The Role of MicroRNAs in the Endometrium

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Thesis presented in partial fulfillment for the degree of Doctor of Philosophy at Imperial College London

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All experiments included in this thesis were performed by myself unless otherwise stated in the text.
Abstract

Decidualization of the endometrium is essential for successful pregnancy and in human females of reproductive age, it occurs every month following the post-ovulatory rise in progesterone levels. The decidualization of primary human endometrial stromal cells (hESCs) can be recapitulated by treatment with cAMP and progestins, which results in changes in gene expression that give rise to phenotypes that favour implantation and survival of the conceptus. MicroRNAs (miRNAs) are a diverse class of small, non-coding RNA molecules that regulate gene expression post-transcriptionally and have important roles in many biological processes. Expression profiling revealed several miRNAs to be regulated during decidualization, and alterations in miRNA pathway components were also found. Although induction of Dicer suggested increased capacity to produce mature miRNAs, endogenous miRNA silencing became restricted upon decidualization of hESCs due to the down-regulation of the Argonaute proteins, catalytic components of the RNA-induced silencing complex. This was reflected in the regulation of miRNA target genes, which only appeared to be subject to miRNA-dependent regulation in undifferentiated hESCs. Moreover, the regulation of the androgen receptor (AR), a nuclear hormone receptor known to modulate the expression of a subset of decidual genes, was not dependent on miRNAs during decidualization. Rather, an RNA binding protein, poly(C)-binding protein 1 (PCBP1), regulated AR expression in hESCs during decidualization and in LNCaP cells. Additionally, decidualizing hESCs export miRNAs in exosome-sized vesicles that can be taken up by a variety of cells, including trophoblast and vascular cells, representing a novel mode of communication that may coordinate responses across different cell types at the feto-maternal interface.
Acknowledgements

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Lastly, but definitely not least, my parents and family deserve a big thank you for their support and love during my PhD (and the previous 22 years leading up to it).
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<td>ERK</td>
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°C degrees Celsius
P4 progesterone
P53 tumor suppressor protein of 53 kDa
PABP poly(A) binding protein
PACT protein activator of protein kinase R
PAGE polyacrylamide gel electrophoresis
PAI-1 plasminogen activator inhibitor 1
PAZ Piwi-Argonaute-Zwille
PBS phosphate buffered saline
PCBP1 poly(C) binding protein 1
PCDH7 protocadherin 7
PCR polymerase chain reaction
PKA protein kinase A
PMSF phenylmethanesulfonyl fluoride
PAZ Piwi-Arcogueta-Zwille
PRE progesterone response element
PRL prolactin
PROK1 prokineticin 1
PVDF polyvinylidene difluoroide
RACE rapid amplification of cDNA ends
RISC RNA induced silencing complex
RNA ribonucleic acid
ROS reactive oxygen species
rpm revolutions per minute
RPMI Roswell Park Memorial Institute
rRNA ribosomal RNA
s second(s)
SDS sodium dodecyl sulphate
siRNA small interfering RNA
SMRT silencing mediator of retinoid and thyroid receptor
SOC Super Optimal Broth with Catabolite Repression
snRNA small nucleolar RNA
SRC1 steroid receptor coactivator 1
SSC saline sodium citrate
STAT5 signal transducer and activator of transcription 5
SUMO small ubiquitin-like modifier
TAMRA carboxytetramethyl rhodamine
TAP tobacco acid pyrophosphatase
TBE Tris borate EDTA
TBS Tris buffered saline
TBS-T Tris buffered saline Tween 20
TE Tris EDTA
TEMED N,N,N',N'-tetraethylmethylethane-1,2-diamine
tissue factor
TF transforming growth factor-beta
TIMP tissue inhibitor of metalloproteinase
TRAIL tumor necrosis factor-related apoptosis inducing ligand
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TRBP</td>
<td>Trans-activator RNA-binding protein</td>
</tr>
<tr>
<td>TRIB2</td>
<td>Tribbles 2</td>
</tr>
<tr>
<td>Tris</td>
<td>Trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>TSG101</td>
<td>tumor suppressor gene 101</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>uNK</td>
<td>uterine natural killer cells</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
<td>weight/volume</td>
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<td>Exportin 5</td>
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<tr>
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<td>5'-3' exoribonuclease 1</td>
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<td>µl</td>
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Chapter 1 - Introduction
1.1 The Endometrium & Decidualization

1.1.1 Structure of the Human Endometrium

The endometrium is the lining of the uterus. It can be divided into three layers; the uppermost epithelium, the functional stromal layer and the basal stromal layer (Fig. 1.1). In addition, tubular uterine glands lined with columnar epithelium extend from the surface through the stroma to the base of the endometrium. The basal layer borders the myometrium and, in women of reproductive age, the overlying functional layer undergoes dramatic cyclical changes in thickness and structure in response to ovarian steroid hormones. Spiral arteries, which are also subject to remodeling during the menstrual cycle, supply the endometrium with blood.

Changes in ovarian hormone levels drive cyclical changes in the endometrium each month (Fig. 1.2). During days 5-14 of the menstrual cycle (the proliferative/follicular phase), increasing levels of circulating estrogen drive cell proliferation in the basal endometrial layer. This regenerates the functional layer following the previous menstruation, which grows from 0.5-1 mm to 5-7 mm thick (1). Just prior to and following ovulation, estrogen levels decline and there is a post-ovulatory rise in progesterone levels, due to the formation of the corpus luteum that is the source of progesterone in the ovary. Progesterone stimulates a cascade of molecular signaling events in the endometrium, which leads to the remodeling that is necessary for implantation of the embryo and pregnancy (2). The glands become highly tortuous and secretory, there is an influx of specialized uterine natural killer (uNK) cells and stromal cells differentiate into specialized epithelioid decidual cells. In the absence of pregnancy, declining progesterone levels, due to corpus luteum decay, leads to breakdown of the functional layer of the endometrium, focal bleeding and cell death (menstruation) (3).

The remodeling of the stromal compartment of the endometrium during the mid-secretory/luteal phase of the cycle is termed decidualization. It is a process that is repeated over 400 times during the reproductive life of a woman. Many reproductive and obstetric complications such as miscarriage, pre-eclampsia, fetal growth restriction and preterm labour have been linked to disorders affecting decidualization. In addition, certain knockout mouse models have revealed that decidualization is indispensable for pregnancy. Understanding this process is therefore important from biological and clinical standpoints.
Figure 1.1 Layers of the Human Endometrium
Hematoxylin and eosin stained sections of proliferative phase endometrium.
(A) Low magnification image of the myometrium and endometrium in cross section.
(B) Higher magnification image of the endometrium showing glands, functional and basal layers.
Figure 1.2 The Menstrual Cycle and Decidual Transformation

The ovarian hormones estrogen and progesterone control the growth of the endometrium. Estrogen promotes the ordered growth of the functional layer in the follicular/proliferative phase of the cycle. Following ovulation, progesterone secreted by the corpus luteum controls decidualization in combination with other factors such as relaxin (RLX), prostaglandin E2 (PGE$_2$), and corticotrophin-releasing hormone (CRH), which increase intracellular cAMP levels. Decidualization can be recapitulated in cultured hESCs by treatment with a progestin, such as medroxyprogesterone acetate (MPA), and a cAMP analogue.
1.1.2 Decidualization in Humans and Other Species

Decidualization only occurs in species where the trophoblast breaches the luminal epithelium following implantation. The extent of decidual transformation in the human endometrium during pregnancy is profound, extending from the epithelium to the inner third of the myometrium (the uterine junctional zone) and affecting all cell compartments of the endometrium. The extent of decidualization correlates with the degree of trophoblast invasion during pregnancy, and in humans this invasion is particularly deep, reaching the uterine junctional zone (4, 5).

Decidualization in humans, induced by rising ovarian progesterone levels, is first apparent in the stromal cells surrounding the spiral arteries approximately 9 days after ovulation in every menstrual cycle (6). If pregnancy occurs, decidualization extends to all endometrial compartments, and is maintained by blastocyst-derived human chorionic gonadotrophin (hCG) signaling and placental progesterone production. By contrast, in mice and most other mammals that have estrous cycles, decidualization only occurs following blastocyst implantation and is first apparent in stromal cells surrounding the implanting blastocysts.

A difference in the magnitude and timing of decidual transformation is correlated with the ability to undergo overt menstruation. In humans, apes and certain fruit bat species, blood and endometrial tissue is shed trans-vaginally following declining progesterone levels. Falling progesterone levels, in the absence of pregnancy, induce a switch in the substances secreted by stromal cells to pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), which then leads to menstrual shedding (7). In rodents and most other placental mammals, however, the endometrial lining is reabsorbed and not externally visible. Theories regarding the purpose of menstruation include the removal of sperm-borne pathogens from the uterus and the metabolic efficiency of discarding a decidualized endometrium when no implantation occurs, rather than maintaining it (8, 9). However, the fact that cycle-dependent decidualization and deep trophoblast invasion are associated with menstruation suggests that menstruation may be involved in preconditioning the uterus for pregnancy (10). Pregnancy and menstruation are both inflammatory processes and the latter may serve as a stimulus to prepare and protect the uterus from the more profound insults of the former. There is as yet little direct evidence for menstrual preconditioning but it is known that pre-eclampsia, an obstetric complication where uterine spiral arteries are inadequately remodeled, is more common in first pregnancies of younger women (11). Whatever the
purpose of menstruation, it only takes place in those species where decidualization is under maternal control and initiated in every cycle prior to implantation. The high degree of uterine remodeling required for the exceedingly invasive trophoblast of humans may mean that the decidual process must be initiated prior to implantation in anticipation of it. This would imply that if defective decidual responses were produced in each cycle, the chance of successful pregnancies would be reduced irrespective of the quality of the trophoblast.

1.1.3 Morphological Characteristics of Stromal Cell Decidualization

Human endometrial stromal cells (hESCs) are mesenchymal in origin and have a fibroblastic appearance in the proliferative phase and in culture (Fig. 1.2). Upon decidualization, they acquire a secretory phenotype, an epithelioid-like morphology, a rounding of the nucleus, expansion of the rough endoplasmic reticulum and Golgi complex, and cytoplasmic accumulation of glycogen and lipid droplets. Multiple processes appear on the surface of the cells that extend freely into the extracellular matrix (ECM). Decidual hESCs also express α-smooth muscle actin, desmin and vimentin, and therefore can be considered myofibroblastic (12). Adherens junctions but not true desmosomes form between stromal cells and ECM proteins produced by decidualized cells include decorin, laminin, type IV collagen, fibronectin, and heparan sulfate proteoglycans. The decidua ECM provides a support for coordinated trophoblast invasion and the properties that decidual hESCs acquire are important for protection of the conceptus from maternal and environmental insults.

1.1.4 Biochemical Changes during Decidualization

The transformation of hESCs into decidual cells in preparation for pregnancy can be considered a differentiation process. In addition to the morphological changes described above, there are biochemical changes, which are also ultimately dependent on hormonal signaling and coordinated alterations in gene expression.

A key trait of decidualized hESCs is their ability to secrete a range of substances, including cytokines (such as interleukin-11 (IL-11) and interleukin-1beta (IL-1β)), chemokines (e.g. CXCL6, CXCL11, CCL4, CCL14), growth factors (e.g. epidermal growth factor (EGF), heparin-binding growth factor (HB-EGF), Lefty-A, activin A) and neuropeptides (e.g. somatostatin and ghrelin). These factors are involved in propagation of the decidual process via autocrine and paracrine signaling. Chemokine expression, for
example, is key to recruiting specialized uNK cells to the feto-maternal interface (13). Two major secretory products are insulin-like growth factor binding protein-1 (IGFBP1) and prolactin (PRL), which have been used in many studies as classical decidual marker genes. Recent studies have also revealed that decidualized hESCs can act as biosensors of embryo quality. Arrested embryos co-cultured with decidualized hESCs caused a reduction in the secretion of various cytokines by hESCs compared to those cultured with developing embryos (14). The underlying mechanisms for these observations, however, remain unclear.

Gene expression profiling with microarray technology has shown that the biochemical reprogramming of hESCs during decidualization is underpinned by reprogramming of gene expression in functionally related gene families. Studies have revealed alterations in the expression of genes involved ECM organization, cytoskeletal organization, metabolism, apoptosis, differentiation, cell cycle regulation and cell adhesion (15-19). These changes serve to promote establishment of the trophoblast in the maternal tissues and formation of the placenta. It is known, for example that decidual cells surrounding the spiral arteries express high levels of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1), both of which are involved in maintaining vascular integrity prior to menstruation and during pregnancy (20, 21). Invasion of the trophoblast through the decidual ECM is regulated by decidualized hESCs. To promote invasion, the extravillous trophoblast secretes MMPs to breakdown ECM proteins. MMP actions are opposed by various tissue inhibitors of metalloproteinases (TIMPs) expressed by decidual cells (22-24). IGFBP1, which is secreted by decidualized hESCs in large quantities, can bind to $\alpha_5\beta_1$ integrin on cytotrophoblast, which inhibits their invasiveness (25). Interestingly, IGFBP1 can also have an autocrine effect by binding $\alpha_5\beta_1$ integrin on hESCs, which can stimulate decidualization even in the absence of MPA (26).

The endometrium must also adapt to protect the semi-allogeneic trophoblast from maternal immune responses and simultaneously protect the maternal and fetal tissues from pathogens. Decidualized stromal cells, via the secretion of chemokines and PRL, recruit (CD56$^{\text{bright}}$/CD16$^-$) uNK cells, which have immunoregulatory effects on other leukocytes in the endometrium (27, 28). Moreover, decidualized hESCs are able to secrete indoleamine 2,3-dioxygenase (IDO), tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) and Fas ligand, which can suppress T cell responses (29-31).

During gestation the remodeling of the spiral arteries generates oxidative stress, which the embryo must be protected from. Early in pregnancy the extravillous trophoblast
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invades the spiral arteries and plugs them to limit exposure of the embryo to excessive reactive oxygen species (ROS), which are by-products of oxidative metabolism and can cause indiscriminate damage to cellular macromolecules. Later on, to support fetal growth, the plugs must dislocate to permit transformation of the spiral arteries into high capacity vessels that perfuse the intervillous space with copious amounts of blood. This results in a dramatic increase in oxygen tension at the feto-maternal interface in the period of a few days and, consequently, a burst of ROS (32).

Decidualized hESCs protect themselves and other cells from the damaging effects of ROS via several mechanisms. Firstly, they induce several free radical scavengers, such as mitochondrial superoxide dismutase 2, thioredoxin, peroxiredoxin (31). Glutathione peroxidase 3, which is a secreted enzyme, is also produced by decidualized hESCs and it has strong extracellular antioxidant activity. Secondly, they express growth arrest and DNA damage inducible protein alpha of 45 kDa (GADD45α), which is involved in responses to oxidative stress, cell cycle arrest and DNA repair. Crucially, down-regulation of the forkhead box O 3a transcription factor (FOXO3a) in decidualized hESCs renders them refractory to oxidative stress-induced cell death (31).

In summary, decidual transformation endows hESCs with the ability secrete an assortment of signaling molecules, regulate trophoblast invasion, regulate endometrial haemostasis, modulate immune responses and resist oxidative stress. All of these functions serve to promote placentation and protect the conceptus from various insults.

1.1.5 Hormonal Signaling during Decidualization

Endocrine signaling in the endometrium is necessary for its ordered growth in the proliferative phase and decidual transformation in the secretory phase. Table 1 shows a list of gene knockout (KO) mouse models with impaired decidual responses. One of the earliest genes to be established as essential for decidualization was the progesterone receptor (PR), which belongs to the nuclear receptor superfamily of transcription factors (33, 34).

Progesterone, a steroid hormone, is able to bind the ligand-binding domain of PR and then promotes its translocation to the nucleus where it can act as a transcription factor in regulating gene expression. Progesterone is synthesized in the corpus luteum and, in pregnancy, by the placenta. Being a steroid it can freely diffuse through the plasma membranes of cells to reach PR in the cytoplasm. Progesterone signaling can be mediated by three isoforms of PR – PR-A, PR-B and PR-C which arise from differential promoter
usage of the PR gene. Figure 1.3 shows the layout of the various domains in PR isoforms. PR-A lacks 164 amino acids at the N-terminus compared to PR-B and has a weaker trans-activation function. However, a further KO mouse specific for PR-A resulted in female sterility and revealed that this isoform is essential for the decidual response (35). PR-C is the shortest isoform, lacking the entire N-terminus and first zinc finger of the DNA-binding domain, such that it cannot bind DNA (36). This isoform is postulated to act as a progesterone “sponge” where it is up-regulated at parturition in the myometrium to sequester progesterone from activating PR-A and PR-B (37).

Table 1. Knock-out mice with impaired decidual reactions

<table>
<thead>
<tr>
<th>Gene Disrupted</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooxygenase-2</td>
<td>(38)</td>
</tr>
<tr>
<td>Leukaemia Inhibitory Factor</td>
<td>(39)</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>(33)</td>
</tr>
<tr>
<td>PR-A</td>
<td>(35)</td>
</tr>
<tr>
<td>Steroid receptor coactivator 1</td>
<td>(40)</td>
</tr>
<tr>
<td>Hoxa-11</td>
<td>(41)</td>
</tr>
<tr>
<td>Hoxa-10</td>
<td>(42)</td>
</tr>
<tr>
<td>Interleukin-11</td>
<td>(43), (44)</td>
</tr>
<tr>
<td>Components of interferon gamma signaling</td>
<td>(45)</td>
</tr>
</tbody>
</table>

Figure 1.3 Domain layout of human progesterone receptor isoforms and human androgen receptor. PR-B is the full-length isoform and PR-A lacks the 164 N-terminal amino acids. DBD, DNA-binding domain; LBD, ligand-binding domain. Transcriptional activation functions, AF1, AF2 and AF3 can be ascribed to different portions of the molecule. Numbers represent amino acids residues.
Upon binding of progesterone, PR undergoes conformational changes which lead to dissociation from heat-shock protein chaperones, phosphorylation, dimerization, translocation to the nucleus and binding to progesterone response elements (PREs) in the promoter regions of target genes. Interaction of PR with coactivators such as steroid receptor coactivator 1 (SRC-1) permits recruitment of the basal transcription machinery and various chromatin remodeling complexes, such as histone acetyltransferases. In keeping with this, the SRC-1 KO mouse also exhibits an impaired decidual response (40). PR can also silence genes by recruiting corepressor proteins, such as silencing mediator of retinoid and thyroid receptor (SMRT) and nuclear receptor corepressor (N-CoR).

Although progesterone signaling is essential for decidualization it is not sufficient. In vivo, differentiation of the functional layer is only apparent about 10 days after progesterone levels start rising, suggesting other signals are necessary. Also, treatment of primary cultures of hESCs with progesterone alone or in combination with estradiol results in decidualization only after 8 to 10 days (46, 47). A key mediator necessary for initiating the decidual process is cyclic adenosine monophosphate (cAMP). It has been established that cAMP levels rise during the menstrual cycle, with higher levels reported in biopsies taken in the secretory phase than those taken in the proliferative phase (48). cAMP is produced from adenosine triphosphate by adenylate cyclase. This enzyme is activated by stimulatory heterotrimeric G proteins, which are in turn coupled to various cell surface receptors known as G protein-coupled receptors (GPCRs). GPCRs are a very diverse family of seven transmembrane domain proteins. They transduce signals from the extracellular environment to the cytoplasm via the binding of specific ligands and coupling this event to G protein activation. GPCRs in stromal cells can bind to various endocrine factors expressed in the endometrium. The expression of ligands such as relaxin (RLX), and corticotropin-releasing hormone (CRH) and/or their cognate receptors increases during the secretory phase (49, 50). Prostaglandin E2 is and its cognate receptors are present throughout the menstrual cycle (51). These factors stimulate adenylate cyclase and therefore intracellular cAMP production. In addition, hCG produced in large quantities by trophoblast can bind to the luteinizing hormone GPCR and further stimulate cAMP production during pregnancy (52). Figure 1.4A illustrates GPCR activation leading to cAMP production and PKA activation.

cAMP activates the protein kinase A (PKA) pathway by binding the regulatory subunits of PKA and permitting the dissociation and activation of the catalytic subunits. These phosphorylate numerous target proteins in both the cytoplasm and nucleus. Major
nuclear substrates for PKA are cAMP response element binding protein (CREB) and the related cAMP response element modulator (CREM) (53). These proteins are basic region/leucine zipper proteins, which dimerize by the leucine zipper and bind cognate DNA sequences (cAMP response elements; CREs) with the basic region. The phosphorylated CREB/CREM complex recruits the histone acetyltransferase CREB binding protein (CBP) to the promoter regions of target genes and this enzyme remodels the chromatin to favour transcription (54, 55). PKA signaling via CREB and CREM is normally subject to negative feedback inhibition because cAMP also stimulates expression of an isoform of CREM known as inducible cAMP early repressor (ICER), which represses CREB/CREM function to down-regulate cAMP-induced genes and its own expression (56). However, in decidualizing hESCs there is a persistent up-regulation of ICER suggesting that ongoing cAMP signaling is occurring (57). The binding of CREB and CREM to CREs may not be the main mechanism for cAMP-mediated induction of decidual specific genes. A recent study highlighted the importance of redox signaling and the nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) in mediating the cAMP-dependent induction of IGFBP-1 and PRL that occurs in the first 12 to 24 h of differentiation (58).

Treatment of primary hESCs with 8-Bromo-cAMP (a cAMP analogue) leads to induction of IGFBP-1 and PRL within a few hours. However, the decidual phenotype is not maintained with cAMP treatment alone and the expression of decidual marker genes declines after 6 to 8 days of culture. Treatment with a combination of 8-Bromo-cAMP and a progestin leads to a much higher induction of marker genes and their expression is sustained (47, 59). Thus cAMP and progesterone signaling have a synergistic effect for prompt and sustained decidual transformation of hESCs. The cross talk of PR and cAMP signaling is integral to the decidual process and it occurs at several levels (Fig. 1.4B). PKA activation is known to disrupt the interaction between PR and co-repressors, permitting PR to associate with coactivators (60). Also, cAMP induces the expression or activation of several transcription factors including Forkhead box O 1a (FOXO1A), CCAAT/enhancer binding protein beta (C/EBPβ) and signal transducer and activator of transcription 5 (STAT5) which are capable of interacting with PR on various promoters that do not necessarily have PREs (61-64). In other words, liganded PR-A serves as a platform for the recruitment of decidual-specific transcription factors on a wide range of genes that would not otherwise be regulated by liganded PR-A alone. Another level of cross talk is the fact that cAMP treatment alters the expression of components of the sumoylation pathway.
This pathway can modify proteins by covalent conjugation of peptides onto lysine residue side chains. PR-A can be modified by small ubiquitin-like modifier 1 (SUMO1), which leads to repression of its transcriptional activity. cAMP treatment of hESCs leads to a gradual increase in PR-A activity through reduced sumoylation of this nuclear receptor (65, 66).

Another nuclear receptor that is regulated during the menstrual cycle is the androgen receptor (AR) (see figure 1.3 for domain layout). AR is most widely recognized for its functions in the male reproductive system and prostate cancer, however, it is expressed in the cycling endometrial stroma, with expression decreasing in the secretory phase but remaining detectable (67). Tissue levels of androgen and conversion of androstenedione to testosterone are higher in the secretory phase too, and in pregnancy circulating androgen levels rise (68, 69). A recent study demonstrated that ligand activation of AR enhanced PRL induction by cAMP and progesterone treatment of primary hESCs. This was associated with a reduction in AR sumoylation and siRNA-mediated AR knockdown revealed that this nuclear receptor regulates a distinct subset of genes involved in cytoskeletal organization and cell cycle regulation in hESCs (70). However, the mechanisms of AR regulation in the endometrium are poorly understood.
Figure 1.4 The cAMP pathway and cross-talk between cAMP and PR-A signalling in decidualizing hESCs
A. Various extracellular ligands can bind G protein-coupled receptors located in the plasma membrane of cells. Ligand-bound receptors activate heterotrimeric G proteins. The GTP-bound Ga subunit then activates adenylate cyclase which catalyses cAMP formation. cAMP binds to the regulatory subunits of PKA and induces the release and activation of the catalytic subunits, which enter the nucleus to phosphorylate different target proteins. B Intracellular cAMP levels are increased by GPCR activation, which stimulates expression and nuclear accumulation of C/EBPβ, FOXO1A and activated STAT5 (*). cAMP-induced ROS production by NOX4 also promotes nuclear accumulation of C/EBPβ. Progesterone binding to PR-A leads to liganded PR (PR-A*) entering the nucleus and forming complexes with cAMP-regulated transcription factors. These complexes activate transcription of various genes associated with decidualization. PIC, preinitiation complex.
1.2 MicroRNAs

1.2.1 An Historical Perspective

MicroRNAs (miRNAs) are evolutionarily conserved non-coding small RNA molecules that regulate gene expression. Despite their simple structure, the story of their discovery and impact on biology is not a straightforward one. In 1989, Victor Ambros’s lab found that a gene called lin-4 regulated developmental timing in the nematode worm *Caenorhabditis elegans* (71). Loss-of-function mutation of *lin-4* resulted in mature worms lacking many adult structures. Loss-of-function mutations in another gene called *lin-14* led to larval stage worms developing many adult features precociously, leading to small, poorly developed adults (71, 72). The opposing phenotypes produced by *lin-4* and *lin-14* mutations revealed that *lin-4* functions as a repressor of *lin-14* (71). *Lin-14* encoded a protein (73), unexpectedly, however, *lin-4* did not encode a regulatory protein but a 22 nucleotide (nt) non-coding RNA molecule derived from a 61 nt short hairpin RNA.

In 1991, Gary Ruvkun’s lab found that the 3' untranslated region (UTR) of the *lin-14* gene contained conserved sequences that regulated the abundance of LIN-14 protein (74). Subsequently, in two seminal 1993 papers, Ambros and Ruvkun demonstrated that the sequence of the small *lin-4* RNA had partial complementarity to sequences in the *lin-14* 3'UTR and that interaction between these sequences was responsible for post-transcriptional down-regulation of LIN-14 protein levels (75, 76) (Fig. 1.5). Thus Jacob and Monod’s 1961 proposal of an RNA molecule regulating the abundance of a specific protein had been realized, although in the context of nematode development and not the bacterial *lac* repressor system as they had originally theorized (77).

For a few years it was assumed that this type of regulation was a peculiarity of *C. elegans* for several reasons. Firstly, no evidence for *lin-4* sequences in organisms other than closely related nematodes was found. Secondly, there was reluctance in the field to explain developmental processes of other organisms in new terms. Transcription factor-mediated gene regulation and post-transcriptional regulation involving RNA binding proteins were thought to be satisfactory paradigms. Finally, the *lin-4* gene itself was for a long time thought to be unique and no other short hairpin RNAs were found in *C. elegans*. 
However, more evidence for RNA-mediated regulation of gene expression was slowly accumulating. Another target of lin-4 was found (lin-28), which suggested that different genes could acquire functional lin-4 sites by evolution (78). In 1998, RNA interference (RNAi) was discovered in C. elegans, where small RNAs (21-25 nt) derived from double-stranded RNA could regulate genes in a sequence specific manner (79). They were designated short interfering RNAs (siRNAs) and functioned as guides to mediate cleavage of target mRNAs near the centre of the region targeted by the siRNA in Drosophila, C. elegans and mammalian cells (80-84). The study of viral resistance in plants also gradually led to the parallel discovery of RNAi (85-87). It became conceivable that the lin-4 small RNA could be derived from its precursor and induce its effects using the RNAi machinery.

