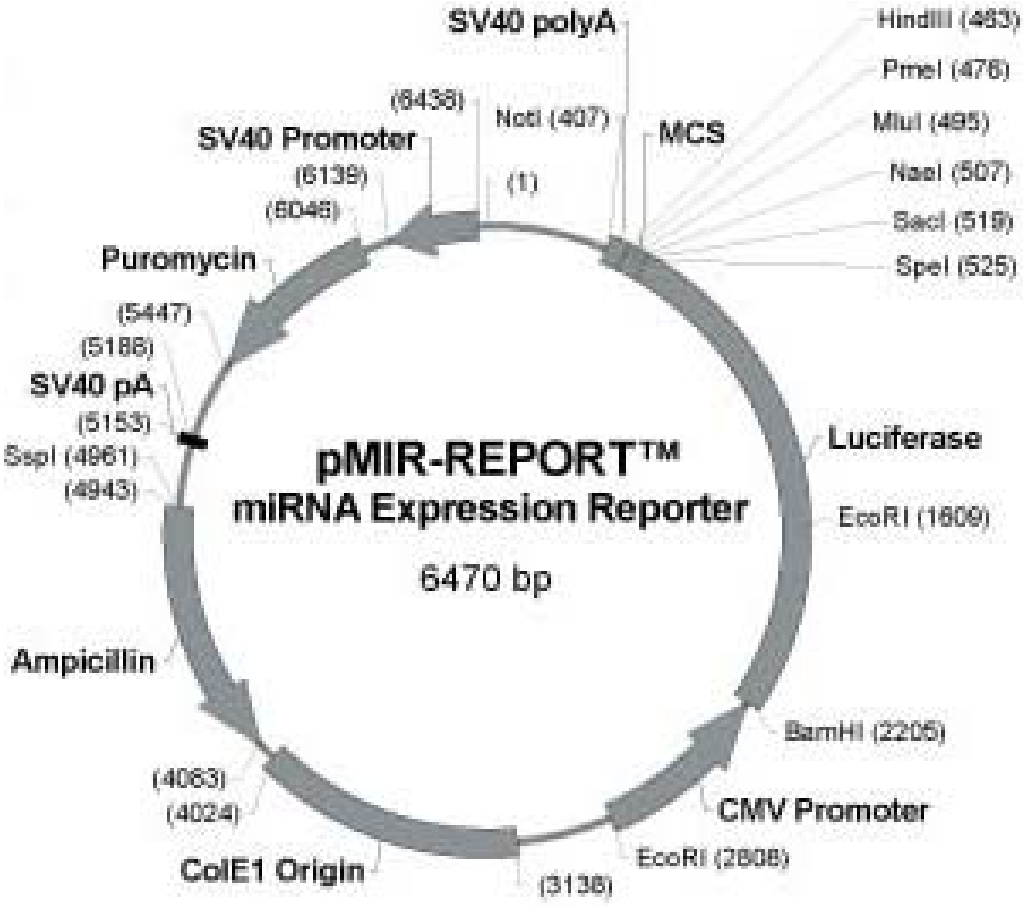

Figure 3-a. pMIR-REPORT vector. (*pMIR-REPORT™ miRNA Expression Reporter Vector System* 2015)