In 2000, the Ruvkun lab identified another small RNA in C. elegans produced from the let-7 gene, again involved in developmental timing, and it appeared to repress its target lin-41 by similar mechanisms to lin-4 repression of lin-14 (88, 89). The watershed moment soon arrived when the same lab showed that let-7 was almost perfectly conserved in a wide range of animals from sea urchins to humans (90). This led to a burst of discovery of new small RNA molecules, now termed microRNAs, using cDNA libraries of small RNA and computational identification in C. elegans, mice and humans (91-96).

Since 2001 the miRNA field has grown enormously and RNAi has become well established as a tool for loss of function studies in many systems. Over 1000 different miRNAs have been identified in humans and the number is continuing to rise. An online registry has been set up to catalog all miRNAs identified in humans and other organisms.
A key area of investigation is computational prediction of microRNA targets to uncover the pathways they are involved in. It has been estimated that up to 60% of mammalian genes are regulated by miRNAs (98).

These small RNAs are engaged in diverse processes of metazoan biology, such as viral replication, cell fate, morphogenesis, physiology and disease. MicroRNA function and biogenesis has been intensively studied in humans and several model organisms. Often miRNAs have tissue or cell type specific expression patterns, implying important roles in development and cell biology. The roles of miRNAs in disease states including cardiovascular disease, cancer, Alzheimer’s disease and diabetes are subject to intense research efforts and the prospect of miRNA-based therapeutics is nearing (99). To summarize, although miRNAs were initially regarded as a nematode oddity, work during the last twenty years, and particularly the last ten, has revealed their ubiquity and pervasive roles in biology, and this work looks set to continue.

1.2.2 Nomenclature

The naming of miRNAs is systematic and each one is given a “miR-” prefix followed by a number (e.g. miR-100). Numbers are assigned sequentially with identical miRNAs having the same number, regardless of organism. However, for historical reasons, some miRNAs have names that do not fit with this scheme, such as let-7. MicroRNA genes in humans are given capitalized names (e.g. MIR100). Closely related miRNAs are grouped into miRNA families, which have the same number but individual letters for each family member (e.g. miR-10a, miR-10b, etc.). To specify the organism, a prefix of the abbreviated species name is added (e.g. cel- for Caenorhabditis elegans, hsa- for Homo sapiens, mmu- for Mus musculus, etc.), (e.g. hsa-miR-100, mmu-miR-100). Often mature miRNAs have multiple locations from the genome from which they originate. To specify this, another number is added as a suffix (e.g. hsa-miR-29b-1 and hsa-miR-29b-2). Primary miRNA transcripts are designated pri-miR-X and hairpin precursors are designated pre-miR-X.
1.2.3 MicroRNA Biogenesis

MicroRNA biogenesis is a stepwise process initiated in the nucleus and continued in the cytoplasm, utilizing components of the RNAi pathway. The various stages can be subject to regulation and the regulatory functions of resultant mature miRNAs on their targets is dependent on a host of proteins. The stages leading up to mRNA targeting are described in this section. Figure 1.6 illustrates the canonical miRNA biogenesis pathway.

MicroRNA Gene Transcription

A proportion of miRNAs genes are intergenic (i.e. located in between annotated protein coding genes), suggesting that they derive from independent transcription units. However, up to 50% of mammalian miRNA genes are intragenic and located in annotated protein coding genes (100). These intragenic miRNA genes are found in the introns of host genes and the expression of the miRNA is presumably correlated with the host gene’s expression. However, intragenic miRNA genes may be transcribed in the opposite direction of the host gene (from the antisense strand) and thus rely on independent promoters and even some sense-oriented intragenic miRNA genes can have their own promoters (101).

Many miRNA genes are isolated, although up to 50% of mammalian miRNA genes are clustered in the genome in ways that suggest they are transcribed together as polycistronic transcripts, which are processed to separate the different mature species. MicroRNAs in genomic clusters are often related to each other, but not always, and related miRNAs can be located from disparate parts of the genome too. The common feature of all miRNA genes is that they produce intermediate RNA transcripts that fold into hairpin structures, which can be recognized by the miRNA processing machinery.

Most mammalian miRNA genes are transcribed by RNA polymerase (pol) II, which is also responsible for transcribing protein-coding genes (102, 103). This means that miRNA gene transcription can be regulated by a vast number of transcription factors that are already known to interact with RNA pol II on the promoters of protein-coding genes. Primary miRNA transcripts (pri-miRNAs) can be of various lengths (usually >1 kilobase), have 5’ 7-methylguanosine caps and 3’ poly(A) tails characteristic of RNA pol II-mediated transcription and all have secondary structures with one or more imperfectly paired stem-loops, which are necessary for further processing steps. A minority of miRNA genes associated with Alu element repeats are transcribed by RNA pol III, which is also responsible for generating transfer RNA (tRNA) and 5S ribosomal RNA (rRNA) (104).
Nuclear processing and Export

In the nucleus, pri-miRNAs are recognized by the Microprocessor complex, which consists of the ribonuclease (RNase) III Drosha and its binding partner DiGeorge syndrome critical region 8 (DGCR8). Drosha homologues are found in worms, flies, mice and humans but not in the yeast species Schizosaccharomyces pombe. Human Drosha is involved in processing ribosomal RNA precursors as well as primary miRNA transcripts (105, 106). DGCR8 binds to the pri-miRNA hairpin, via its two double-stranded RNA binding domains (dsRBDs), at the single stranded RNA-double stranded RNA (ssRNA-dsRNA) junction. This spaces Drosha such that it cleaves the 5’ and 3’ strands of the hairpin RNA approximately 11 bp away from the ssRNA-dsRNA junction, releasing a hairpin stem loop that is 60-70 nt in size (106-109). The excised hairpin is referred to as a precursor miRNA (pre-miRNA) and has a 2 nt 3’ overhang and a 5’ phosphate group that are characteristic of RNase III-mediated cleavage. Cleavage occurs co-transcriptionally and, if the miRNA is intronic, prior to pre-mRNA splicing (110, 111).

The Microprocessor complex also contains several cofactor proteins such as the DEAD box RNA helicases p68 and p72 and various heterogeneous nuclear ribonucleoproteins (hnRNPs) (107). Although not essential for cleavage activity, these proteins promote fidelity and specificity of cleavage and they also serve as regulators of the complex. Several studies have demonstrated that other nuclear proteins can regulate Microprocessor activity by interacting with these auxiliary factors (112-115). Additionally, a mechanism for auto-regulation of the Drosha/DGCR8 complex has been discovered; DGCR8 stabilizes Drosha protein by direct interaction and Drosha can cleave hairpin structures in the 5’ UTR and coding region of the DGCR8 mRNA, rendering the mRNA unstable (116).

A small minority of miRNAs do not require processing by Drosha/DGCR8 (117-119). These miRNAs, referred to as “mirtrons”, are in short introns and the termini of the pre-miRNAs coincide with splice donor and acceptor sites. The activity of the mRNA splicing machinery is sufficient to generate the pre-miRNA hairpin stem loops that are exported to the cytoplasm.

Exportin 5 (XPO5), a member of the nuclear transport receptor family, mediates export of pre-miRNAs to the cytoplasm (120). This protein was originally known as a minor export factor for tRNAs (121, 122), but its major cargo is now known to be pre-miRNAs. Exportin 5 binds to its cargo and the guanosine triphosphate (GTP)-bound form of Ran, the cofactor necessary for transport. Cargo is transported through the nuclear pores.
and is released in the cytoplasm upon hydrolysis of GTP (123). Export of miRNA precursors is again dependent on correct RNA secondary structure for recognition by the export machinery. The presence of a >14 bp dsRNA stem and 3’ overhang is necessary for exportin 5 recognition, but loop and sequence variations do not affect binding (124).

Cytoplasmic processing and RNA induced silencing complex (RISC) loading

In the cytoplasm, the RNase III enzyme Dicer processes pre-miRNAs. Dicer is a highly conserved protein found in almost all eukaryotes, including *Schizosaccharomyces pombe*. It was first recognized for ‘dicing’ long dsRNA into siRNA molecules, and is therefore necessary for RNAi (125). In some organisms there are multiple Dicer homologues, each with specialized roles. For example in *Drosophila*, Dicer 1 is required for miRNA biogenesis and Dicer 2 is required for siRNA production (126). In mammalian species there is only one isoform of Dicer and the importance of microRNAs in development is demonstrated by the fact that Dicer KO mice are embryonic lethal (127).

Human Dicer is approximately 220 kiloDaltons (kDa) in size and contains an N-terminal DEXH-box helicase domain, a domain of unknown function (DUF283), a Piwi Argonaute Zwille (PAZ) domain, two RNase III domains and a dsRBD. Dicer interacts with other dsRNA binding proteins; trans-activator (TAR) RNA-binding protein (TRBP, also known as TARBP2) and protein activator of protein kinase R (PACT) (128, 129). Although not necessary for cleavage of the substrate, TRBP stabilizes Dicer and couples the miRNA pathway to the mitogen-activated protein kinase (MAPK) pathway (130). The MAPK extracellular signal-regulated kinase (ERK), which is best known for promoting cell growth, phosphorylates TRBP and this stabilizes the TRBP-Dicer complex.

The way in which Dicer binds to the pre-miRNA substrate determines the length of the mature miRNA product. The PAZ domain recognizes the 3’ end of the pre-miRNA and then the RNase III domains cleave both strands of the stem at a position approximately two helical turns away (131, 132). The resultant products are a duplex RNA ~22 nt in length (the mature miRNA duplex) and the terminal bases and loop. Again the products have 2 nt 3’ overhangs that are a feature of RNase III-mediated cleavage. In other words, the structure of Dicer determines the lengths of mature miRNAs and siRNAs, and these molecules are known to be of roughly the same size.

Mature miRNAs do not function in isolation but are associated with an effector complex, which is also utilized by siRNAs, known as the RNA induced silencing complex (RISC). The core component of the RISC is an Argonaute (Ago) protein. Four isoforms
exist in mammals – Ago 1 to Ago 4 – which bind miRNAs with largely equal propensities. Argonaute 2, however, is the only isoform that has endonuclease activity and is capable of cleaving mRNA. Only one strand from the mature miRNA duplex is incorporated into the RISC to mediate silencing. Strand selection for loading onto Argonaute is largely dependent on the relative thermodynamic stability of the two ends of the duplex. The strand with more unstable base pairs at its 5’ end is usually the one that is selected (133, 134). The strand most often incorporated into RISC is known as the miRNA or guide strand and the other strand is referred to as the passenger strand or miRNA*. The passenger strand is often degraded, however, this is not a stringent rule and sometimes the passenger strand can be incorporated into RISC.

To date, only one miRNA has been found that is processed independently of Dicer. Vertebrate miR-451 has a highly conserved terminal loop but poor conservation in the stem, which is unusual for miRNAs (135). Drosha processing of pre-miR-451 yields a short hairpin with an 18 bp stem that is too short for Dicer processing. The pre-miRNA bypasses Dicer processing and is loaded onto Ago proteins. If the hairpin is loaded onto Ago2, the 3’ hairpin arm is cleaved by Ago2’s endonuclease activity and the resultant 30 nt species (ac-pre-miR-451) is further trimmed by an unknown nuclease to give mature miR-451 (136, 137).

The sequential enzymatic processing of miRNAs by Drosha and Dicer results in a double stranded miRNA/miRNA* duplex. However, the mature RISC complex, which is able to target mRNAs, contains only a single stranded small RNA. Thus separation of the two strands of the duplex is necessary before a functional RISC is formed. The RISC loading complex, consisting of Dicer, TRBP and Argonaute, couples Dicer-mediated cleavage of pre-miRNA with loading and unwinding of the duplex (138, 139). The heat shock proteins 70 and 90 (HSP70 and HSP90) were also found to assist in RISC loading by driving an open conformation of Argonaute through ATP hydrolysis (140). The loading of the mature miRNA duplex onto Argonaute proteins is therefore ATP-dependent but the unwinding of the duplex is not.
Figure 1.6 The canonical miRNA biogenesis pathway
MicroRNA genes are transcribed usually by RNA polymerase II to generate primary miRNA transcripts. The nuclear microprocessor complex, consisting of Drosha and DGCR8, then processes the pri-miRNA to release a ~70 nt stem-loop pre-miRNA. Pre-miRNA molecules are exported from the nucleus to the cytoplasm by Exportin-5 and Ran-GTP. To generate functional miRNAs, pre-miRNAs are further processed by Dicer, which dimerises with TRBP and PACT. Dicer removes the loop region to generate a ~22 nt mature miRNA duplex that is loaded onto Argonaute (AGO) proteins that form the core component of the RNA-induced silencing complex (RISC). The duplex is unwound with one strand (the miRNA strand) remaining complexed with AGO to mediate mRNA targeting. The miRNA* strand is usually degraded.
1.2.4 Mechanisms of miRNA Action

*Interactions between miRNAs and mRNAs and bioinformatic prediction*

The specificity of miRNA action is due to the base pairing between bases in 3’ UTR of mRNAs and the miRNA bases. This base pairing is rarely perfect but some rules have been established from experimental and bioinformatic analyses. The most crucial feature of the miRNA-mRNA interaction is contiguous and perfect Watson-Crick base pairing in the seed region, which is between nucleotides 2 to 7 at the the 5’ end of the miRNA. Indeed, the most conserved regions of metazoan miRNAs are their 5’ regions, implying that they are under high selective pressure and therefore important for function (141). An adenosine opposite position 1 of the miRNA can further improve miRNA activity, although it need not base pair with miRNA nucleotides (142). Even so, perfect complementarity in the seed region is not essential for interaction, as exemplified by let-7’s interaction with lin-41 mRNA in *C. elegans*, where bulges and mismatches are present in the seed region (143). The 3’ end of the miRNA may also contribute to base pairing with the mRNA, and this can often stabilize the interaction when pairing in the seed region is suboptimal. The main region of mismatch in miRNA:mRNA pairings is in the central portion of the miRNA. This central bulge prevents cleavage of the mRNA by Ago2 and is the main difference in the action of miRNAs and siRNAs, which are fully complementary to their targets. Figure 1.7 shows typical generic miRNA-target interactions. Although most miRNAs have predicted and validated target sites positioned in the 3’ UTRs of transcripts, there have been reports of miRNAs targeting the 5’ UTR and coding regions of mRNAs (144-146). Furthermore, some of the associations between miRNAs and 5’ UTRs appear to activate rather than repress translation (146).

The prediction of miRNA targets is a complex affair. As 3’ UTRs are typically several hundred nt in size, they can harbour potential sites for many different miRNAs. As a first step, current prediction algorithms search the 3’ UTRs of genes for stretches of 7 bases that are complementary to miRNA seed regions. Since a given 7mer can occur every 16,000 bases by chance, this searching will generate many false positives. To reduce false positives, algorithms also consider cross-species conservation of sites. This assumes that true miRNA binding sites are more likely to be conserved during evolution due to selective pressure, while random 3’ UTR sequence is assumed to mutate at a higher rate. These assumptions were confirmed in several studies (142, 147-150), but cross-species comparisons are obviously restricted to those species where the miRNAs in question are
conserved too, and species-specific target genes are also missed by this method. Further considerations include the accessibility of the sites in the 3’UTR, since mRNA molecules can fold up to prevent miRNA access, and whether specific miRNAs and putative mRNA targets are co-expressed for there to be any interaction \textit{in vivo}.

There are three widely used algorithms for miRNA target prediction: \textit{miRanda}, \textit{TargetScan} and \textit{PicTar}. \textit{MiRanda} (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) does not consider cross-species conservation of binding sites and just searches for complementary base pairing between miRNAs and the 3’UTRs in a given genome. Scores are calculated based on the degree of base pairing and seed region interactions are given extra weight. Energy values are also computed based on the thermodynamic stability of the miRNA:mRNA duplex. \textit{TargetScan} (http://www.targetscan.org/) predicts targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA. Conservation of targets is also considered and miRNA families are categorized into groups based on the degree of conservation. The \textit{PicTar} algorithm (http://pictar.mdc-berlin.de/) is similar to \textit{TargetScan}, with orthologous 3’ UTRs subjected searches for miRNA sites and species conservation included, but it also accounts for multiple 3’ UTR binding sites for one miRNA or the potential for co-operation of different miRNAs on one 3’ UTR. Overall, bioinformatic algorithms are useful for initial exploration of miRNA function and gaining evolutionary insights, but experimental validation of miRNA:mRNA interactions, using reporter gene assays, mutagenesis and miRNA overexpression/anti-miR studies, is the only current way to be confident that \textit{in silico} predictions are valid \textit{in vivo}.
Figure 1.7 Typical miRNA-target interactions.
The interactions highlighted in blue are regarded as having the highest efficacy, with 8mer >> 7mer-m8 > 7mer-A1. 6mer sites are less effective but interactions between the 3' region of the miRNA and mRNA can supplement seed region interactions and also compensate for any mismatches in the seed region. ORF, open reading frame. 'N' denotes any RNA base.
Silencing by miRNAs

At the heart of miRNA function is interaction between the mRNA and miRNA, but many proteins are necessary for generating the effects of the miRNA on protein output and mRNA stability. Argonautes are essential components of RISC and they contain three conserved domains: PAZ, MID and PIWI (P-element-induced wimpy testis) (151). The PIWI domain has an RNase H-like fold which, in Ago2, is catalytically active and able to cleave mRNA. The PAZ domain is able to interact with the 3’ end of the miRNAs (152). The 5’ end of the miRNA is anchored in a deep pocket at the junction of the MID and PIWI domains and bases in the seed region contact the Ago residues via the ribose-phosphate backbone and are displayed in a semi-helical conformation on the protein’s surface. These bases are thus readily available for pairing with mRNA bases. The PIWI domain of Ago is also responsible for interacting with GW182 proteins, which are essential for miRNA-mediated silencing in animal cells (153, 154). This group of proteins is named after the glycine (G) and tryptophan (W) repeat-containing domains in their N terminal regions. The GW repeat domain is responsible for Ago interaction and disruption of the interaction by point mutations or peptide competition abrogates miRNA-mediated repression (154, 155).

Many studies have examined the mechanisms of repression downstream of miRNA binding to mRNA. However, it appears that there are multiple and overlapping ways in which protein output can be down-regulated. Studies in a variety of organisms, have found that miRNAs block mRNA translation either at the initiation or elongation stages or promote mRNA destabilization and decay, brought about by removal of the 5’ cap (decapping) or 3’ poly(A) tail (deadenylation). Figure 1.7 illustrates several mechanisms of how miRNAs regulate protein abundance.

Translation of mRNA to protein is a highly coordinated process requiring many factors to ensure initiation at the correct codon, elongation and termination. Initial studies of miRNA function in C. elegans showed that lin-4 caused an inhibition of lin-14 and lin-28 translation without a reduction in mRNA levels (156, 157). These studies and further work on mammalian cells found that miRNAs and their targets associated with polysomes when fractionated on sucrose sedimentation gradients (156, 158). A polysome is a functional unit of protein synthesis consisting of several ribosomes attached along the length of an mRNA molecule. The polysomes containing miRNA-repressed mRNAs were considered to be actively involved in translation because treatment with translation inhibitors led to dissociation into monosomes or ribosomal subunits. Hence, miRNAs were
Figure 1.8 Mechanisms of miRNA-mediated gene silencing in animals.
The top figure shows circularised mRNA, which is ready for translation in polysomes with the cap complex interacting with PABP. The RISC complex containing miRNA, AGO and GW182 proteins targets the 3'UTR and can disrupt the cap complex, preventing translation initiation. RISC may also interrupt translation post-initiation, by promoting ribosome drop-off or proteolytic degradation of the nascent polypeptide. mRNA deadenylation is mediated by the CAF1-CCR4-NOT complex, which is recruited by interaction with GW182. Deadenylated mRNA is susceptible to decapping by the DCP1-DCP2 complex aided by EDC4 and DDX6. Removal of the 5' cap promotes 5' to 3' decay of the mRNA, which is catalysed by the XRN1 exonuclease.
initially though to block translation post-initiation and during elongation. Further work proposed that miRNAs promoted ribosome drop off or degradation of the nascent polypeptide chain during the elongation step (159, 160).

However, other conflicting data indicated that miRNAs inhibit translation at the initiation stage. Pillai et al. found that mRNAs bound by miRNAs do not co-sediment with polysomes but with lighter fractions containing few ribosomes (161). Furthermore, miRNA-mediated repression was dependent on the 5’ cap (162, 163), which is essential for initiation, and those mRNAs containing internal ribosome entry sites (IRESs) that are not translated in a 5’ cap-dependent manner were refractory to miRNA repression. The 5’ cap and 3’ poly(A) tails on eukaryotic mRNAs interact with eukaryotic initiation factor eIF4E and poly(A) binding protein (PABP), respectively. eIF4E, is part of the eIF4F complex that also contains a large scaffolding protein called eIF4G. PABP binds to eIF4G and this circularizes the mRNA, protecting it from degradation and cap recognition by eIF4E is essential for translation initiation (164, 165). GW182, in association with Ago, can also interact with PABP and may interfere with eIF4E function or 5’ cap binding and thereby inhibit translation initiation (166).

siRNAs can cause degradation of their mRNA targets by Ago2 cleavage. However, due to the generally mismatched base pairing between miRNAs and target mRNAs, Ago2 is unable to cleave mRNA. Despite this, there is evidence for miRNAs inducing mRNA degradation. For example, several studies have shown that over-expression of particular miRNAs in cultured cells leads to a depletion of transcripts that contain seed matches for those miRNAs (167-172). Conversely, depletion of miRNAs with anti-miRs or depletion of components of the miRNA pathway (such as Dicer, Drosha and Argonautes) leads to an up-regulation of corresponding target transcripts (173-175).

Degradation is thought to follow deadenylation and decapping (Fig. 1.8). MicroRNAs first induce deadenylation of mRNAs through the action of the CAF1-CCR4-NOT deadenylase complex. The GW182 recruits the CAF1-CCR4-NOT complex to the poly(A) tail which removes the adenosine residues (176). Indeed, knocking down CAF1 or NOT1 has been shown to block the majority of miRNA-induced deadenylation and mRNA destabilization (177). Deadenylation leaves mRNAs unable to circularize and so the cap complex is no longer stabilized. Decapping is thus promoted and is catalysed by the DCP1-DCP2 complex, which is also associated with enhancers including EDC4 and DDX6. Removal of the 5’ cap leaves the mRNA vulnerable to 5’ to 3’ decay that is catalysed by the major cytoplasmic 5’-to-3’ exonuclease XRN1 (178).
The question of whether translational repression or mRNA degradation is the dominant mechanism for miRNA action has been addressed in several studies by monitoring global mRNA and protein levels in parallel and ribosome profiling (167-169, 172). Most of the evidence points towards mRNA degradation being the major contributor to the silencing mediated by animal miRNAs, with only a minority of targets having steady mRNA levels but reduced protein levels. However, whether mRNA degradation is a consequence of an initial block in translation is still an open question (178).

1.2.5 Extracellular MicroRNA

Cell-to-cell communication is necessary for co-ordinating the responses of groups of cells in multicellular organisms and is integral for scores of biological processes, ranging from development to immune responses and angiogenesis. Intercellular communication that takes place without cell-to-cell contact is mediated by soluble messengers such as peptide hormones (e.g. cytokines, insulin, EGF), lipid or phospholipid-derived hormones (e.g. progesterone and prostaglandins) or small molecules (e.g. nitric oxide). The biological roles of these factors have been well studied and they continue to be explored. In recent years, miRNAs have emerged as new class of intercellular messengers but their roles outside of the cell are still poorly understood.

Small RNA was first detected in the conditioned medium of 3T3 cells 40 years ago (179). Furthermore, over 40 years ago it was demonstrated that RNA could be transferred between cells (180, 181). More recently, miRNA has been detected in plasma, serum, and other bodily fluids (182-187). However, the fact that free RNA is highly susceptible to degradation by widespread stable RNases suggests that it must be somehow protected when outside of cells. Also, RNA could be released via passive leakage from dead or dying cells, or actively secreted, implying a novel mode of communication.

Serum RNA from cancer patients and RNA from conditioned media of various malignant cell lines was found to be complexed with proteolipids, thereby suggesting a protective mechanism (188). With regards to extracellular miRNA, protection from RNases has been shown to occur by association with high-density lipoprotein (HDL) (189), Ago2 (190, 191), nucleophosmin-1 (NPM1) (192), or encapsulation in extracellular vesicles (193, 194) (Fig. 1.9A). Of these, HDL-associated and vesicle-encapsulated miRNAs were shown to be actively released by cells and taken up by recipient cells. Ago2-associated miRNAs are so far regarded as being by-products of dead cells and it is not yet known if other cells can take up NPM-1-associated miRNAs.
The umbrella term ‘microvesicle’ has been used in the literature to denote a sub-micron extracellular vesicle that is surrounded by a phospholipid bilayer. However, this term can refer to exosomes or shedding vesicles, which have distinct cellular origins and sizes. Exosomes are <100 nm in diameter, have an endocytic origin and are released by the fusion of multivesicular bodies (MVBs) with the plasma membrane (Fig. 1.9B) (195). Many cell types have been shown to produce exosomes but they are most well characterized in immune cells. By contrast, shedding vesicles are generally larger and up to 200 nm in diameter and are formed by outward budding off of the plasma membrane (196). The physico-chemical properties of exosomes and shedding vesicles are quite similar which means that isolates from cell culture supernatants or biological fluids prepared by centrifugation are likely to contain mixed populations of vesicles. In addition to miRNA, microvesicles contain numerous proteins in their lumen and membranes that vary between cell types. Exosome markers include the tetraspanin CD63 and chaperone HSP90, although characterization is far from complete (197). β1 integrin is marker of shedding vesicles from diverse sources and shedding vesicles derived from tumours and neutrophils are enriched with MMPs (196). Purification methods based on immunological techniques are likely to produce the most homogenous preparations of vesicles. The roles of exosomal proteins are also under investigation. For example, exosomes from various cancer cell lines can trigger fibroblast to myofibroblast differentiation through transforming growth factor-beta (TGF-β) being expressed on the exosome surface (198).

MicroRNA secretion and transfer by exosomes/exosome-like vesicles has been studied in the greatest depth. Valadi et al. were the first to demonstrate exosome-mediated transfer of miRNA between cells (193). They characterized exosomes from mouse and human mast cell lines and found that they contained functional mRNAs as well as selected miRNAs. Gibbings et al. found that GW182, Ago2 and miRNAs were associated with endosomes and exosome-like vesicles contained miRNAs and high levels of GW182 (199). By small RNA cloning and sequencing, this study found cellular and exosomal miRNA profiles were similar, which is in contrast to the Valadi study where some miRNAs were found to be more abundant in exosomes than cells. Pegtel et al. demonstrated that Epstein-Barr virus (EBV)-infected B cells secrete exosomes that transfer EBV miRNAs to non-infected cells (200). Furthermore, this group showed that target reporter gene expression in recipient cells could be repressed by exosomal miRNAs. Kosaka et al. also demonstrated transfer of miRNA by exosomes in cell lines and implicated the ceramide biosynthesis pathway in secretion (201). Treating cell lines with GW4869, an inhibitor of neutral
sphingomyelinase 2 (nSMase2) which is the rate-limiting enzyme of ceramide synthesis, or transfecting with siRNA against nSMase2 led to a reduction in miRNA secretion. A recent study examined miRNA transfer from vascular endothelial cells to vascular smooth muscle cells in the context of vascular sheer stress and atherosclerosis (202). This study found that extracellular vesicles from sheer stress exposed endothelial cells could induce an atheroprotective phenotype in smooth muscle cells and in mice that were fed a high-fat diet. 

To summarize, evidence for miRNAs acting in paracrine or endocrine manner is accumulating and careful characterization of the modes of transfer is still ongoing. MicroRNAs in serum and other biological fluids are currently being investigated for their potential as biomarkers for a range of pathologies (203). Vesicle-mediated transfer may be the most versatile transfer method as many proteins in the vesicle membrane may dictate targeting via interaction with specific cell surface receptors. Little is still known about how uptake of vesicles occurs and it is unclear if there is selection of particular miRNAs for export and, if so, how.
Figure 1.9 Extracellular MicroRNAs and Exosome production

(A) Forms of extracellular miRNA. miRNA can leak passively out of dying cells into the extracellular environment and maybe Ago associated. MicroRNAs that are secreted in a controlled manner may be enclosed in extracellular vesicles (i.e. exosomes or shedding vesicles) or associated with HDL or nucleophosmin1 (NPM1). HDL and vesicle-associated export has been shown to transfer miRNA to recipient cells.

(B) The origins of exosomes. Invagination of the membrane surrounding endocytic cisterna leads to the accumulation of internal vesicles. Thus multivesicular bodies (MVB) are produced and, as an alternative to lysosomal targeting, MVBs may fuse with the plasma membrane releasing exosomes into the extracellular milieu.

1.2.6 MicroRNAs and the Endometrium

With the endometrium being such a dynamic tissue and its significance in the origins of many reproductive disorders, research into the role of miRNAs in this tissue has been proceeding industriously. Numerous studies have focused on expression profiling during the cycle and in pathological conditions such as endometriosis and endometrial cancer.

Kuokkanen et al. profiled miRNA expression in endometrial epithelial cells isolated from late proliferative phase and mid-secretory phase biopsies. They found 24 miRNAs to be more abundantly expressed in mid-secretory samples and proposed that a proportion of these regulated genes involved in the cell cycle (204). Pan et al. compared miRNA expression in endometrial epithelial cells and stromal cells and found 32 miRNAs to be differentially expressed (205). This study also found changes in the expression of particular miRNAs upon treatment of cells with estradiol, MPA or ER and PR antagonists (ICI-182780 and RU-486, respectively), thus highlighting hormonal control of certain miRNA genes.

The Pan study also examined miRNA expression in endometriosis patients. This disease is characterised by the growth of endometrial tissue in regions of the body outside the uterine cavity. It occurs in 10-15% of women of reproductive age and its symptoms include chronic pelvic pain, irregular uterine bleeding and in some cases infertility. The ectopic lesions are thought to become established by the retrograde passage of endometrial fragments in the menstrual fluid through the fallopian tubes (206). The fragments then implant in the peritoneum, ovary or pelvic structures and are sensitive to estrogen and progesterone, much like eutopic tissue.

However, despite retrograde passage of endometrial fragments being quite common, only a proportion of women develop endometriosis. The factors thought to contribute to the establishment of endometriosis include genetic predisposition, aberrant immunological responses and environmental factors. Also, studies have shown that there are fundamental differences between eutopic tissues from women with and without endometriosis. For example, gene expression microarray studies have shown that there is an impaired proliferative to secretory transition in the endometrium of women with endometriosis (207). The functional genomic analysis by Burney et al. found a signature of enhanced cell survival, and high expression of genes involved in DNA synthesis and mitosis. Microarray-based profiling, next generation sequencing and real-time quantitative
PCR has also revealed differential expression of many miRNAs between normal and endometriosis samples and ectopic and eutopic endometrium (205, 208-212).

The functions of some miRNAs in endometriosis have been explored. MiR-20a has been found to be up-regulated by hypoxia in endometriotic stromal cells and it regulates the expression of dual specificity phosphatase 2 (DUSP2) (213). This resulted in greater phosphorylation of ERK and the up-regulation of many angiogenic genes. Angiogenesis is thought to be crucial for the establishment of ectopic lesions, and this study implicated miRNA-mediated regulation of important signalling factors for this process. The transcription factor homeobox A10 (HOXA10) is involved in uterine development and is also regulated during the menstrual cycle with expression crucial for embryo implantation. HOXA10 expression is decreased in the endometrium of women with endometriosis and Petracco et al. found that miR-135a and miR-135b target HOXA10, and the expression of these miRNAs is elevated in endometriosis (214). MiR-29c was one of several miRNAs up-regulated in endometriomas (endometriotic cysts of the ovary) (212). This miRNA was shown to target ECM genes such as collagen type VII Al (COL7A1).

Grechukhina et al. recently published an interesting study that tied in genetic predisposition to endometriosis with miRNA function (215). Women with and without endometriosis were screened for a single nucleotide polymorphism (SNP) in one of 10 known let-7 binding sites in the 3’ UTR of KRAS. In mice activation of this gene can cause spontaneous endometriotic lesions to develop (216). The study found that women with endometriosis were much more likely to have a SNP that disrupts let-7 binding and thus increased KRAS expression.

The study of miRNAs in cancer is proceeding rapidly and their roles in endometrial cancer, which is the most common gynaecological cancer, are being investigated too. Again expression profiling by several groups has revealed many miRNAs are dysregulated in endometrial cancer (217-221) and the functional consequences are now being uncovered (222). FOXO1A is a transcription factor and tumour suppressor gene that is down-regulated in endometrial cancer. Several miRNAs (including miR-9, miR-27, miR-96, miR-153, miR-182, miR-183 and miR-186) that are up-regulated in endometrial cancer have been shown to bind the 3’UTR of FOXO1A and mediate its repression (223). MiR-181a and miR-98 were found to be altered in the transition to cancer with effects on several target genes including PR (224). MiR-205, which several profiling studies reported being over-expressed in endometrial cancer, has now been shown to be a significant prognostic marker (225). Thus, data on miRNAs are filling in some of the gaps in knowledge.
regarding existing models of endometrial carcinogenesis and also opening up new possibilities.

With regard to decidualization of the endometrium, the role of miRNAs less clear. Genetically modified mice in which cre recombinase is expressed from the anti-Müllerian hormone receptor 2 (Amhr2) promoter have been crossed with Dicer^{Δ/Δ} mice. In the female offspring, Dicer was specifically ablated in the reproductive tract and mice were infertile (226). These conditional KO mice exhibited decreased ovulation rates, hypertrophic uterine horns and oviductal cysts that blocked the passage of embryos into the uterus for implantation. Intriguingly, despite having smaller uteri than wild type mice, mechanically induced decidualization in the conditional KO mice was found to be macroscopically and histologically normal. However, the authors did point out that Amhr2 is more weakly expressed in endometrial stromal cells than myometrial smooth muscle cells, and perhaps Dicer deletion in the endometrial stromal cells might not have been robust.

Additional work on the mouse uterus has shown differential miRNA expression in implantation sites, which are the sites of decidual transformation, verses inter-implantation sites (227). This mouse study found, miR-143, miR-298, miR-26a, miR-21 miR-20a and let-7 family members were increased in abundance at implantation sites and miR-290 and miR-292 were down-regulated. Another study found that treatment of ovariectomized mice with estradiol or progesterone led to changes in transcript and protein levels of key miRNA pathway components such as Dicer1, Drosha, Dgcr8 and Xpo5 in the uterus (228). The expression of Xpo5 and Dgcr8 increased with estradiol treatment, a change that was blocked by treatment with the ER antagonist ICI-182780. Dicer1 and Xpo5 expression levels were increased by progesterone treatment and these inductions were blocked by treatment with the PR antagonist RU-486. With regards to human decidualization and implantation, one study has found 13 miRNA to be differentially expressed the secretory endometrium of women who have repeated implantation failure during in vitro fertilisation (IVF) compared to fertile women (229).
Hypotheses & Aims

MicroRNA expression is altered in endometrial tissue during the menstrual cycle. Therefore, one hypothesis to be tested is that miRNAs are also differentially expressed in cultured endometrial stromal cells when differentiated with cAMP and progestin. Given decidualization of hESCs is associated with dramatic changes in cell cycle regulation, cytoskeletal organization, signal transduction, stress responses and metabolism, a further hypothesis to be tested is that the miRNA synthesis/effector pathway is also subject to regulation.

Having determined which miRNAs are differentially expressed, the regulation of the targets of some of these will be examined. Furthermore, the decrease in AR protein levels that has been observed by Cloke et al. (70) could be a result of transcriptional or post-transcriptional mechanisms. The contributions of miRNAs and ribonucleoproteins in AR regulation in hESCs have not been explored. Therefore, we hypothesize that miRNAs or RNP are involved in AR regulation during decidualization, which has consequences for AR function.

Finally, the highly secretory nature of decidualized hESCs prompted us to hypothesize that they are also able to secrete miRNAs into the extracellular milieu and other cell types in the endometrium can take up that secreted miRNAs. In summary, the aims of this thesis are as follows:

• To explore changes in miRNA pathway components in hESCs.
• To examine the regulation of potential targets of differentially expressed miRNAs.
• To examine the role of miRNAs and ribonucleoproteins (RNPs) in the regulation of AR.
• To examine the possibility of miRNA secretion and transfer by decidualized hESCs.
Chapter 2 - Materials & Methods
2.1 Materials

2.1.1 Antibodies

*Primary Antibodies*

AGO1 (raised in rat) (a kind gift from Dr. Nick Dibb)
AGO2 (raised in rat) (a kind gift from Dr. Nick Dibb)
Alix (raised in rabbit) (Cell Signaling 2171)
AR (raised in mouse) (Biogenix MU256-UCE)
Calnexin (raised in rabbit) (Cell Signaling 2679)
DICER (raised in rabbit) (Cell Signaling 3363)
DICER (raised in mouse) (Abcam ab14601)
DNMT3B (raised in rabbit) (Cell Signaling 2161)
Drosha (raised in rabbit) (Cell Signaling 3364)
GAPDH (raised in mouse) (Millipore MAB374)
HSP90 α/β (raised in rabbit) (Santa Cruz sc-7947)
P53 (raised in rabbit) (Santa Cruz sc-6243)
PCBP1 (raised in mouse) (Santa Cruz sc-16504)
TSG101 (raised in mouse) (Santa Cruz sc-7964)
α-tubulin (raised in rat) (Abcam ab6161)
β-actin (raised in mouse) (Abcam ab6276)

*Secondary Antibodies*

HRP conjugated Goat Anti-Rabbit IgG (Invitrogen 656120)
HRP conjugated Rabbit Anti-Goat (Dako P0449)
HRP conjugated Rabbit Anti-Rat (Invitrogen 619520)
HRP conjugated Goat Anti-Mouse (Invitrogen 626520)

2.1.2 Plasmids

pcDNA3.1 (+) (Invitrogen)
pGL3 basic (Promega)
pGL3 miR-29b2 4kb prom (created during the PhD)
pch110 (a kind gift from Dr. Brianna Cloke) (230)
TetGFP (a kind gift from Dr. Ariel Poliandri)
pCMV Luc miR-30 (P) (a kind gift from Prof. Bryan Cullen)(231)
pSuper miR-30 (a kind gift from Prof. Bryan Cullen) (232)
pSuperior.puro (Oligoengine)
pSuperior.puro cel-miR-39 (created during the PhD)
p3XFLAG-AGO2 (a kind gift from Dr. Nick Dibb)
pSG5-PCBP1 (a kind gift from Dr. Brianna Cloke) (233)
pSG5-AR (a kind gift from Dr. Brianna Cloke)
PLightswitch AR 3’ UTR (purchased from Switchgear genomics)
pcDNA3.1 Luc AR-3'UTR (created during the PhD)
pcDNA3.1 Luc AR-3'UTR AUC-rich (created during the PhD)

2.1.3 siRNA, anti-miRs and synthetic miRNA

siRNAs
siGENOME non-targeting siRNA pool (Dharmacon D-001206-13)
Non-targeting siRNA (NBS Biologicals B01001)
human DICER siGENOME SMARTpool (Dharmacon M-003483-00)
human PCBP1 siGENOME SMARTpool (Dharmacon M-012243-01)

Anti-miRs
Anti-miR Negative control (MSY0002505)
Anti-miR-100 (Qiagen MIN0000098)
Anti-miR-29b (Qiagen MIN0000100)
Anti-miR-29c (Qiagen MIN0000681)

Synthetic miRNA
Synthetic cel-miR-39 (Qiagen MSY0000010)
2.1.4 Bacterial Strains and Media

*Lysogeny Broth (LB)*
1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) glucose

*LB-agar*
1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) glucose, 1.5% (w/v) bactoagar

*Super Optimal Broth with Catabolite Repression (S.O.C. Medium) (Invitrogen)*
2% w/v bactotryptone, 0.5 % w/v yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM glucose

*Transformation and Storage Solution (TSS)*
LB-broth containing: 10% w/v polyethylene glycol, 10% v/v DMSO, 50 mM MgCl₂

*DH5a Escherichia coli*
F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phosphoA supE44 λ– thi-1 gyrA96 relA1

*One Shot TOP10 Chemically Competent E. coli* (Invitrogen)
F’ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 lacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 supG λ-

2.1.5 Cell Culture Reagents

0.05% Trypsin/ethylenediamine tetraacetic acid (EDTA) (Invitrogen)
100x Antibiotic-antimycotic (Invitrogen)
100x L-glutamine (Invitrogen)
2% Gelatine (Sigma)
Collagenase type IA (Sigma)
Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (1:1) 1X Nutrient mix with HEPES and L-glutamine and phenol red (Invitrogen)
DMEM/F12 (1:1) 1X Nutrient mix with HEPES and L-glutamine without phenol red (Invitrogen)
Dimethyl sulfoxide (DMSO) (Sigma)
DNase I (Roche)
Endothelial cell Growth Supplement (Sigma)
Ham’s F12 1X Nutrient mix (Invitrogen)
Heat Inactivated Fetal Bovine Serum (Invitrogen)
Heparin from porcine mucosa (Sigma)
Insulin from bovine pancreas (Sigma)
Opti-MEM Medium (Invitrogen)
Plasticware and cell culture plates/flasks (Corning)
Roswell Park Memorial Institute (RPMI)1640 Medium (Invitrogen)
Sterile disposable scalpels (Swann-Morton)

2.1.6 Media Formulations

10% DCC DMEM/F12
DMEM/F12 with phenol red supplemented with 10% v/v DCC-FBS, 1x L-glutamine, 1x antibiotic-antimycotic, 1nM estradiol, 2ug/ml insulin

5% DCC DMEM/F12
DMEM/F12 with HEPES without phenol red supplemented with 5% v/v DCC-FBS, 1x L-glutamine, 1x antibiotic-antimycotic

2% DCC DMEM/F12
DMEM/F12 with HEPES without phenol red supplemented with 2% v/v DCC-FBS, 1x L-glutamine, 1x antibiotic-antimycotic

HUVEC Medium
Ham’s F12 media with phenol red supplemented with 10% v/v FBS, 0.75 mg/ml sodium bicarbonate, 1x L-glutamine, 1x antibiotic-antimycotic, 0.1 mg/ml heparin, 30 mg/ml EGCS
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*BeWo/Ishikawa Medium*

DMEM/F12 with phenol red supplemented with 10% v/v FBS, 1x L-glutamine and 1x antibiotic-antimycotic

*LNCaP Medium*

RPMI 1640 supplemented with 10% v/v FBS, 1x L-glutamine with or without antibiotic-antimycotic

2.1.7 Chemicals and Reagents

3M Sodium Acetate (Ambion)

20x Saline-sodium citrate (Invitrogen)

10mM dNTP mix (Invitrogen)

10x MMLV buffer (Sigma)

100x ROX reference dye

17α-Medroxyprogesterone acetate (Sigma)

17β-estradiol (Sigma)

5X Reporter lysis buffer (Promega)

8-Bromo-cAMP (Sigma)

Agarose, molecular biology grade (Roche)

Ammonium persulphate (APS) (Sigma)

Ampicillin (Sigma)

Beta-mercaptoethanol (Sigma)

Bovine serum albumin (BSA) (Sigma)

Bromophenol blue (Sigma)

Chloroform (Sigma)

Complete EDTA-free protease inhibitors tablets (Roche)

Cycloheximide (Calbiochem)

Dharmafect 4 (Dharmacon)

Diethylpyrocarbonate (DEPC)-treated water (Ambion)

Dihydrotestosterone (DHT) (Sigma)

Dimethyl sulfoxide (DMSO) (Sigma)

Dithiothreitol (Sigma)

Ethanol (BDH)
Ethidium Bromide Solution (Invitrogen)
Ethylenediamine tetraacetic acid (EDTA) (Sigma)
Glycerol (BDH)
Glycine (Sigma)
HEPES (Sigma)
Imidazole (Sigma)
JumpStart SYBR Green I Mix (Sigma)
Luciferase Assay Reagent (Promega)
MG132 (Calbiochem)
MMLV Reverse Transcriptase (Sigma)
N,N,N’,N’-tetraethylmethylethane-1,2-diamine (TEMED) (Sigma)
Nonidet P-40 (NP-40) (BDH)
Phenol:Chloroform:isoamyl alcohol 24:25:1 (Sigma)
Phenylmethanesulfonyl fluoride (PMSF) (Sigma)
Polyethylene glycol (PEG) (Sigma)
Potassium Chloride (Sigma)
Propan-2-ol (BDH)
Protogel 30% acrylamide (37.5:1 acrylamide to bisacrylamide) (National Diagnostics)
Random hexamers (Invitrogen)
Restriction endonucleases and buffers (New England Biolabs)
RNAse OUT Ribonuclease Inhibitor (Invitrogen)
Non-fat milk powder (Sigma)
Sodium azide (Sigma)
Sodium chloride (Sigma)
Sodium deoxycholate (BDH)
Sodium dodecylsulphate (SDS) (BDH)
TRI reagent (Sigma)
TRI reagent LS (Sigma)
Trishydroxymethylaminomethane (Tris) (Sigma)
Triton X-100 (Sigma)
Tween-20 (Sigma)
2.1.8 Buffers and Solutions

Phosphate Buffered Saline (PBS)
140 mM NaCl, 2.5 mM KCl, 1.5 mM KH$_2$PO$_4$, pH 7.2, 10 mM Na$_2$HPO$_4$, pH 7.2

Tris Buffered Saline (TBS)
130 mM NaCl, 20 mM Tris, pH 7.6

Tris Buffered Saline-Tween 20 (TBS-T)
0.1 % v/v Tween-20 in TBS

Tris Borate EDTA (TBE)
90 mM Tris Borate, 2 mM EDTA pH8.0

10X DNA loading buffer
0.2% (w/v) Bromophenol Blue, 40% (v/v) Glycerol, 100mM EDTA pH8.0

Radio-immunoprecipitation buffer (RIPA buffer)
150 mM NaCl, 50 mM Tris pH 8.0, 0.1% v/v SDS, 0.5% v/v sodium deoxycholate
1% v/v NP-40, 5 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail

High Salt Buffer
0.4 M KCl, 20 mM HEPES pH 7.4, 20% v/v glycerol, 1 mM DTT, 1 mM PMSF
1x protease inhibitor cocktail

2x Lammeli gel loading buffer
50 mM Tris-HCl, pH 6.8, 50 mM imidazole, pH 6.8, 1 % w/v SDS, 10 % v/v glycerol
2 % v/v 2-mercaptoethanol, 0.002 % w/v bromophenol blue

SDS Running Buffer
25 mM Tris, 190 mM glycine, 0.1 % w/v SDS

Transfer Buffer
25 mM Tris-base, 192 mM Glycine, 20% v/v methanol
**Western Blocking solution**
5% w/v non-fat milk powder in TBS-T

**Antibody Dilution Buffers**
5% w/v BSA in TBS-T with 0.1% v/v NaN₃
5% w/v non-fat milk powder in TBS-T with 0.1% v/v NaN₃

**Plasmid prep suspension buffer P1 (Qiagen)**
50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A

**Plasmid prep lysis buffer P2 (Qiagen)**
200 mM NaOH, 1 % w/v SDS

**Plasmid prep neutralization buffer P3 (Qiagen)**
3 M CH₃COOK, pH 5.5

**Plasmid prep equilibration buffer QBT (Qiagen)**
750 mM NaCl, 50 mM MOPS, pH 7.0, 15 % v/v isopropanol, 0.15% v/v Triton X-100

**Plasmid prep wash buffer QC (Qiagen)**
1 M NaCl, 50 mM MOPS, pH 7.0, 15 % v/v isopropanol

**Plasmid prep elution buffer QF (Qiagen)**
1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15 % v/v isopropanol

**Plasmid prep elution buffer EB (Qiagen)**
10 mM Tris-HCl, pH 8.5

**Endotoxin free TE Buffer (Qiagen)**
10 mM Tris, pH 8.0, 1 mM EDTA

**NE buffer 1 (New England Biolabs)**
10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1 mM DTT
NE buffer 2 (New England Biolabs)
10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT

NE buffer 3 (New England Biolabs)
50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT

NE buffer 4 (New England Biolabs)
20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT

Annealing Buffer
200 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA

5x T4 Ligase Buffer (Invitrogen)
250 mM Tris HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) Polyethylene Glycol-8000

10x DNase I Reaction buffer (Sigma)
200 mM Tris-HCl, pH 8.3, 20 mM MgCl₂

DNase Stop solution (Sigma)
50 mM EDTA

MMLV 10x reaction buffer (Sigma)
500 mM Tris-HCl, pH 8.3, with 500 mM KCl, 30 mM MgCl₂, 50 mM DTT

10x Hotstart plus Taq PCR reaction buffer (Qiagen)
10x concentrated. Contains Tris·Cl, KCl, (NH₄)₂SO₄, 15 mM MgCl₂ pH 8.7

Array Wash Solution 1
2x Saline Sodium Citrate (SSC) (0.3M NaCl, 30mM trisodium citrate pH 7.0)
0.2% v/v SDS

Array Wash Solution 2
2x SSC

Array Wash Solution 3
0.2x SSC
2.1.9 SDS gels

*Resolving gels*

375 mM Tris pH 8.9
0.1% v/v SDS
7.5%, 10%, 12% or 14% v/v Acrylamide/Bisacrylamide
0.03% v/v ammonium persulphate (APS)
0.06% v/v TEMED

*Stacking gels*

80 mM Tris pH 6.8
80 mM Imidazole pH 6.8
0.1% v/v SDS
4.1% v/v Acrylamide/Bisacrylamide
0.09% v/v APS
0.09 % v/v TEMED

2.1.10 Kits

Profection Mammalian cell Transfection kit (Promega)
HiSpeed Maxiprep kit (Qiagen)
QIAprep Spin Miniprep kit (Qiagen)
Galacto-Light Plus β-galactosidase reporter gene assay kit (Applied Biosystems)
Amplification grade DNase I kit (Sigma)
First Choice RLM-RACE kit (Ambion)
KOD DNA polymerase (Novagen)
Luciferase assay kit (Promega)
Accuprime Taq polymerase (Invitrogen)
TOPO TA Cloning Kit with pCR4 TOPO vector (Invitrogen)
HotStart Plus Taq DNA polymerase (Qiagen)
REDTaq DNA polymerase (Sigma)
T4 DNA ligase (Invitrogen)
Quick Ligase (New England Biolabs)
GenElute DNA Gel Extraction kit (Sigma)
GeneJET PCR purification kit (Fermentas)
Fluorescent DNA quantitation kit (Bio-Rad)
Platinum Taq Supermix (Invitrogen)
PureLink miRNA isolation kit (Invitrogen)
NCode miRNA Labelling kit (Invitrogen)
MEGAscript T7 in vitro transcription kit (Ambion)
Retic Lysate IVT kit (Ambion)

**2.1.11 Primer sequences**

QPCR primers for protein coding genes and U6 snRNA

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<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>DICER1</td>
<td>TTGCTTGGAATGGAACCAGAAA</td>
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<td>EIF2C1 (AGO1)</td>
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<td>EIF2C2 (AGO2)</td>
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<td>PRL</td>
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<td>U6 snRNA</td>
<td>CTCGCTTTCGCACGAAAA</td>
<td>AACGCTTACGAATTTGGG</td>
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**Pri-miRNA qPCR primers**

<table>
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<th>Pri-miRNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Pri-miR-29b-1</td>
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### Chapter 2

#### Materials & Methods

#### Mature miRNA RT and qPCR primers

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<th>Mature miRNA</th>
<th>Stem loop RT primer</th>
<th>Forward PCR primer</th>
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<td>TGGTACAGTACAGACATCTG</td>
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#### 2.1.12 Miscellaneous

GlycoBlue (Ambion)

PageRuler PreStained Protein Ladder (Fermentas)

Hyperladder I and V DNA ladders (Bioline)

Immobilon P PVDF membrane (Millipore)

RNase ZAP (Ambion)

Super RX Autoradiography Film (Fujifilm)

96 well qPCR plates ABI compatible Low profile (Appleton Woods)

Optically clear adhesive seals (Appleton Woods)

Quick-Seal Ultracentrifugation tubes (Beckman Coulter)
2.2 Methods

2.2.1 Bacterial Propagation of Plasmids and Plasmid Isolation

*Preparation of chemically competent DH5α E. coli*

DH5α *E. coli* was streaked out close to a flame onto an LB agar plate containing no antibiotics. Following overnight incubation at 37°C, a single colony was picked and inoculated into 1 ml of LB broth without antibiotic. This was cultured at 37°C for 3 h and then stored at 4°C. The following day 100 ml of LB without antibiotics in a 500 ml conical flask was inoculated with the 1 ml starter culture. This was allowed to grow at 37°C with shaking until the optical density at 600 nm (OD$_{600}$) reached 0.3. OD$_{600}$ was determined on a photometer and using fresh LB broth as a blank. Upon reaching the required OD, the flask was immediately placed on ice for 30 min to stop bacterial growth. Bacteria were then pelleted by centrifugation at 3900 rpm for 10 min at 4°C. Supernatant was removed and the pellet was resuspended 10 ml of ice-cold TSS. Bacteria were then aliquoted into Eppendorf tubes kept on ice and tubes were flash frozen in liquid nitrogen. Competent bacteria were stored at -80°C.

*Transformation of chemically competent DH5α E. coli*

To prepare large quantities of plasmid DNA, chemically competent DH5α *E. coli* was first transformed with the relevant plasmid. A vial of bacteria was thawed on ice and in an Eppendorf tube 50 µl of bacteria was added to 1 µl of plasmid DNA. The tube was flicked to mix and incubated on ice for 30 min. This was followed by a heat shock where the tube was immersed in a 42°C water bath for 30 seconds and then immediately placed on ice again. After 2 min on ice, 250 µl of SOC medium was added to the bacteria and then the bacteria were incubated at 37°C for 1 h. The culture was spread at a high density and low density on LB agar plates containing the appropriate antibiotic to select for transformants. Plates were incubated at 37°C overnight and the following day colonies were picked.

*Transformation of electrocompetent DH5α E. coli*

For cloning, ligated DNA was used to transform electrocompetent *E. coli* by electroporation. Dr. David Barneda Ciurana prepared these bacteria. These are more competent than the chemically competent bacteria, which increased the likelihood of propagating rare vector-insert ligations. A vial of bacteria was thawed on ice and in an Eppendorf tube, 50 µl of bacteria was added to 1 µl of ligation reaction and mixed gently.
The bacteria were incubated on ice for 30 min and then pipetted into a chilled electroporation cuvette. Electroporation was carried out with an Eppendorf 2510 electroporator at 1670 V. The bacteria were mixed with 250 µl of SOC medium, incubated at 37°C for 1h and then spread on LB-agar plates containing the appropriate antibiotic to select for transformants. Plates were incubated at 37°C overnight and the following day colonies were picked.