b. pRL-SV40 vector

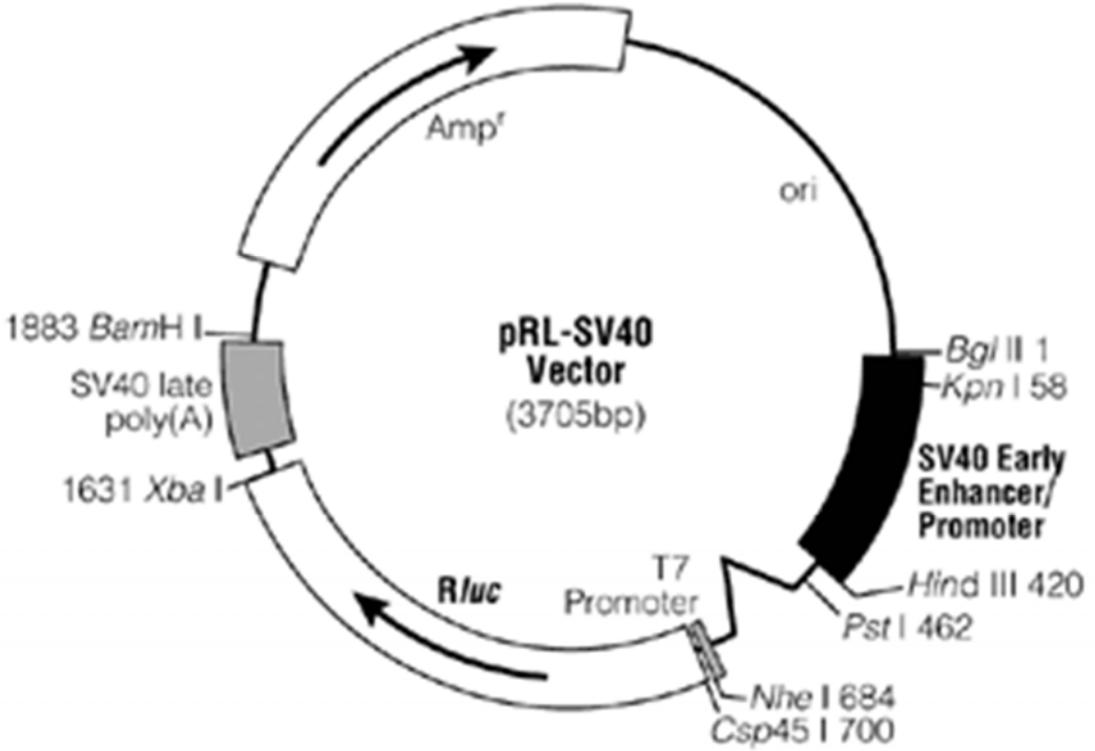

Figure 3-b. pRL-SV40 vector. (*pRL Renilla Luciferase Control Reporter Vectors* 2015)

4. *PROS1*, *F3* and *F8*-3'UTR sequence (source: Ensembl; format: Genebank)

> *PROS1*-3'UTR (1212bp)

```
   1  GGCATCTTTT CTCTGCTTAT AATACCTTTT CCTTGTGTGT AATTATACTT ATGTTTCAAT
  61  AACAGCTGAA GGGTTTTATT TACAATGTGC AGTCTTTGAT TATTTTGTGG TCCTTTCCTG
 121  GGATTTTTAA AAGGTCCTTT GTCAAGGAAA AAAATTCTGT TGTGATATAA ATCACAGTAA
 181  AGAAATTCTT ACTTCTCTTG CTATCTAAGA ATAGTGAAAA ATAACAATTT TAAATTTGAA
 241  TTTTTTTCCT ACAAATGACA GTTTCAATTT TTGTTTGTAA AACTAAATTT TAATTTTATC
 301  ATCATGAACT AGTGTCTAAA TACCTATGTT TTTTTCAGAA AGCAAGGAAG TAAACTCAAA
 361  CAAAAGTGCG TGTAATTAAA TACTATTAAT CATAGGCAGA TACTATTTTG TTTATGTTTT
 421  TGTTTTTTTC CTGATGAAGG CAGAAGAGAT GGTGGTCTAT TAAATATGAA TTGAATGGAG
 481  GGTCCTAATG CCTTATTTCA AAACAATTCC TCAGGGGGAA CAGCTTTGGC TTCATCTTTC
 541  TCTTGTGTGG CTTCACATTT AAACCAGTAT CTTTATTGAA TTAGAAAACA AGTGGGACAT
 601  ATTTTCCTGA GAGCAGCACA GGAATCTTCT TCTTGGCAGC TGCAGTCTGT CAGGATGAGA
 661  TATCAGATTA GGTTGGATAG GTGGGGAAAT CTGAAGTGGG TACATTTTTT AAATTTTGCT
 721  GTGTGGGTCA CACAAGGTCT ACATTACAAA AGACAGAATT CAGGGATGGA AAGGAGAATG
 781  AACAAATGTG GGAGTTCATA GTTTTCCTTG AATCCAACTT TTAATTACCA GAGTAAGTTG
 841  CCAAAATGTG ATTGTTGAAG TACAAAAGGA ACTATGAAAA CCAGAACAAA TTTTAACAAA
 901  AGGACAACCA CAGAGGGATA TAGTGAATAT CGTATCATTG TAATCAAAGA AGTAAGGAGG
 961  TAAGATTGCC ACGTGCCTGC TGGTACTGTG ATGCATTTCA AGTGGCAGTT TTATCACGTT
1021  TGAATCTACC ATTCATAGCC AGATGTGTAT CAGATGTTTC ACTGACAGTT TTTAACAATA
1081  AATTCTTTTC ACTGTATTTT ATATCACTTA TAATAAATCG GTGTATAATT TTAAAATGCA
1141  TGTGAATATC TTTATTATAT CAACTGTTTG AATAAAACAA AATTACATAA TAGACATTTA
1201  ACTCTTCATA CA
```