Small-scale plasmid DNA preparation - Miniprep

To test for positive clones by sequencing or diagnostic digestion, plasmid DNA was prepared from small-scale bacterial cultures. This involved inoculating 5 ml of LB containing selective antibiotic with a single colony and allowing the bacteria to grow in a shaking incubator at 37°C overnight. Following overnight culture, bacteria were pelleted by centrifugation for 15 min, at 3900 rpm at 4 °C. Supernatants were poured off and bacterial pellets were stored at -20°C until needed. QIAprep Spin MiniPrep Kit was used to extract up to 20 µg of high-copy plasmid DNA. The protocol involved lysis of bacteria (suspended in buffer P1) under alkaline conditions (buffer P2) followed by lysate neutralization and adjustment to high-salt binding conditions in one step (buffer N3). After clearing the precipitated genomic DNA and cellular debris by centrifugation, the lysates were passed through a silica membrane. The high-salt conditions ensured that only DNA was adsorbed, while RNA, cellular proteins, and metabolites flow through. To ensure plasmid DNA was not degraded, endonucleases were removed by a wash step with a buffer containing chaotropic salts (buffer PB). The membrane was then desalted (buffer PE) to allow elution of plasmid DNA with 50 µl buffer EB.

Large-scale plasmid DNA preparation – Maxiprep

The Qiagen HiSpeed Maxi Kit was used to obtain large quantities of plasmid DNA that were suitable for mammalian cell transfection. A single colony was picked and 5 ml of LB broth containing ampicillin at 50 µg/ml was inoculated and allowed to grow at 37°C for approximately 8 h with continuous shaking. This was then used to inoculate 200 ml of LB broth with ampicillin, which was incubated overnight at 37°C with continuous shaking. The large culture was then centrifuged at 3900 rpm for 15 min at 4°C to pellet the bacteria. Supernatants were removed and pellets were stored at -80°C until DNA extraction. The protocol is based on a modified alkaline lysis procedure in which bacterial cells (suspended in buffer P1) were lysed in sodium hydroxide/SDS in the presence of RNase A (buffer P2). SDS solubilised the phospholipid and protein components of the cell membrane, leading to
cell lysis. Sodium hydroxide denatured chromosomal DNA, plasmid DNA and proteins, while RNase A digested the liberated RNA. The lysate was neutralized by the addition of acidic potassium acetate (buffer P3). The high salt condition caused SDS to precipitate with the denatured proteins, chromosomal DNA and cellular debris, trapped in salt-detergent complexes. The smaller, covalently closed plasmid DNA re-natured and remained in solution. The lysates were passed through a pre-equilibrated (buffer QBT) anion-exchange resin operating by gravity flow under low salt and low pH conditions to trap the plasmid DNA, RNA, protein, dyes and low-molecular weight impurities were removed by a medium salt wash (buffer QC). Plasmid DNA was eluted in a high salt buffer (buffer QF) and then concentrated and desalted by isopropanol precipitation. The purified DNA was washed with 70 % v/v ethanol, air-dried and redissolved in TE buffer.

Polyethylene glycol DNA precipitation

Plasmid DNA prepared by maxiprep was precipitated with polyethylene glycol (PEG) and sodium chloride prior to use in transfection. This precipitation removes non-supercoiled plasmid DNA, which would otherwise reduce transfection efficiency. To one volume of DNA solution 0.6 volumes of 20% w/v PEG 2.5 M NaCl solution was added and mixed. The DNA was then left on ice for 1 h to precipitate and then centrifuged at 12,000 g at 4°C for 25 min to pellet the DNA. The supernatant was removed and the DNA was washed two times in ice-cold 70% v/v ethanol. The pellet was air dried for 5 min and then redissolved in a suitable volume of TE buffer before storing at 4°C. DNA concentration and purity was measured with the Nanodrop ND1000 spectrophotometer. An A260/A280 ratio close to 1.8 indicated that DNA was free of protein and phenol, which absorb light strongly at 280 nm.

Preparation of Glycerol Stocks

 Cultures of bacteria that had been transformed with the relevant plasmid were stored at -80°C to provide stocks that could be readily propagated when needed. This involved mixing 500 µl of culture with 500 µl of a sterile solution of 40% v/v glycerol in LB broth. The mixture was then placed in a cryovial and snap frozen in a dry ice-ethanol bath. Cryovials were then transferred to the -80°C freezer.
2.2.2 DNA manipulation and Molecular Cloning

*Agarose Gel Electrophoresis*

Molecular biology grade agarose was weighed out and mixed with 1x TBE in a conical flask to give the appropriate percentage w/v concentration. Agarose was melted in a microwave for 2 min on a medium setting and allowed to cool to 50°C. Ethidium bromide solution (10 mg/ml) was added to give a final concentration of 0.5 µg/ml. Gels were cast in appropriate trays with combs to produce wells. Cooled gels were then placed in electrophoresis tanks and 1x TBE was added to cover the gel and electrodes. DNA samples to be run were mixed with 10x gel loading buffer and loaded into wells along with a suitable DNA ladder. A current was applied across the gel at a constant voltage of 10V/cm of the gel. Gels were imaged under ultraviolet light.

*Restriction Endonuclease Digestion*

Plasmid DNA was digested at 37°C using the relevant restriction enzymes and 10X reaction buffer diluted to 1X. Guidelines recommended by New England Biolabs were followed. Where BSA was necessary, a 100x stock was diluted 1:100 in the reaction mix. Digestion was generally carried out for 1-2 h.

*PCR for cloning*

The Accuprime Taq DNA polymerase High Fidelity kit was used to amplify miR-29b-2 promoter region from human genomic DNA. This kit contains an enzyme mixture composed of recombinant Taq DNA polymerase, Pyrococcus species GB-D polymerase, and Platinum Taq Antibody. Taq DNA polymerase has a nontemplate-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3’ ends of PCR products, which makes them suitable for TOPO cloning. The *Pyrococcus* species GB-D polymerase is proofreading and has a 3’ to 5’ exonuclease activity, so PCR products are of high fidelity. In 0.2 ml PCR tubes, 50 ng of human genomic DNA was mixed with 5 µl of 10x Buffer II (specified for genomic DNA templates), 1 µl each of forward and reverse primers (at final concentrations of 200 nM), 0.2 µl of enzyme mix and water was added to a final volume of 50 µl. Non-template control reactions containing water instead of human genomic DNA were also set up. PCRs were run with the following conditions: 94°C for 1 min followed by 35 cycles of 20 s at 94°C, 20 s at 55°C and 1 min per kilobase at 68°C.

KOD DNA polymerase was used to produced PCR products containing a luciferase cassette coupled to a full length AR 3’UTR or one with a deletion of the UC-rich region.
KOD DNA polymerase is a high fidelity enzyme derived from *Thermococcus kodakaraensis*. The template used was a commercially available construct (pLightswitch AR 3’UTR) purchased from Switchgear genomics which contained 634 bp of the AR 3’UTR cloned downstream of luciferase. PCR reactions had a total volume of 25 µl, containing 2.5 µl of 10x Reaction buffer, dNTPs (at 200 nM final concentration each), 1.5 mM MgCl₂, forward and reverse primers at 400 nM final concentrations and 0.2 µl of KOD enzyme. Reactions were run at 98°C for 15 s, 45°C for 5 s and 72°C for 30 s for 30 cycles.

**TOPO cloning**

To enable efficient digestion of PCR products produced by Accuprime Taq polymerase, they were first cloned into the pCR4 TOPO vector using the TOPO TA cloning kit. This method relies on the Taq-generated PCR product having single 3’ deoxyadenosine (A), which can base pair with single 3’ deoxythymidine (T) residues in the linearised TOPO vector. Ligation of PCR product and vector occurs when the associated topoisomerase I is released from the vector. PCR products produced with KOD DNA polymerase were cloned into the pcDNA 3.1 directional TOPO vector. For these products the forward primer contained an extension to enable directional TOPO cloning.

In an Eppendorf tube, 0.5 µl of PCR product was mixed with 0.5 µl of TOPO vector and 2 µl of water. This mixture was incubated at room temperature for 5 min for ligation to occur and then placed on ice. One Shot TOP10 chemically competent *E. coli* were then transformed with the ligation. 1 µl of ligation was mixed gently with 50µl of TOP10 cells on ice. The bacteria were left on ice for 30 min before heat-shock at 42°C for 30 s. Bacteria were placed on ice for 2 min and then 250 µl of SOC medium was added close to an open flame. Bacteria were allowed to recover at 37°C for 1h and then spread onto LB agar plates containing ampicillin at 100 µg/ml. Plates were incubated at 37°C overnight. Plasmid DNA was extracted from transformants by miniprep and diagnostic digests carried out with the appropriate restriction enzymes to identify positive clones.

**Gel extraction of DNA**

To extract DNA from agarose gels the GenElute DNA Gel Extraction kit was used. DNA bands of the desired molecular weight were cut out from the gel with a scalpel whilst visualizing under UV light. The gel slice was weighed and 3 volumes of gel solubilization solution were added (e.g. for a 200 mg gel, slice 600 µl of solution was added). Incubating at 50°C for 10 min with occasional mixing dissolved the gel. Meanwhile, the Binding
Column was prepared by adding 500 µl of Column Preparation Solution and centrifuging at 13,000 rpm for 1 min and discarding the flow-through. To the dissolved gel, one gel volume of 100% isopropanol was added and mixed. The solubilized gel was then added to the prepared Binding Column and centrifuged at 13,000 rpm for 1 min at room temperature. DNA fragments adsorb onto the silica membrane, which was then washed with 700µl of Wash Solution. Residual Wash Solution was removed by a further centrifugation for 1 min and then DNA was eluted by adding 30-50µl of Elution Buffer to the membrane, allowing it to stand for 1 min and then centrifuging at 13,000 rpm for 1 min to collect the eluate in a tube. Following gel extraction, a small volume of eluate was run on an agarose gel to check that DNA had been recovered.

*T4 DNA ligase*

Ligation of insert and vector was carried out with T4 DNA ligase. 2 µl of vector and insert were first electrophoresed on an agarose gel to assess their relative concentrations. The optimal ratio of insert to vector in the ligation reaction was 3:1. Vector was mixed with insert at this approximate ratio and 5X T4 ligase buffer was added. To this, 1 µl of T4 DNA ligase was added and made up to 20µl final volume with water. Vector only ligation reactions were also set up to assess vector only recircularization. Ligations with T4 DNA ligase were incubated at 16°C overnight.

*Oligonucleotide annealing*

10µl of each complementary oligonucleotide (at a concentration of 1µg/µl) was mixed with 80 µl of annealing buffer. This was boiled for 15 min and then placed in a water bath at 70°C for 10 min. The water bath was then turned off and the oligonucleotides were allowed to cool to room temperature. Annealed oligonucleotides were stored at -20°C until needed.

*T4 Polynucleotide kinase (PNK) treatment*

Annealed oligonucleotides were 5’ phosphorylated with T4 polynucleotide kinase to enable ligation into dephosphorylated cut vector. Seven microlitres of annealed oligonucleotides were mixed with 5µl of 10X NEB ligase buffer (containing 10mM ATP), 2.5µl of T4 PNK and 35.5 µl of DEPC-treated water. This was incubated at 37°C for 30 min and then at 70°C for 15 min to inactivate the kinase.
**Phenol-Chloroform extraction of DNA**

This method was used to purify DNA from proteins and other components of enzymatic reactions. The volume of the DNA was adjusted to 150µl with water and then an equal volume of phenol:chloroform:isoamyl alcohol was added and vortexed to mix. This mixture was then centrifuged at 13,000 rpm for 5 min at room temperature. The upper aqueous layer was transferred to a new tube. This was mixed with an equal volume of chloroform and centrifugation was repeated. The resultant aqueous layer, containing DNA, was removed to a new tube and the volume noted. The addition of 0.1 volume of 3M sodium acetate and 2 volumes of absolute ethanol precipitated DNA. The mixture was incubated on ice for 30 min before centrifuging at 13,000 rpm at 4°C for 25 min to pellet DNA. The DNA was washed twice with cold 70% v/v ethanol, air-dried and redissolved in Elution Buffer or DEPC-treated water.

**Bacterial Alkaline Phosphatase treatment**

This treatment removed 5’ phosphate moieties from DNA. It was carried out on digested plasmid to reduce vector recircularization upon ligation that would otherwise increase cloning background (vector without any insert ligated). Ten microlitres of cut vector was mixed with 4 µl of 10X dephosphorylation buffer, 0.5 µl of bacterial alkaline phosphatase (BAP) and DEPC-treated water to a final volume of 40 µl. This mixture was incubated for 40 min at 65°C. BAP treatment was stopped by phenol:chloroform extraction of DNA and DNA was redissolved in 6 µl of water.

**Quick Ligase**

Quick Ligase from New England Biolabs was used to ligate 5’phosphorylated annealed oligonucleotides to BAP-treated vector. 1µl of dephosphorylated vector was mixed with 2µl of oligonucleotides, 10µl of 2x Quick ligase buffer, 7µl of water and 1µl of Quick ligase. Vector to insert ratio was approximately 1:3. The mixture was incubated at room temperature for 5 min before being placed on ice. A vector only ligation was also carried out to assess vector only recircularization.

**DNA sequencing**

The Core Genomics Facility in the Commonwealth Building at Hammersmith Hospital performed DNA sequencing. Plasmid DNA or PCR product was mixed with water and 3.2 pmol of sequencing primer in a total volume of 10 µl and then delivered to the Core Genomics Facility. Chromatograms were observed with the 4Peaks software.
2.2.3 5’ Rapid Amplification of cDNA ends (5’ RACE)

To identify the transcription start sites of genes we employed a technique that amplifies the very 5’ ends of cDNA molecules. The amplified ends can then be sequenced and the transcription start site mapped on the chromosome. RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) is designed to amplify cDNA only from full length, capped mRNA. For 5’RACE Ambion First Choice RLM RACE kit was used according to the manufacturer’s instructions. Briefly, 10 µg of total RNA from decidualized hESCs was treated with calf intestinal phosphatase (CIP) to remove 5’ PO₄ from degraded mRNA, rRNA and tRNA. 7-methyl guanosine capped mRNAs are unaffected by CIP. CIP treated RNA was then phenol:chloroform extracted and treated with tobacco acid pyrophosphatase (TAP) which removes the 5’ cap structure from full length mRNA. This was followed by ligation of a 5’ RACE adapter to CIP/TAP treated RNA using T4 RNA ligase. RNA was then reverse transcribed to cDNA using random hexamers and MMLV reverse transcriptase.

The 5’RACE cDNA was then used as a template in nested PCR reactions using reverse primers specific to the gene of interest and forward primers specific for the 5’ RACE adaptor sequence. An outer PCR was first conducted with the 5’ outer RACE primer, outer gene specific primer and HotstarTaq Plus polymerase. A small amount of this PCR was then used as a template in the inner PCR reaction again using the HotstartTaq plus polymerase, a 5’ inner RACE primer and a gene specific primer. Inner 5’RACE products were run on agarose gels to estimate their size and then extracted for DNA sequencing.

2.2.4 Endometrial Biopsies

Endometrial biopsies were obtained from normally cycling women aged 24-40 years awaiting in vitro fertilization treatment, for either tubal or male-factor infertility, at the time of diagnostic laparoscopy and hysteroscopy. All women had regular menstrual cycles and were not on hormonal treatment at the time of biopsy. Where the tissue was used directly for experiments, samples were classed as ‘proliferative' or 'secretory' depending on the stage of the cycle at which the biopsy was taken. For establishment of primary cultures, biopsies from any phase of the cycle were used. All patients gave informed written consent before collection of samples and the Ethics and Research
Committee of Hammersmith and Queen Charlotte’s Hospital NHS Trust approved the study.

2.2.5 Mammalian Cell Culture

*Dextran coated-charcoal stripped serum preparation*

Fetal bovine serum contains steroid hormones, which could mask the effects of exogenously added hormones. Therefore to remove steroid hormones, FBS was treated with dextran coated-charcoal. To 500 ml of FBS, 1.25 g of activated charcoal and 125 mg of dextran was added and mixed. The mixture was incubated in a 56°C water bath for at least two hours with occasional inversion to mix. Charcoal was pelleted by centrifugation at 3000 rpm for 30 min and the supernatant was then filtered through a 0.22 µm bottle-top filter into a sterile bottle. Serum was then aliquoted and stored at -20 °C. DCC-FBS was used at concentrations of 10%, 5% or 2% v/v in media preparations.

*Endometrial biopsy processing and primary cell culture*

To establish cultures of primary hESCs, endometrial biopsies were finely chopped in a Petri dish with scalpels before being placed in a 25 cm² flask with 10 ml of digestion media. Digestion media consisted of serum-free DMEM/F12 with 1x antibiotic-antimycotic, 0.5 mg/ml collagenase type 1A, and 0.1mg/ml DNase I to remove viscous DNA released by some cells that die. Digestion was carried out for 1 h in a 37°C 5% CO₂ incubator with occasional shaking of the mixture. To stop digestion, 10 ml of pre-warmed 10% DCC DMEM/F12 media was added. The cell suspension was then transferred to a 50 ml centrifuge tube and centrifuged at 180g for 5 min. Following centrifugation, the supernatant was aspirated away and the pellet, containing blood cells, stromal and epithelial cells, was resuspended in 12 ml of 10% DCC DMEM/F12 and plated in a 75 cm² flask. If the pellet was large, the cells would be plated in two 75 cm² flasks. Following 1 h of incubation at 37°C, the media, containing mostly blood and epithelial cells would be plated in a fresh flask and the initial flask, containing mostly stromal cells, would be washed and 10% DCC DMEM/F12 media would be replenished. 10% DCC DMEM/F12 would be replenished every other day.

hESCs were passaged when they reached confluency. Cells in 75 cm² flasks were washed with 10ml of pre-warmed PBS and then 2 ml of 0.05% trypsin/EDTA was added and incubated 37 °C for 3-4 min. Flasks were knocked to dislodge cells. To stop trypsin activity, 8 ml of warm 10% DCC DMEM/F12 was added and the cell suspension was pipetted up and down to disperse clumps of cells. The cells were then collected by
centrifugation at 180g for 5 min and the supernatant was aspirated away. The cells were then resuspended in 10% DCC DMEM/F12 and plated in the formats desired. Cells were used for experiments up to the third passage.

**Hormone treatments**

To decidualize hESCs *in vitro* they were treated with 0.5 mM 8-Bromo-cAMP and 1 µM medroxyprogesterone acetate (MPA). Treatments were added to 2% DCC DMEM/F12 or, where necessary, to serum-free DMEM/F12. Vehicle treatments consisted of a volume of ethanol equivalent to the MPA volume. Where used, dihydrotestosterone (DHT) was added to cultures at a final concentration of 0.1 µM.

**Cell Line Culture**

All cell lines were cultured in 37°C incubators with 5% CO₂. Cell lines were passaged when they reached confluency in a similar way to methods described for hESCs. Cell lines were also prepared for storage in liquid nitrogen. This involved collecting cells from 75 cm² flasks by trypsinisation and centrifugation as above and then resuspending cell pellets in media containing 10% v/v DMSO (freezing medium). 1 ml aliquots of cells in cryogenic vials were placed in Mr. Frosty isopropanol-containing freezing containers to ensure gradual cooling at 1°C/min. Mr. Frosty containers were placed at -80°C and the next day vials were transferred to liquid nitrogen. To recover cells from storage, cells were quickly thawed by placing cryovials in a 37°C water bath and plating in 20 ml of medium, which was then replaced as soon as cells had adhered to the plastic.

HUVEC cells were plated in flasks and plates that had been coated in gelatine. This involved diluting 2% gelatine to 1% in PBS and adding it to the plastic cell culture surface. Plates were kept at room temperature for at least 10 min before removing the gelatine and allowing the plates to dry for 10 min. Cells were then plated in the coated plates.

**2.2.6 Human oocytes and Embryo culture**

Human oocyte collection and embryo culture was performed by Dr. Deborah Taylor at the University of Warwick. Women underwent ovulation induction and oocytes were collected by transvaginal ultrasound guided aspiration and inseminated with prepared sperm (day 0). Oocytes were examined the following morning, 19-20 hours post insemination, and classified by the presence of two pronuclei. Fertilised embryos were cultured in MediCult media (Origio Ltd, UK) to day 5 of development. Following embryo transfer, surplus embryos were donated to research by couples giving informed consent.
Day 5 embryos, including embryos reaching the blastocyst stage and those that had arrested, were included in this study. This work was carried out under a Human Fertilisation and Embryology Authority licence, with local approval from the Research Ethics Committee.

A total of 11 human embryos were used in this study, three embryos at the blastocyst stage and 8 arrested, day 5 embryos. Embryos were cultured individually in 50 µl drops of MediCult BlastAssist media (Origio Ltd, UK) containing hESC conditioned medium ultracentrifugation pellet extracts. Following overnight culture (16 hours) embryos were washed through several drops of PBS prior to the removal of the zona pellucida with acidified Tyrode’s solution. Denuded arrested embryos and blastocysts were again washed in PBS, then individual embryos were lysed in 10 µl of Lysis solution (Ambion Single Cell Lysis Kit, Life Technologies) and stored at -20°C.

2.2.7 Cell Transfection

*Transient transfection of hESCs*

The Profection Mammalian transfection kit was used to transfect primary hESCs with plasmid DNA and RNA by the calcium phosphate co-precipitation method. Experiments were performed in 6- or 24-well plates or 10 cm dishes. When cells reached 80% confluency, media was changed from 10% to 2% DCC DMEM/F12 on the day before transfection. The following day at least 1h prior to transfection, media was changed to 5% DCC DMEM/F12 to half the normal volume. Transfection mixes were prepared as follows: in a 15 ml or 50 ml Falcon tube the appropriate volume of 2X HEPES-buffered saline (HBS) was added and in another tube plasmid DNA or RNA was mixed with nuclease-free water and 2M CaCl₂. The DNA/CaCl₂ mix was then added dropwise to the 2X HBS whilst agitating the 2X HBS either by vortexing or bubbling air through it with a 1 ml serological pipette. The final concentration of CaCl₂ in the combined mix was 122 mM. The mixture was then incubated for 20 min at room temperature to allow the precipitate to form and then added to cells dropwise. Cells were incubated with transfection mixes for 4 to 5 h and then washed two to three times with 2% DCC DMEM/F12 and allowed to recover in 2% DCC DMEM/F12 overnight in the presence or absence of treatments.
Dharmafect transfection of siRNA

LNCaP cells in 6 well plates were transfected with siRNA at a final concentration of 100nM using DharmaFECT4. Cells were plated in RPMI media with 10% FBS, L-glutamine but without antibiotics. Cells were transfected two days after plating.

In one tube siRNA was mixed with Opti-MEM and incubated at room temperature for 5 min. In a second tube, DharmaFECT4 was mixed with Opti-MEM and incubated at room temperature for 5 min. The siRNA mix was then added to the DharmaFECT4 mixture and incubated for 20 min. The combined mixture was then added to fresh LNCaP medium without antibiotics. Spent media was removed from the cells and media containing the transfection mix was added to the cells.

2.2.8 Reporter Gene Assays

Firefly Luciferase assays

Cells in 24 well plates that had been transfected with the relevant constructs were first washed with PBS before being lysed in 1x Reporter Lysis Buffer containing 1mM PMSF and 1x protease inhibitor cocktail. To each well 60 µl of lysis buffer was added and the plates were placed on a shaker in the cold room for 10 min at low speed to ensure the lysis buffer was well spread over the bottom of the wells. Plates were then placed at -80°C until needed for luciferase and β-galactosidase assays. The freezing step was necessary as the lysis buffer requires a freeze-thaw cycle to properly lyse the cells. The Luciferase Assay kit from Promega was used to perform the assay. Prior to assaying, plates were thawed at room temperature on a shaker and 10-20 µl of each lysate was transferred to white 96 well plates. Luciferase activity was measured by adding 50 µl of Luciferase Assay Reagent to each well of the white 96 well plate with a distributor pipette and immediately reading luminescence setting on the Victor2 microplate reader.

Beta-galactosidase assays

This assay was carried out on lysates remaining from luciferase assays to normalize luciferase activity measurements. The Galacto-Light Plus β-galactosidase reporter gene assay kit was used. Lysates were transferred to 96 well PCR plates and plates were sealed and incubated at 48 °C for 50 min to inactivate endogenous β-galactosidase. Following incubation, 10-20 µl of each lysate was transferred to a white 96 well plate and 70 µl of 1x Galacton-Plus substrate diluted in Reaction Buffer Diluent (100 mM sodium phosphate pH 8, 1 mM MgCl₂) was added per well. The reaction was incubated in the dark for 30-60 min.
at room temperature. To read β-galactosidase activity, 100 µl of Accelerator II solution was added per well with a distributor pipette and the plate was read immediately for luminescence on the Victor2 microplate reader.

2.2.9 SDS Polyacrylamide Gel Electrophoresis & Western Blotting

Cell lysis

To produce whole cell lysates for immunoblotting, cells grown in 6 well plates were washed in cold PBS and then 80-100µl of cold RIPA buffer (containing 1mM PMSF and 1x protease inhibitor cocktail) was added to each well. Cells were scraped on ice and lysates were transferred to Eppendorf tubes. Lysates were kept on ice for 5-10 min before centrifuging at 12,000g for 5 min at 4°C to pellet insoluble material. Supernatants were transferred to new Eppendorf tubes and snap frozen in a dry-ice/ethanol bath. Lysates were stores at -80 °C until needed.

Extraction of protein from tissue

Endometrial biopsies that had been flash frozen in liquid nitrogen were used for protein extraction. A small piece of tissue approximately 0.5 cm in size was cut off and placed in an Eppendorf tube on ice. Then 120-200 µl of high salt buffer was added and the tissue was manually homogenized with an Eppendorf micropestle on ice. Homogenates were briefly sonicated to break up viscous DNA and then centrifuged at 12,000g for 5 min at 4°C to pellet debris. Supernatants were transferred to new tubes and snap frozen in a dry ice-ethanol bath.

Measuring protein concentration

To determine the protein concentration of lysates to be used for Western blotting, the bicinchoninic acid (BCA) assay kit was used. This is a colorimetric assay that relies on reduction of Cu²⁺ to Cu⁺ by proteins and the ability of Cu⁺ to then form an intensely coloured complex with BCA. Assays were performed in 96 well microplates. A five point standard curve of bovine serum albumin (BSA) was constructed by dilution of a 2 mg/ml stock to 1.6, 1.2, 0.8, 0.4 and 0.2 mg/ml concentrations in lysis buffer. Lysates were mixed 1:1 with lysis buffer. BCA reagent kit consisted of Solution A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide and Solution B, containing 4% cupric sulfate. Solution A and B were mixed in a ratio of 50:1 and 100 µl of the mixture was added to each standard and sample in the microplate. The plate was incubated at 37°C for 30 min to allow the colour to develop and
the plate was then allowed to cool. Absorbance at 562 nm was measured with the OPTImax microplate reader and data was analysed with Softmax Pro 4.0 software.

Casting of denaturing polyacrylamide gels

Polyacrylamide gels used were of the discontinuous variety, with a stacking gel of mildly acidic pH layered above a resolving gel of alkaline pH. The Bio Rad MiniProtean system was used to cast gels. First, the resolving gel mix was made up and poured in between the glass plates and this was overlayed with butanol to eliminate bubbles and create a level surface. The resolving gel was allowed to set for at least 20 min and then the butanol was removed. The top of the resolving gel was rinsed with distilled water before adding the stacking gel mixture and a comb to form wells. The stacking gel was allowed to set for 20 min and gels were wrapped in damp tissue and stored at 4°C for a maximum of 2 days or used immediately.

Electrophoresis

Gels were assembled onto the electrophoresis equipment and the tank was filled with SDS running buffer. Protein samples to be run on gels were mixed with 2X or 4X Laemmli buffer and boiled at 95°C for 5 min. They were then briefly centrifuged and loaded using thin gel-loading pipette tips. Gels were run at 80 V for 10 min before increasing the voltage to 120-150 V. Gels were run until the dye fronts reached the bottom of the gels.

Electroblotting

Gels were removed from the glass plates and the stacking gels were sliced off. PVDF membranes were activated by soaking in methanol for 10 s and then immersed in cold transfer buffer. “Sandwiches” consisting of a sponge pad, two filter papers, the gel, the PVDF membrane, two more filter papers and another sponge pad were constructed in the transfer buffer and placed in the blotting cassette. Air bubbles were removed from the sandwich by rolling a pipette over it. Cassettes were inserted onto the transfer apparatus such that the gel was closest to the cathode and the PVDF closest to the anode. Transfer was performed at 110 V for 1.5 h with an ice pack immersed in the buffer to cool. A magnetic stirrer agitated the transfer buffer during the transfer. Following transfer, PVDF membranes were removed from sandwiches and allowed to air dry. They were then re-soaked in methanol and put in blocking buffer for at least 1 h at room temperature or overnight at 4°C. Blocking was performed with gentle shaking.
**Antibody incubations**

Following blocking, blots were rinsed in TBS-T then placed into 50 ml Falcon tubes containing diluted primary antibodies. Antibodies were diluted in 5% v/v BSA in TBS-T or 5% v/v non-fat milk in TBS-T together with sodium azide. Antibody tubes were placed on a tube roller at 4°C overnight. Following incubation with primary antibodies, membranes were placed in trays and washed four times in TBS-T (once for 1 min and then three times for at least 5 min each). Washed blots were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in 5% v/v non-fat milk in TBS-T for 1 h at room temperature with gentle shaking. Washing with TBS-T was repeated as above and then enhanced chemiluminescent (ECL) substrate was added to the membrane. Chemiluminescence was captured with radiography film or imaged on the ImageQuant imager (GE Healthcare).

**Densitometry**

Densitometry was carried out by analyzing images of Western blots with ImageJ software (rsbweb.nih.gov/ij/).

**2.2.10 RNA extraction**

**Cellular RNA**

Media was removed and cells were washed with PBS and TRI reagent was added to well. For each well of a six-well plate 0.5 ml of TRI reagent was used and for a 10 cm dish 3 ml was used. Lysates in TRI reagent were stored at -80°C until needed for RNA extraction. To extract RNA, 0.2 volumes of chloroform was added and tubes were vigorously shaken for 15 s. Mixtures were allowed to stand at room temperature for 3 min before centrifugation at 12,000g for 15 min at 4°C to separate the organic and aqueous phases. Aqueous phases, containing RNA, were then transferred to new RNase-free Eppendorf tubes and 0.5 volume of isopropanol was added to precipitate the RNA for 10 min at room temperature. RNA was pelleted by centrifugation at 12,000g for 25 min at 4°C. Supernatants were removed from pellets and ice-cold 75% ethanol was used to wash pellets. Following centrifugation at 7,500g for 15 min at 4°C, supernatants were removed and pellets were air-dried for 5 min and dissolved in DEPC-treated water. RNA was incubated at 55°C for 5-10 min to promote solubilization and then stored at -80°C. RNA concentration was determined using the Nanodrop ND1000 spectrophotometer.
Media RNA

Media samples were cleared by centrifugation at 300g for 10 min and then at 12,000g for 30 min. They were then stored at -80°C until extraction. Media was thawed and then 3 volumes of TRI reagent LS were added for every one volume of media. 4 or 5 µl of Glycoblu co-precipitant was also added and, where necessary, 2 µl of a 1 nM solution of cel-miR-39 was spiked in too. Samples were thoroughly vortexed and allowed to stand at room temperature for 5 min. Next, 0.2 volumes of chloroform were added for every one volume of TRI reagent LS used and the samples were vortexed for 15 s. Samples were left to stand at room temperature for 5 min before centrifuging at 12,000g for 15 min at 4°C to separate the aqueous and organic phases. Aqueous layers were transferred to new tubes and 0.5 volumes of isopropanol were added and vortexed to precipitate RNA and Glycoblu. Centrifugation at 12,000g for 25 min at 4°C was performed to pellet the Glycoblu, which co-precipitates with RNA. Glycoblu/RNA pellets were washed with ice-cold 75% ethanol, air-dried and redissolved in DEPC-treated water. Samples were incubated at 55°C for 5 min to promote RNA solubilization and then stored at -80°C.

2.2.11 Reverse Transcription

Random Hexamer reverse transcription

Before reverse transcribing to cDNA, 0.5-1 µg of RNA was treated with amplification grade DNase I. This removed any contaminating DNA that may have carried over from the RNA extraction process. RNA was mixed with DEPC-treated water to a final volume of 4 µl and then 0.5 µl of 10x DNase I buffer and 0.5 µl of DNase I was mixed in. Samples were incubated at room temperature for 15 min before the reaction was stopped by adding 0.5 µl of stop solution and incubating at 65°C for 10 min to inactivate the DNase I.

DNase-treated RNA was then annealed with random hexamers. To each RNA sample, 1µl of dNTPs and 1µl of random hexamers were added and made up to 10µl with DEPC-treated water. Samples were incubated at 70°C for 10 min before placing on ice. Next a master mix of RT reaction components was prepared. For each sample, 2 µl of 10x MMLV buffer, 0.5 µl of RNaseOUT and 0.5 µl of MMLV reverse transcriptase. The volumes of the components were scaled up for n+1 samples. The master mix was distributed among the RNA samples and the reactions were incubated at 25°C for 10 min, 37°C for 50 min and 85°C for 5 min. cDNA was stored at -20°C.
**Stem loop miRNA reverse transcription**

To reverse transcribe miRNAs, the stem loop primer system developed by Chen et al., was used (234). Reverse transcription primers have a stem loop structure with a 3’ overhang that is complementary to the 3’ portion of the mature microRNA (Fig. 2.1). MiRNA molecules are converted to cDNA by reverse transcriptase, which is then used as a template for quantitative real-time PCR using a miRNA-specific forward primer and a universal reverse primer, which is complementary to sequences in the stem of the RT primer. A linear U6 reverse primer was used to reverse transcribe U6 snRNA for normalization.

For cellular RNA, DNase I treatment was carried out as above but for 200-300 ng of RNA. RNA extracted from media was not DNase I treated. For embryos lysed in single cell lysis solution, the entire 10µl of lysate was included in the reverse transcription reaction. In a total reaction volume of 20 µl, DNase-treated cellular RNA, media RNA or embryo lysate was mixed with a pool of miRNA stem loop primers (each at a final concentration of 5 nM), 1 µl dNTPs, 2 µl 10x MMLV buffer, 0.5 µl RNase OUT and 0.5µl of MMLV. The components were assembled as a master mix for n+1 samples before adding to the RNA samples. Reverse transcription reactions were run on a thermal cycler using the conditions established by Tang et al., for multiplex miRNA reverse transcription (235). The reaction conditions were as follows: 16°C for 30 min, followed by 60 cycles at 20°C for 30 s, 42°C for 30 s and 50°C for 1 s. Heat inactivation at 85°C for 5 min was performed at the end. MicroRNA cDNA was stored at -20°C
2.2.12 Real time Quantitative PCR (qPCR)

**Principle**

This technique is used to quantify, in relative terms, the abundance of specific mRNAs, and therefore gene expression. It has also been applied to quantify miRNA expression. Prior to qPCR, mRNA/miRNA is converted to cDNA, as described the random hexamer reverse transcription and stem loop miRNA reverse transcription sections. Specific primer pairs and Taq DNA polymerase are used to amplify targets in cDNA samples and fluorescent chemistry is used to quantify the progress of the PCR reaction. Specifics of the chemistries are described in sections below but all qPCR assays were run.
on the ABI Step One Plus instrument in 96 well plates sealed with optically clear seals. Briefly, the system comprises of a thermal cycler to which laser light is distributed via optical fibres. This leads to excitation of fluorophores in the samples and the resulting fluorescence emission passes back through the optical fibres and is directed to a spectrograph attached to a charged coupled device camera detector. The signal is then analysed by software. With every cycle, fluorescence signal intensity increases in proportion to the abundance of amplicon. The fluorescence intensity is read by the detector during each cycle and the software calculates the change in intensity ($\Delta R_n$) as $\Delta R_n = R_{n+} - R_{n-}$, where $R_{n+}$ is the fluorescence emission at each point in time and $R_{n-}$ is the fluorescence emission of the baseline. The baseline is a measure of the background fluorescence and is usually determined between cycles 3 and 15. The software constructs an amplification plot of $\Delta R_n$ versus the cycle number. During the early stages of the reaction, $\Delta R_n$ does not exceed the baseline. As the reaction proceeds, the change in fluorescence intensity reaches a threshold, which is defined as statistically significant level above the baseline. The threshold cycle (Ct) is the cycle number at which the increase fluorescence intensity crosses the threshold and it is used as a measure of gene expression. The more highly expressed a gene is, the lower the Ct value. Monitoring the progress of the reaction with each cycle allows comparison of rates of amplification when they are proceeding in an exponential manner, as opposed to assessment of end product in conventional PCR. This allows for a more accurate and reliable quantification with greater sensitivity.

Primer Design

For protein coding genes, primers were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) so that one flanked an exon-exon junction in the mRNA, thereby eliminating the possibility of amplifying any genomic DNA not eliminated from the cDNA. Amplicons were 100-200 bp. Percentage GC content of primer pairs was 40-60% and melting temperatures (Tm) were around 60°C.

For mature microRNAs, primers were designed in a similar way to those used by Chen et al., (234). Forward miRNA specific primers were designed with 4 to 5 bases extra at the 5’ end and the universal reverse primer was the same as used by Chen et al., (234). For pri-miRNAs forward and reverse primers were designed to be complementary to sequences flanking the stem loop pre-miRNA.
All primers were ordered from Invitrogen and upon arrival were dissolved in DEPC-treated water to a stock concentration of 100 µM. Reconstituted primers were stored at -20°C.

**SYBR Green I based qPCR**

In this form of qPCR, the reaction mix contains SYBR Green I dye which, upon binding double stranded DNA, is able to fluoresce when excited with light of a particular wavelength. As PCR product accumulates after every cycle, the fluorescence intensity increases and thus is a measure of product abundance.

Master mixes containing 2X Jumpstart SYBR Green I mix, forward and reverse primers at (200-500 nM final concentration), ROX reference dye and DEPC-treated water were prepared. Master mixes were aliquoted into 96 well plates and then cDNA was added to plates. Each PCR had a 20 µl volume. For every target assayed, standard curves consisting of serial dilutions of pooled cDNA were also added to plates. Non-template control reactions, where water was added instead of cDNA, were also included. Standards and samples were assayed in duplicate PCRs. The PCR conditions consisted of an initial denaturation at 94°C for 2 min and then 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. SYBR Green I signal was measured at the 60°C step of each cycle. Melting curves were also run following PCR cycling to check for single melting temperatures (indicative of single products) in PCRs.

**miRNA Pre-Amplification**

To increase qPCR sensitivity for cel-miR-39 in microRNA transfer studies, cDNA for cel-miR-39 was pre-amplified prior to qPCR. This involved setting up 12.5 µl PCR reactions consisting of 11.25 µl of Platinum Taq Supermix, 0.125 µl of cel-miR-39 forward primer, 0.125 µl reverse primer (final concentration of each primer was 200mM) and 1 µl cDNA. A master mix of primers and Supermix was first prepared and aliquoted into 0.2 ml PCR tubes. cDNA was then added and negative control reaction with water instead of cDNA was set up too. PCR reactions were run on a thermal cycler with the following conditions: 94°C for 2 min 20 s followed by 16 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s. For embryo cDNA samples, the number of cycles was extended to 25.
Taqman probe based qPCR

Pre-amplified cDNA for cel-miR-39 was used as a template for Taqman probe-based qPCR. This chemistry is based on a third primer that binds to the template in between the forward and reverse primers. This primer is termed a probe and is labeled at the 5’ end with the fluorophore 6-carboxyfluorescein (6-FAM) and at the 3’ end with the quencher dye carboxyltetramethyl rhodamine (TAMRA). The assay relies on the 5’ to 3’ exonuclease activity of Taq polymerase. As Taq polymerase extends the forward primer and synthesizes the nascent strand, it degrades the probe and releases the 6-FAM moiety such that its fluorescence is no longer quenched by the TAMRA dye. Thus 6-FAM fluorescence signal intensity increases as the PCR progresses and is measure of PCR product accumulation. This form of assay has increased specificity as it requires a third primer to bind to the template and the fluorescence signal is specific to that template.

Master mixes containing 2X Jumpstart Taq mix, forward and reverse primers (each at 1 µM final concentration), Taqman probe (at 200 nM final concentration), ROX reference dye, and DEPC-treated water were prepared. Master mixes were aliquoted into 96 well plates and then cDNA was added to plates. Each PCR had a 15 µl volume. Non-template control reactions, where water was added instead of cDNA, were also included. Standards and samples were assayed in duplicate PCRs. The PCR conditions consisted of an initial denaturation at 94°C for 2 min and then 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s.

2.2.13 Bioanalyzer Analysis of RNA

Cellular RNA and RNA extracted from ultracentrifugation pellets was loaded onto Agilent RNA 6000 Pico chips at the Core Genomics Facility at Hammersmith Campus (http://genomics.csc.mrc.ac.uk/Booking.html). Chips were also loaded with Agilent RNA 6000 Pico Ladder. Chips were run on the Agilent 2100 Bioanalyzer to size profile RNA.

2.2.14 miRNA Microarray

We used the NCode multispecies miRNA microarray version 2.0 to screen for differentially expressed miRNAs in undifferentiated and decidualized hESCs. First, small RNAs were isolated from samples of total RNA and then a polyadenosine (A) tail was added to the miRNA using poly(A) polymerase. Then a highly specific tag was ligated to each tailed miRNA using a bridging oligo. The tagged miRNAs were then purified and hybridized to the microarrays overnight. The following day the arrays were washed and Alex Fluor 5 capture reagents were hybridized to the array. These bind to the ligated tags.
Chapter 2

Materials & Methods

on the microRNA. The arrays were then washed and scanned. Figure 2.2 shows the experimental outline. Components for poly(A) tailing, tag ligation, tagged miRNA purification and capture reagent hybridization were included in the NCode miRNA Labelling kit.

![Figure 2.2 miRNA microarray sample processing](image)

**Figure 2.2 miRNA microarray sample processing**  
Adapted from Invitrogen NCode miRNA Labeling System

**Small RNA isolation**

To extract small RNA, 10 µg of total RNA from each sample was processed with the PureLink miRNA Isolation kit. RNA was mixed with 300 µl of L3 binding buffer and 300µl of 70% v/v ethanol. This was then added to the spin cartridge, which was centrifuged at 12,000g for 1 min. To the flow through, 700 µl of 100% ethanol was added and vortexed. This was then added to another spin cartridge and centrifuged at 12,000g for 1 min. The flow through was discarded and the cartridge was washed twice with 500 µl of Wash Buffer W5. Residual wash buffer was removed by centrifugation for 2 min at 13,000g. Small RNA was eluted from the column by adding 60 µl of RNase-free water supplied with the kit. Approximately 300 ng of small RNA was recovered from each 10 µg total RNA sample.

**Poly(A) tailing**

Small RNA was concentrated with a SpeedVac concentrator on a low heat setting. 232 ng of each sample was made up to 14.5µl with DEPC-treated water. Each sample was
mixed with 1 µl of NCode multispecies microarray controls, 1 µl of 0.5 mM ATP, 2.5 µl of 25mM MnCl$_2$, 5 µl of 5x poly(A) polymerase buffer and 1 µl of poly(A) polymerase (final volume of 25 µl). The mixtures were centrifuged briefly and incubated at 37°C for 15 min. Subsequent capture sequence ligation was performed immediately afterwards.

**Capture sequence ligation and Purification of tagged miRNA**

To each 25 µl poly(A) polymerase reaction, 6 µl of Alexa Fluor 5 ligation mix, 2 µl of T4 DNA ligase and 3 µl of DEPC-treated water was added. Following mixing and centrifugation, the reactions were incubated at room temperature for 30 min. The reaction was stopped by adding 4 µl of 0.5 M EDTA and mixing. Finally 60 µl of TE buffer was added to make the final volume of each sample 100 µl. The tagged RNA was then purified using the NCode miRNA Purification Module according to the manufacturer’s instructions.

**Hybridization of tagged miRNA**

Purified tagged miRNA was mixed with 2x Hybridization Solution that had been pre-heated to 70°C. The mixtures were incubated at 75°C for 10 min and then kept at 52°C until loading on the array. Arrays, consisting of epoxy-coated glass slides printed with miRNA probes, were loaded with sample and covered with LifterSlips. Arrays were placed in humidified hybridization chambers and incubated overnight at 52°C. Following overnight incubation, arrays were removed from hybridization chambers and LifterSlips were removed. Arrays were successively washed in array wash solutions 1, 2 and 3 according to the manufacturer’s instructions and dried by brief centrifugation.

**Hybridization of Alexa Fluor 5 Capture Reagent**

With the tagged miRNA hybridized to the arrays, they were ready for probing with the Alexa Fluor 5 Capture Reagent, which fluorescently labels hybridized miRNAs. The hybridization mix was prepared by adding 2.5 µl of Alexa Fluor 5 Capture Reagent to 22.5µl of DEPC-treated water and 25µl of 2X hybridization solution. A master mix was prepared where volumes were scaled up for n+1 number of arrays. The mix was incubated in the dark for 10 min at 75°C and kept in the dark at 62°C until adding to the arrays. Keeping away from direct light, the hybridization mix was added to arrays and covered with new LifterSlips. Arrays were again placed in humidified hybridization chambers and incubated at 62°C for 4 h in the dark. Following this, arrays were washed successively in array wash solutions 1, 2 and 3 according to instructions and dried by brief centrifugation.
**Scanning and Analysis**

Arrays were scanned on an Agilent microarray scanner at the Core Genomics Facility within 30 min of the final wash. Signal intensities from scanned images were derived using Agilent’s Feature Extraction software. Mr. Geraint Barton of the Bioinformatics Facility performed data analysis using Agilent’s GeneSpring GX software.

### 2.2.15 Exosome Isolation

To isolate extracellular vesicles from conditioned media, a serial centrifugation protocol, developed by Théry et al, was used (197). Media was collected and centrifuged at 300g for 10 min at room temperature. The supernatant was then collected in 14 ml round bottom polypropylene tubes and centrifuged at 12,000g for 30 min at 4°C to pellet all cell debris. The supernatants from this centrifugation were placed in ultracentrifugation tubes, which were heat sealed and ultracentrifuged for 70 min at 100,000g at 4°C in a SW28 swing-bucket rotor. Tops of ultracentrifugation tubes were carefully cut off and supernatants removed. A small amount of supernatant (~100 µl) was allowed to remain in each tube and the bottoms of the tubes were flushed with 0.22 µm-filtered PBS. The PBS-resuspended vesicles were then placed in ultracentrifugation-grade 1.5 ml tubes and ultracentrifuged again at 100,000g for 70 min at 4°C in a fixed angle T110 rotor. Supernatants were removed and pellets were resuspended in 50 µl of PBS or TBS and stored at -80°C. Alternatively, pellets were lysed in TRI Reagent or RIPA buffer for extraction of RNA and protein, respectively, and lysates stored at -80°C.

### 2.2.16 Electron microscopy

Electron microscopy was performed by Dr. Raffaella Carzaniga at the Electron Microscopy Facility at South Kensington campus. A small amount of exosomal sample in TBS was loaded onto continuous carbon 300 mesh copper grids. Negative staining was performed with a 2% aqueous solution of uranyl acetate. Images were taken using a Tecnai transmission electron microscope at an operating voltage of 120kV. Images were recorded using an Eagle CCD camera.

### 2.2.17 Nanosight analysis

NanoSight’s “Nanoparticle Tracking Analysis” (NTA) detects and visualises populations of nanoparticles in liquids down to 10 nm, dependent on material, and measures the size of each particle from direct observations of diffusion. Dr. Jason Webber of Cardiff University analysed conditioned media samples from hESCs using the
Nanosight apparatus. For Nanosight analysis, 2% DCC-FBS medium was ultracentrifuged at 100,000g overnight to deplete serum exosomes before adding to cells. Small vesicles present in conditioned culture media were analysed using the Nanosight LM10 system, configured with a 405nm laser and a high sensitivity digital camera system. Videos of 60 s durations were analysed by the NTA-software (version 2.2), with the minimal expected particle size set at 50 nm. Each sample was diluted in nanoparticle free water, so that the concentration was between $2 \times 10^8$ and $9 \times 10^8$ particles/ml. The presented data, which corrects for dilution, represent the size distribution profile and concentration values from triplicate measurements.

2.2.18 DNA Quantitation

The Bio-Rad DNA quantitation kit was used to assess DNA content of cells and thereby provide a relative estimation of cell number. This kit contains Hoechst 33258 dye, which is relatively selective for dsDNA and in high salt does not show fluorescent enhancement in the presence of either protein or RNA. The dye binds specifically to the A-T base pairs in dsDNA resulting in an increase in fluorescence and a shift in the emission maximum from 500 to 460 nm. Cells in 10 cm dishes were washed in PBS and then lysed in 800 µl of 0.02% v/v SDS in TE buffer. The lysates were briefly sonicated and stored at -20°C until needed. To assay DNA content, a standard curve of serially diluted calf thymus DNA was added to a 96 well plate. 10 µl of each lysate was also added. To each well, 200 µl of a 2µg/ml solution of Hoechst 33258 dye in 1x TEN buffer (part of the kit) was added. Fluorescence at 460nm was measured on a Lumistar Optima plate reader.

2.2.19 Immunohistochemistry (IHC)

Dr. Jennifer Steel performed this procedure and I took images of the slides on a light microscope. Endometrial tissue was fixed in formalin and then embedded in paraffin wax. Sections were cut on a microtome and these were deparaffinised by incubating slides in Histoclear for 2 x 5 min. Histoclear was removed by putting slides in 100% ethanol for 2 x 5 min and the sections were rehydrated by immersing in 70% ethanol for 1 min followed by distilled water for 1 min.

Endogenous peroxidase was next quenched by the immersing slides in PBS containing 0.3% hydrogen peroxide for 30 min. Slides were then rinsed 2 x 2 min in PBS before blocking. The sections were incubated with PBS containing 2% normal serum from the species that the secondary antibody was raised in. Blocking was carried out in a humidified chamber for 30 min at room temperature. Blocking solution was then removed
and primary antibody was added, diluted 1 in 50 in the blocking solution. Negative control slides were incubated with blocking solution only. The incubation was carried out at 4°C overnight in a humidified chamber. Excess primary antibody was removed by washing slides in PBS for 2 x 2 min. For Dicer staining, Vector biotinylated goat anti-rabbit antibody diluted 1 in 100 in PBS was added to slides and incubated for 30 min at room temperature.

Secondary antibodies were then washed off with 2 x 2 min rinses in PBS. For Dicer IHC the third layer was now added to the slides, which consisted of avidin-conjugated HRP. This was incubated for 30 min and would bind to the biotinylated secondary antibody. Dicer IHC colour development was performed after rinsing off excess avidin-conjugated HRP. Colour development involved adding AEC substrate and incubating for 15 min at room temperature. This was followed by a rinse in distilled water, staining with hematoxylin for 2-3 s and rinse in tap water. Sections were then dehydrated by immersing in 70% and then 100% ethanol, dried and mounted with coverslips using xylene and Permount.

2.2.20 In vitro transcription and translation

In vitro transcription

The MEGAscript T7 kit was used to generate large quantities of mRNA from linearised plasmid constructs. First, 3 µg of plasmid was linearised by digesting with a restriction enzyme that cut the backbone of the vector outside the protein coding sequence and promoter region. Following digestion, DNA was cleaned up using the GeneJET PCR purification kit. Small volumes of digested plasmids were run on agarose gels to check that linearization had occurred. To 1 µg of linearised plasmid, 2 µl of each NTP solution (i.e. ATP, GTP, CTP and UTP), 2 µl of 10x Reaction buffer, 2 µl of enzyme mix was added. DEPC-treated water was added to a final volume of 20 µl. The reaction was incubated at 37°C for 4 h. To each reaction, 30 µl of Lithium Chloride Precipitation solution and 30µl DEPC-treated water was added and mixed. Samples were incubated overnight at -20°C to precipitate RNA. Tubes were centrifuged at 13,000 rpm for 15 min at 4°C to pellet RNA and pellets were washed in 70% v/v ethanol. The RNA was re-dissolved in DEPC-treated water and concentration determined on the Nanodrop.
In vitro translation

RNA generated using the MEGAscript T7 kit was translated using the Retic Lysate IVT kit. Reactions were carried out in 25 µl, 10 µl or 5 µl scale. RNA was mixed with 20x translation mix, methionine (final concentration of 2 µM), DEPC-treated water and Retic Lysate. The reactions were incubated at 30°C for up to 2 h. Reactions were stopped by either adding hot 2x Laemmli buffer (for Western blotting) or placing on ice (where luciferase assays were to be performed).
Chapter 3 - Results

MicroRNA expression and miRNA pathway regulation in decidualizing hESCs
Chapter 3

Results

Introduction

Decidualization in the non-pregnant human endometrium is first apparent in the stromal cells surrounding the spiral arteries during the mid-secretory phase of the cycle. The decidual transformation of hESCs is underpinned by dramatic changes in gene expression, which reprogram the cells to acquire the phenotype necessary to support implantation and pregnancy. Progesterone and cAMP signaling drive the process synergistically and a host of transcription factors collaborate to modulate gene expression. The role of miRNAs in this process is poorly understood but the fact that miRNAs are involved in many other differentiation processes and that their defining purpose is post-transcriptional gene regulation justifies investigation of their role in hESCs.

Although miRNA expression profiling of endometrial tissue containing mixed cell populations and endometrial epithelial cells during the cycle has been reported previously (204, 205), no such profiling has been undertaken for primary human endometrial stromal cells differentiated in vitro with cAMP and progestin. This treatment has been shown to closely recapitulate the gene expression changes that are observed in vivo and primary cultures are amenable to further manipulation such as transfection or drug treatments. Identification of differentially expressed miRNAs would be a first step to identifying miRNA targets whose regulation may be important for decidual transformation.

The miRNA pathway contains various proteins that collaborate to generate mature miRNAs and enable them to regulate target genes. It is unclear if miRNA pathway components are at all regulated during decidualization of hESCs. Major changes in these components are likely to have important effects on miRNA function regardless of miRNA species.

In this chapter we present data on the differential expression of miRNAs in undifferentiated versus decidualized hESCs and also examine the transcriptional regulation of one miRNA gene cluster that was found to be up-regulated in decidualizing hESCs. Data regarding the regulation of key miRNA pathway components is also presented.
3.1 Expression profiling of miRNAs in decidualizing hESCs

We used a microRNA microarray approach to screen for differentially expressed miRNAs in vehicle-treated hESCs and those treated with 8-Bromo-cAMP and medroxyprogesterone acetate (C+M) for 8 days. Total RNA was extracted and then small RNA was isolated, labeled and hybridized to miRNA microarrays. Following scanning, the background-subtracted median fluorescence values for each probe were processed with Genespring GX software. Firstly, to normalize the data across all arrays, signal intensities were subjected to global linear scaling to the 75\textsuperscript{th} percentile. This was achieved by multiplying each intensity signal by an array-specific scaling factor. To obtain this factor the software, for both the whole series and each array individually, log transforms signal intensities and then ranks them in increasing order. It then calculates the scaling factors from the ratio of the whole series to each array’s 75\textsuperscript{th} percentile value.

Secondly, technical variation between the arrays was assessed in several ways: with principle component analysis, pairwise scatter plots and determining correlation coefficients for these pairwise plots. Correlation coefficients between each array are shown in table 2. Coefficients between arrays with the same treatment were higher than those with different treatments, indicating adequate reproducibility between replicate arrays in the same treatment groups. Significantly differentially expressed miRNAs were determined using an unpaired Student’s t-test with a cut off value of \( p = 0.05 \).