> *F3*-3'UTR (1235bp)

```
   1  AGGAAGCACT GTTGGAGCTA CTGCAAATGC TATATTGCAC TGTGACCGAG AACTTTTAAG
  61  AGGATAGAAT ACATGGAAAC GCAAATGAGT ATTTCGGAGC ATGAAGACCC TGGAGTTCAA
 121  AAAACTCTTG ATATGACCTG TTATTACCAT TAGCATTCTG GTTTTGACAT CAGCATTAGT
 181  CACTTTGAAA TGTAACGAAT GGTACTACAA CCAATTCCAA GTTTTAATTT TTAACACCAT
 241  GGCACCTTTT GCACATAACA TGCTTTAGAT TATATATTCC GCACTCAAGG AGTAACCAGG
 301  TCGTCCAAGC AAAAACAAAT GGGAAAATGT CTTAAAAAAT CCTGGGTGGA CTTTTGAAAA
 361  GCTTTTTTTT TTTTTTTTTT TTTTTTGAGA CGGAGTCTTG CTCTGTTGCC CAGGCTGGAG
 421  TGCAGTAGCA CGATCTCGGC TCACTGCACC CTCCGTCTCT CGGGTTCAAG CAATTGTCTG
 481  CCTCAGCCTC CCGAGTAGCT GGGATTACAG GTGCGCACTA CCACGCCAAG CTAATTTTTG
 541  TATTTTTTAG TAGAGATGGG GTTTCACCAT CTTGGCCAGG CTGGTCTTGA ATTCCTGACC
 601  TCAGGTGATC CACCCACCTT GGCCTCCCAA AGTGCTAGTA TTATGGGCGT GAACCACCAT
 661  GCCCAGCCGA AAAGCTTTTG AGGGGCTGAC TTCAATCCAT GTAGGAAAGT AAAATGGAAG
 721  GAAATTGGGT GCATTTCTAG GACTTTTCTA ACATATGTCT ATAATATAGT GTTTAGGTTC
 781  TTTTTTTTTT CAGGAATACA TTTGGAAATT CAAAACAATT GGCAAACTTT GTATTAATGT
 841  GTTAAGTGCA GGAGACATTG GTATTCTGGG CACCTTCCTA ATATGCTTTA CAATCTGCAC
 901  TTTAACTGAC TTAAGTGGCA TTAAACATTT GAGAGCTAAC TATATTTTTA TAAGACTACT
 961  ATACAAACTA CAGAGTTTAT GATTTAAGGT ACTTAAAGCT TCTATGGTTG ACATTGTATA
1021  TATAATTTTT TAAAAAGGTT TTCTATATGG GGATTTTCTA TTTATGTAGG TAATATTGTT
1081  CTATTTGTAT ATATTGAGAT AATTTATTTA ATATACTTTA AATAAAGGTG ACTGGGAATT
1141  GTTACTGTTG TACTTATTCT ATCTTCCATT TATTATTTAT GTACAATTTG GTGTTTGTAT
1201  TAGCTCTACT ACAGTAAATG ACTGTAAAAT TGTCA
```