Of the several hundred miRNA probes on the arrays, only 17 miRNAs were significantly (\( p < 0.05 \)) differentially expressed, and of these 14 were up-regulated and 3 were down-regulated upon C+M treatment (Table 3). Expression of PRL was also monitored in the samples with qPCR to assess differentiation and it is clear that with C+M treatment there was a massive induction of this classical decidual marker (Fig. 3.1).

The array platform used contained probes for mouse, rat, zebrafish, fruitfly and nematode miRNAs as well as human miRNAs. We found four differentially expressed miRNAs that are not annotated in humans (mmu-miR-290, mmu-miR-201, dme-miR-316 and cel-miR-240). Of these, cel-miR-240 has some homology to hsa-miR-193b* and mmu-miR-290 has some homology to hsa-miR-371. Thus there may be some cross hybridization of these human miRNAs to the \textit{C. elegans} and mouse miRNA probes on the array. With regards to dme-miR-316 and mmu-miR-201, these might be homologous to novel, as yet unregistered, human miRNAs.

To confirm some of the changes observed in the array, the expression levels of four representative differentially expressed miRNAs, miR-29b, miR-29c, miR-100 and miR-
143, were assayed with real-time quantitative PCR (qPCR) in independent time course samples of primary hESCs differentiated for up to 8 days (Fig. 3.2A). These miRNAs were chosen for the following reasons: miR-143 and miR-29c had some of the lowest p-values, moreover, of all the human miRNAs, miR-29c exhibited the greatest fold change; miR-29b is known to be clustered on the genome with miR-29c and it also increased in abundance upon decidualization; additionally miR-29b and miR-29c were found by Kuokkanen et al to be down-regulated in late proliferative endometrial epithelium compared to mid-secretory epithelium; miR-100 has been examined keeping with the microarray data, miR-29b, miR-29c and miR-100 increased in abundance and miR-143 levels were diminished by 8 days of C+M treatment.

MicroRNA abundance can be determined by the rate of transcription from miRNA genes and also by regulation of post-transcriptional processing by the miRNA pathway. To further explore the regulation of differentially expressed miRNAs, we assayed with qPCR the pri-forms of miR-29b, miR-29c, miR-100 and miR-143 (Fig. 3.2B). MiR-29b can originate from two loci; miR-29b-1 is part of a cluster with miR-29a on human chromosome 7 and miR-29b-2 is clustered with miR-29c on human chromosome 1. We observed pri-miRNA transcripts originating from the chromosome 1 cluster (pri-miR29b-2 and pri-miR-29c) increasing in abundance with C+M treatment. In fact the primers used for each pri-miR amplify different regions of the same polycistronic transcript, which is why the changes on the graphs are so similar. By contrast, the expression of pri-miR-29b-1 actually fell with treatment. Thus the increase in mature form of miR-29b (Fig. 3.2A) can be accounted for by increased transcription from the chromosome 1 cluster during decidualization, rather than the cluster on chromosome 7. Furthermore, as we did not observe miR-29a to be differentially expressed in the microarray data, it implies that this locus is not activated during decidualization.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle 1</th>
<th>Vehicle 2</th>
<th>Vehicle 3</th>
<th>C+M 1</th>
<th>C+M 2</th>
<th>C+M 3</th>
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*Table 2 Pairwise correlation coefficients between each miRNA microarray.*

Correlation coefficients were calculated based on pairwise scatter plots for normalized log-transformed signal intensities.
Table 3. Differentially expressed mature miRNAs in undifferentiated and decidualized hESCs.
Triplicate small RNA samples from vehicle-treated or 8 day C+M-treated hESCs were analysed using miRNA microarrays. miRNAs differentially expressed with p<0.05 (according to the Student's t-test) are listed. Mean fold-change in miRNA expression relative to the vehicle-treated sample is shown for each significantly differentially expressed miRNA. hsa, Homo sapiens; cel, C. elegans; mmu, Mus musculus; dme, Drosophila melanogaster

<table>
<thead>
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<th>miRNA</th>
<th>Fold-change upon C+M treatment</th>
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Figure 3.1. Prolactin induction in samples used for miRNA microarray The graph shows the expression of PRL, a decidual marker, assayed with qPCR in the samples used for array analysis. PRL expression was normalised to L19 levels, which served as a housekeeping gene. The error bars represent standard error of the mean (n=3) for one biological replicate.
The expression of pri-miR-100, which is transcribed from a single locus on human chromosome 11, increased dramatically with 2 days of C+M treatment and then gradually diminished by 8 days. The expression of pri-miR-143, transcribed from human chromosome 5, fell by >90% by 8 days of C+M treatment and therefore reflected the change in expression of the mature form but more markedly. This miRNA is clustered with miR-145. However, the levels of mature miR-145 do not change during decidualization as it was not flagged up in the array and qPCR revealed its expression did not significantly change in the time course (Fig. 3.2C).

Overall, the expression of the four mature miRNAs assayed by qPCR reflected the expression of the pri-forms, suggesting that transcriptional regulation is an important determinant of miRNA abundance in decidualizing hESCs. However, the contrasting changes of mature miR-143 and miR-145, which originate from the same primary transcript, points towards potential differential post-transcriptional regulation of the individual miRNAs on this cluster.
Figure 3.2 QPCR profiling of mature and pri-miRNAs in decidualizing hESCs
Cultured hESCs were treated with vehicle (day 0) or C+M for 2, 4 and 8 days. RNA was extracted and QPCR was used to profile the mature (A) and pri forms (B) of miR-29b, miR-29c, miR-100 and miR-143. (C) Levels of mature miR-145, a miRNA clustered with miR-143, were also monitored during decidualization. Mature miRNA levels were normalised to U6 snRNA levels and pri-miRNA levels were normalised to L19 transcript levels. Bars show the mean of triplicate samples and data is representative of three biological replicates. Error bars denote SEM. * means p < 0.05 according to the ANOVA test, NS means not statistically significant.
3.2 5’ RACE of miR-29b-2/miR-29c transcription start site

Most miRNA genes are transcribed by RNA polymerase II and so are likely to be regulated by many of the transcription factors already known to regulate protein-coding genes. Identification of transcription factors responsible for regulating genes can be achieved by searching for consensus binding motifs in their promoter regions. The promoter regions of most genes are proximal to their transcription start sites (TSS) and so mapping the TSS of a gene is an important first step in elucidating gene regulation.

The miR-29b-2/miR-29c cluster on chromosome 1 is not located within a protein-coding gene and is therefore considered an independent transcription unit. It has been previously been studied by Chang et al., (236). They examined repression of this cluster by the transcription factor c-Myc, and using rapid amplification of cDNA ends (5’ RACE), they found that the 5’ ends of two previously catalogued expressed sequence tags (ESTs) that contain the pre-miRNAs coincided with regions of high c-Myc binding, as determined by Myc chromatin immunoprecipitation-sequencing (ChIP-sequencing). On the chromosome, the miR-29b and miR-29c hairpin loops are located approximately 20 kb from the 5’ ends of the ESTs, The hairpin loops themselves are only roughly 500 bp apart. The RNA transcribed from this locus can be alternatively spliced to give two transcripts – one 2247 bp long with 6 exons and another 2062 bp long with 5 exons – which can be considered the polycistronic pri-miRNAs for miR-29b and miR-29c.

To further examine the regulation of the miR-29b-2/miR-29c cluster in hESCs, whose expression was increased during decidualization, we performed 5’ RACE to identify the TSS using primers that were complementary to the ESTs. Figure 3.3 illustrates the position of the TSS for the miRNA cluster in decidualized hESCs relative to the annotated ESTs. It mapped to the second exon specific to EU154352. The RACE method used can only convert mRNAs that have an intact 5’ cap to cDNA, so the product obtained represents the true 5’ end of the transcript and hence the TSS on the chromosome. It may be that this miRNA cluster has alternative TSSs, which may vary between different cell types given that the ESTs start further upstream.
Chapter 3

Results

3.3 The miR-29b-2/miR-29c cluster is regulated by C/EBPβ isoforms in decidualizing hESCs

With the data obtained from 5' RACE, the region extending from +65 to -4024 bp relative to the TSS was cloned into pGL3-basic vector upstream of the luciferase reporter gene cassette. Figure 3.4 shows the sequence of genomic DNA that was cloned and also the positions of the exon and TSS. Figure 3.5 (left panel) shows luciferase reporter assays conducted in undifferentiated hESCs and those treated with C+M for 3 days. As expected, where empty pGL3 was transfected, reporter activity was minimal in both vehicle and C+M-treated cells. However, transfection of pGL3 containing the cloned 4.1kb insert (pGL3 miR-29b2 4kb prom) gave a dramatic increase in luciferase activity in C+M treated cells, implying that treatment stimulates transcription from the cloned fragment.

The 4.1 kb cloned sequence was analyzed for transcription factor binding sites using the Alibaba 2.1 online search tool (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). Two C/EBPβ binding sites were predicted by Alibaba 2.1 (highlighted in Fig. 3.4). Although many other factors were predicted to bind, we chose to test if the over-expression of the C/EBPβ could enhance the activation of the reporter because this factor is known to be induced in hESCs treated with cAMP and it has important roles in the activation of other decidual genes such as PRL (61, 237). There are two isoforms of C/EBPβ; the full length liver-enriched activating protein (LAP) and the truncated liver-enriched inhibitory protein (LIP). Co-expression of either

Figure 3.3 5’ RACE of the miR-29b-1/miR-29c cluster.
The decidual TSS obtained by 5 RACE mapped to the second exon of the EU154352 EST. This EST contains the miR-29b-2 and miR-29c hairpins in the 6th exon. Another EST, designated EU154351, lacks exon 2. The region of genomic DNA extending approximately 4kb upstream of the TSS (incorporating exon 1) was cloned into pGL3 basic vector.
isoform enhanced the C+M-induced reporter activity (Fig. 3.5 right panel). Indeed, even in vehicle treated cells over-expression of LAP or LIP increases reporter activity, although the effect is more than two-fold enhanced by C+M treatment.

To summarize, the 5'RACE data identified a new TSS for the miR-29b-2/miR-29c cluster and reporter assays showed that a 4 kb region upstream of this site harbours cis-acting elements that promote transcription upon C+M treatment. Although C/EBPβ has a positive effect on reporter activity, other transcription factors may also be involved in promoting transcription. Interestingly, there was one consensus site for PR identified by Alibaba 2.1 in the 4.1 kb sequence and it may also contribute to transcriptional activation.

Figure 3.4 The 4.1 kb genomic DNA region cloned into pGL3 basic
Highlighted in yellow is the first exon of EU154352 and in blue is the start of the second exon. The TSS mapped to the junction of the blue and green sequences, i.e. the +1 base is a ‘G’. In red are the putative C/EBPβ binding sites predicted by Alibaba 2.1.
Figure 3.5 Luciferase reporter assays with the pGL3 miR29b2 4kb construct.
Left panel hESCs in 24 well plates were transfected with 200ng/well of pGL3 empty vector or pGL3 miR29b2 4kb, and 100ng/well of pcH110. Cells were then treated with vehicle or C+M for three days before harvesting. Right panel pGL3 miR29b2 4kb Reporter and pcH110 were transfected along with 50 ng/well of pcDNA 3.1 (reporter only) or pcDNA LAP or pcDNA LIP. Cells were again treated for 3 days with vehicle or C+M before harvesting. Luciferase values were normalized to beta-galactosidase activity. Bars show the mean of six replicates and error bars denote the standard error of the mean. Data is representative of two biological replicates.
3.4 Regulation of miRNA pathway components in during decidualization

The ribonuclease Dicer is essential for generating mature miRNAs. Using Western blotting, we profiled Dicer protein levels in endometrial biopsies taken during the proliferative or secretory phase of the cycle (Fig. 3.6A). In all four of the proliferative stage biopsies Dicer levels are low, however in three out five secretory stage samples there were higher levels of Dicer. Additionally, sections of the endometrium from the early, mid and late secretory phases were stained for Dicer using immunohistochemistry (Fig 3.6B). There appeared to be positive staining in the stroma at all three stages but Dicer was only apparent in the glandular epithelium during the mid and late stages of the secretory phase.

Given that decidualization in humans occurs in the mid-secretory phase, this result prompted us to look at Dicer and other components of the miRNA pathway in hESCs decidualized in vitro with 8-Br-cAMP and MPA (Fig. 3.7A and B). Primary hESCs were treated with vehicle (day 0) or C+M for up to 8 days and harvested for RNA and total protein. Figure 3.6B shows qPCR results for Drosha, Dicer, Ago1 and Ago2. Also shown are transcript levels of PRL and IGFBP-1, key decidual markers, which were massively induced upon C+M treatment, as expected. We observed no change in Drosha mRNA levels during treatment and Dicer mRNA levels increased modestly by 8 days of treatment. However, the levels of Ago1 and Ago2 decreased by approximately 40% by 8 days of C+M treatment. Similar but more striking changes were observed at the protein level (Fig. 3.7B). Drosha protein levels decrease somewhat by 8 days whereas 4 and 8 days of C+M treatment markedly increased Dicer levels. By contrast, the levels of both Ago1 and Ago2 were dramatically diminished by 8 days of treatment. Densitometric analyses of protein levels of Drosha, Dicer, Ago1 and Ago2 from undifferentiated and 8 d C+M-treated hESCs confirmed these changes.

We found the mature miRNA levels of several differentially expressed miRNAs to reflect changes in the corresponding pri-miRNA levels, ultimately suggesting transcriptional regulation (Fig 3.2). The increase in Dicer levels upon decidualization may also be contributing to mature miRNA levels by post-transcriptional regulation as the majority of the differentially expressed miRNAs flagged up in the microarray were up-regulated upon decidualization (Table 2). It is interesting to see that upon decidualization there are diminished levels of Ago1 and Ago2, proteins critical for miRNA function, but increased expression of Dicer, an essential enzyme for miRNA biosynthesis.
Figure 3.6 Regulation of Dicer in vivo.
(A) Western blotting for Dicer and GAPDH as a loading control on protein from endometrial biopsies collected in either the proliferative (n=4) or secretory (n=5) phases.
(B) Dicer IHC in sections of human endometrium from the early, mid or late secretory phases. Brown staining indicates positive Dicer expression. For the no antibody control anti-Dicer antibody was omitted from the staining procedure.
Figure 3.7 Regulation of miRNA pathway components in hESC cultures.
Primary hESCs were vehicle treated (day 0) or treated for 2, 4 or 8 days with C+M before harvesting for RNA or protein. (A) QPCR was performed for Drosha, Dicer, Ago1 and Ago2. The decidual markers PRL and IGFBP1 were also assayed. Expression levels were normalized to L19 levels and bars represent the means of triplicate samples ± SEM and data are representative of three biological replicates. (B) Protein samples from the time course were immunoblotted for Drosha, Dicer, Ago1 and Ago2. GAPDH acted as a loading control. Graph shows densitometry analysis for protein levels of Dicer Drosha, Ago1 and Ago2 in day 8 C+M treated hESCs relative to the vehicle treated sample for three independent blots. * indicates p<0.05 according to ANOVA and NS means not significant.
3.5 TARBP2 increases in abundance upon decidualization

The dramatic and opposing changes observed in Dicer and Ago protein levels during decidual transformation led us to investigate the stability of these proteins in hESCs. Preliminary experiments with the commonly used translation inhibitor cycloheximide suggested that in decidualized hESCs, the half-life of Dicer is increased by nearly two-fold, which may partly explain the higher absolute levels observed upon differentiation.

The stability of Dicer is influenced by its binding partner, TARBP2. Paroo et al., found that phosphorylation of TARBP2 by ERK led to increased stability of the miRNA generating complex and over-expression of a phospho-mimic version of TARBP2, where the phosphorylated serine had been replaced with an aspartic acid residue, increased Dicer protein levels (130). Although ERK phosphorylation is not induced by C+M treatment (238), we nevertheless profiled TARBP2 expression in decidualizing hESCs as increased levels may be responsible for the stabilization of Dicer that we observed. Figure 3.8 shows that TARBP2 mRNA levels do indeed rise upon C+M treatment with levels increasing more than two-fold by 8 days of treatment. The greater abundance of TARBP2 may in part explain the higher levels of Dicer detected upon decidualization of hESCs.

![Figure 3.8 TARBP2 levels increase upon decidualization.](image)

Figure 3.8 TARBP2 levels increase upon decidualization.
Primary hESCs were vehicle treated (day 0) or treated for 2, 4 or 8 days with C+M before harvesting for RNA. QPCR was performed for TARBP2 and data was normalized to L19 expression. Data shows the mean of triplicate samples ±SEM and is representative of three biological replicates. p<0.05 according to the ANOVA test.
Summary

The transformation of hESCs into decidual cells is characterized by vast alterations in gene expression with important consequences for cellular function that are key to successful pregnancy. From the results presented in this chapter, we can conclude that miRNA expression is altered in decidualizing hESCs but only a relatively small number of miRNAs were differentially expressed between undifferentiated and decidualized hESCs. As the miRNA platform used was based on miRbase release 9.0, it contains probes for just 553 human miRNAs. On the current version of miRbase (release 19) there are over 2042 mature human miRNA, so there may be more differentially miRNAs in decidualizing hESCs that could have been missed. Furthermore, the data showed two miRNAs with no currently known human homologues to be differentially expressed in decidualizing hESCs – dme-miR-316 and mmu-miR-201. Although these could represent novel human miRNAs, their detection would need to be confirmed independently with Northern blotting and other criteria detailed by Ambros et al., would need to be satisfied, which includes identification of the hairpin precursor and accumulation of this precursor in cells with reduced Dicer function (239).

The expression of miR-29c, miR-29b and miR-30d has been shown to be up-regulated in endometrial epithelial cells in the secretory phase versus the proliferative phase (204), and we have observed the same miRNAs to be up-regulated in stromal cells treated with cAMP and MPA, a stimulus that mimics the signaling that occurs in vivo during the mid-secretory phase.

Some of the changes in miRNA expression were confirmed in independent time courses with qPCR and we were able to link changes in mature miRNA levels to changes in corresponding primary transcripts. The qPCR results for the two primary transcripts encoding miR-29b pointed towards specific up-regulation of the cluster on chromosome 1 that also contains miR-29c. With 5’ RACE a novel TSS for this miRNA cluster was identified in hESCs. Cloning of a 4.1 kb genomic region upstream of this TSS was carried out and this cloned fragment drove luciferase reporter expression upon C+M treatment, which was enhanced by co-expression of C/EBPβ isoforms.

The regulation of miRNA pathway proteins has been observed in the uterus of ovariectomized mice treated with progesterone and estrogen (228). However, no data has been published for the human endometrium or primary hESCs differentiated in vitro. Decidualization of hESCs was also associated with up-regulation of Dicer but a down-regulation of Argonaute isoforms. The potential targets of differentially expressed
miRNAs and consequences of miRNA pathway alterations are examined in the following chapter.
Chapter 4 - Results

The Regulation of Androgen Receptor by Poly(C)-binding Protein-1 and the Regulation of miRNA Targets during decidualization of hESCs
Introduction

From siRNA and microarray studies, the androgen receptor is known to regulate a subset of genes involved in cytoskeletal organization and cell cycle control in decidualizing hESCs (70). Therefore, despite not being essential for decidualization, unlike PR, AR activity makes specific contributions to the overall decidual phenotype. Like several other nuclear receptors, AR activity is induced by binding of specific ligands, in this case androgens such as testosterone. The levels of androgens in the endometrium fluctuate during the cycle, as does expression of the AR itself. Mertens et al., studied expression in endometrial tissue samples taken throughout the menstrual cycle by immunohistochemistry (240). They found strong nuclear staining in stromal cells of both basal and functional layers and highest AR expression was observed in the early proliferative phase, with levels generally lower in secretory phase samples. The mechanism of AR regulation in the endometrium, however, is poorly understood.

MicroRNA-mediated regulation of gene expression is a phenomenon that relies on sequence-specific interactions between miRNAs and mRNAs and on the actions of the RISC complex. In addition to binding miRNAs, transcripts may also directly interact with ribonucleoproteins (RNPs). These are a diverse group of proteins but all members contain one or more domains that bind RNA. Several different types of RNA binding domain have been identified, including the RNA recognition motif (RRM), K-homology (KH) domain, the RGG (arginine-glycine-glycine) box, the DEAD/DEAH box, the zinc finger (ZnF), and PAZ domain. Some of these domains are also found in proteins that are part of the miRNA biogenesis pathway. The diversity of RNPs in eukaryotes is thought to reflect need for proteins to control the highly evolved post-transcriptional modifications of mRNAs including, pre-mRNA splicing, RNA editing, polyadenylation, transport of mRNA to the cytoplasm, translation control, and mRNA localization and turnover.

One example of an RNP is the heterogeneous nuclear ribonucleoprotein D (hnRNP D). This protein was first identified as binding A+U rich elements in the 3’ UTR of c-myc mRNA, where it destabilizes the mRNA and therefore reduces c-myc expression (241). Sequences in the mRNA that regulate aspects of mRNA function through interaction with RNPs are designated cis-acting elements, and A+U rich elements (AREs) are the best described of these. AREs usually consist of AUUUA pentamers or UUAUUUA(U/A)(U/A) nonamers. Another cis-acting element is the U-rich polypyrimidine tract with the C(U),C motif. The c-myc mRNA also contains this motif and it interacts with the HuD, a member of the Elav/Hu family of RNPs.
The AR mRNA does not contain AREs, however, the 3’ UTR of AR mRNA contains a C(U)nC motif and a CCCUCCC motif which, in other transcripts, is known to interact with poly(C)-binding proteins (PCBPs) (242). PCBPs are a highly conserved family of RNPs which contain three KH domain repeats. Yeap et al., found that another Elav/Hu family member, HuR (Human antigen R), could interact with a 51 nucleotide UC-rich region and poly(C)-binding proteins PCBP1 and PCBP2 interacted with the CCCUCCC motif within this region (242). Thus their data suggested a role for these proteins in post-transcriptional regulation of AR.

As AR is critical to the development of prostate cancer, its regulation by miRNAs has been studied in the context of this disease. Using high throughput screening, Östling et al., found 71 miRNAs influence AR protein levels in a panel of prostate cancer cell lines (243). Many of those that decreased AR levels did not target the 436 nt 3’ UTR listed on the TargetScan entry but an extended 6.8 kb 3’ UTR. This group validated the targeting of AR by 13 miRNAs using 3’ UTR binding assays.

In the previous chapter, we described changes in both miRNA expression and the miRNA synthesis/effecter pathway during the decidualization of hESCs. In this chapter we explore the contributions of miRNAs and the RNP PCBP1 (also known as hnRNPE1) to the regulation of AR levels in decidualizing hESCs. Also presented is data on the role of miRNAs in establishing the decidual phenotype and the regulation of targets of decidual miRNAs.
Chapter 4

4.1 The regulation of AR in decidualizing hESCs

To explore AR regulation in hESCs, expression of AR protein was examined in undifferentiated and decidualized hESCs (Fig. 4.1A). Given that medroxyprogesterone acetate (MPA) also has androgenic effects, we decidualized hESCs with progesterone (P4) instead to eliminate any confounding ligand-dependent effects on AR levels. It was clear that there was a remarkable reduction in AR protein levels upon decidualization, as shown previously (70). To determine if the reduction could be accounted for by decreased transcription of AR, mRNA levels were examined with qPCR (Fig. 4.1B). However, in contrast to protein levels, transcript levels remained quite stable upon C+P4 treatment.

Increases in proteasomal degradation could account for the decreased AR protein levels upon decidualization. However, treatment with MG132 did not restore AR levels in decidualized hESCs to those of undifferentiated hESCs, indicating AR protein degradation is not higher upon decidualization (Fig. 4.1C). The stability of AR protein in undifferentiated and decidualized hESCs was evaluated with cycloheximide treatment (Fig. 4.1D). The half-lives of AR protein in undifferentiated and decidualized hESCs were similar, (6.88 h and 5.56 h, respectively). These results pointed towards translational repression of AR occurring in decidualized hESCs.
Figure 4.1 AR is down-regulated in decidualizing hESCs

(A) and (B) HESCs were cultured in the absence or presence of 8-Br-cAMP and P4 (C+P4), as indicated. Treatment media were replenished every 2 d and protein and RNA harvested after 8 d. (A) Western blotting for AR protein and β-actin as a loading control. (B) AR mRNA levels were assayed by qPCR and normalized to L19. Data show the mean of triplicate samples ± SEM and are representative of three biological replicates. (C) HESCs cultured in the absence or presence of C+P4 for 8 d were treated with MG132 at 20 µM final concentration for up to 3 h, as indicated. A Western blot was performed for AR protein expression with β-actin serving as a loading control. (D) hESC were treated with or without C+P4 for 2 days and then treated with cycloheximide (CHX) at 10 µg/ml for up to 12 hours. Western blotting was performed for AR protein expression. Densitometry was performed and relative AR protein levels were plotted against time. Half-lives (t_{1/2}) were calculated using the formulae t_{1/2} = ln2/k and lnC = lnC_0 – kt, where k is the slope and C is the relative amount of protein. Data represent the means of three independent samples. Inset blot shows AR bands at the same exposure in vehicle and C+P4-treated hESCs with β-actin as a loading control.
4.2 AR-targeting miRNAs are not regulated in hESCs and Dicer knock-down does not reverse AR inhibition upon decidualization.

Although most evidence points towards miRNAs destabilizing mRNAs and consequently reducing protein levels of targets, they can also decrease protein levels by translational repression whilst having little effect on mRNA levels. We first addressed whether hESCs express the short 436 nt AR 3’ UTR or the longer 6.8 kb version which can be targeted by many more miRNAs. Figure 4.2A shows the locations of primer pairs that were used in PCRs of cDNA from undifferentiated and decidualized hESCs. Primer pair one is located in the 436 nt UTR and would amplify from both long and short UTRs. Primer pair two is specific to the longer 6.8 kb UTR. Figure 4.2A shows PCR products were obtained with both primer pairs in both undifferentiated and decidualized hESCs. Thus hESCs express the long form of the AR 3’ UTR and possibly also the short form.

In prostate cancer cell lines, miR-9, miR-34a and miR-34c were found to downregulated AR protein levels and a luciferase reporter gene coupled to the AR 3’UTR (243). We profiled the expression of miR-9, miR-34a and miR-34c in hESC time courses with qPCR (Fig 4.2B). We chose to profile these miRNAs out of the 71 studied by Ostling et al because they caused significant reductions in AR protein levels with little change in AR transcript levels in LNCaP and 22Rv1 prostate cancer cells – an observation also found in decidualizing hESCs (Fig. 4.1). Moreover, miR-9 and the miR-34 families have been detected in endometrium by a previous study examining their expression in endometriosis (208). Notably, miR-9 is predicted to bind at six separate sites in the 6.8 kb 3’ UTR: 2x 8mer sites, 2x 7mer-m8 sites and 2x 7mer-1A sites. No significant change in any miRNA was seen during C+M treatment. If anything, miR-9 expression was slightly decreased by 8 days of decidual treatment. If any of these miRNAs were to reduce AR expression we would expect their abundance to increase upon decidualization.

To fully test if miRNAs at all regulate AR expression in hESCs we knocked down Dicer with siRNA and monitored AR expression with qPCR and Western blotting (Fig. 4.2C). Dicer knockdown did not reverse the inhibition of AR protein upon treatment with C+P4. Interestingly, depletion of Dicer did lead to an increase in AR mRNA levels and perhaps protein levels in undifferentiated hESCs, but no increase was observed in differentiated hESCs. Altogether the data suggests minimal involvement of the miRNA pathway in the down-regulation of AR protein levels that occurs in decidualizing hESCs, although AR may be regulated by miRNAs in undifferentiated hESCs.
Figure 4.2 AR regulation by miRNAs

(A) Upper panel: Schematic showing the positions of primer pairs 1 & 2 on the long and short 3’ UTRs of the human AR mRNA. Amplicon sizes are indicated. Lower panel: PCRs using each primer pair were conducted on genomic DNA as a positive control and no RT controls and water as negative controls as well as cDNA from undifferentiated (Undiff.) and decidualized (Decid.) hESCs. Products were resolved on a 2% TBE agarose gel along with a DNA marker (M).

(B) HESCs were treated with vehicle (day 0) or decidualized for 2, 4 and 8 days. qPCR was performed for miR-9, miR-34a and miR-34c. miRNA levels were normalized to U6 snRNA levels. Data represent the means of triplicate samples ± SEM and are representative of two biological replicates.

(C) hESCs were transfected with non-targeting (NT) or Dicer siRNA and treated with vehicle or C+P4 for 8 days. Cells were harvested for RNA and protein and AR expression was assayed with qPCR (left panel) and Western blotting (right panel). QPCR data, normalized to L19, represents means for triplicates ± SEM and are representative of two biological replicates. Dicer expression was also examined with Western blotting to check for knock-down and β-actin acted as a loading control.
4.3 PCBP1 is regulated in decidualizing hESCs and it represses AR levels.

One of the proteins identified by Yeap et al., to interact with the AR 3’ UTR was poly(C)-binding protein-1 (PCBP1). Previous studies had found that during in vitro decidualization of hESCs, PCBP1 was up-regulated at both the mRNA and protein level (Fig. 4.3A and B). Previous studies also found that transfection of undifferentiated hESCs with a plasmid construct over-expressing PCBP1 (pSG5-PCBP1) led to a reduction in AR protein levels but mRNA levels were unchanged, thus mimicking the effect seen when differentiating hESCs with C+P4 (Fig 4.3C). To test if loss of PCBP1 is sufficient to increase AR levels in differentiated hESCs, we transfected hESCs, that had been decidualized for four days, with non-targeting siRNA or siRNA targeting PCBP1 (Fig. 4.3D). Following four further days of treatment, Western blotting revealed that PCBP1 knock-down led to increased AR protein levels and qPCR showed that AR transcript levels were unchanged. This result suggested that loss of PCBP1 in decidualized hESCs releases the AR mRNA from translational repression.

The effect of PCBP1 over-expression on the induction of AR target genes was also tested. hESCs were transfected with empty vector or pSG5-PCBP1 and then treated with vehicle or dihydrotestosterone (DHT), a potent AR ligand, for 48 h before harvesting for RNA. QPCR of genes identified previously as being AR dependent (70) – wingless-type MMTV integration site 4 (WNT4), potassium channel subfamily K, member 3 (KCNK3) and protocadherin 7 (PCDH7) – revealed that over-expression of PCBP1 blunted their DHT-dependent induction (Fig. 4.3E).
Figure 4.3 PCBP1 is up-regulated upon decidualization and regulates AR expression

(A) & (B) hESCs were untreated or treated with C+P4 for the times indicated and harvested for RNA and protein. (A) QPCR was carried out to assess PCBP1 transcript levels and normalized to L19. Data represents mean of triplicates ± SEM. (B) Western blotting for PCBP1 and β-actin as a loading control.

(C) HESC cultures transiently transfected with the pSG5 control plasmid or a pSG5-PCBP1 were harvested 48 h later for RNA and protein analyses. The abundance of AR transcripts (mean ± SEM) was normalized to L19, whereas β-actin served as a loading control for the Western analysis.

(D) Primary cultures were decidualized with C+P4 for 4 d and then transfected with either NT or PCBP1 siRNA. After another 4 d of treatment, PCBP1 mRNA and protein levels were determined by qPCR and Western blot analyses, respectively. The abundance of AR transcripts (mean ± SEM) was normalized to L19, whereas β-actin served as a loading control for the Western analysis.

(Panels A-D courtesy of Dr. Brianna Cloke) (E) HESCs were transfected with a pSG5 or pSG5 PCBP1. After 48 h of treatment with dihydrotestosterone (DHT), RNA samples were assessed for AR target gene expression. The qPCR data are presented as fold change (±S.E.M.) compared to non-treated cells. * denotes p<0.05 according to the student’s t-test.
Given that the 3’ UTR of AR contains a UC-rich motif that binds to PCBP1, we cloned a section of the UTR containing this motif downstream of a luciferase reporter cassette. We also produced a clone that lacks the UC-rich motif. Using these constructs, we generated mRNA using a T7 MEGAscript kit and then performed in vitro translation with rabbit reticulocyte lysate in the presence or absence of PCBP1 protein. The resultant mixtures were assayed for luciferase activity in order to gauge the efficiency of translation. Figure 4.4A shows that PCBP1 was able to modestly inhibit translation of the luciferase mRNA with or without the presence of the UC-rich motif. Indeed, in vitro translation of an AR mRNA that is devoid of any 3’ UTR (generated from pSG5-AR construct) was inhibited by spiking the reaction with PCBP1 protein (Fig. 4.4B).

To show that PCBP1 was part of a general mechanism for AR regulation, the ability of PCBP1 to modulate AR expression was also demonstrated in LNCaP cells, a prostate cancer cell line whose growth is highly dependent on AR signaling. Transfection of PCBP1-targeting siRNA in LNCaPs led to a marked up-regulation of AR protein levels (Fig. 4.5C), similar to the effect observed in hESCs.

In summary, during decidual transformation AR expression is maintained at the mRNA level but dramatically reduced in terms of protein. Increased AR protein degradation or decreased stability could not account for this change and neither could miRNA-mediated regulation. Over-expression of PCBP1, an RNA binding protein, however, could replicate the effect seen upon decidualization and although the 3’ UTR contains a PCBP1 binding motif this appeared to be unnecessary for inhibiting translation in vitro. Moreover, PCBP1 is up-regulated during decidualization and its abundance has consequences for AR target genes in hESCs.
Figure 4.4 PCBP1 inhibits translation and is also able to regulate AR in LNCaP cells.

(A) Luciferase activity from protein generated by a 1.5 h \textit{in vitro} translation reaction, in the presence of \textit{in vitro} translated PCBP1 (+) or an unprogrammed \textit{in vitro} translation reaction (-), with RNA transcribed prepared from pcDNA3.1 Luc-AR3UTR or pcDNA3.1 Luc-ΔUC rich. Data represent the fold change in luciferase activity relative to the (-) reaction. * denotes \textit{p}<0.05 according to the student’s t-test. The assay was performed in triplicate with the mean (B) Western blot for AR that was \textit{in vitro} translated for increasing lengths of time from RNA derived from pSG5-AR in the presence of \textit{in vitro} translated PCBP1 (+) or an unprogrammed \textit{in vitro} translation reaction (-). (C) LNCaP cells were transfected with non-targeting (NT) siRNA or PCBP1 siRNA and then harvested after 48h. Western blotting was performed for PCBP1, AR and \textit{β}-actin.
4.4 siRNA-mediated Dicer knock-down does not block decidual marker gene induction

Dicer was found to be up-regulated at the protein level upon decidualization of hESCs partly due to increased protein stability. We therefore tested the effect of Dicer knock-down on the induction of decidual marker genes PRL and IGFBP-1. This would provide a read-out of how well hESCs can differentiate when depleted of a crucial miRNA pathway component as the induction of these markers depends on a host of transcription factors including PR, C/EBPβ and FOXO1A, which also regulate many other decidual genes.

In figure 4.5A hESCs were treated with vehicle or C+M for 2 days and then transfected with non-targeting (NT) or Dicer siRNA. Treatments were continued for a further 5 days before harvesting for RNA and protein. Western blotting revealed significant depletion of Dicer upon transfection of Dicer siRNA, as well as the previously observed increase in Dicer levels upon C+M treatment in NT siRNA-transfected cells. The expression of PRL and IGFBP-1 was monitored with qPCR and we found that upon Dicer knockdown PRL induction was increased and IGFBP-1 induction was somewhat blunted.

PRL and IGFBP-1 are induced in hESCs within hours of C+M treatment. We therefore tested the effect of knocking down Dicer prior to C+M treatment (Fig. 4.5B). Primary hESCs were transfected with NT or Dicer siRNA and then vehicle-treated or treated with C+M for 10 h, 24 h, 48 h or 8 d. As shown by Western blotting, Dicer knockdown was achieved and again an increase in Dicer levels was observed by 8 days of C+M treatment. The induction of PRL up to 48 h of C+M treatment was similar in NT siRNA-transfected and Dicer siRNA-transfected hESCs. By 8 days there was slightly lower PRL expression where Dicer siRNA was transfected. IGFBP-1 expression was higher in Dicer siRNA-transfected than NT siRNA-transfected cells by 48h of C+M treatment, but by 8 days the trend had reversed. Overall, the depletion of Dicer prior to or following initiation of C+M treatment certainly does not block the induction of PRL and IGFBP-1, and so the ability to decidualize is maintained.
Figure 4.5 Dicer knock-down does not block induction of PRL and IGFBP1

(A) Primary hESCs were treated with vehicle or C+M for 2 days and on the third day transfected with non-targeting (NT) or DICER siRNA. Treatments were continued for a further five days (8 days in total) before harvesting for protein and RNA. Dicer levels were assessed with Western blotting with β-actin acting as a loading control. QPCR was conducted for PRL and IGFBP1 and data were normalized to L19 levels. Data depicts the means of three technical replicates ± SEM and is representative of two biological replicates.

(B) Primary hESCs were transfected with NT or DICER siRNA and then treated with vehicle (-) or C+M for the indicated times. Cells were harvested for RNA and protein. Dicer levels were examined with Western blotting with β-actin as a loading control. PRL and IGFBP1 mRNA levels were assayed with qPCR and normalized to L19 levels. Data depicts the means of three technical replicates ± SEM and is representative of two biological replicates.
4.5 Upon decidualization miRNA targets are refractory to the effects of anti-miRs

The observation that in undifferentiated cells, but not in decidualized hESCs, AR expression was increased by Dicer knock-down prompted us to investigate the regulation of targets of some of the differentially expressed miRNAs identified in Table 1. Shown in figure 4.6 are interactions of miR-29b, miR-29c and miR-100 (three miRNAs up-regulated in decidualized hESCs) with mRNA targets. We chose genes that were known to decrease in abundance during decidualization as these could be subject to regulation by the up-regulated miRNAs predicted to target them.

The epigenetic regulator DNA methyltransferase 3B (DNMT3B) is down-regulated in decidualized hESCs (244), and is an experimentally validated target of the miR-29 family, as described previously in work on lung cancer cells (245). Tribbles 2 (TRIB2) is a pseudo-kinase that negatively regulates FOXO transcription factors (246). We have found that it is down-regulated in decidualized hESCs (Fig. 4.7) and is a predicted target of miR-29b/c and miR-100 according to the PicTar, TargetScan and miRANDA algorithms. Prokineticin 1 (PROK-1) is, according to miRANDA, a predicted target of miR-100, and is a pro-implantation cytokine that in highly up-regulated early in the decidual response but is then down-regulated later on (247).

We utilized anti-miRs to inhibit specific miRNAs and monitored the expression of targets. In figure 4.8A hESCs were transfected with anti-miR-29b/c, anti-miR-100 or a negative control (NC) anti-miR and then vehicle-treated or C+M-treated for 3 days. DNMT3B expression was assessed with qPCR and Western blotting. It was clear that upon C+M treatment DNMT3B was down-regulated. However, only in undifferentiated cells was anti-miR-29b/c able to increase DNMT3B expression at the protein and mRNA level. The effect of the anti-miR was completely lost in C+M treated cells. As expected, NC anti-miR and non-targeting anti-miR-100 did not have an effect on DNMT3B levels.

A similar effect was observed for TRIB2 (Fig. 4.8B). Here, hESCs were transfected with NC anti-miR or a pool of anti-miRs against miR-29b/c and miR-100. Only in undifferentiated hESCs did the anti-miR pool increase TRIB2 expression. Moreover, this trend in TRIB2 expression was also seen in hESCs transfected with DICER siRNA as Dicer knock-down only increased TRIB2 expression in undifferentiated hESCs (Fig. 4.8C). Transfection of the anti-miR pool (containing anti-miR-100) increased PROK1 expression in undifferentiated hESCs but paradoxically led to a decrease in C+M treated hESCs (Fig 4.8D). Altogether, the data for AR, DNMT3B, TRIB2 and PROK1 suggested
that miRNA activity is high in undifferentiated hESCs and consequently target repression can be relieved by anti-miR or DICER siRNA transfection, but upon decidualization miRNA target genes are refractory to miRNAs as both anti-miRs and Dicer knock-down failed to produce the expected effects.

Figure 4.6 Interactions of miR-29b, miR-29c and miR-100 with targets.
Two sites in the 3’ UTR of the DNMT3B mRNA are targeted by miR-29b and miR-29c. The TRIB2 mRNA contains three sites that are predicted to bind miR-29 family members and one site predicted to bind miR-100. PROK1 3’UTR contains one miR-100 site that has G:U wobble base pairs in the seed region. For DNMT3B TargetScan was used to predict binding sites which were the same as those validated in reference 245. For TRIB2 TargetScan, miRANDA and PicTar were used to predict binding sites. For PROK1 miRANDA was used to predict binding sites.
**Figure 4.7 TRIB2 mRNA is down-regulated during decidualization**

HESCs were treated with vehicle (day 0) or C+M for 2 or 8 days and then RNA was extracted. QPCR for TRIB2 was carried out and expression levels normalized to L19 levels. Data represents the mean of triplicate samples ±SEM and is representative of three biological replicates. * means p < 0.05 according to the ANOVA test.
Figure 4.8 Upon decidualization miRNA targets are refractory to anti-miRs and DICER knock-down.

(A) hESCs were transfected with negative control (NC) anti-miR, anti-miR-29b/c or anti-miR-100 and then treated with vehicle or C+M for 2 days before harvesting for RNA and protein. DNMT3B and L19 mRNA levels were assessed by qPCR (left panel) and DNMT3B protein levels were examined by Western blotting (right panel). Alpha-tubulin was also probed as a loading control. (B) Cultured hESCs were transfected with negative control anti-miR or a pool of anti-miRs against miR-100, miR-29b and miR-29c. Cells were then treated with vehicle or C+M for 2 days before harvesting for RNA. Expression of TRIB2 at the mRNA level was then determined with QPCR and normalized to L19. (C) hESCs were vehicle treated or decidualized for 2 days and then transfected with NT siRNA or DICER siRNA. Treatments were continued for a further 5 days before harvesting RNA. QPCR for TRIB2 was performed and data normalized to L19. (D) Cultured hESCs were transfected with negative control anti-miR or a pool of anti-miRs against miR-100, miR-29b and miR-29c. Cells were treated with vehicle or C+M for 2 days. Expression of PROK1 was determined with QPCR and normalized to L19. QPCR data show the mean of triplicates ± SEM and are representative of three biological replicates. * indicates p<0.05 according to the student’s t-test.
4.6 MicroRNA reporter assay revealed that silencing capacity is reduced upon decidualization.

To further explore generic miRNA activity in undifferentiated and decidualized hESCs we used a miR-30 reporter assay (Fig. 4.9). This consisted of a reporter construct containing a luciferase gene with eight miR-30 binding sites in the 3’ UTR (pCMV Luc miR-30 (P)) and another plasmid encoding miR-30 as a short hairpin RNA that can be processed by Dicer to give mature miR-30 (pSuper-miR-30) (231). We found that co-transfecting hESCs with increasing amounts of pSuper-miR-30 led to greater miR-30-dependent repression of luciferase activity. However, where low doses of pSuper-miR-30 were transfected (25 ng/well), the level of repression was greater in undifferentiated hESCs than in cells treated with C+M (Fig. 4.9A). Furthermore, over-expression of Ago2 from the p3XFLAG-AGO2 construct increased miR-30-dependent repression in differentiated hESCs to match levels observed in undifferentiated hESCs (Fig 4.9B). Thus the miRNA reporter assay revealed a reduced silencing capacity in decidualized hESCs, which could be increased by over-expression of Ago2.
Figure 4.9 MicroRNA reporter assay.

(A) Cultured hESCs were treated with vehicle or C+M for two days and then transfected with pCMV Luc miR-30 (P) (300ng per well), pcH110 and increasing amounts of pSuper miR-30. hESCs were then treated with vehicle or C+M for two more days before harvesting. (B) Cultured hESCs were transfected with pCMV Luc miR-30 (P) (300 ng/well), pcH110, 20ng/well of pSupermiR-30 and 50ng/well p3XFLAG-AGO2, alone or in combination as shown. hESCs were treated with vehicle or C+M for three days before harvesting. For (A) and (B) luciferase activity levels were normalised to beta-galactosidase levels (derived from pcH110). Data are expressed as fold change relative to the reporter only sample. Each bar denotes the mean of 4 replicates and the error bars represent the SEM. Data is representative of two biological replicates * indicates p<0.05 according to student’s t-test.
Summary

MicroRNA-mediated gene regulation is dependent on the biogenesis of miRNAs and their targeting to 3’ UTRs of mRNAs in the context of the RISC. Dicer activity is the gateway for the production of functional mature miRNAs and RISC loading, and is therefore crucial to this form of gene regulation. Depletion of Dicer from hESCs did not prevent the induction of the classical decidual marker genes PRL and IGFBP1 upon treatment with 8-Br-cAMP and MPA, implying that the differentiation process is not blocked following disruption to the miRNA pathway. This result is in keeping with the data on the conditional Dicer knockout mouse where mechanically induced decidualization of the uterine horns was macroscopically normal.

Although our results suggested that miRNAs are not essential for decidualization, genes that are regulated during the decidual process and important for certain aspects of the decidual phenotype might nevertheless be subject to miRNA-mediated post-transcriptional regulation. We considered AR to be a potential target of miRNAs in decidualizing hESCs because the disparity between mRNA and protein levels in decidual cells could not be accounted for by increased AR protein turnover, leaving open the possibility that translational repression by miRNAs might be responsible for regulation. However, although hESCs expressed the 6.8 kb version of the AR 3’ UTR that can be targeted by many miRNAs, miR-9, miR-34a and miR-34c levels did not change during decidualization and Dicer knock-down did not restore AR protein levels in differentiated hESCs. Instead we found that PCBP1, an RNP known to interact with the AR 3’UTR, was up-regulated during decidualization and its depletion or over-expression led to AR protein levels increasing and decreasing, respectively. Interestingly, however, it appeared that in vitro translation of AR mRNA could be inhibited by PCBP1 in the absence of the UC-rich motif and indeed the entire AR 3’UTR. It may be that other pyrimidine-rich sequences in the AR mRNA coding region or part of the 5’ UTR, that is included in the pSG5-AR construct, could interact with PCBP1. There are several CCUCC motifs in these other regions of the transcript but their involvement in binding PCBP1 and regulating AR expression is currently unknown.

The lack of involvement of miRNAs in AR regulation during decidualization did not rule out other targets being regulated by miRNAs. We focused on targets that are predicted to be regulated specifically by those miRNAs whose abundance increased during decidualization (miR-29b, miR-29c and miR-100). Often miRNA and target abundances inversely correlate, so we chose target genes whose expression decreased during
decidualization either early on (DNMT3B and TRIB2), or later (PROK1). Anti-miRs against miR-29b/c and miR-100 had the expected effects of increasing target gene expression, but only in undifferentiated hESCs, with no effect observed in hESCs treated with C+M. This result was similar to the effect of Dicer knock-down on AR and TRIB2. Finally, the miR-30 reporter assay demonstrated a reduced capability for miRNA-dependent silencing in decidualized hESCs, which could be increased by Ago2 over-expression.

The down-regulation of Ago1 and Ago2 that occurs during decidualization renders hESCs less sensitive to miRNAs, but Dicer up-regulation suggests that there is greater capacity to produce mature miRNAs. In the next chapter we therefore examined the possibility of miRNAs being exported from decidualized hESCs as their function within hESCs is reduced.
Chapter 5 - Results

MicroRNA secretion by decidualizing hESCs
Introduction

The recent discovery of extracellular miRNAs opens up the possibilities of a new mode of intercellular communication and novel biomarkers of physiological and pathological processes. Although RNA itself is highly susceptible to hydrolysis due to the extra hydroxyl group in comparison to DNA, it is the presence of various stable RNases in the extracellular environment that lead to the degradation of naked RNA. The high stability of miRNAs found thus far in biological fluids is a result of their encapsulation in vesicles or association with lipoprotein complexes.

The remodelling of the endometrium requires cooperative interactions between several cell types to produce an environment that is favourable to implantation and pregnancy. Paracrine signalling by decidualizing hESCs is central to driving the remodelling of the entire endometrium and is achieved by secretion of various soluble molecules, which influence immune cells, endothelial cells, epithelial cells and invading trophoblast. In the previous chapters we described dramatic changes in miRNA expression and components of the miRNA pathway. The most notable change was the reduction in silencing capacity in decidualized hESCs, owing to reductions in Argonaute abundance. Given that decidualized hESCs are highly secretory cells but have reduced intracellular miRNA function, in this chapter we examined the possibility that miRNAs are exported from hESCs and taken up by other cell types.
5.1 Upon decidualization miRNAs are actively secreted by hESCs into cell culture medium.

To determine if hESCs could secrete miRNAs into cell culture medium upon differentiation, primary cultures were treated with vehicle or C+M for 2, 4 and 8 days. For the final two days of culture, hESCs were washed in PBS three times and cultured in serum-free medium containing the appropriate treatments. This was done to avoid the detection of bovine miRNAs that could be present in the DCC-FBS. The induction of PRL and IGFBP-1 was found to be robust under these conditions, suggesting that differentiation is not impaired by an absence of serum for two days. The conditioned medium was then collected and RNA was extracted from equal volumes of each sample. A synthetic *C. elegans* miRNA that is not found in mammalian species (cel-miR-39) was spiked into each sample prior to RNA extraction. Cel-miR-39 was assayed with qPCR and used to normalize for differences in extraction efficiency between samples.

Figure 5.1A shows that the levels of all five mature miRNAs that featured in Figure 3.1 (miR-29b, miR-29c, miR-100, miR-143 and miR-145) were detectable in media samples and were most abundant in conditioned medium from cells differentiated for 8 days. The data for miR-143 is particularly interesting as in the cell this miRNA, both at pri- and mature levels, is down-regulated upon decidualization, but in the media its abundance increased. MiR-143 secretion is a relatively early event compared to other miRNA species, which suggests that this miRNA may be stored in undifferentiated hESCs and readily released upon decidualization. Moreover, the presence of miRNA in culture medium was unlikely to be due to cell lysis as U6, a highly abundant snRNA, could not be detected in conditioned medium, whereas it could be detected in cellular RNA (Fig 5.1B).
Figure 5.1 Decidualizing hESCs secrete miRNAs into cell culture medium.
(A) Primary hESCs were treated with vehicle (day 0) or C+M for 2, 4 and 8 days. For the final two days of culture for each sample, hESCs were incubated in serum-free medium. Spent medium was collected, centrifuged at low speed to remove cell debris and RNA was extracted. QPCR was carried out for the miRNAs indicated and data was normalized to levels of cel-miR-39 that had been spiked into the media just prior to RNA extraction. The data represents mean of triplicates and error bars show the SEM. * denotes p<0.05 according to the ANOVA test.
(B) QPCR was carried out for U6 snRNA on cDNA made from the templates indicated. PCR products were resolved on a 3.5% agarose gel alongside a DNA marker (M) and visualized under UV light.
5.2 miRNA in conditioned medium can be depleted by ultracentrifugation and ultracentrifugation pellets are enriched in small RNAs

Extracellular vesicles that contain miRNAs have been described by several previous studies (193, 194, 200, 201). Ultracentrifugation has been routinely used to isolate microvesicles and exosomes from various sources (197), and we chose to test if miRNAs could be depleted from conditioned medium by ultracentrifugation and thereby supporting the hypothesis that decidual miRNAs are secreted in extracellular vesicles.

Serum-free conditioned medium was ultracentrifuged at 100,000xg for 70 min and RNA subsequently extracted from the supernatants. RNA was also extracted from unconditioned medium and conditioned medium not subjected to ultracentrifugation, in parallel. Figure 5.2A shows levels of miR-100 in unconditioned medium and the conditioned medium pre- and post-ultracentrifugation. We found that ultracentrifugation was able to deplete most miR-100 from conditioned medium. Additionally, miR-100 could be detected in the ultracentrifugation pellet of conditioned medium (Fig. 5.2B). As expected, no miR-100 could be detected in unconditioned medium.

RNA extracted from ultracentrifugation pellets of decidual cell conditioned media was size profiled using an Agilent Bioanalyzer (Fig. 5.2C). The analysis revealed enrichment for small RNA and this profile contrasted with the cellular RNA profile, which typically contained peaks for the 18S and 28S ribosomal RNAs.
Figure 5.2 Ultracentrifugation depletes miRNA from supernatants and pellets are enriched in small RNAs
(A) Serum-free conditioned medium from decidualized hESCs was ultracentrifuged. RNA was extracted and QPCR carried out for miR-100, normalizing to cel-miR-39 levels. Unconditioned medium and non-ultracentrifuged conditioned medium (pre-spin) were also tested in parallel. Data shows the means of triplicates ± SEM for two biological replicates (B) QPCR for miR-100 was also conducted on ultracentrifugation pellets from unconditioned and conditioned medium. (C) RNA from decidualized hESCs and ultracentrifugation pellets of conditioned medium were loaded onto an RNA Pico chip and analyzed on an Agilent Bioanalyzer. Representative RNA profiles are shown from 3 repeats.
Chapter 5

Results

5.3 Endogenous miRNAs in decidual cell conditioned medium are relatively resistant to RNase A

Naked RNA is highly susceptible to degradation, however, extracellular RNA detected in biological fluids and conditioned cell culture medium has been shown to be highly stable. We sought to test if the miRNA detected in serum-free conditioned medium from decidualized hESCs were also resistant to RNase-mediated degradation.

Conditioned medium from decidualized hESCs was divided into equal volumes and left untreated, mixed with RNase A, or mixed with RNase A and 0.1% Triton X-100. Samples were incubated at 37°C for 35 min. As a control to assess degradation of naked miRNA, conditioned medium was spiked with synthetic cel-miR-39 and RNase A added before incubating at 37°C for 35 min. Reactions were stopped by adding Trizol LS and freezing at -80°C. Figure 5.3 shows the levels of miRNA (either endogenous miR-100 or spiked cel-miR-39) relative to untreated controls. RNase A treatment did lead to some degradation of miR-100 but the scale of the depletion was much smaller that that observed for spiked cel-miR-39. Furthermore, co-treatment of conditioned medium with Triton X-100 and RNase A led to even lower levels of miR-100 being detected, presumably through this detergent solubilising membrane bound vesicles and facilitating access of RNase A to miRNAs.

Figure 5.3 miRNA secreted by decidualized hESCs is relatively resistant to RNase A.

500 µl of serum-free conditioned medium from decidualized hESCs was untreated or mixed with 2.5 ul of RNase A (20mg/ml stock) alone or in combination with 0.1% Triton X-100. Separate media samples were spiked with cel-miR-39 and incubated with RNase A. Samples were incubated at 37°C for 35 min. RNA was extracted and qPCR performed for miR-100 and cel-miR-39. Data are presented as relative expression compared to untreated medium. Data represent the mean of triplicate samples ± SEM.

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5.4 Extracellular vesicles in the size range of exosomes were isolated from decidual cell conditioned medium.