> *F8*-3'UTR (1802bp)

```
   1  GGGTGGCCAC TGCAGCACCT GCCACTGCCG TCACCTCTCC CTCCTCAGCT CCAGGGCAGT
  61  GTCCCTCCCT GGCTTGCCTT CTACCTTTGT GCTAAATCCT AGCAGACACT GCCTTGAAGC
 121  CTCCTGAATT AACTATCATC AGTCCTGCAT TTCTTTGGTG GGGGGCCAGG AGGGTGCATC
 181  CAATTTAACT TAACTCTTAC CTATTTTCTG CAGCTGCTCC CAGATTACTC CTTCCTTCCA
 241  ATATAACTAG GCAAAAAGAA GTGAGGAGAA ACCTGCATGA AAGCATTCTT CCCTGAAAAG
 301  TTAGGCCTCT CAGAGTCACC ACTTCCTCTG TTGTAGAAAA ACTATGTGAT GAAACTTTGA
 361  AAAAGATATT TATGATGTTA ACATTTCAGG TTAAGCCTCA TACGTTTAAA ATAAAACTCT
 421  CAGTTGTTTA TTATCCTGAT CAAGCATGGA ACAAAGCATG TTTCAGGATC AGATCAATAC
 481  AATCTTGGAG TCAAAAGGCA AATCATTTGG ACAATCTGCA AAATGGAGAG AATACAATAA
 541  CTACTACAGT AAAGTCTGTT TCTGCTTCCT TACACATAGA TATAATTATG TTATTTAGTC
 601  ATTATGAGGG GCACATTCTT ATCTCCAAAA CTAGCATTCT TAAACTGAGA ATTATAGATG
 661  GGGTTCAAGA ATCCCTAAGT CCCCTGAAAT TATATAAGGC ATTCTGTATA AATGCAAATG
 721  TGCATTTTTC TGACGAGTGT CCATAGATAT AAAGCCATTT GGTCTTAATT CTGACCAATA
 781  AAAAAATAAG TCAGGAGGAT GCAATTGTTG AAAGCTTTGA AATAAAATAA CAATGTCTTC
 841  TTGAAATTTG TGATGGCCAA GAAAGAAAAT GATGATGACA TTAGGCTTCT AAAGGACATA
 901  CATTTAATAT TTCTGTGGAA ATATGAGGAA AATCCATGGT TATCTGAGAT AGGAGATACA
 961  AACTTTGTAA TTCTAATAAT GCACTCAGTT TACTCTCTCC CTCTACTAAT TTCCTGCTGA
1021  AAATAACACA ACAAAAATGT AACAGGGGAA ATTATATACC GTGACTGAAA ACTAGAGTCC
1081  TACTTACATA GTTGAAATAT CAAGGAGGTC AGAAGAAAAT TGGACTGGTG AAAACAGAAA
1141  AAACACTCCA GTCTGCCATA TCACCACACA ATAGGATCCC CCTTCTTGCC CTCCACCCCC
1201  ATAAGATTGT GAAGGGTTTA CTGCTCCTTC CATCTGCCTG ACCCCTTCAC TATGACTACA
1261  CAGAATCTCC TGATAGTAAA GGGGGCTGGA GACAAGGATA AGTTATAGAG CAGTTGGAGG
1321  AAGCATCCAA AGATTGCAAC CCAGGGCAAA TGGAAAACAG GAGATCCTAA TATGAAAGAA
1381  AAATGGATCC CAATCTGAGA AAAGGCAAAA GAATGGCTAC TTTTTTCTAT GCTGGAGTAT
1441  TTTCTAATAA TCCTGCTTGA CCCTTATCTG ACCTCTTTGG AAACTATAAC ATAGCTGTCA
1501  CAGTATAGTC ACAATCCACA AATGATGCAG GTGCAAATGG TTTATAGCCC TGTGAAGTTC
1561  TTAAAGTTTA GAGGCTAACT TACAGAAATG AATAAGTTGT TTTGTTTTAT AGCCCGGTAG
1621  AGGAGTTAAC CCCAAAGGTG ATATGGTTTT ATTTCCTGTT ATGTTTAACT TGATAATCTT
1681  ATTTTGGCAT TCTTTTCCCA TTGACTATAT ACATCTCTAT TTCTCAAATG TTCATGGAAC
1741  TAGCTCTTTT ATTTTCCTGC TGGTTTCTTC AGTAATGAGT TAAATAAAAC ATTGACACAT
1801  AC
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