To investigate if decidualized hESCs produced extracellular vesicles, ultracentrifugation pellets from conditioned medium were observed with transmission electron microscopy (EM). Figure 5.4A shows cup-shaped structures that were less than 100 nm in diameter. These structures were in the size range of exosomes as described in EM studies by others and we further studied them using the Nanosight nanoparticle tracking system. This instrument can size profile particles down to 10 nm and determine the concentration of particles in liquid samples. Primary hESCs were vehicle-treated or decidualized with C+M in 2% DCC medium that had been depleted of serum exosomes by overnight ultracentrifugation. Media was exposed to cells for four or eight days and then collected for Nanosight analysis. Figure 5.4B (left panel) shows there was an accumulation of particles between four and eight days of culture. By comparison, unconditioned medium had a particle concentration of 4.47 ($\pm$ 0.32) x $10^8$ per ml and so the vast majority of particles detected in conditioned medium originated from hESCs. Between undifferentiated and decidualized hESCs there was a modest but significant increase in particle concentration in media from C+M-treated hESCs at four days but no significant difference was observed after eight days. Size profiling (Fig. 5.4B right panel) revealed that a significant proportion of particles were less than 100 nm in diameter in both four and eight day conditioned medium from decidualized hESCs. The Nanosight size distribution profiles obtained from hESC-conditioned media were similar to those reported by Vallhov et al., in their studies of B cell exosomes (249). The larger particles detected by the Nanosight are likely to be aggregates of exosomes.

Exosomes and microvesicles have distinct cellular origins and, although both types of vesicle have been reported to contain miRNAs, their protein constituents are different. Although limited by relatively small numbers of cells and exosomes compared to other studies that examined protein constituents of exosomes, with Western blotting we were able to detect TSG101 and HSP90 in ultracentrifugation pellets from decidual cell conditioned medium (Figure 5.4C). These proteins have been identified as exosome markers in several studies.

Exosomes are derived from multivesicular bodies, which in turn originate from endosomes (195). Another exosome marker is Alix, a member of the endosomal sorting complex required for transport (ESCRT) pathway (197, 250). A commonly used protein marker that has been found to be absent in exosomes is calnexin, a chaperone resident to
the endoplasmic reticulum (197). Preliminary immunoblotting experiments on cell lysates and conditioned medium ultracentrifugation pellets from undifferentiated and decidualized hESCs showed a result opposing what was expected; Alix was not detected in the ultracentrifugation pellets but only in cell lysates and calnexin was detected in both the ultracentrifugation pellets and cell lysates.
Figure 5.4 hESCs secrete exosomes into the extracellular medium.
(A) Transmission electron micrograph of an ultracentrifugation pellet of decidual cell conditioned medium. The scale bar represents 100 nm. Image taken by Dr. R. Carzaniga. (B) Nanosight analysis of conditioned medium. Medium was collected from vehicle or C+M treated hESCs after 4 or 8 days of culture. Dr. J. Webber analyzed the medium with the Nanosight apparatus and tested unconditioned medium in parallel (data not shown). Left panel shows mean particle concentrations in conditioned medium samples (SEM) * denotes p<0.05 according to t-test. Right panel shows size distributions of particles from 4 and 8 day conditioned media from C+M treated hESCs. (C) hESCs were treated with C+M for 7 days and serum-free conditioned medium from the final 2 days was collected and ultracentrifuged. Pellets were lysed in RIPA buffer and Western blots for TSG101 and HSP90 were performed alongside whole cell lysates.
5.5 MicroRNA secreted by decidualized hESCs can be taken up by other cell types present at the feto-maternal interface

The exosomal secretion of miRNAs by decidualized hESCs suggested a novel mode of communication with other cells. We therefore sought to test if secreted miRNA could be transferred to other cell types present at the feto-maternal interface and early human embryos.

Figure 5.5 illustrates the experimental procedure. Primary hESCs were first transfected with pSuperior.puro cel-miR-39, a plasmid from which cel-miR-39 is expressed as an shRNA and then processed by Dicer to give mature cel-miR-39. hESCs were then differentiated with C+M for 6 days and for the final 4 days of culture then medium was not changed. Conditioned medium was collected and applied to various cells that represented cell types present in the pregnant endometrium – Ishikawa cells (an endometrial epithelial cell line), BeWo cells (a trophoblast cell line), HUVECs (to represent endothelial cells) and also undifferentiated and decidualized hESCs. Where human embryos were tested as recipients, hESC conditioned medium was ultracentrifuged and the resulting pellet was resuspended in embryo culture medium for incubation with embryos. Recipient cells were incubated with conditioned medium for 24 h and embryos were incubated for 16 h. Before lysis in Trizol, recipient cells were washed 3 times in PBS and the last wash was kept. cel-miR-39 could not be detected in the last PBS wash (data not shown).

Figure 5.6A shows that cel-miR-39 was abundant in the conditioned medium and in the embryo culture medium. In Figure 5.6B, qPCR revealed that cel-miR-39 could be detected in BeWo cells, undifferentiated and decidualized hESCs, HUVECs and to a lesser extent in Ishikawa cells following incubation with conditioned medium. Despite testing six embryos, some of which had breached zona pellicudas, no cel-miR-39 could be detected, even though endogenous U6 snRNA could be detected. No difference in uptake could be observed between undifferentiated and decidualized hESCs. We observed that HUVECs contained the greatest levels of cel-miR-39 that were approximately one thousandth of the levels detected in the donor hESCs transfected with pSuperior.puro cel-miR-39. Thus, we concluded that miRNA secreted by decidualized hESCs can be transferred to various cell types that are present at the feto-maternal interface, with some cells able to take up more than others.
Figure 5.5 Schematic for experiment to test miRNA transfer from decidualized hESCs to various cell lines and human embryos.
Figure 5.6 Uptake of miRNA from decidual cell conditioned medium by various cell types at the feto-maternal interface.
(A) cel-miR-39 levels in conditioned medium from hESCs transfected with pSuperior.puro cel-miR-39 and in embryo medium (containing resuspended ultracentrifugation pellet). Control medium from cells not transfected with pSuperior.puro cel-miR-39 was also tested in parallel. ND means not detected. (B) cel-miR-39 levels in various recipient cells, human embryos and donor hESCs. Levels were normalized to endogenous U6 snRNA levels. Data the mean of triplicates ± SEM and represents three biological replicates. ND means not detected.
Chapter 5

Results

Summary

The differentiation of hESCs endows them with properties that favour embryo implantation, placentation and ultimately successful pregnancy. A key phenomenon necessary for these effects is intercellular communication within the endometrium. The roles of cytokines, chemokines and other classical signalling molecules have been well studied in the endometrium. However, the role of extracellular miRNAs is currently unknown. This chapter describes two novel properties of hESCs – that they secrete miRNAs upon decidualization and they produce exosomes.

Increased secretion of miRNAs into cell culture medium upon decidualization was apparent for all miRNAs tested. As miRNAs from different families were all increased in abundance in conditioned medium from cells decidualized for 8 days, it suggested that there is little selectivity for particular miRNAs for secretion. However, secretion may still be an active process as the data for miR-143 suggests – this miRNA decreased in abundance in the cell during decidualization but its levels increased in conditioned medium. Nevertheless, there does appear to be some selectivity in terms of the types of RNA molecule exported from decidualized hESCs; U6 snRNA, which is located in the nucleus of cells, was not detected in cell culture medium and the 18S and 28S ribosomal RNAs, which are abundant in cells, did not appear to be enriched in conditioned medium ultracentrifugation pellets. It is currently unknown if messenger RNAs are also secreted by decidualized hESCs, although evidence from other studies shows that exosomes can contain mRNAs as well as miRNAs (193).

Ultracentrifugation was able to deplete miRNAs from cell culture supernatants and pellets contained vesicular structures up to 100 nm in diameter and proteins known to be present in exosomes. Furthermore, we found a resistance to RNase degradation of miRNA in conditioned medium, a result observed by other studies where exosomes were found to contain miRNAs (193, 194, 200, 201). Although HSP90 and TSG101 were present in the exosomal fraction from decidualized hESCs, the lack of Alix may be due to lower sensitivity of the antibody or a lack of sufficient exosomal material for detection. It is probably the latter reason as some exosome studies isolate exosomes from large volumes (up to 200 ml) of conditioned media where cells are grown at high density or use specialized culture apparatus which increase exosome yields up to 12-fold (200, 251).

The unexpected detection of calnexin in the conditioned medium ultracentrifugation pellet may also be due to differences in methodology – some studies perform an additional purification step where, during ultracentrifugation, a 30%
sucrose/D$_2$O cushion underlays the supernatant (252). Omission of this step from our exosome isolation procedure may have led to calnexin contaminating the preparation. Indeed, calnexin has been reported to be released from cells into cell culture medium (253).

The Nanosight data showed that roughly equivalent numbers of particles are released from undifferentiated and decidualized hESCs. It therefore suggests that there is a mechanism for recruiting miRNAs into exosomes that is specifically activated during decidualization. It is possible that the changes in the miRNA pathway components that occur during decidualization may be involved in this mechanism.

Our data showing the transfer of miRNAs from decidualized hESCs to various recipient cell types indicates that miRNA may have the potential to act in a paracrine fashion in the endometrium. For example, the high uptake of secreted miRNAs by HUVECs may have consequences for vascular remodelling in the endometrium, which is a key event during placentation. We did not observe miRNA uptake by human embryos, however, this finding must be considered preliminary in view of the presence of the zona pellucida in our cultured embryos, which could have prevented exosomes reaching the embryo plasma membrane. The mouse zona pellucida is permeable to macromolecules (254), but data on larger particles is lacking. The permeability of human zona pellucida has not been characterised. For this reason, the zona pellucida was deliberately breached in some of our embryos using micromanipulation to facilitate exosome access, however, these particular embryos did not become blastocysts. Implanting blastocysts may also secrete microRNAs, therefore, the potential for communication between embryonic and maternal cells via miRNA signalling requires further investigation.
Chapter 6 - Discussion
**General Summary**

In addition to embryo development and placentation, successful pregnancy depends on decidualization of the endometrium. This process is maternally initiated in humans and is dependent on the signalling mediated by progesterone and several factors that lead to increases in intracellular cAMP levels (2). Arguably, decidualization is the most crucial change necessary for successful pregnancy because without it blastocyst implantation is impaired, which precludes pregnancy altogether. Decidualization confers various characteristics on hESCs, including a resistance to oxidative stress, the ability to maintain vascular integrity during trophoblast invasion and the ability to induce immunological tolerance to fetal alloantigens. Driving the transformation of hESCs into decidual cells are changes in PR activity, epigenetic modifications, sumoylation, ROS signalling and alterations in the activity or abundance of several other transcription factors that ultimately depends on crosstalk between cAMP and progesterone signalling (58, 61, 62, 66, 238, 255-257). Over 3000 genes exhibit altered expression in hESCs treated in vitro with cAMP and MPA (2) and decidualization can be considered a differentiation process, akin to the differentiation that occurs in stem cells.

MicroRNAs have emerged as important gene regulatory molecules in several aspects of cellular biology including differentiation. They constitute a highly diverse class of small RNAs conserved in all animals and they are uniquely placed to post-transcriptionally modulate gene expression in a sequence specific manner, although their exact mechanism of action is still under investigation. The study of extracellular miRNAs is a rapidly growing sub-field which has the potential to uncover novel biomarkers and intercellular communication.

The results presented in this thesis reveal changes in miRNA expression during decidualization and regulation of the miRNA pathway, with important functional consequences. Although we expected miRNAs to have important roles in the decidualization process itself, Dicer depletion did not inhibit decidualization and data on the regulation of several miRNA target genes and reporter gene assays showed that miRNA-mediated regulation is diminished during decidualization. Furthermore, we found exosomal miRNA export is activated upon decidualization, opening up the possibility of intercellular communication in the endometrium being mediated by miRNAs.
Differential miRNA Expression

We observed changes in the abundance of specific miRNAs upon C+M treatment of hESCs. Some of these differentially expressed miRNAs were also shown to change at the primary transcript level indicating that, much like protein-coding genes, miRNA genes undergo transcriptional regulation during decidualization. It would be interesting to delineate the pathways controlling transcription of specific miRNA genes in hESCs as much work has been conducted on the regulation of key decidual genes and there may be common transcription factors involved.

Of the 13 human miRNAs identified by the microarray to be differentially expressed in undifferentiated compared to decidualized hESCs, the majority are clustered on the genome with other miRNAs. However, only in the case of the miR-29b-2/miR-29c did all members of cluster come up in the microarray. QPCR for miR-145, which is clustered with miR-143, showed that it was not regulated in a similar manner to miR-143 upon C+M treatment. Furthermore, in addition to miR-29b, miR-19b and let-7a are transcribed from multiple genomic locations; miR-19b can be expressed from chr. X and 13, and let-7a can be expressed from chr. 9, 11 and 22. Further studies are required to determine if the changes in the levels of mature miR-19b and let-7a are due to changes in transcription from all or a subset of their genomic loci.

We showed that C+M treatment and a key decidual transcription factor, C/EBPβ, induced luciferase expression from a reporter construct containing promoter sequence from the miR-29b-2/miR-29c cluster. More precise definition of the promoter region could be achieved by performing reporter assays with constructs containing smaller fragments of the 4.1 kb cloned sequence. Further characterization of C/EBPβ binding using ChIP and electrophoretic mobility shift assays (EMSAs) may confirm its involvement in regulating this miRNA cluster. Additionally, through 5’ RACE we found that this miRNA cluster had a decidual-specific TSS. MiR-29c was previously studied in the context of endometriosis and found to regulate ECM genes in hESCs (212). This study did not find miR-29c to be up-regulated in hESCs treated with progesterone alone. However, the induction of C/EBPβ in hESCs is dependent on cAMP signalling (237), so there may have been insufficient levels of this transcription factor to induce miR-29c with P4 treatment alone. A recent study by Estrella et al., used combined E2 and P4 treatment to induce decidualization of hESCs (258). They found, with miRNA PCR arrays, that 26 miRNA were differentially expressed upon treatment and only miR-22* was in common with our results. Although PCR is more sensitive than microarray-based methods, it may be that
C+M treatment leads to different miRNAs being regulated compared to E2 + P4 treatment of hESCs.

The identification of four differentially expressed non-human miRNAs in the microarray could be spurious, but the result may be confirmed with Northern blotting techniques and deep sequencing. Indeed, next generation sequencing was recently used to identify seven confirmed novel miRNAs in various tissues from the female reproductive system and an additional 51 novel miRNAs, which were predicted with high confidence (259). The array platform we used was based on release 9.0 of miRbase, which contained 4,361 entries, but the current release of miRbase (miRbase 19 as of August 2012) contains 21,264 entries. Therefore, there may be significantly more differentially expressed miRNAs in undifferentiated and decidualized hESCs that were missed by our array because the probes were not present.

A further consideration is the temporal nature of gene expression; several decidual genes are known to have biphasic expression patterns with levels increasing early in the decidual process and declining later on. The same may be true of certain miRNAs, implying that the 8 day time point chosen for the miRNA array would only screen for those miRNAs differentially expressed late in the decidual process or whose change is maintained from early to late stages. Any miRNAs differentially expressed at just early stages of the decidualization would be missed by the 8 day time point.

The miRNA pathway

The changes observed in miRNA pathway components were striking. Dicer protein levels were increased several fold by 8 days of C+M treatment. Dicer catalyzes the processing of pre-miRNAs to mature miRNAs. Although we observed increased levels of Dicer, to confirm that pre- to mature miRNA processing is increased in decidualized hESCs Northern blotting studies could be used as the same probe can both detect pre- and mature forms for a particular miRNA on the same blot. A study of Dicer regulation in melanocytes found the transcription factor MITF up-regulated Dicer expression and Northern blotting showed this promoted processing of pre-miRNAs to mature miRNAs (260). Even so, most of the mature miRNAs identified by the microarray were up-regulated upon decidualization, and even though transcriptional changes may also occur, the processing of pre-miRNAs to mature miRNAs may occur at an increased rate in decidualized hESCs. We found expression of TARBP2 mRNA to increase by 8 days of C+M treatment, which could contribute to greater stability of Dicer in decidualized hESCs.
Examining the transcription factors responsible for TARBP2 induction in hESCs would be an important step in determining how a key part of the miRNA pathway is regulated. A study examining the alternative transcription start sites of the TARBP2 gene, which produce TRBP1 and TRBP2, found C/EBPβ consensus sites in the promoter region (261). This transcription factor is already known to function in decidualizing hESCs and it may regulate expression of this gene too.

The recent paper by Estella et al., also found Dicer to be induced upon C+M treatment of hESCs, a change not induced by E2 and P4 treatment (258). Additionally, in common with our study, Dicer expression was increased in the glandular and luminal epithelium during the late secretory phase of the cycle. In agreement with our data, they also showed siRNA-mediated knockdown of Dicer did not affect PRL or IGFBP1 induction upon C+M treatment. Moreover, they demonstrated that several transcription factors important for decidualization (e.g. FOXO1, C/EBPβ, SP1) were unperturbed by depletion of Dicer. They observed HOXA10 levels to be decreased upon Dicer knockdown but their overall conclusion was that Dicer plays a minor role in decidualization.

Treatment of hESCs with C+M led to down-regulation of Argonautes 1 and 2, suggesting that the capacity for miRNA silencing was reduced in decidualized hESCs, an effect that was confirmed with the miR-30 reporter assay. There are more components of the miRNA pathway that have yet to be examined in hESCs. For example, exportin 5 and GW182 may also be subject to regulation during decidualization. GW182 proteins closely interact with Argonautes and regulation of their expression in decidualizing hESCs may be important for reduced miRNA-dependent silencing.

Another aspect of miRNA-mediated regulation that may be important in hESCs is the abundance, distribution and composition of processing bodies (P-bodies). These cytoplasmic foci are where translationally repressed mRNAs reside and are also the sites of mRNA degradation. Indeed, they were first discovered when XRN1, the exonuclease responsible for mRNA degradation, was shown to have a punctate localization within the cytoplasm of mouse fibroblasts (262). P-bodies contain several miRNA pathway proteins including Argonautes and GW182 (263-265) and also factors necessary for nonsense-mediated mRNA decay (NMD), mRNA deadenylation and decapping (266). Even though the presence of visible P-bodies is not essential for miRNA silencing, given that miRNA-dependent regulation is decreased in decidualized hESCs, it is tempting to speculate that decidual cells may have fewer or perhaps smaller P-bodies than undifferentiated hESCs.
Dicer is a key enzyme for the generation of mature miRNAs and numerous studies demonstrate that it is necessary for the survival or differentiation of a range of cell types. The *Amhr2-cre Dicer* conditional knockout (cKO) mice specific to the female reproductive tract were infertile but did not display an impaired decidual response (226). Our data and that of Estella *et al.*, on Dicer knock-down in hESCs is in keeping with this as PRL and IGFBP1 were still highly induced upon C+M treatment. Recently, data on a novel *Dicer* cKO mouse has been published (267). Unlike the previous cKO model, cre recombinase is expressed from the *PR* gene and this resulted in strong ablation of Dicer expression in the uterine luminal epithelium and stroma. Again female *Dicer* cKO were sterile and adult mice had greatly reduced uterus size compared to wild type littermates. However, this study did not explicitly examine the decidual response as previous studies have but instead focussed on expression profiling of miRNAs and target genes dysregulated by *Dicer* deletion. *PR-cre* starts to be expressed in the uterus in two-week-old mice. The *PR-cre Dicer* cKO mice exhibited more apoptosis of uterine stromal cells at postnatal day 15 but if the infertility is a consequence of insufficient stroma and poor uterine development or a defective decidual response is still unresolved. Importantly, at this stage of development they found only modest decreases in the levels of most mature miRNAs, possibly due to their high intracellular stability, which has previously been studied in-depth in inducible *Dicer* KO mouse embryo fibroblasts (268). The long half-life of mature miRNAs (on average 119 h, according to (268)) should be borne in mind when depleting Dicer with siRNA or genetic engineering.

**Androgen Receptor Regulation**

Previous studies established a limited but nonetheless significant role for AR in regulating specific genes in decidualizing hESCs (70). The regulation of the AR expression in decidualizing hESCs suggested a potential role for miRNAs, as AR protein levels were dramatically reduced but mRNA levels were maintained. However, AR-targeting miRNAs did not exhibit regulation and Dicer knock-down was not able to restore AR protein levels upon decidualization. Instead, the RNA binding protein PCBP1 was shown to be sufficient to repress AR protein levels in vivo and also repress translation in vitro. This RNP was increased in abundance during decidualization of hESCs and it was also found to repress AR expression in LNCaP cells. The global effects of PCBP1 depletion by siRNA were recently examined in human SH-SY5Y cells and 375 transcripts were affected (269). Accordingly, in decidualized hESCs PCBP1 is likely to be regulating many other targets in
addition to AR. Translational silencing by PCBP1 has been studied for the 15-
lipoxygenase mRNA and PCBP1 inhibits translation of this transcript by inhibiting the 60S
ribosomal subunit from joining to the 40S subunit at the initiation codon (233, 270). Whether or not PCBP1 represses AR translation by a similar mechanism remains to be determined.

There may exist in hESCs cross-talk between RNPs and the miRNA pathway. Recently, it was demonstrated that the RNP HuR can relieve miRNA-dependent repression of transcripts containing AREs, the motif HuR interacts with (271). HuR is expressed in hESCs, and its over-expression leads to increases in AR mRNA and protein levels (272). Despite the AR mRNA lacking AREs, HuR interacts with the UC-rich region of the AR 3’UTR (242), so HuR may relieve miRNA-dependent repression of AR.

**MicroRNA function, secretion and the fetal-maternal conflict**

Although depletion of Dicer did not block decidualization or implicate miRNAs in the down-regulation of AR, we did observe another effect; Dicer knock-down increased AR and TRIB2 levels in undifferentiated hESCs but not in decidualized hESCs. Similar findings were observed for other miRNA target genes when inhibiting specific miRNAs with anti-miRs and with the generic miR-30 reporter assay. Grimaldi et al., showed that upon decidualization DNMT3B along with its regulator, helicase lymphoid-specific (HELLS), are down-regulated but global levels of DNA methylation are unchanged (244). By employing anti-miRs we found regulation of DNMT3B by the miR-29 family was apparent in undifferentiated hESCs but not in decidualized hESCs. The mechanisms causing down-regulation of DNMT3B in decidualized hESCs may therefore be transcriptional or perhaps post-transcriptional but mediated by RNPs. Our overall conclusion was that miRNA activity was higher in undifferentiated hESCs than in decidualized hESCs.

The physiological relevance of reduced miRNA activity in decidualized hESCs is unclear. One existing paradigm that this finding may fit in with is that of the fetal-maternal conflict (273). It has been known for some years that, far from being a fully co-operative enterprise between mother and fetus, pregnancy entails a certain level of conflict between mother and fetus due to differences in the optimal level of nutrient transfer from mother to offspring for each party. The fetus’s optimal amount of maternal investment is likely to be higher than the level the maternal tissues find optimal because maternal investment in the fetus must be balanced by the need to supply adequate nutrients to the maternal tissues.
This conflict originates from the genetic difference between mother and fetus; only 50% of the maternal genes are present in the fetus, so the maternal investment in fetal survival can only guarantee transmission of 50% of her genes to the next generation in any one pregnancy, whereas 100% of the fetus’s genes stand to gain from maternal investment in the fetus’s survival (273). One of the most well known manifestations of this conflict is the genomic imprinting where paternally or maternally derived alleles for certain genes are specifically silenced in offspring. Another manifestation is the evolution and expression of placental hormone gene families (274). For example, human placental lactogen is produced by the syncytiotrophoblast at very high levels and it stimulates glucose availability to the fetus. This gene has been duplicated in primates suggesting strong selective pressure, however the action of placental lactogen is opposed by increased maternal production of insulin during pregnancy.

It has been demonstrated that the trophoblast, an embryonic tissue, can secrete miRNAs in exosomes and placenta-specific miRNAs have been detected in the maternal circulation (275). Being inhibitory molecules, miRNAs are well placed function in the fetal-maternal conflict and trophoblast-derived miRNAs taken up by maternal tissues may inhibit specific genes, potentially those involved in inhibiting the transfer of nutrients from mother to fetus. It could be that the down-regulation of miRNA activity upon decidualization we have observed is a defence mechanism to protect hESCs from the effects of trophoblast-derived miRNAs that may be taken up by maternal cells during pregnancy.

This study provides the first evidence for miRNA secretion by decidualizing hESCs and it is mediated in part by extracellular vesicles, which were in the size range of exosomes and expressed at least two exosome markers. Other studies have shown that extracellular vesicles from diverse sources can be enriched for miRNAs. From the results in chapter 5, we have shown that although undifferentiated hESCs produce vesicles, they are relatively poor in miRNAs and that the differentiation process somehow leads to an increase in miRNA abundance in vesicles, with little increase in vesicle production. This suggests that a mechanism may exist to divert miRNAs to vesicles and that it is activated during decidualization. A mechanism to increase miRNA abundance in exosomes could entail co-localization of Dicer and the RISC loading complex to multivesicular bodies. Studies by Gibbings et al., have already shown that GW-bodies (cytoplasmic bodies containing GW182, Ago2 and miRNA) are closely linked to endosomes and multivesicular bodies (199). Further work to study miRNA recruitment to exosomes in hESCs could
involve immunocytochemistry of RISC and ESCRT components in undifferentiated and decidualized hESCs. Co-localization of exosome and miRNA machinery would prompt further functional studies utilising siRNA against various components and monitoring miRNA activity, exosome biogenesis and miRNA secretion.

Although our study has focused on exosomal miRNA the results shown in figure 5.3, suggest that a proportion of the extracellular miRNA from decidualized hESCs is susceptible to RNase and presumably not contained within vesicles. Therefore, an important question that remains is if the increase in miRNA secretion observed upon decidualization is a result of increased exosomal or non-exosomal secretion.

**Exosome biology in the Endometrium**

Decidualized hESCs are already known secrete a range of proteins which coordinate responses throughout the endometrium. In addition to miRNAs, exosomes contain various proteins that may have specific signalling functions in recipient cells. For example, exosomes from various cancer cell lines can trigger fibroblast to myofibroblast differentiation through transforming growth factor beta (TGF-β) being expressed on the exosome surface (198). Therefore, further characterisation of exosomes from decidualized hESCs would shed light on other macromolecules that may be important for paracrine signalling in the endometrium. Given that decidualization of hESCs has been found to be dramatically impaired in hESCs sampled from women with recurrent pregnancy loss (247), the secretion of miRNAs may also be altered in these patients.

The uptake of miRNA secreted by decidual cells was demonstrated using a *C. elegans* specific miRNA and we found that various recipient cell types contained different amounts of secreted cel-miR-39. Leaving aside any potential differences in cel-miR-39 stability within the different recipients, this result points towards different cell types having differing abilities to interact with decidual cell exosomes. The interactions of exosomal surface proteins and plasma membrane proteins are likely to be important for this although the mechanisms governing exosome uptake are poorly understood. Although not examined in hESCs, a well-known class of exosome surface proteins are the tetraspanins (e.g. CD63). Tetraspanins can interact with a variety of integrins and characterisation of integrin expression in recipient cells and tetraspanins on hESC exosomes may elucidate the factors important in exosome targeting at the feto-maternal interface (276). Given that we observed miRNA uptake by BeWo cells, miRNA secretion by decidualizing hESCs may represent another part of the fetal-maternal conflict where maternal miRNAs are targeting
genes in the fetus as part of a “counter-attack” to the secretion of miRNAs by the trophoblast.

To summarize, human endometrial stromal cells acquire a range of characteristics upon decidualization, which favour embryo implantation and successful pregnancy and changes in miRNA expression and the miRNA pathway are also characteristic of decidualization. Even though down-regulation of Argonautes upon decidualization renders hESCs less sensitive to the silencing effects of miRNAs, exosomal miRNA secretion is activated, implying a novel form of intercellular communication in the endometrium. However, further work is required to understand the detailed mechanisms and wider implications of these findings, including work in the context of gynaecological and obstetric disorders.
References


**Publications**
